NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été daclylogra phiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

ISBN 0-315-62302-0

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ACKNOWLEDGEMENTS

I am grateful for having been given the opportunity to enter the Physiology program at University of Ottawa. The time I spent in the Department of Physiology was enjoyable and rewarding. Several people helped at various stages throughout my project, freely offering their time and expertise; I am thankful to everyone who participated.

My supervisor Dr. George Biro has been very kind, supportive and always encouraging. Dr. Biro is an excellent teacher who offered his time freely, patiently introducing me to difficult concepts in a manner I could understand. He helped continuously throughout the development of the protocols, the experiments, the interpretation of the data and the writing of this thesis. He has very high personal and professional morals and I feel privileged to have had the opportunity to learn under his guidance.

Dr. Peter Anderson spent a great amount of time helping develop the biochemical protocols and discussing the results of the biochemical analysis. Dr. Anderson was an excellent and very patient teacher with a wonderful sense of humor; working in his lab was most enjoyable.

Dr. Graham Mainwood, also on my committee was very kind. He had great insight to my work continuously offering suggestions and explanations.

Other department members especially Dr. D. Perry were very helpful. He generously provided us with myosin monoclonal antibody, and spent much time discussing my results.

Dr. Michael Munroe from the Department of Mechanical Engineering was very helpful discussing the results of the mechanical data.

Dr. Eamon Tierney a visiting fellow in Anaesthesia spent much time helping to develop the anaesthetic protocol. It is because of him that our surgical success rate was so high.

The surgical portion of my experiments was quite long, and involved much work for the staff of the Animal Facility and Experimental Surgery. The preoperative and postoperative care of the animals was expertly provided for by Dr. Marilyn Keeney and her staff: Mr. P. Bradley, Ms. J. Craigie, Ms. S. Dyer, Ms. C. Groulx, and Ms. A. Hogarth. Ms. Miza Bosc-Davie from Experimental Surgery patiently taught me surgical skills required to perform these experiments. Ms. J. Sistek, and Mr. R. Seymour, also from Experimental Surgery, assisted in performing the surgeries.

Technical expertise with some of the biochemical procedures was cheerfully offered by Ms. Diane Mauldin, Mr. Steve Knight, Mr. Richard Dalpe, and Ms. Cynthia Gwillam.

Histological staining was expertly done by Ms. Ute Portner-Davis. Help in interpreting the histology slides was kindly given by Dr. K. Sarkar from the Department of Pathology.

Dr. Michael McBurney from the Department of Medicine generously provided actin monoclonal antibodies.

The computer expertise which was continously offered by Mr. James Evans is greatfully acknowledged. Jim spent much time teaching me to use a PC, typing the thesis, preparing the graphs and preparing the final manuscript. I thank him very much.

This thesis is dedicated to my brother Andrew.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

This study followed the temporal changes of the healing of infarcted myocardium over a twenty-eight day time period. Permanent ligation of the circumflex coronary artery at its origin was performed on pigs. Animals were randomly allocated to one of seven groups; they were sacrificed at either one, three, seven, fourteen, twenty-one, or twenty-eight days after occlusion. A control group received only a sham surgery. Radionuclide-labelled microspheres were injected pre and post-occlusion and prior to sacrifice. Ten minutes after permananent occlusion blood flow to the infarct zone decreased to 6.5% of the control value, and remained significantly below preocclusion flows for the entire duration. Flow to the noninfarct zone increased above control one day after occlusion, and remained significantly elevated above control until day twenty-one post-operatively.

The rate of disappearance of the contractile proteins actin and myosin and the appearance of collagen were followed using several biochemical techniques. 3-methylhistidine, an amino acid marker of actin was measured using high performance liquid chromatography (HPLC). It remained unchanged in both the infarct and noninfarct zones for the duration of the experiment. The presence of actin and myosin in twenty-eight day old infarcts was also confirmed using poly-acrylamide gel electrophoresis and monoclonal antibodies. Hydroxyproline, an amino acid marker of collagen, which comprises approximately 10% of the protein, was measured using several methods. In the noninfarct zone hydroxyproline increased from 0.4% to 0.6% of the total protein. In the infarct zone the percentage of hydroxyproline increased significantly with the age of the infarct from 0.5% to 3.3% at twenty-eight days, as measured with the Berg and o-phthalaldehyde (OPA) assays. Histologically, tissues were stained with several stains to demonstrate the appearance of collagen. New collagen was visible by day seven in the infarct zone; it appeared fine and randomly oriented. Subsequent mature collagen was thick, and oriented parallel to the circumference of the heart.

Stiffness was assessed by *in vitro* stress-strain analysis (average maximum strain was 160% of length at zero tension) in full thickness strips of infarct and noninfarct zone tissue, and epicardial and endocardial strips of infarct tissue from all hearts. A decreased tensile strength was observed in three-day infarcts only. The total stiffness did not change significantly from control in either region of the heart at any measured time. However, when the components of stiffness were compared within the same heart, the elastic component of stiffness increased and the viscous component decreased in the infarct zone. In the noninfarct zone the changes were opposite, a decrease in the elastic component of stiffness and increase in the viscous component were observed.

In conclusion, changes in blood flow, and mechanical and biochemical properties occurred in both the infarct and noninfarct zones in pig hearts after permanent coronary artery occlusion. Both intra-animal and inter-animal differences in the rate of healing were observed. As well, changes did not occur simultaneously; the blood flow and biochemical changes preceeded the mechanical ones. These findings also suggest that hypertrophy of the noninfarct zone occurs, taking approximately twenty-one days. Although repair of the infarct zone is not complete by twenty-eight days, significant myocyte clearing and collagenous replacement has occurred which maintains tensile strength and total stiffness.

1.0 INTRODUCTION

1.1 PREAMBLE

The heart is an essentially spherical pump whose wall is composed of myocytes supported within a collagenous weave. It is supplied with blood by a capillary network. The pumping action results from force generated by the contractile apparatus of the myocytes and the force is mainly transmitted by the collagenous weave. If the blood supply to the myocytes is compromised by loss of patency of a coronary artery, contractile activity ceases rapidly. If the lack of blood supply is more protracted, functional loss becomes irreversible and structural disintegration of the myocytes follows. They are then cleared and replaced by a fibrous scar tissue. While this is happening, the rest of the myocardium must continue to function and may in fact, assume an increased workload to compensate for the area lost. This is the process of myocardial infarction.

The healing of the heart with an infarct has been examined histologically, mechanically and biochemically, but the documented information is not complete. Most studies describe only a single parameter. Although short term changes have been well described, long term changes have not been as extensively documented, and longitudinal studies are scarce. There is therefore an uncertainty of the time course of many changes. The following is a brief synopsis of the available studies.

1.2 GENERAL WOUND HEALING

Healing of the infarcted myocardium has similarities to the healing of other injured body tissues. The basic steps of wound repair are the same, and can be divided into events occurring days, weeks and months after the initial damage (Hunt *et al.*, 1984). Within days there is an infiltration of the damaged area by granulocytes, monocytes and macrophages. The necrotic tissue, tissue which has been irreversibly damaged, is cleared by the phagocytic white blood cells. Two to four

weeks later, this is followed by a proliferation of fibroblasts which synthesize new collagen. There is an ingrowth of capillaries into the area. This combination of capillaries and fibroblasts is called granulation tissue. The fibroblasts produce enough collagen to refill the space left by the damaged cells (Cohen *et al.*, 1986, Hunt *et al.*, 1984). This replacement tissue is called scar tissue. Over subsequent months remodelling of the scar occurs. This involves collagen turnover and cross linking of the fibers. The scar is remodelled so that it can withstand the forces to which it is subjected (Anders-Carlstedt, 1987, Nimni, 1983, Viidek, 1973).

A series of different collagen types appear after wounding, (Chiarello *et al.*, 1986, Glynn and Houck, 1981) . Initially there is an increase in a very fine reticular form of collagen, believed to be produced by locally stimulated fibroblasts in the wound area. This collagen type is not believed to contribute to the tensile strength of the developing scar (Nimni, 1983). The initial collagen is followed by the laying down of a thick stranded type collagen (Chiarello *et al.*, 1986, Clore *et al.*, 1979). It is not yet determined whether this is also produced by the original local fibroblasts, or by the new fibroblasts which enter the area (Mallory *et al.*, 1939).

1.3 HEALING OF INFARCTED MYOCARDIUM

Three characteristics become evident from the histology and blood flow literature which describes the healing of infarcted myocardium (Cohen, 1985, Mallory *et al.*, 1935, Weisman and Healy, 1987). First, as in other tissues, the healing of the damaged area occurs in an organized and predictable way. Regional infarct expansion is followed by infarct contraction. Second, global changes occur in the undamaged myocardium during the healing process, namely cavity dilatation, and hypertrophy. Third, the changes which occur during healing are not dependent on the presence of a large and continuous blood flow to the necrotic area. In the following sections these concepts are expanded.

1.3.1 HISTOLOGY OF HEALING MYOCARDIUM

The process of healing of infarcted myocardium is well documented histologically. Mallory *et al.* (1939) initially described the main features, based upon their examination of human autopsy specimens. More recent histological descriptions of human infarct healing (Fishbein *et al.*, 1978, Olsen, 1980) support the earlier findings. A problem with these descriptions however, is the necessity of dating the infarct retrospectively; consequently there is some uncertainty as to the actual time course of the observed events. The descriptions of experimental animal infarct healing have attempted to define the time course changes more precisely (Reimer, 1979).

Inflammation is defined as the response of living tissue to damage and the resulting necrosis (Robbins, 1984). Like the healing of "general wounds", the healing of an infarction can be divided into phases. The initial period of acute inflammation associated with the clearing of the necrotic material is followed by a more prolonged period of chronic inflammation associated with the laying down and remodelling of collagenous replacement material. These phases will be briefly described.

The area of cardiac muscle severely deprived of blood flow becomes necrotic about 45 to 90 minutes after occlusion (Klein *et al.*, 1984, Schaper *et al.*, 1986). Early cellular changes are characterized by general intracellular disorganization including broken sarcolemma, myofibrillar disruption, and nuclear and mitochondrial changes (Schaper, 1986). By twenty-four hours there is an influx of polymorphonuclear leukocytes to the area. Granulocytes are the initial predominant inflammatory cell (Fishbein *et al.*, 1978, Olsen, 1980). The myocytes appear hyper-eosinophilic with routine histological staining.

The second phase, chronic inflammation, is associated with an ingrowth of blood vessels, connective tissue and inflammatory cells. Monocytes and macrophages gradually become the predominant inflammatory

cells. About four to five days after the occlusion, beginning at the periphery and progressing inwards towards the center, necrotic tissue is removed and blood capillaries and fibroblast proliferation start to appear. At two weeks, the ingrowth of capillaries and connective tissue is still increasing.

The third phase is associated with a proliferation of collagen. Between nine days (Fishbein *et al.*, 1978, Olsen, 1980) and three weeks (Mallory *et al.*, 1939) after occlusion new, fine collagenous fibers are visible histologically at the periphery of the infarct. These fibers gradually become more "dense" and appear to be arranged in series with muscle (Carroll *et al.*, 1989). At four to six weeks there may be still a necrotic central mass of tissue which has not yet been cleared. Apart from this, collagen is the prominent feature of the infarcted area (Olsen, 1980). In most hearts, by about two to three months the infarcted area has become contracted, and is a firm white scar. The scar will not be completely resorbed, but rather remains permanently (Mallory *et al.*, 1939).

There is no clear demarcation of stages. Since healing progresses from the periphery inwards, at any time different areas of the infarct may be at different stages of healing; the healing of the core lagging behind healing at the edges. However, in all areas the orderly sequence of myocyte clearance followed by collagen deposition occurs. Several factors may influence the rate of healing, including the size of the infarction, the nature of the occlusion, and the status of the remaining coronary collateral bed (Mallory *et al.*, 1939, McDonough *et al.*, 1984).

1.3.1.1 HISTOLOGICAL CHANGES IN THE NONINFARCTED AREA

The changes in the noninfarcted areas of the heart have not been described as extensively as those in the infarcted area. Nevertheless changes do occur. It is well documented that a compensatory hypertrophy of the noninfarcted area develops. This involves proliferation of both

the myocytes and connective tissue (Roberts *et al.*, 1984). This process is detailed further in Section 1.6.

1.3.2 DEVELOPMENT OF BLOOD VESSELS - HISTOLOGICAL DESCRIPTION

The coronary circulation has been studied in man and in many animals, including the pig, dog, guinea pig, and rabbit (Maxwell *et al.*, 1987, Patterson and Kirk, 1983, Weaver *et al.*, 1986). Significant differences in the location, number and size of communications between coronary vessels, called collateral vessels, occur between species. These vessels which normally are potential channels, can provide an alternative pathway for blood flow if a major vessel becomes stenosed or occluded. Besides the presence of preformed vessels, collaterals can also form in response to myocardial stress (Bloor, 1974, Cohen, 1985).

To examine the development of collateral vessels, ischemia and subsequent infarction is usually produced with an ameroid constrictor (O'Konski *et al.*, 1986). This causes a gradual narrowing of the coronary vessel, with occlusion occurring in seven to fourteen days. The area which was gradually deprived of blood may or may not infarct (Cohen,1985). The occurrence of infarcted tissue depends upon the degree of collateral vessel development. This development can be studied by angiography, by microsphere injection, and by post-mortem barium sulphate gelatin injection of the vascular bed. The major stages of this vessel development in the threatened region are summarized by Cohen (1985). Immediately after coronary artery occlusion the native collateral vessels are thought to dilate. This is followed by a phase of radial growth which increases the vessel diameter, then by an increase in the wall thickness of the vessel. There is then a regression of these vessels (Castor, 1981).

Histologically, vessel ingrowth to the infarcted area is observed about four days after occlusion (Castor, 1981, Fishbein *et al.*, 1978, Lautsch, 1973, Mallory *et al.*, 1939). The appearance of new vessels is

too slow to preserve viability of the myocytes, especially if occlusion is sudden (Savage *et al.*, 1981, Cohen, 1985). It does however allow for the ingrowth of granulation tissue. The ingrowth of capillaries is associated with an influx of fibroblasts to the area (Mallory *et al.*, 1939, Olsen, 1980).

The stimuli for vessel ingrowth are not precisely understood, however several contributing factors are believed to influence it (Mallory *et al.*, 1939, Schaper et al., 1967). Many have been hypothesized including altered wall stress, hypoxia, and humoral factors. Growth factors from fibroblasts, platelets and macrophages have also been associated with vessel growth (Cohen, 1985, Hunt, 1984, Takemura *et al.*, 1984). All of these may be present after infarction.

1.3.2.1 BLOOD FLOW IN INFARCTED TISSUE

The development of the radioactive microsphere method (Rudolph and Heymann, 1967) to measure blood flow experimentally has generated much research on blood flow distribution patterns. The principle of the microsphere technique is that after injection microsphere distribution throughout the body is in proportion to the amount of blood flow to the area. Microspheres become trapped in the capillary bed at the site where the vessel size is smaller than the sphere. This is about the precapillary level.

Blood flow values to infarcting regions of the heart have been well documented for the initial hours after occlusion (Cohen, 1985, Fedor *et al.*, 1978, Hearse *et al.*, 1986, Sjoquist *et al.*, 1984). It has been repeatedly shown that an area of myocardium will become necrotic when the blood flow is suddenly and permanently decreased.

Savage *et al.* (1981) reported blood flow changes in the porcine heart at fifteen minutes, and twenty-four and forty-eight hours after permanent coronary artery occlusion. Fifteen minutes after occlusion flow to the infarct zone decreased to 12% of normal, and there was a

small (10%) but significant increase in flow to noninfarcted tissue. At twenty-four and forty-eight hours, flow to the infarcted area remained unchanged, while flow to the region adjacent to the infarct increased. Flow to the noninfarcted area increased to 142% of the control levels. Consequently, it appears the blood flow pattern to both the infarcted and noninfarcted tissue changes after a coronary occlusion.

Long term blood flow patterns to both the infarcted and noninfarcted regions after sudden complete occlusion are not well documented. The lack of documentation of the long term blood flow changes is probably due to the initial uncertainty of the fate of microspheres trapped in the tissue bed for extended periods. There was an initial concern that significant microsphere loss could occur from infarcted regions. To address this, Reimer and Jennings (1979) injected microspheres into animals immediately prior to permanent coronary vessel occlusion. The animals were killed eight days later. These investigators expected to find no significant differences in preocclusion blood flows to any area of the heart, greater than those expected from normal temporal and spatial differences. However, the preocclusion ratio of microspheres in the infarct to noninfarct areas was not 1.0 as expected, but was significantly less. The lowest ratio reported was 0.43. This implied that a significant loss of microspheres from the infarcted tissue had occured. This belief was supported experimentally by others (Capurro *et al.*, 1979, Jugdutt *et al.*, 1979, Lekven and Anderson, 1980).

When these investigations were extended to periods as long as twenty-eight days, the preocclusion infarct-to-noninfarct ratio of microsphere content increased to values greater than 1.0. Ratios as high as 3.39 were reported (Reimer and Jennings, 1979). This significant "gain", a technical impossibility, implied that microsphere loss was minimal (Consigny *et al.*, 1982, Kiewiet de Jonge *et al.*, 1980, Reimer and Jennings, 1979). The conclusion from these studies was that microspheres do remain lodged in the infarcted tissue. The apparent changes which are reported in control blood flows are probably due to

changes in the tissue itself. If the mass of the tissue in which the microspheres initially lodged changed from the time the microspheres were injected until the time when microsphere content was measured, there would be post-facto changes in the microsphere concentration and the blood flow determinations. This is because blood flow measurements are expressed per gram of tissue weight at the time of sacrifice. The initial edema which characterizes the early stages of healing increases the tissue weight and dilutes the microspheres. This results in an underestimation of the real blood flow accounting for the infarct-to-noninfarct ratio of less than 1.0. With increasing time between preocclusion microsphere injection and postocclusion sacrifice, the subsequent scar formation and tissue contraction results in a concentration of the microspheres and the infarct-to-noninfarct ratio rises. As a result the infarct flow becomes overestimated. Therefore, there is a continually changing reference tissue mass. Long-term blood flow estimates must take this into account. Murdock and Cobb (1980) have proposed that the actual degree of apparent loss or gain of microspheres can be easily calculated, by comparing the preocclusion flows of the infarct to noninfarct zone using a simple equation; ((NIZ - IZ)/NIZ)*100), where NIZ is noninfarct flow, and IZ is infarct flow, in the same transmural level. A positive value indicates a dilution of spheres and a negative value suggests their concentration. All of this is based on the assumption that blood flow to the noninfarct zone equals that to the infarct zone before coronary artery ligation. This is a reasonable assumption, since blood flow in the normal ventricle is fairly evenly distributed (Murdock and Cobb, 1980).

1.4 MYOCARDIAL PROTEINS IN INFARCTION

The turnover rate of individual proteins is unique. Normally the protein environment is in a constant state of flux. There is a continous balance between protein synthesis, an energy demanding process and protein degradation. When a protein has incurred even a single insult it is damaged and must be cleared. It is completely replaced, rather than repaired (Gevers, 1984).

Myocardial proteins are located both intracellularly and extracellularly. The majority of intracellular proteins are found in the myocytes which comprise about 30% of the total number of cells but over 70% of the volume (Gevers, 1984). The myocytic proteins are those of the myofibrils, mitochondria, sarcolemma, sarcoplasmic reticulum, cytoplasm and nucleus. The proteins of the myofibers comprise approximately 50% of the total proteins of the heart. Actin and myosin are the major myofibrillar proteins contributing 12 and 25% of the total myocardial protein respectively, (Gevers, 1984). Others include tropomyosin, troponins, C-protein, and M-line proteins. The major extracellular protein is collagen, contributing approximately 80% of the extracellular protein (McClain, 1974) and 4% of the total protein (Gevers, 1984). Other extracellular proteins include elastin, proteoglycans, and fibronectin.

Early in ischemic damage many proteolytic enzymes are released and activated, damaging the proteins of both the intracellular contractile apparatus and the extracellular matrix. It was believed that after intracellular damage proteolytic enzymes were released from intracellular lysosomes, their release resulting in extracellular protein damage. There is now evidence that proteolytic enzymes are located in the extracellular matrix as well, which become activated by ischemia, and can disrupt the matrix within minutes (Sato *et al.*, 1983, Zhao *et al.*, 1987).

Damage to either intracellular or extracellular proteins negatively affects the performance of the heart. The disintegration of the myocytes results in loss of function of the contractile units. The damage to the extracellular matrix alters the efficiency of tension-transmission, as it is the matrix which is important in transmitting the force developed by the myocytes during contraction (Caulfield and Borg, 1979, Zhao *et al.*, 1987).

Although in many studies the total concentration of collagen is usually estimated by measuring one of its marker amino acids

(hydroxyproline), this will clearly provide only inadequate description of the functional changes.

1.5 COLLAGEN

1.5.1 GENERAL DESCRIPTION

Collagen is the most abundant protein in the body accounting for approximately 30% of the total body protein, (Eyre, 1980). The fundamental unit of collagen is its polypeptide chain. Each polypeptide chain has approximately 1055 amino acids. The profile of these amino acids is unique; unlike other proteins, collagen has a repeating triplicate pattern of amino acids. The general sequence is $(Glycine\text{-}X\text{-}Y)_{340}$. Glycine contributes one third of the amino acids. Proline and hydroxyproline each contribute about 10% of the molecule, approximately 100 amino acids each. Proline is in the X position and hydroxyproline is in the Y position. This repetitive sequence is required for proper formation of the collagen helix. Each polypeptide collagen chain is twisted into a left handed helix, with about three amino acids per turn. This is called an alpha (α) chain. Three α chains are coiled together in a right handed helix forming the collagen molecule. The molecule is large, and rod shaped, approximately 300 nm long, and 1.4 nm in diameter. It has a molecular weight of about 340,000. Several collagen molecules are joined together to form a collagen fibril. The fibril is 10-200 nm in diameter, depending upon the collagen type.

The process of collagen formation is well described by several authors (Nimni, 1983, Prockop, 1979). Briefly, the polypeptide chain is produced within fibroblasts initially in a long precursor form (Eghbali *et al.*, 1988). Prior to being secreted from the fibroblasts, post-translational modifications are made to the polypeptide. These include the hydroxylation of proline and lysine and the addition of glucose and galactose to hydroxylysine. Three polypeptide chains then coil together, forming a helical arrangement, and disulphide bonds form at the end regions. The protein is then secreted. Extracellularly the end

regions are enzymatically cleaved leaving the collagen molecule. Intramolecular and intermolecular cross links form, connecting the individual polypeptide chains and collagen molecules. These cross links are essential for the functioning of the molecule, providing it with structural strength. The collagen molecules spontaneously assemble into fibrils; the arrangement is believed to be a quarter-staggered pattern (Nimni, 1983).

The regulation of collagen synthesis is complex and still not entirely understood. Fibroblasts, although very numerous, are generally in the resting or G_0 state of the cell cycle (Weber at al., 1989). This means they are present, but are not actively producing collagen. They can, however, be stimulated to activity. The exact signals which regulate the type and amount of collagen to be synthesized and/or degraded are not yet elucidated (Hunt, 1984, Nimni, 1983). It is believed that enzymes, especially prolyl hydroxylase, the intracellular enzyme causing the hydroxylation of proline, may be important in regulating the rate of collagen synthesis (Prockop *et al.*, 1979). Other proposed stimuli include hypoxia (Foidart, 1981), altered wall stress (Weber *et al.*, 1987), and macrophage derived growth factors (Hunt *et al.*, 1984). The primary regulation is at the level of transcription (Duance and Bailey, 1981), but subsequent steps can also be affected (Pinnell, 1982).

Proteolytic enzymes are responsible for the degradation of collagen. These include collagenases, cathepsins and other proteases (Duance and Bailey, 1981); the collagenases being the major enzymes responsible for the process. They are found in several sources including polymorphonuclear leukocytes, macrophages, platelets and fibroblasts (Duance and Bailey, 1981, Krane, 1982). They are also located in an inactive form in the extracellular matrix (Caulfield and Wolkowicz, 1988, Montfort and Perez-Tamayo, 1962, Zhao *et al.*, 1987,). Proteases located within leukocytes, such as cathepsins, are believed to have a synergistic role with the collagenases.

The turnover rate of collagen is highly variable, with different collagen types having different rates from about fifteen days for type III, to months or years for type I (Nimni, 1974, Prockop and Kivirikko, 1968, Weber, 1989).

1.5.2 BIOCHEMICAL QUANTITATION OF COLLAGEN

The general ability of cells to synthesize collagen is assessed by measuring the changing hydroxyproline concentration, or by measuring the activities of specific enzymes. Prolyl and lysyl hydroxylases are those most often assayed.

The presence of collagen in a sample can be determined by several techniques, including histology, immunohistochemistry, and gel electrophoresis. The present "gold standard" is to assay the amount of hydroxyproline in the sample. Hydroxyproline is an amino acid found primarily in collagen. Other minor sources of this amino acid include acetylcholinesterase and Cl_q which is a component of the complement system. They contain one and five percent hydroxyproline respectively (Chiariello *et al.*, 1986). Although complement is present in the ischemic region within minutes after the occlusion (Rossen *et al.*, 1985), its continued presence in scar tissue is unlikely (Prockop *et al.*, 1979). The biochemical determination of hydroxyproline is an accurate and simple colourimetric reaction, with high reproducibility and is a well accepted procedure (Berg, 1982). Many investigators then extrapolate the percentage of collagen in the sample by multiplying the concentration of hydroxyproline by 7.46, a factor proposed by Neuman and Logan in 1950. This was based on the assumption that 13% of the collagen molecule was hydroxyproline. Although this will yield a good approximation, it is not entirely accurate, as not all collagen types have exactly the same proportion of hydroxyproline (Miller and Gay, 1982).

1.5.3 COLLAGEN IN THE NORMAL HEART

The collagen in the myocardium contributes approximately 3-5% of the total protein. This has been documented in human and animal hearts (Caspari *et al.*, 1977, Chiariello *et al.*, 1986, Fredericksen *et al.*, 1978, Gevers, 1984, Montfort and Perez-Tamayo, 1962).

1.5.3.1 COLLAGEN TYPES IN THE NORMAL HEART

There are several genetically distinct collagen types (Eyre, 1980, Prockup *et al.*, 1979). At present twenty-three different alpha chains and twelve different collagen types have been identified (Uitto *et al.*, 1986). Four of these collagen types, designated types I, III, IV, and V have been found in the myocardium. Weber *et al.* (1987) reported that in the primate myocardium, more than 85% of the collagen was type I, approximately 10% was type III, and approximately 5% was type V.

Type I collagen forms thick fibers (Foidart, 1981). It is the presence of this collagen which is believed to be associated with the tensile strength of a tissue (Nimni, 1983). Type III collagen, described by histologists as reticulin fibers (Eyre, 1980), is a fine collagen. It is the earliest form which appears in a healing wound. It is not believed to contribute to the tensile strength but rather to provide a lattice for subsequent healing (Duance and Bailey, 1981). It may also guide inflammatory cells and fibroblasts to the area (Clore *et al.*, 1979). It is found associated with type I collagen, and is abundant in scar tissue (Nimni, 1983). Type V collagen is found primarily in blood vessels and smooth muscle cells (Prockop, 1986).

1.5.3.2 COLLAGEN LOCATION IN THE NORMAL HEART

The collagen network of the heart includes the leaflets of the valves, the chordae tendinae and an extracellular matrix (Weber, 1989). The location of this collagen matrix was first documented in 1907 by Holmgren. He described both vertebrate and invertebrate silver stained

specimens using light microscopy. The location and significance of the myocardial collagen was addressed again by Caulfield and Borg (1979) who examined human and animal hearts by light, scanning and transmission electron microscopy. The following is a summary of the location of the collagen in the ventricle.

There are three main locations of collagen in the myocardium: 1) bundles connecting myocytes to myocytes, 2) myocyte to capillary bridges and 3) bundles around groups of myocytes. Bundles of collagen fibers or "struts" connect individual myocytes. In rodent hearts these struts are 120-150 nanometers (nm) in diameter, and are inserted perpendicularly into the basal laminae of adjacent myocytes close to the Z band. Struts also connect individual myocytes to capillaries. These bundles, also 120 - 150 nm in diameter, are inserted perpendicularly into the basement membrane of the capillary, and tangentially into the basal laminae of adjacent myocytes, also close to the Z line (Borg and Caulfield, 1979). A collagen envelope surrounds groups of myocytes. Thin layers of collagen bundles, 30-70 nm in diameter, form a weave about groups of myocytes (Robinson *et al.*, 1983, 1985). The weave network is interconnected to other networks by long bundles of collagen 80-100 nm in diameter. On the surface of the myocyte are small bundles of collagen arranged parallel to the long axis of the myocyte. These are about 1-3 sarcomeres in length, 10-12 nm in diameter. (Orenstein *et al.*, 1980). There is also a minute lattice of fibrils, microthreads, and ground substance matrix (Robinson *et al.*, 1983, Weber, 1989).

The precise type of collagen associated with each structure is not yet fully described. The collagen found in these different locations of the matrix are believed to be associated with different roles. The myocyte-to-myocyte struts become lax as the myocyte shortens and thickens during contraction, and become taut during relaxation (Caulfield and Borg, 1979). This prevents myocyte slippage during contraction and thereby preserves their orientation. Neighbouring myocytes are stretched to about the same length, thereby the following contraction will have uniform force.

The myocyte-to-capillary "struts" become taut during contraction, and become lax with relaxation. This occurs because of the angle of their insertion. It is believed this acts to keep the capillary open during myocardial contraction. As the myocyte shortens, tension would be placed on the strut, keeping the capillary open (Caulfield and Borg, 1979).

During diastole the orientation of the myocytes stays the same but the orientation of the collagen weave changes. As the sarcomere length is increased the weave becomes aligned along the long axis of the myocyte. It is believed that this reorientation prevents overstretching of the sarcomere and thereby maintains mechanical advantage. (Robinson *et al.*, 1985).

1.5.4 COLLAGEN AND WOUND HEALING

Collagen is the major structural component of scar tissue (Ross and Reith, 1985). The collagen of scar tissue is newly synthesized and laid down (Nimni, 1983), filling the space left by the necrotic cells (Caulfield *et al.*, 1985, Cohen *et al.*, 1986, Hunt *et al.*, 1984). Scars contain two types of collagen, the relative percentage of each changing with the duration of healing. The appearance of different collagen types in the healing wound follows the same pattern as the appearance of collagen in the embryo (Nimmi, 1983). The collagen initially appearing contains a high percentage of type III; this is followed by a gradually increasing percentage of type I. Eventually the scar contains about the same ratio of types I-to-III normally found in that tissue (Chiariello *et al.*, 1986, Clore *et al.*, 1979, Duance and Bailey, 1981).

1.5.5 COLLAGEN AND MYOCARDIAL INFARCTION

The above is the general pattern seen in wound healing. It has been documented that the collagen content in the region of a healing infarct increases (Frederiksen *et al.*, 1978). The characteristics (i.e. its type, its organization) of this collagen have not yet been defined.

Infarction provides two potential stimulators for both collagen degradation and synthesis: altered wall stress (Weber at al., 1987) and tissue hypoxia (Foidart *et al.*, 1981). Changes in collagen concentration are reported after coronary vessel occlusion in both infarcted and noninfarcted regions of the heart (Anversa *et al.*, 1985, 1986). These have been described both biochemically and histologically. The most apparent changes occur in the infarcted region. Histologically the early disruption of the collagen matrix is well described. This is discussed in Section 1.5.5.2. Biochemically, large increases in collagen concentration in the infarcted territory have been quantitated in several animal species including the rat, rabbit, pig and dog, and in the human (Cannon *et al*,. 1983, Frederiksen *et al.*, 1987, Jugdutt *et al.*, 1986, Lerman *et al.*, 1983, Przyklenk *et al.*, 1987).

1.5.5.1 BIOCHEMICAL QUANTITATION

There appears to be an initial 25% decrease in hydroxyproline concentration in the infarcted region during the first twenty-four hours after occlusion (Cannon *et al.*, 1983). This initial decrease in hydroxyproline concentration is correlated with the histological appearance of the disruption of the collagen matrix (Cannon *et al.*, 1983). This decrease is then followed by rising concentrations, with an approximately 1-to-5 fold increase reported seven days after permanent vessel occlusion, 7-fold after fourteen days, 10-fold after twenty-eight days and 13-fold after forty-two days (Chiarello *et al.*, 1986, Jugdutt *et al.*, 1986, Lerman *et al.*, 1983).

Although the concentration of collagen increases with time, these studies all expressed the amount of hydroxyproline found relative to the dry weight of the tissue. This may introduce an error, since it is known that the healing process does involve a changing of the constituents of the damaged tissue. Clearly, as necrotic cells and their constituents are removed, relative collagen concentration could be increased simply by the removal of the myocytes or their

constituents. Therefore, the observed increases in concentration may only be relative and not absolute increases. Although not very significant in the early stages of healing, this concern becomes increasingly problematic as the time between infarction and biochemical study increases, because of continuing changes in the tissue's composition. The problem of trying to quantitate the degree of the changes in a tissue which is constantly changing is similar to that encountered with respect to the microspheres and blood flow (Section 1.3.2.1).

1.5.5.2 HISTOLOGICAL APPEARANCE

The damaged wall of the heart often becomes thinner soon after an infarction. This is called infarct expansion and is defined as the acute dilatation and thinning of the infarcted area which occurs without any additional cell necrosis (Weisman and Healy, 1987). Histologically thinning is not accounted for by removal of necrotic tissue in infarcts less than one week old (Hutchins and Bulkley, 1978, Weisman and Healy, 1987). Thinning is believed to be related to the disruption of the extracellular matrix, resulting in its failure to hold the myocardial cells together. This may be macroscopically manifested by subsequent aneurysm formation and infarct rupture (Jugdutt *et al.*, 1986, Factor et al., 1986). It is believed that expansion could explain the wall motion changes seen early after infarction (Hutchins and Bulkley, 1978). These will be discussed further in Section 1.6.4.1.

There are several reports of disruption of the matrix occurring within hours of the onset of ischemia (Ganote and Hiede, 1987). Crozatier *et al.* (1977) described an increase in the length of sarcomeres in the ischemic region fifteen minutes after coronary vessel occlusion. This was confirmed by Sato *et al.*, (1983) who reported that in pigs the extracellular matrix was disrupted within twenty minutes after permanent coronary artery occlusion. After two hours the collagen "struts" and the weave in the infarct regio· ·ere broken. The

sarcomere lengths in this area increased from 2.3 to 3.6 microns, indicating failure of myocytes to resist passive stretching.

Zhao *et al.*, (1987) reported similar damage occurring very early after occlusion in dogs. They performed twelve 5-minute periods of left anterior descending coronary artery ligation, with a ten minute rest between each. They reported that on microscopic examination the collagen cables had become rough and uncoiled, the dense collagen weave around groups of myocytes was patchy or absent, and the myocyte-to-myocyte struts were broken. The compliance of the ventricle was also increased. They concluded that the collagen which is a mechanical coupler, had been disrupted resulting in an increased compliance and that this process was strikingly rapid.

The collagen matrix in dogs subjected to left anterior descending coronary artery ligation showed similar extensive disruption after two hours. After three hours the loss was greater and extended beyond myocytes showing visibly ischemic damage (Caulfield *et al.*, 1985).

Spontaneous acute infarcts in humans also demonstrate the same disruption of the collagen network. The earliest hearts examined were from persons who died eighteen hours after the onset of chest pain (Caulfield *et al.*, 1985). Factor *et al.* (1986) examined twenty-four hour human infarcts. Both authors found that collagen fibers were absent in the infarcted areas.

The mechanism of the disruption of the extracellular matrix within minutes after coronary occlusion is not yet understood. Apparently, it occurs before there is leakage of intracellular proteolytic enzymes or before there is a major infiltration by leukocytes to the area. (Caulfield et al., 1985, Factor *et al.*, 1986). Although it is possible that the altered mechanics which occurs immediately after infarction could result in altered stress patterns on the collagen weave and struts, it is not felt that this is the cause of the disruption (Zhao *et al.*, 1987). It is believed that inactive extracellular

collagenases or a collagenolytic system becomes activated by ischemia, and subsequently destroys the matrix (Caulfield and Wolkowicz, 1988, Zhao *et al.*, 1987).

The inflammatory cell proteases, although not entirely responsible for the matrix rupture, do contribute to the subsequent collagen degradation (Romson *et al.*, 1983), since leukopenic rats show a less intense degradation up to three days after coronary vessel occlusion, when compared to "normal" rats (Cannon *et al.*, 1983).

1.5.6 COLLAGEN CONCLUSION

There is much still to be understood about the collagen matrix. Questions concerning the exact location and mechanical properties of the different collagen types, the effect of "switching" types and location of collagen, and the exact interaction between the different types must still be addressed. However collagen is the major protein of the extracellular matrix and even though it is present in relatively small amounts in the normal heart, it exerts the major influence on its passive mechanical properties.

1.6 REMODELLING AFTER INFARCTION

There are two "remodelling" changes reported after myocardial infarction: thinning of the infarcted zone and hypertrophy of the noninfarcted region. Evidence for infarct expansion and wall thinning, was described by Roberts *et al.* (1984), who documented changes in the left ventricle after infarction in rats. Two days after permanent vessel occlusion, the left ventricle was dilated. This dilatation was associated with an increased endocardial circumference and no change in the epicardial circumference. By twenty-one days there was still no change in the epicardial circumference, however the infarcted segment lengths of left ventricular epicardium and endocardium and the area of the infarcted tissue were all decreased. They concluded that after myocardial infarction there is both a contraction of the infarcted

tissue and a hypertrophy of the noninfarcted tissue. The wall thinning in the infarct zone could be due to two processes: an initial infarct expansion due to the rupture of the collagen matrix, and subsequently due to clearing of the necrotic myocytes. There are numerous other reports of wall thinning and segment lengthening in the ischemic zone (Fishbein *et al.*, 1978, Weisman and Healy, 1987).

Several investigators reported that a compensatory hypertrophy of the noninfarcted area of the myocardium develops after permanent coronary vessel occlusion. The ability of the noninfarcted area to hypertrophy appears to be related to the original amount of tissue lost. The limit of infarct size appears to be between 30-40% of the left ventricular volume. Infarctions larger than this are believed to be too great to be completely compensated by the remaining noninfarcted myocardium (Anversa *et al.*, 1985, 1986, Rubin *et al.*, 1983, Jugdutt *et al.*, 1986).

1.7 INTRODUCTION TO MECHANICS

As stated previously, contraction of the myocytes develops tension in the wall of the heart. It is now increasingly recognized (Weber, 1989) that the extracellular matrix, including the collagen network, is the major structure whose function is to withstand, store and transmit the forces developed by the contracting myocytes. In addition, they also must allow adequate compliance to facilitate diastolic filling. The application of engineering principles and materials science has contributed significantly to the understanding of the passive mechanical properties of the heart and to their functional importance (Mirsky, 1976).

The following definitions are helpful to the review of the mechanical properties:

An <u>elastic</u> material acts as a perfect spring; ideally, it is extensible without limits, and its response to loading is independant

of the *rate* of load application. Loading and unloading follow the same path.

Elasticity is the property of a material to recover from a deformed state to its original conformation when the deforming forces are removed. Deformation is caused by a force applied to overcome the resistance to deformation of the object. Thus, a load applied to a spring will result in its deformation.

A viscous material's behavior can be simulated by a dashpot. The behavior of a dashpot is to dampen displacement. The amount of dampening is dependent on the viscosity of the fluid and on the *rate* of force application.

Stress is defined as force per unit cross sectional area. The extension (elongation) resulting from the application of a load is referred to as strain; it is expressed as the percentage elongation of the resting length. Stress and strain are usually used when cylindrical or slab-like structures are subjected to unidirectional loading.

Stiffness is calculated by determining the slope of a stress-strain relationship. If the relationship is linear, it is also called Young's modulus.

If a material maintains its stress under constant strain, and returns to its original length when that strain is removed, it is said to be "elastic". If however, the stress changes during a sustained strain, the material is said to exhibit "visco-elastic" properties. If, under constant strain, the stress decreases, it exhibits "stress-relaxation", whereas if the stress increases under sustained strain, it exhibits "creep".

Tensile strength is generally defined as the stress which causes the structure to break.

Compliance is a measure of the distensibility of three-dimensional hollow structures, defined as the change in volume induced by a unit change in transmural (or distending) pressure.

1.7.1 GLOBAL MECHANICS OF THE HEART

Although admittedly, the mechanical properties of the intact heart have great physiological significance in determining the efficiency of force transmission during systole and of filling during diastole, a complete description is extremely complex, because of the complex geometry, finite wall thickness and multi-directional orientation of the structures within the wall (Mirsky, 1976). This becomes even more complex when a part of the ventricular wall becomes ischemic and/or undergoes infarction (Bogen, 1984, Mirsky, 1976, Rankin, 1977). It is simpler to consider the principles of materials testing when cylindrical or slab-like samples are subjected to unidirectional loading along the long axis of the structure (Harkness, 1968).

1.7.2 PRINCIPLES OF MATERIALS TESTING

The mechanical properties of a material, (as distinct from a structure, like the heart) are usually determined by subjecting the material to unidirectional loading; the developed stress is measured as a result of strain increments applied (Fung, 1981, Viidek, 1973).

When strain is applied to a material, the developed stress may remain constant, or creep or stress relaxation may occur. These behaviors may be simulated by models utilizing springs and dashpots. These elements can be arranged in simple or complex ways. The simplest arrangements are illustrated in Figure 1.1 on the following page.

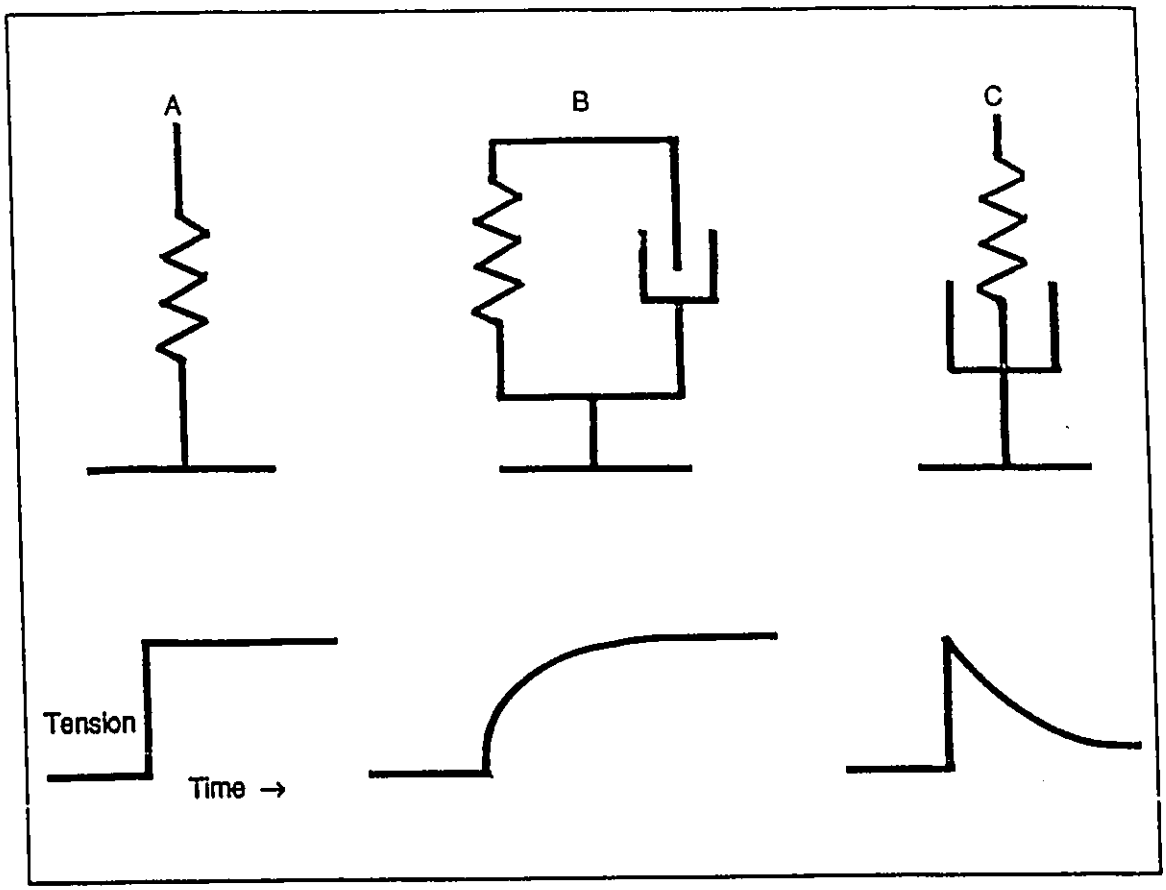


Figure 1.1

Diagrammatic representation of three models which simulate the behaviours of different materials, (after Viidek, 1973 and Fung, 1980). The properties of the material are described in terms of its time-dependent behaviour in response to a rapidly applied stretch. A purely elastic material, as represented by a spring, attains its new tension instantaneously and maintains its tension. A viscoelastic material, represented by a parallel arrangement of a spring and a dashpot, attains its tension slowly. A viscoelastic material, represented by a series arrangement of a spring and dashpot, attains the maximum tension instantaneously, "losing" tension thereafter. For more details, see text.

In reference to Figure 1.1, situation "a" is represented by a single spring; the tension changes instantaneously when the length is increased and remains constant during sustained strain. This is purely elastic behavior. The second situation "b" is modelled by a spring in parallel with a dashpot, often referred to as the Kelvin model (Viidek, 1973). At the moment of application of a load, the spring is stretched but the extension is limited by the dashpot. Gradually as the dashpot is extended loading the spring further, there is an increased tension on the spring, resulting in "creep". The third model, "c" is represented by a spring and a dashpot in series. It is referred to as the Maxwell model (Viidek, 1973). The applied load is primarily taken by the spring at the moment of its application; with time, the dashpot is extended, taking tension off the spring, resulting in "stress relaxation".

Simple materials consisting of a single constituent, are relatively easy to test because the stiffness of their single constituent is unique. Composite materials are more difficult to describe, because their multiple constituents may have different stiffnesses. The total stiffness of the composite depends on the relative concentration, orientation and stiffness of the constituent materials (Cox, 1978, Harkness, 1968, Lee and Boughner 1981). As a general rule, the greater the concentration of parallel elements of a given stiffness, the greater is the overall measured stiffness, ie. a load applied to a large number of "springs" within a unit cross section, is distributed among all these, resulting in a smaller extension of each. However if the "springs" are in series, the application of a given load will extend all elements, resulting in a decreased stiffness. When, in composite materials, the constituents are arranged in a random orientation or in a weave, the situation becomes more complex. In a weave arrangement there are varying orientation angles of the fibers. Two things therefore happen. First, at a given orientation, an individual fiber bears a fraction of the load, which is a cosine function of the orientation angle; fibers which are parallel to the line of force are loaded, fibers perpendicular to the force carry no

load, and those at varying angles between carrying a fraction of the load. Second, with progressively increasing loading the orientation changes, bringing more and more fibers into fuller alignment, and thereby each taking a greater percentage of the load. The load on an individual fiber is actually decreased (Cox, 1978, Daly, 1982). Effectively this results in more parallel components being loaded, increasing the effective or apparent stiffness.

The behavior of a material during both loading and unloading may be described. A material that is purely elastic will follow the same stress-strain pattern on both deformation and relaxation. Many materials, however take different routes. Hysteresis is the failure of the material to move along the identical stress-strain path with loading and unloading.

Many materials do not return to their original resting length, but rather assume a longer length than the original resting length. The process of straining the material thus results in a permanent deformation of the material, and sequential stress - strain curves are shifted to the right (Fung, 1981, Viidek, 1973). In this case the material is described as having a "plastic" component.

Materials are usually subjected to repeated cycles of strain application and removal. The first cycle is called the "conditioning loading" and may cause some permanent structural changes. The results of this conditioning cycle generally are not included in the data analysis (Fung, 1981, Lee and Boughner, 1981, 1985, Viidek, 1973). Subsequent cycles, called "preconditioned" are eventually identical and provide the data used to describe the behavior of the material (Fung 1981).

1.7.3 BIOLOGIC MATERIALS

Most biological materials exhibit viscoelastic behavior (Daly, 1982, Fung, 1981). They are generally composites in which the mechanical properties are due to more than one constituent of different inherent stiffness. The individual constituents are arranged in a complex pattern which is analogous to multiple springs and dashpots in a complex series and parallel disposition.

Most biological materials including muscle, become stiffer as they are progressively extended. Each additional length increment requires greater force. Thus, the relationship is not linear but tends to become exponential (Fung, 1967, 1981). The general pattern of the stress-strain relationship of biological materials can be separated into three phases. The first phase is exponential. This is followed by a nearly linear phase. The final phase is irregular, ending with failure or rupture of the material. Stiffness is generally determined as the slope of the stress-strain relation over its lower linear range.

Most biologic materials exhibit three properties: elasticity, viscosity, and plasticity (Viidik, 1973). The extent to which these properties are expressed depend upon the rate and the magnitude of the applied deformation, and of the history of the stress, ie. a single loading vs repeated loading. Therefore, when reporting the stiffness of a biologic material, the strain over which it is calculated must be expressed (Fung, 1967). The slope of the stiffness/strain curve is constant over the physiologic range for many biologic materials. The relationship between stiffness and stress is also used when a large strain range is examined.

1.7.3.1 MECHANICAL PROPERTIES OF THE MYOCARDIUM

The myocardium displays viscoelastic behavior (Caulfield and Borg, 1979, Fung, 1981, Gilbert and Glantz, 1989, Mirsky, 1976). The anatomical structures accounting for this behavior have not been

precisely defined (Williams *et al.*, 1982), however, the extracellular matrix is believed to be the major component (Borg *et al.*, 1981, Pryzlenek *et al.*, 1987). The notion that both the stiffness and viscoelastic properties of the ventricle could be due to the collagen arrangement was first proposed in the late 1930's by Nagel, Sichel and Bairati (Borg *et al.*, 1981). It is suggested that it is not the absolute concentration of collagen which is important, but rather its alignment and arrangement which largely determine stiffness (Carroll *et al.*, 1989). The collagen fiber itself is very stiff and can be extended only minimally. However, when the fibers are arranged in a weave, the weave behaves less stiffly and extension occurs by realignment of the constituents of the weave. In effect a greater number of fibers are brought into a series alignment. Therefore it is the presence of the collagen weave in the myocardium which may account for the viscous and elastic properties (Caulfield and Borg, 1979).

The nature of the extracellular collagen weave is believed to influence the stiffness of the ventricle. Borg *et al.* (1981), compared the collagen network of rats and hamsters, because previous investigators had shown that the ventricle of the rat was approximately twice as stiff as that of the hamster. They found the only difference in the collagen between these two species was in the nature of the weave surrounding groups of myocytes, the rats having a more extensive weave.

The experiments of O'Brien and Moore (1966) suggest further that the collagen matrix is largely responsible for the passive properties of the heart. In these experiments the pressure-volume relation of the excised heart was determined after treatment with trypsin, elastase, and collagenase. Only the last treatment resulted in a significant increase in compliance.

Both viscous and elastic components of stiffness are characteristic of materials. Although these properties have been investigated in the pericardium (Lee and Boughner 1981, 1985), they have not yet been

examined in normal or infarcted myocardium. Although the location and orientation of collagen in a healing myocardial infarct has not been described, it is known that collagen in the types of healing wounds examined initially is randomly oriented. It subsequently lines up, assuming a parallel arrangement. This implies that changes in the functional properties of a healing infarct should occur. These have not been investigated however, the changes in the histological appearance of collagen would suggest an increased elastic stiffness may occur.

1.7.4 MECHANICS AND HEALING

The majority of wound healing literature describes the healing of dermis and of tendons after experimental lesions. Tensile strength, defined as the tension per cross sectional area within a strip at rupture, is used as an endpoint of time course changes. In both of these tissues the tensile strength of the healing wound appears to have three phases. These mechanical phases parallel the histological phases of wound healing.

There is initially a period of decreased tensile strength, associated with edema and destruction of the components of the tissue. This is followed by gradually increasing strength of the wound which is associated with fibroplasia. The strength of the scar increases progressively and plateaus about sixteen days after wounding. The remodelling phase starts about three weeks after wounding. It is associated with the organization and maturation of collagen. Tensile strength continues to increase further (Anders-Carlstedt, 1987, Mustoe *et al.*, 1987). About four weeks after wounding the tensile strength of tendons is only about 15-20% normal; after twenty-six weeks strength is about 50% normal. All authors correlated the increase in tensile strength of the wound with the onset of collagen deposition (Anders-Carlstedt, 1987, Harkness, 1968, Mustoe *et al.*, 1987). The time course of the phases appears to be unique to individual tissues. Consequently one would expect to see this same pattern in the healing of the heart but not necessarily at the same rate (Duance and Bailey, 1981).

1.7.4.1 MECHANICS AND INFARCTION

The changes in active contractile activity of the ventricle immediately after coronary occlusion are extensively described both experimentally (Kittleson *et al.*, 1987, Sakai *et al.*, 1985, Tennant and Wiggers, 1935, Theroux *et al.*, 1977) and clinically (Bardet *et al.*, 1977, Chen *et al.*, 1986, Lamas and Pfeffer, 1986, Sabbah *et al.*, 1987).

Immediately after occlusion, contraction of the infarcted area ceases. This may be followed by systolic bulging of the ischemic area (Swan, 1979, Tennant and Wiggers, 1935, Theroux *et al.*, 1977, Tyberg *et al.*, 1974). The loss of contracting myocardium causes a decreased stroke volume and cardiac output and an increased left ventricular end-diastolic volume (Pantley *et al.*, 1984). The initial dyskinesia of the area, is followed a period later by akinesis (Theroux *et al.*, 1977). The decreased contractility of the infarcted region appears to be balanced by an increased contractility of the noninfarcted region, in an attempt to preserve global function (Swan, 1979). Hyperkinesis of the nonischemic region of the left ventricle distant from the ischemic zone has been demonstrated in experimental animals during acute ischemia (Goto *et al.*, 1988, Sakai *et al.*, 1985, Smalling, 1986, Yew, 1987).

The passive changes are less well characterized. Within seconds after coronary artery occlusion an increased global compliance has been measured in excised hearts (Forrester, 1972, Mirsky, 1976). This lasts for approximately 1 to 3 days after infarction. The compliance of the ventricle then gradually decreases, often becoming less than that in control hearts (Connelly *et al.*, 1985, Diamond *et al.*, 1972, Gaasch *et al.*, 1976, Hood *et al.*, 1970, Forrester *et al.*, 1972, Kumar *et al.*, 1980, Lerman *et al.*, 1983, Swan, 1972).

Besides compliance, the bursting pressure of the isolated ventricle has also be examined. This is determined by adding known volumes of fluid to the ventricle at a constant rate. The pressure within the

ventricle is monitored continuously until it ruptures. The pressure within the ventricle when it ruptures is defined as the bursting pressure, and is used to represent the absolute tensile strength of the weakest point of the entire ventricle. Lerman *et al.* (1983) measured this pressure in rabbits after coronary occlusion. They reported that the bursting pressure in control animals was much greater than pressures to which the ventricle would be exposed *in vivo*: 664 mm Hg. This value did not change in infarcted hearts during the initial eight days of healing. However the site of the rupture changed. 59% of early infarcts (1 to 4 days post-occlusion) ruptured through the infarct, whereas only 18% of those examined later (8 days post-occlusion) ruptured through the infarct. This implies that within days after sudden and permanent coronary artery occlusion the infarcted region is no longer the weakest section of the heart.

The question then arose as to whether the observed changes in wall motion and the altered global behavior were due to a changing of the inherent properties of the infarcted region. Many investigators have theorized that a change in the structural organization of the myocardium could be responsible for an altered mechanical behavior (Caulfield *et al.*, 1985, Pearlman *et al.*, 1982, Weisman and Healy, 1987).

1.7.4.2 REGIONAL PROPERTIES OF INFARCTED MYOCARDIUM

There is very little documentation of the passive elastic properties of the myocardium after coronary vessel occlusion. The two main measurements which define the regional properties are regional stiffness, defined as the relationship between stress and strain, and tensile strength, defined as the tension per cross sectional area within the strip at rupture.

The passive mechanical properties of infarcted myocardial strips were examined twenty-four hours after infarction in the rabbit (Laird and Vellekoop, 1977, Przyklenk *et al.*, 1987). Tensile strength, strain

at rupture, and stiffness were not significantly different between control strips and those from twenty-four hour infarcts. By eight to ten days after the occlusion the stiffness of the infarct zone was still not different from the control group (Laird and Vallekoop, 1977), however, the tensile strength was increased 2.4 fold over control (Lerman *et al.*, 1983).

1.7.5 MECHANICS AND COLLAGEN

After sudden coronary artery occlusion, an area of the myocardium becomes necrotic. The myocytes in that area are no longer capable of generating any tension. Over a period of days to weeks, these are removed and the area scars. The infarcted region thins, causing a change in the geometry of the ventricle. As the radius from the center to the infarct zone increases, wall stress increases, and wall thickness decreases. The external circumference of the myocardium may or may not be altered. The global compliance changes, but the bursting pressure is unaltered; the regional stiffness is unaltered but the absolute tensile strength increases.

An initial examination of the global and regional changes at first seems contradictory. However, it is possible that the presence of the same infarcted material can confer all of these changes. The early increased global compliance is most probably due to destruction of the collagen matrix. Without an intact matrix, compliance will increase. As well, the disruption would make that area weaker, and ventricular rupture would occur through the infarcted area. The subsequent decrease in global compliance is most probably due to the laying down of collagenous scar tissue in the infarcted region. The unchanged bursting pressure is most probably a reflection of the high tensile strength of the scar, equal to, or higher than, that of the surrounding myocardial tissue.

Stiffness and tensile strength are not necessarily analogous; one may change without necessarily changing the other. Stiffness is the degree of reproducible and reversible extension under loading. The stiffness of a biologic material (i.e. myocardium) is believed primarily to be due to the presence of collagen. Increasing stiffness is most probably due to a regional increase in the density of collagen fibers. Tensile strength is the ability to withstand loading without rupture. It is related to individual attachments, not to the inherent stiffness of a material. Rupture of a material represents the failure of such attachments. Therefore it is possible to have an unchanged stiffness and a decreased or increased tensile strength or, an altered stiffness with normal tensile strength.

Changes in the location, orientation and amount of collagen may explain the above mechanical changes. Lerman *et al.* (1983) reported that the decreased ventricular compliance observed eight days after infarction correlated with increasing hydroxyproline concentrations. Przylenk *et al.* (1987) reported a correlation between tensile strength and hydroxyproline content. More useful information would be obtained by correlating the collagen per unit cross sectional area, with the regional properties of stiffness and tensile strength (Harkness, 1968).

1.8 STATEMENT OF THE PROBLEM

The time course of the process of healing of infarcted myocardium is not well documented. No comprehensive study appears in the literature which describes the time course of the many changes occurring in different regions of the heart as it heals after an infarction. The available studies generally document only the initial period after an infarct, and describe only a single parameter.

It is important that the heart be studied as a whole. Infarcted and noninfarcted regions need to be examined in the same heart. This is important because both regions in the heart are affected by the infarction.

It seems desirable that the following general areas be described:

1. What is the time course of the regional blood flow distribution in the left ventricle after a permanent occlusion of the circumflex coronary artery at its origin?
2. Are there regional alterations in the protein profile of the ventricle, with specific reference to collagenous and contractile proteins, which occur during the healing process?
3. Are the regional passive mechanical properties of the ventricle altered in response to the presence of scar tissue formed after infarction?

The following specific questions were examined:

1. What is the time course of:
   a) blood flow changes to the infarct and noninfarct zones
   b) degree of change from initial mass (i.e. contraction of scar) determined by relative changes in microsphere concentration
   c) hydroxyproline content in the infarct and noninfarct zones
   d) 3-methylhistidine content in the infarct and noninfarct zones
   e) water content of infarct and noninfarct regions
   f) the histological appearance of collagen in the infarct zone
   g) mechanical properties

i) as a first approximation of stiffness, Young's modulus determined from stress-strain relation of longitudinal strips from infarct and nonfarct zones.

ii) viscoelastic behavior: the contribution of viscous and elastic components of stiffness

2. What correlations can be made between the time course of these variables.

To this end, we utilized a chronic porcine animal model. An acute coronary occlusion was performed surgically. The animals were allowed to recover and then were subsequently killed at either one, three, seven, fourteen, twenty-one or twenty-eight days after infarction. Measurements were made on the hearts recovered at the time of sacrifice.

2.0 METHODS

2.1 SURGICAL PROCEDURE

The aim of the project was to examine the time course of healing of infarcted myocardium. It was desireable to choose the most appropriate animal model that reflected the human response to a sudden coronary artery occlusion. It was also important to produce an infarct of similar size, location and one which was comparably damaging to all hearts. The infarct had to be sufficiently large to provide adequate material for subsequent analysis, however it should not be so large as to induce congestive heart failure, since survival of the animal to specified periods was necessary. In the following sections the procedures will be described whereby an acute coronary occlusion was caused in pigs, and a variety of measurements were made at specified intervals thereafter.

The blood supply to the heart is similar in the human and the pig; approximately 80% - 85% have a right dominant coronary circulation (Cohen, 1985, Weaver *et al.*, 1986). The left anterior descending coronary artery (LAD) supplies the free wall and is the major supplier of the septal wall (Weaver *et al.*, 1986). It was felt that to produce an infarct by LAD occlusion sufficiently large for the analytical requirements, the pumping ability of the heart would be compromised and the conducting system would be damaged. The circumflex artery supplies between 25 to 30% of the left ventricle (Weaver *et al.*, 1986). There are essentally no functional collaterals between the left anterior descending and circumflex arteries (Patterson and Kirk, 1983, Schaper *et al.*, 1967, White and Bloor, 1981). Therefore permanent ligation of the circumflex artery near its origin would produce a large transmural infarction of the left ventricular free wall and would present a reasonable experimental compromise.

2.1.1 ANIMAL MODEL

Landry - Yorkshire - Hampshire cross bred pigs, of both sexes and weighing 30 to 50 kg were obtained from Animal Diseases Research

Institute, Agriculture Canada. On arrival to the University of Ottawa animal facility all animals received a vitamin and mineral injection (MU-SE), (1 ml/200 lbs; 1 ml contained 10.95 mg sodium selenite, 50 mg Vit. E, 250 mg polysorbate 80). Prior to surgery they were housed individually for a minimum of seven days. They received free access to water and standard pig chow, (Growena).

2.1.2 ANESTHETIC PROCEDURE

Food, but not water was withheld from the animal twenty-four hours prior to surgery. The following anesthetic procedure was developed, to provide a surgical level of anesthesia, to prevent reflex sympathetic activation without excessive direct myocardial depression and to provide adequate muscle relaxation. This was best achieved using small doses of a combination of agents. Drug dosages were determined using the fasted weight. The animals were preanaesthetised with atropine sulfate, (0.05 mg/kg), injected intramuscularly (IM). Thirty minutes later, Ketamine hydrochloride, (ROGARSETIC, 10.0 mg/kg) and Diazepam, (VALIUM, 8.0 mg/kg) were injected intramuscularly.

Anesthesia was induced 15 minutes later with 3% Methoxyflurane gas applied by mask. Xylocaine aerosol spray was applied to the larynx, and an appropriately sized endotracheal tube was inserted. An ear vein was cannulated for the administration of fluids and drugs. The pig was then transferred to the operating room. The endotracheal tube was attached to a Boyle anesthetic machine (Model Mark III). The ventilator used was a Penlon Anesthesia Ventilator (Model 500). The pig was ventilated with a gas mixture of nitrous oxide and oxygen, 50 : 50%, ten to fifteen times per minute, with a tidal volume of 10 - 15 ml/kg. Maintenance levels of methoxyflurane gas, 0.1 to 0.5%, were delivered to the anesthetic circuit for the duration of the surgical procedure.

Fentanyl citrate, (SUBLIMAZE, 3 μg/kg), a narcotic analgesic, was administered intravenously (IV) prior to initiating surgery. Subsequent doses of 1 μg/kg were given, when required, as indicated by a rise in

arterial blood pressure. This was presumably indicative of catecholamine release due to the sensation of pain. Pancuronium Bromide (PAVULON, 0.1 mg/kg), a neuromuscular blocking agent, was administered intravenously prior to the initial skin incision over the chest. Additional doses of 0.025 mg/kg were administered as required, as assessed by muscle twitching. Sodium chloride solution (0.9% NaCl) was administered throughout the entire procedure at the rate of 12 ml/kg/hr. Arterial blood gases were measured regularly, using a pH/Blood Gas Analyser (Instrumentation Laboratory, Model 513). Sodium bicarbonate was administered to correct any developed base deficit estimated as (0.3 × body weight × (measured bicarbonate - 25)).

The administration of methoxyflurane was stopped before the end of the procedure; 10 minutes per hour of anesthesia time. At the completion of the surgery the animal was ventilated with nitrous oxide and oxygen until it was coughing and swallowing spontaneously. Before disconnecting the pig from the respirator, 100% oxygen was administered, for several minutes. The pig was then transferred to the recovery area.

Immediately post-operatively, animals received 1 ml of Derapen-C (PENICILLIN-G BENZATHINE, 150,000 I.U.). To manage post operative pain a 50μg Fentanyl bolus was administered intravenously. The endotracheal tube was removed when the pig would no longer tolerate it, approximately two hours later.

2.1.3 SURGICAL PROCEDURE TO PRODUCE TRANSMURAL MYOCARDIAL INFARCTION

The surgery was performed using aseptic technique. Prior to draping the animal, ice packs were placed near the abdomen, and a rectal thermometer was inserted. Electrodes were placed on the four limbs, to record the electrocardiogram. The electrodes were attached to a Grass EKG Pulse Preamplifier, (Model 7P6C), which was attached to a Grass D.C. Driver Amplifier, (Model 7DAG). The output was recorded by a 3 channel Grass pen recorder.

The incision sites were shaved, washed, and then cleaned with Povidine (10% IODINE). The animal was then draped, leaving only the surgical sites exposed (groin, left chest). The femoral artery was exposed in the groin and cannulated using 20 gauge Canus tubing, (inside/outside diameter, 0.03/0.06 mm). This was attached to a pressure transducer, (Gould and Statham Transducer, Model P23D6) and was used for the monitoring of arterial blood pressure, and provided access for arterial blood sampling.

The chest was opened on the left side, in the fourth intercostal space. The skin was incised, and the intercostal muscles were cut through. Bleeding was controlled by electrocautery (Bovie Electrosurgical Unit, Model CSV). One rib was removed. The pleura was opened. The lungs were then packed away from the operative field, using 4" X 4" gauze pads soaked in warm saline. The pericardium was opened.

A catheter having multiple side holes (#5 French Levine feeding tube) was inserted into the left atrial appendage. It was held in place with a #3-0 vascular purse string suture and a # 2-0 silk ligature. The line was attached to a Statham P23D pressure transducer, and was used to measure left atrial pressure, and to inject radioactive microspheres. By lifting the left atrial appendage, the bifurcation of the left main stem coronary artery was identified. The circumflex coronary artery was dissected free near its origin. A # 2-0 silk was passed under the artery.

After the vessel was isolated, there was a rest period of ten minutes. The initial radionuclide-labelled microsphere was injected into the left atrium. The circumflex coronary artery was then permanently ligated. Ten minutes later a second microsphere with a different label was injected into the left atrium.

Twenty minutes after the permanent ligation, the left atrial cannula was removed. The pericardium was left open and the chest was sutured closed in layers, using # 1 Prolene to approximate the ribs, # 0

Prolene to close the muscle and subcutaneous layers, and # 3-0 Prolene to close the skin. A chest tube was left in the pleural space. This remained in place for several hours after the procedure, and was used to evacuate the chest of any air or fluid. The arterial line was removed, and the groin incision was closed in layers. The animal was allowed to recover to consciousness.

2.1.4 ANTIARRHYTHMIC DRUGS DELIVERED DURING SURGERY

The procedure of coronary artery ligation is highly arrhythmogenic. with ventricular arrhythmias often developing (Cohen, 1985, Janse *et al.*, 1986, Klein *et al.*, 1984). The protocol therefore included the administration of antiarrhythmic drugs to protect the myocardium, during this period while it was electrically vulnerable. All drugs were administered intravenously.

Procainamide hydrochloride, (PRONESTYL), a class 1 antiarrhythmic, was administered, (10 mg/kg, 25 mg/min infusion rate). The administration was started as soon as the animal was brought into the operating room.

A second class 1 antiarrhythmic, Lidocaine, was administered in two bolus doses of 1.5 mg/kg each. The first bolus was given as the pleura was being opened, the second was given 20 minutes later. A Lidocaine drip, (3 to 4 mg/min) was started immediately after the first bolus. A Sotalol infusion was started as soon as the animal was brought into the operating room, (0.85 mg/kg, 1 mg/min). This drug has both class II and III properties (Lindsay, 1989).

Nifedipine, a calcium channel blocker, was given if it became apparent that the left coronary artery was developing spasm. This was decided if the left anterior descending artery territory suddenly became akinetic and dark purple or blanched. It was given in 0.1 mg amounts by slow IV infusion.

If ventricular arrhythmias developed after the permanent ligation of the circumflex artery defibrillation was attempted, (Stimulator Defibrillator Model 10334, Measurement Engineering Ltd, Ontario). If death occurred it was always within thirty minutes of permanent ligation. Using this protocol the survival rate from occlusion of the circumflex coronary artery at its origin, in the pig, was 80%.

2.1.5 PROCEDURE PRIOR TO SACRIFICE

After a specific period of time, one to twenty-eight days, the pig was again anesthetised, following the above described anesthetic protocol. The femoral artery and ear vein were cannulated. A catheter, connected to a pressure transducer, (Gould and Statham Transducer, Model P23DG) was inserted in the carotid artery and its tip advanced to the left ventricle. The third injection of radionuclide-labelled microspheres was made directly into the left ventricle.

The animal was injected with an overdose of sodium pentobarbital, (EUTHANYL, 110 mg/kg), administered directly into the left ventricle. The chest was opened and the heart was then removed.

2.1.6 SURGICAL PROCEDURE AND PROTOCOL FOR CONTROL GROUP

The design of the experiment involved infarcting the free wall of all animals. To reduce variability, it was felt advisable to use each animal as its own control. This could only be accomplished by using the noninfarcted septal wall for comparison against the infarct zone in the anterior free wall. Before doing this, it was necessary to demonstrate that there were no differences in the parameters measured between the anterior free wall and the interventricular septum in normal pigs.

To satisfy this concern, a group of animals was subjected to only a sham surgery, consisting of the complete surgical procedure with the exception of the permanent ligation of the circumflex artery. Three

different randomly chosen radionuclide-labelled microsphere injections into the left atrium were administered, ten minutes apart. The animal then received a lethal overdose of EUTHANYL, and the heart was removed.

2.2 EXPERIMENTAL DESIGN OVERVIEW

Following successful ligation of the proximal circumflex coronary artery, the animals were randomly assigned to one of six groups, the groups only being different in the time at which the animals were sacrificed. This occurred at one, three, seven, fourteen, twenty-one or twenty-eight days after occlusion. After receiving a lethal injection at the above times, the heart was removed. Four major measurements were carried out on both infarcted and noninfarcted myocardium.

1. The regional blood flow distribution to the myocardium was measured, by counting the radioactivity of microspheres injected at the time of initial surgery and sacrifice.

2. A biochemical analysis of infarcted and noninfarcted myocardium was undertaken.

3. A histological examination of the infarcted and noninfarcted myocardium was performed.

4. The mechanical stiffness of infarcted and noninfarcted regions of the heart was examined.

The procedures employed are described in the subsequent sections.

The analysis of the data included two types of comparisons:

a.) data obtained in each infarcted group sacrificed at specific periods after the occlusion were compared to the control group ("horizontal" comparison) and,

b.) in each group, data obtained from infarcted free wall tissue were compared to data obtained from noninfarcted septal wall tissue, ("within-group" comparison).

2.3 MEASUREMENT I: USE OF RADIONUCLIDE-LABELLED MICROSPHERES FOR REGIONAL BLOOD FLOW DETERMINATION

Microspheres are inert plastic spheres, containing a lattice structure composed of an ion exchange resin to which an unstable radionuclide is bound (Heymann *et al.*, 1977).

Regional myocardial blood flow was determined by injection of microspheres directly into the left side of the heart. The method initially described by Rudolph and Heymann (1967) is based on the principle that microspheres larger than the capillary diameter are trapped in the microcirculation in proportion to the blood flow, during their first circulation.

15μm Nen-Trac^{R} microspheres labelled with ^{57}Cobalt, ^{46}Scandium, ^{113}Tin, ^{95}Niobium, ^{153}Gadolinium, ^{85}Strontium, and ^{141}Cerium were purchased from New England Nuclear, Canada. They were obtained in quantities of 1 mCi, and were suspended in 20 ml of 10% Dextran, containing 0.01% Tween. They were then divided into 20 aliquots, each containing approximately 50 μCi. The volume was brought to 20 ml with 0.9% sterile saline, containing 0.01% Tween-80. The Tween-80 was added to minimize the aggregation of microspheres. Prior to use, random batches were tested to determine that there was no leaching of radioactivity from the microspheres into the supernatant.

Each animal received three injections of approximately two million microspheres, labelled with approximately 50 μCi of specific radioactivity. At each injection, the microspheres selected had been labelled with a different nuclide. Prior to injection the microsphere suspension was vortexed for 15 minutes, using a Sybron Thermolyse Maxi Mix (Model M16715). The microspheres were injected slowly and evenly, over 30 seconds. The injection line was then flushed with 5 ml of warm saline. With each injection a reference arterial blood sample was taken. The sample was collected over 2 minutes, starting 10 seconds prior to the microsphere injection, at a rate of 7.5 ml/minute, using a

Harvard Apparatus infusion/withdrawal pump. The blood was transferred to a polypropylene tube containing 5 ml anticoagulant, (containing 0.73 gm Citric Acid, 2.2 gm Sodium Citrate Dihydrate, 2.2 gm Dextrose, in 100 ml H_2O). It was stored at 4°C until its specific activity was determined.

2.3.1 PREPARATION OF TISSUE FOR DETERMINATION OF RADIOACTIVITY

After sacrificing the animals, full thickness sections of the heart from all regions were taken. Sections were divided into epicardial and endocardial regions, through the midwall. The pieces which averaged 1 gram each, were weighed wet. The tissue samples were air dried, taking three to four days. They were then reweighed. The water content of the section was calculated from the difference between the wet and dry weights.

2.3.2 COUNTING OF TISSUE SAMPLES

All tissue samples had three gamma emitting energies. Prior to determining the specific activity within each sample, a standard aliquot of each injected nuclide was scanned on the LKB - 1282 - Compugamma Universal Gamma Counter equipped with a 256 channel pulse height analyser. The most appropriate windows for counting were determined. The counter was programmed such that all nuclides were counted simultaneously, correcting for background and spillover. Tissue samples were counted twice for 5 minutes, and the counts per minute were averaged.

2.3.3 CALCULATION OF REGIONAL BLOOD FLOW

The radioactivity of the tissue was counted and a correction made to account for the decay of the isotope label. The radioactivity of the reference blood sample was determined and a correction made to account for both the decay of the isotope label, and the dilution of blood with anticoagulant. The calculation of tissue blood flow was based on the

principle that the blood flow to that tissue sample was proportional to its radioactivity. Absolute blood flows were expressed in milliliters of blood flow per gram weight of tissue per minute (Bartrum *et al.*, 1974). The equation used was:

$$\frac{\text{reference blood flow (ml/min)}}{\text{specific activity (cpm) of the blood}} = \frac{\text{tissue blood flow (ml/min)}}{\text{specific activity (cpm) of the tissue}}$$

There were several regional measurements of blood flow made in the infarct and noninfarct zone in each heart. The mean flow value for these regions was determined by averaging the flow to the appropriate tissue samples.

2.4 MEASUREMENT II: BIOCHEMICAL ANALYSIS OF INFARCTED MYOCARDIUM

The objectives of this section were to document the changing protein profile of the infarcted myocardium, with particular reference to the time course of the disappearance of actin and myosin and the appearance of collagen. These three proteins were specifically chosen. No attempt was made to determine if the proteins were functional.

Several biochemical techniques were employed in the determinations. Briefly the general methods employed were: polyacrylamide gel electrophoresis in SDS, a method which separates proteins on the basis of their apparent molecular weight; reverse phase high performance liquid chromatography, a method which separates amino acids on the basis of hydrophobic interactions with the bound phase; and biochemical assays, established methods which measure specific constituents of experimental samples.

The determination of actin and myosin was qualitative only, while that of collagen was quantitative. The specific procedures employed are outlined below.

2.4.1 PREPARATION OF ACETONE POWDER FROM PORCINE HEART

Immediately after harvesting, the myocardial tissue could not be completely analysed, therefore samples were prepared for long term storage. Full thickness samples of infarcted and noninfarcted left ventricle, weighing approximately 5 grams were made into acetone powders.

Tissue samples were individually homogenized for several seconds in distilled water (dH_2O), 10:1, (v/w) using a Polytron Homogenizer (Brinkmann Instruments). Suspensions were diluted with 10 volumes of cold acetone and centrifuged at 5000 rpm for 5 minutes in a Sorvall RC2B centrifuge. The supernatant was suctioned off and discarded. The pellet was twice again resuspended in acetone and recentrifuged. The final pellet was air dried on Whatman No. 3 filter paper, and stored in capped vials at -20°C. This procedure removed water, small molecules and nonpolar substances, leaving macromolecules, primarily proteins, in a dry, stable state.

Protein and amino acid analysis of these prepared acetone powders was subsequently undertaken.

2.4.2 MYOSIN EXTRACTION

In addition to acetone powders, a sample enriched in myosin was prepared by high salt extraction which solubilized myosin filaments in the presence of ATP.

This extraction was performed on ice. The myosin from a finely minced tissue sample was extracted using 3 volumes (v/w) of an extraction buffer containing 0.4 M KCl, 0.05 M imidazole, 0.4mM ATP, at pH 6.8. Extraction was continued for 15 minutes. The mince was then filtered through Miracloth, saving the liquid. Myosin was precipated from the supernatant by the addition of 9 volumes of cold dH_2O and

pelleted by centrifugation. This was then centrifuged at 15,000 rpm for 15 minutes in a Sorvall RC2B centrifuge.

The supernatant was discarded, then the pellet was dissolved in 0.6M KCl, containing 30% glycerol. As degradation of protein can occur by repeated thawing and refreezing of samples, (Kozlovskis *et al.*, 1984), the dissolved extract was aliqoted into Eppendorf tubes, each containing approximately 200 μg protein. The samples were stored at -70°C, until they were analysed for the presence of myosin,. This was done using gel electrophoresis and immunoblotting techniques.

2.4.3 GEL ELECTROPHORESIS TO SEPARATE PROTEINS

The presence of major tissue proteins was initially examined using sodium dodecyl sulfate - polyacrylamide slab gel electrophoresis, (SDS-PAGE) following the method of Laemmeli (1970).

A 14 cm 6% separating gel, (acrylamide/bisacrylamide 40:1 pH 8.8, containing 0.1% SDS) was prepared and poured between two cleaned glass plates. This was carefully overlaid with isobutanol. Once polymerization was complete, the overlay was removed and a 1 cm 4% stacking gel (pH 6.8) was poured on the separating gel. A multi well lane marker was inserted.

Acetone powders of infarcted and noninfarcted tissues, obtained at different stages of healing, were dissolved in sample buffer, containing 2-mercaptoethanol (1%), SDS (0.1%) and Bromophenol Blue (0.001%) to a concentration of 1 - 4 mg/ml, then denatured, by boiling. Samples were centrifuged for 2 minutes in an Eppendorf Centrifuge, (Model 5414). 10 - 20 μl of supernatant were loaded into individual wells. Appropriate standard proteins were loaded on all gels. Standards included Bio-Rad High and Low Molecular Weight Standard, pig heart actin, rabbit muscle myosin, and type I collagen, all which were obtained from Sigma Biochemicals (St. Louis, Mo.).

Electrophoresis was carried out at 100 volts, until the proteins were through the stacking gel, then increased to 200 volts until the Bromophenol Blue dye front had migrated to 1 centimeter (cm) from the bottom of the gel.

Protein bands were visualized, by staining with Coomassie Brilliant Blue, R-250, or silver stain. Gels were then dried down on a Savant Slab Gel Dryer (Model SGD-200), or packaged in air sealed bags for long term storage. Detailed solutions and methodology are described in appendix II.

2.4.4 PROTEIN IDENTIFICATION USING MONOCLONAL ANTIBODIES

In an SDS gel the presence of a protein band from a tissue sample which migrates to the same position as a protein standard does not positively identify that protein. Comigration and positive staining are highly suggestive of, but not definitive for, protein identification. To identify individual protein bands, the proteins were transferred onto nitrocellulose and reacted with specific monoclonal antibodies. This procedure was used for identifying what appeared to be actin and myosin on SDS-PAGE.

Protein transfer according to the technique of Towbin was used (1979). Proteins, initially separated by SDS-PAGE and contained within the acrylamide slab gel were transferred to Bio-Rad nitrocellulose membranes, (Transblot^{R} Transfer Medium, 0.45 μ pore size) in the cold, using a Bio-Rad Trans-Blot apparatus. To transfer the proteins to the nitrocellulose membrane, either 500 mA of current for 3 hours, or 70 mA of current overnight, was used. The electric current was applied in a direction perpendicular to the gel. Proteins within the gel migrated out, and were adsorbed onto the nitrocellulose.

Their presence on the membrane was confirmed by visualization with Ponceau Red stain. After the nitrocellulose was destained, the indirect method of protein identification with antibody was employed, as described by Bourne (1983).

The nitrocellulose was initially overlaid with 3% Bovine Serum Albumin in 0.5 M Tris, 0.85 M NaCl, 0.02 M $CaCl_2$, pH 8.0. The protein adsorbed to the unbound sites on the surface of the membrane to prevent background staining. After extensive washing of the nitrocellulose, the membrane was overlaid with primary antibody. All primary antibodies used were IgG.

HUC, an anti-striated actin antibody, and C4, an anti-generic actin antibody were obtained from Dr. M. McBurney, Department of Medicine, University of Ottawa. The monoclonal antibody BF-32 used to identify myosin heavy chains was obtained from Dr. D.J. Parry, Department of Physiology, University of Ottawa.

After washing again, the nitrocellulose membrane was treated with a second antibody, also an IgG, labelled with horse radish peroxidase that bound to the first antibody. After subsequent washing, the complex was detected using the peroxidase substrate diamino benzidine tetrahydrochloride, (DAB), which forms a coloured precipate in the presence of peroxidase. The details of the procedure are described in appendix II.

2.4.5 ACTIN DETERMINATION

SDS-PAGE (method described in section 2.4.3.) indicated that there was still a protein in twenty-eight day infarcted tissue, which migrated with the same mobility as actin. Experiments were done to: (a) identify whether this protein was actin and (b) to determine its origin knowing that actin is present in both striated muscle (Garrels and Gibson, 1976) and fibroblasts (Bray and Thomas, 1975). These involved reacting blotted proteins with the specific actin antibodies previously described.

2.4.6 TOTAL PROTEIN DETERMINATION

Total protein content was initially determined using Peterson's modified Lowry total protein assay (Peterson, 1983). The basis of the

assay is that the chromogen in Folin-Phenol will be reduced by tyrosine and tryptophane, and by the copper chelates of the peptide chain or polar side chains. The absorbance of the chromogen in solution was measured at 750 nm, with a Beckman DU-7 Spectrophotometer. Protein concentration was expressed as mg protein/mg acetone powder.

This method has serious limitations when used for the analysis of infarcted tissue samples, underestimating the true protein concentration for two reasons. First, this assay assumes that all proteins in the sample are soluble in the reagent. However as healing progresses, the infarcted tissue samples contain increasing amounts of insoluble collagen which will not be measured. Second, when the protein has a low tyrosine and tryptophane content, this assay underestimates the amount of protein in the sample. Since collagen is low in these amino acids, the assay underestimates the true amount of collagen which can be dissolved.

To ensure that all proteins in the sample were assayed, the amino acid content was measured using the o-phthalaldehyde (OPA) technique. This method described by Peterson (1983) was specifically chosen because it permits valid measurement of both insoluble and soluble proteins. The samples were initially hydrolyzed overnight at 106°C in 6N HCl. Total amino acid determinations were subsequently performed on hydrolysed acetone powders of all samples. An o-phthalaldehyde molecule was attached to the amino terminal of each individual amino acid, forming a fluorescent derivative. The fluorescence of the modified amino acids was determined by excitation at 340 nm and measuring the light emitted at 450 nm, using a Turner Spectrofluorometer, (Model 430). The exact details of the above assays are described in appendix II.

All protein and amino acid assays were performed in duplicate. If the variability between sample duplicates was greater than 5%, the sample was reassayed. Standard curves for the Peterson total protein assay were prepared using known increasing concentrations of bovine serum albumin (range 0 - 100 μg). A protein hydrolyzate standard

(Amino Acid Standard-H) obtained from Peirce was used for the preparation of the OPA standard curve, (range 0 - 34 nmoles). This standard contains an equimolar mixture (2.5 μl/ml) of the abundant amino acids found in proteins (composition in Appendix II).

This provided information on the quantity of amino acids in an individual sample. This was used with subsequent assays to express the concentrations of individual amino acids in the tissue samples.

2.4.7 AMINO ACIDS

The protein profile of the myocardium changes as it is transformed from healthy myocardium to scar tissue (Frederickson, 1978). A common method used for identification of a protein is to quantitate unique amino acids, specific for individual proteins. It was felt that if a ratio between two unique amino acids, one representative of scar tissue, and a second representative of the myofibrillar proteins was obtained, then a new and valid method of expressing the changing protein profile would be defined.

A general amino acid profile was performed on all the tissue samples after hydrolysis, using the HPLC amino acid column (method described in sections 2.4.7.2 and 2.4.7.3). This was done to observe major directional changes in amino acid concentrations indicative of protein pattern changes.

Three amino acids were specifically followed over the twenty-eight day healing period. These were hydroxyproline which is a collagen marker, (Berg, 1982), and 3-methylhistidine, which is found only in actin and myosin (Asatoor and Armstrong, 1967). As well, proline which constitutes about 10% of the collagen molecule and is also found in noncollagenous proteins was followed.

2.4.7.1 HYDROXYPROLINE ASSAY

The amount of hydroxyproline in the tissue samples was quantitated by two methods: first the Berg protocol (1982) and second high performance liquid chromatography (HPLC) were done.

The principle of the Berg procedure is that hydroxyproline, which is a water soluble amino acid, can be oxidized and converted to pyrrole, which is toluene soluble. The pyrrole can then be reacted with para-dimethylaminobenzaldehyde (PABA), forming a chromophore having an absorbance maximum at 560 nm and so becomes specific for hydroxyproline in the presence of other amino acids. The complete details of this procedure and the reagents used are described in appendix II.

Knowing the total amount of protein using the Lowry assay results, or amino acid using OPA assay results, in each sample the proportion of that which was hydroxyproline could be calculated. Depending on the type of collagen, the number of hydroxyproline amino acids varies between 91 and 125 residues per 1000 (Miller and Gay, 1982). Exact quantification of collagen is therefore not possible knowing only hydroxyproline concentration. However since collagen contributes the overwhelming majority of hydroxyproline, and its percentage in all collagen types is quite close, the method allows estimation of the relative proportion of protein that is collagen. The complete details of this procedure and the reagents used are described in appendix II.

2.4.7.2 QUANTITATION OF HYDROXYPROLINE AND 3-METHYLHISTIDINE USING REVERSE PHASE LIQUID CHROMATOGRAPHY

To quantitate the amount of individual amino acids in a mixture, precolumn derivitization with phenylisothiocyanate (PITC), a molecule which absorbs light at 260 nm, was carried out following the method of Bidlingmeyer *et al.* (1984), and the Waters Pico Tag™ Manual.

Known weights of acetone powders to which a known amount of norleucine had been added were hydrolyzed. Norleucine is not a naturally occurring amino acid, and could therefore be used as an internal standard (Lehninger, 1975). To remove the ammonia which was produced during hydrolysis, and to make the sample basic, the dried hydrolysate was dissolved in 25 μl of H_2O - Ethanol (EtOH) - triethylamine (TEA), (2:1:1, v/v/v), then dried in a Savant Speed Vac Concentrator. The sample was then derivatised with 20 μl of EtOH - H_2O - TEA - PITC, (7:1:1:1, v/v/v/v). After thirty minutes it was again dried down. The derivatised sample was stored at -70°C until analysed. Amino acid standards from Pierce containing a known amount of internal standard were simultaneously prepared.

The derivatized amino acids were separated using a Varian 5400 Liquid Chromatograph, and the Vista system data for aquisition and analysis. A Waters Pico Tag™, C-18 -reverse phase column (300 mm length, 3.9 mm internal diameter, 300 Angstrom pore size) was used to separate derivitized amino acids. Amino acids were resolved by gradient elution. Eluent "A" was 0.14 M Sodium Acetate, 0.05% Triethylamine, 6% acetonitrile in Milli-Q grade water, adjusted to pH 5.5 with glacial acetic acid, and Eluent "B" was 60% acetonitrile. The flow rate was 1 ml/minute. The column initially was equilibrated with Eluent "A". A gradient of the second eluent (0 - 100% in 80 minutes) was used to elute the amino acids from the column at room temperature. The column was then washed with Eluent "B" followed by Eluent "A", each for ten minutes, between runs.

The absorbance of the eluted, derivitized amino acids was measured at 260 nm with an Ultraviolet (UV) detector. The relative amounts of each were determined from the integrated areas under the peaks, compared to the areas determined when known amounts of derivitized amino acids were separated and detected.

2.4.7.3 IDENTIFICATION AND QUANTITATION OF AMINO ACIDS FROM CHROMATOGRAMS

Amino acids were identified from their elution times. Pierce amino acid standards were derivatised and eluted from the column several times to determine the exact order and timing of individual amino acid elution. Identification of the additional amino acids, norleucine, hydroxyproline, and 3-methylhistidine were made by spiking the amino acid standard prior to column separation.

Norleucine, the internal standard, was used to calculate recoveries. The amount of an individual amino acid in a sample was quantitated relative to norleucine. Concentrations were expressed as nmoles of amino acid per run, or per mg acetone powder, or as a percentage of the total amino acids.

2.5 MEASUREMENT III: A HISTOLOGICAL DESCRIPTION OF THE MYOCARDIUM

Histological sections were prepared to illustrate the results of the mechanical and biochemical measurements with a morphological description of the healing infarct. The primary aim of examining the tissue histologically was to visualize the location and orientation of the collagen fibers.

2.5.1 PREPARATION AND STAINING OF TISSUE

Full thickness sections of infarcted and noninfarcted areas of most hearts were taken. Each section was approximately 5 mm thick by 1 cm^2 in size. The tissue was fixed in 20 volumes (w/v), of 10% Neutral Buffered Formalin. After fixation the specimens were washed to remove all traces of formalin. Dehydration was achieved by processing tissue through ascending concentrations of alcohol (by percent; 70, 80, 90, 100, 100, 100, for two hours each). After clearing with three changes of toluene (two hours each), the tissue was infiltrated with paraffin (Paraplast^R Plus Tissue Embedding Medium) and embedded for sectioning at 5 microns. A Spencer 820 Rotary Microtome (American Optical) and AO blades were used for cutting the tissue sections. Prior to staining, the sections were deparaffinized with toluene and then brought to water through descending concentrations of Alcohol. The following four histological stains were prepared:

1. Hematoxylin and Eosin, a routine nuclear and cytoplasmic stain which demonstrates general cellular morphology;
2. Goldner's Trichrome, a stain for connective tissue;
3. Herovici's Polychrome, a stain which differentiates collagen of different ages;
4. Gomori's Modified Reticulum, a stain which demonstrates collagen and reticular fibers.

The procedures were unmodified and are detailed in appendix II. After staining, routine dehydration, clearing and coverslipping were performed. The sections were examined using a Zeiss Axioplan microscope. Photographs were taken using Kodak Gold film.

2.6 MEASUREMENT IV:
MECHANICAL DESCRIPTION OF HEALING INFARCTED MYOCARDIUM

The relationship between an applied strain and the resultant developed stress within a material can be used to describe the mechanical behavior of a material. Stiffness, the linear relationship between stress and strain, was determined from regional strips of infarcted and noninfarcted tissues. Information on the response of individual strips to both rapid and slow straining was obtained. The methods employed are described below.

2.6.1 DETERMINATION OF REGIONAL MYOCARDIAL STIFFNESS

Strips of infarcted and noninfarcted material were cut circumferentially from the core of the infarct and the septal wall respectively. Two infarct strips were taken, one of which was divided into two, through the midwall into epicardial and endocardial regions. One noninfarct strip was taken from the midwall. Sections were cut to roughly 2 cm in length, and 9 mm^2 cross-sectional area. The strip ends were placed into Tygon tubing and fixed with cyanoacrylate glue. A #1-0 silk ligature was passed through each end and knotted. Strips were placed in saline after preparation. They were tested in random order.

To test, the strip was mounted into the straining apparatus. This consisted of an adjustable muscle stand with a micrometer having a 20 mm travel (Mitutoyo) and an isometric force transducer (Grass Model FT03C) attached. As well there was a rack and pinion stand, a stand with attached adjustable "tongs", and a saline filled plexiglass cylinder with a wire hook through the bottom. To mount the strip, the cylinder was placed directly under the force transducer. The bottom hook of the cylinder wire was attached to the rack and pinion stand, thus rendering it fixed. The muscle strip was attached by the silk threads, between the force transducer and the cylinder wire.

Clockwise turning of the handle of the muscle stand resulted in an elevation of the muscle stand platform. One complete turn, 360°, resulted in a 5 mm change in absolute length as measured by the micrometer; causing a similar elongation of the strip. The tension in the strip caused by the elongation was measured by the force transducer. The signal was amplified by a preamplifer (Grass DC-Low Level Preamplifier Model 7P1), and then by a driver amplifier, (Grass Polygraph DC Driver Amplifier Model 7DAG). The output was recorded on a Grass 3 channel pen recorder, (Model 7H 2560). Prior to beginning each experiment the transducer-preamplifier system was calibrated at two levels by adjusting the sensitivity of the preamplifier such that full scale deflection of the pen recorder corresponded to an absolute load of 20 and 100 grams respectively, on the force transducer. Data were collected on Grass recording paper movirg at 25 mm per minute, the x axis being time, the y, tension, as measured by the force transducer.

The protocol used to stress the tissue was developed so that one test would yield information on both the rapid and slow responses to strain application. The strip was stretched rapidly in 1 mm length increments. This change of length was obtained in approximately 100 msec. The strip exhibited stress relaxation on loading and creep on unloading. After the initial change in length, additional length changes were not performed until the tension in the strip had plateaued (approximately two minutes). Stepwise increments in length were continued until either full scale deflection of the pen recorder was observed, or until the strip fractured. If full scale deflection was obtained the strip was then unloaded in 1 mm steps.

There were two ranges of strain application. The first, a low strain range, representing 0 to 20% strain, was representative of the strain the section would be subjected to *in vivo*, (Babu *et al.*, 1988). The second strain range corresponded to 20 to 60% elongation of the strip. Three loading and unloading cycles were performed at each strain range. The length change from the resting length of each series, was determined by the maximum elongation of the strip during the first

straining cycle. With subsequent cycles the strip was stretched to the same length, regardless of the tension within the strip at that length.

2.6.2 CALCULATION OF MECHANICAL PARAMETERS

The following are definitions of the measurements taken:
Resting length was defined as that length of material between the Tygon tubing sleeves, at which any extension would result in an increased tension. This was measured with a ruler along the long axis of the strip.

The cross sectional area of the strip was measured at this resting, nonstressed length, in the plane at right angles to the direction of the applied force. The width and thickness of the sample were measured.

The elongation, or strain of the material strip was defined as the percentage length change from the original nonstressed resting length:

Strain = ((100 X new length) / original length) - 100

The stress within the material was defined as the load on the strip per unit cross sectional area. It was calculated at specific strains and expressed in gm/cm^2:

$$\text{stress} = \frac{\text{load}}{\text{cross sectional area}}$$

Two values of stress, an "*initial*" and "*final*", were obtained at each strain application. The "*initial*" stress was defined as the immediate tension within the strip at each new length change. The "*final*" stress was defined as that final tension within the strip, after stress-relaxation or creep had occurred.

The major assumptions of the straining procedure employed were:

1. The stepwise stress test could be used to describe the response of the material to a given absolute strain which was applied both quickly and slowly.
2. Each step wise change in length could be considered an individual mini test.

3. The stress-relaxation of one mini test would not influence the subsequent response to strain application.
4. The results of all mini tests were additive.

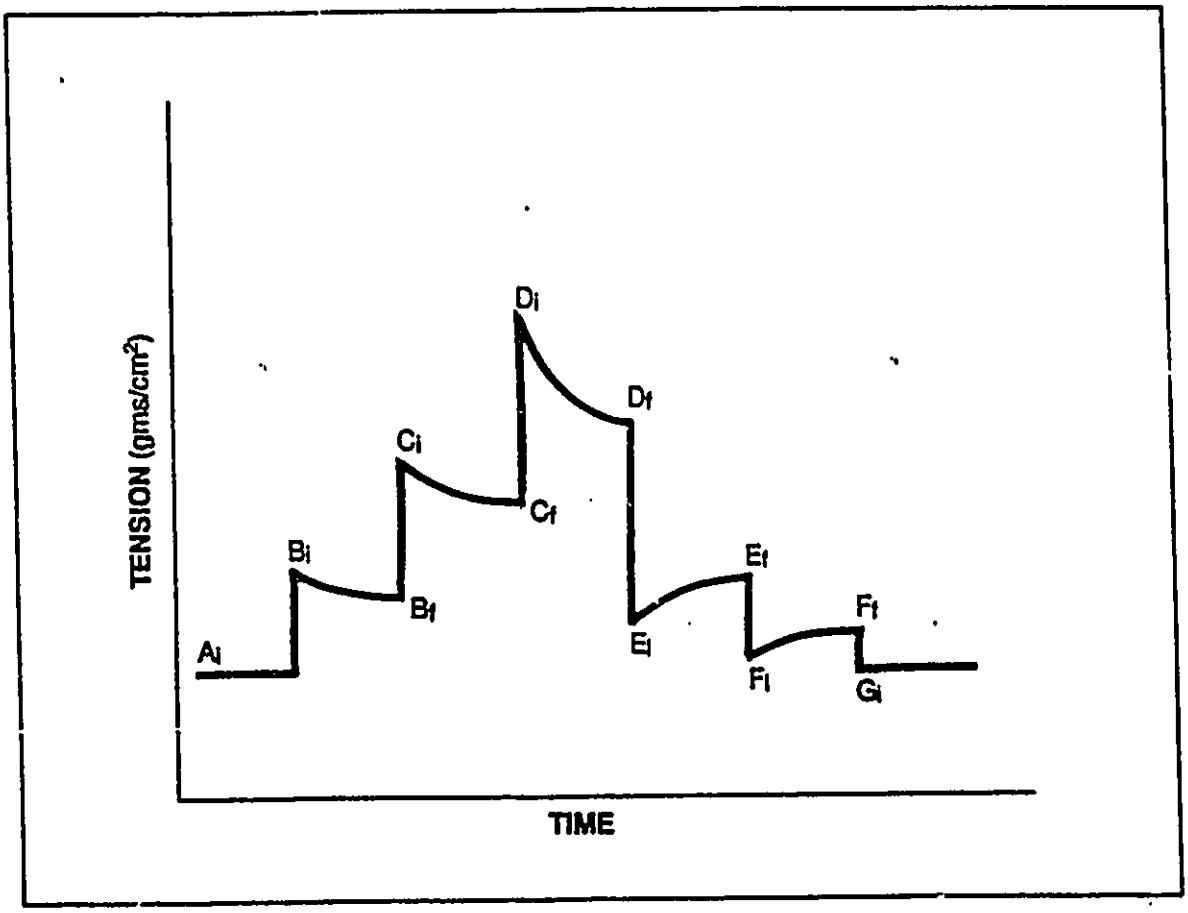


Figure 2.1

Diagrammatic representation of the data obtained from the examination of the mechanical properties of regional strips of myocardial tissue. The time of each length change is indicated on the "x" axis. Tension in the material was allowed to stabilize before the length was again changed. For details please refer to Section 2.6.2 in the text.

The calculation of initial tension on loading was determined from the strip chart recording (refer to Figure 2.1). The initial corrected tension was equal to the previous corrected initial tension, plus the difference between the present initial tension and the previous final tension. In reference to Figure 2.1:

$A_i = A_i$
$B_i = B_i$
$C_i = B_i + (C_i - B_f)$
$D_i = C_i + (D_i - C_f)$

The initial unloading corrected tension was equal to the previous corrected unloading tension minus the difference between the previous final tension and the present initial tension.

D_i = recalculated loading value
$E_i = E_i$
$F_i = E_i - (E_f - F_i)$

These obtained corrected initial tension values were then divided by the cross-sectional area of the strip to determine the initial stress.

$$\frac{\text{Initial Stress}}{\text{cm}^2} = \frac{\text{Initial Tension}}{\text{cross-sectional area}}$$

The final stress obtained after relaxation on loading, or creep on unloading, was calculated directly.

$$\frac{\text{Final Stress}}{\text{cm}^2} = \frac{\text{Final Tension}}{\text{cross-sectional area}}$$

Stress was plotted as a function of strain. The stiffness of the specimen was determined over the linear region of the relationship, as the slope. The initial cycle was the preconditioning cycle. Stiffness was determined from the loading of subsequent cycles. Only strips which were successfully loaded three times were included in the group means. Results were compared to the control group of animals, and to areas within the same heart. The initial stiffness reflected all components of stiffness, viscous and elastic, the final stiffness reflected only the elastic component of stiffness (see Figure 2.2).

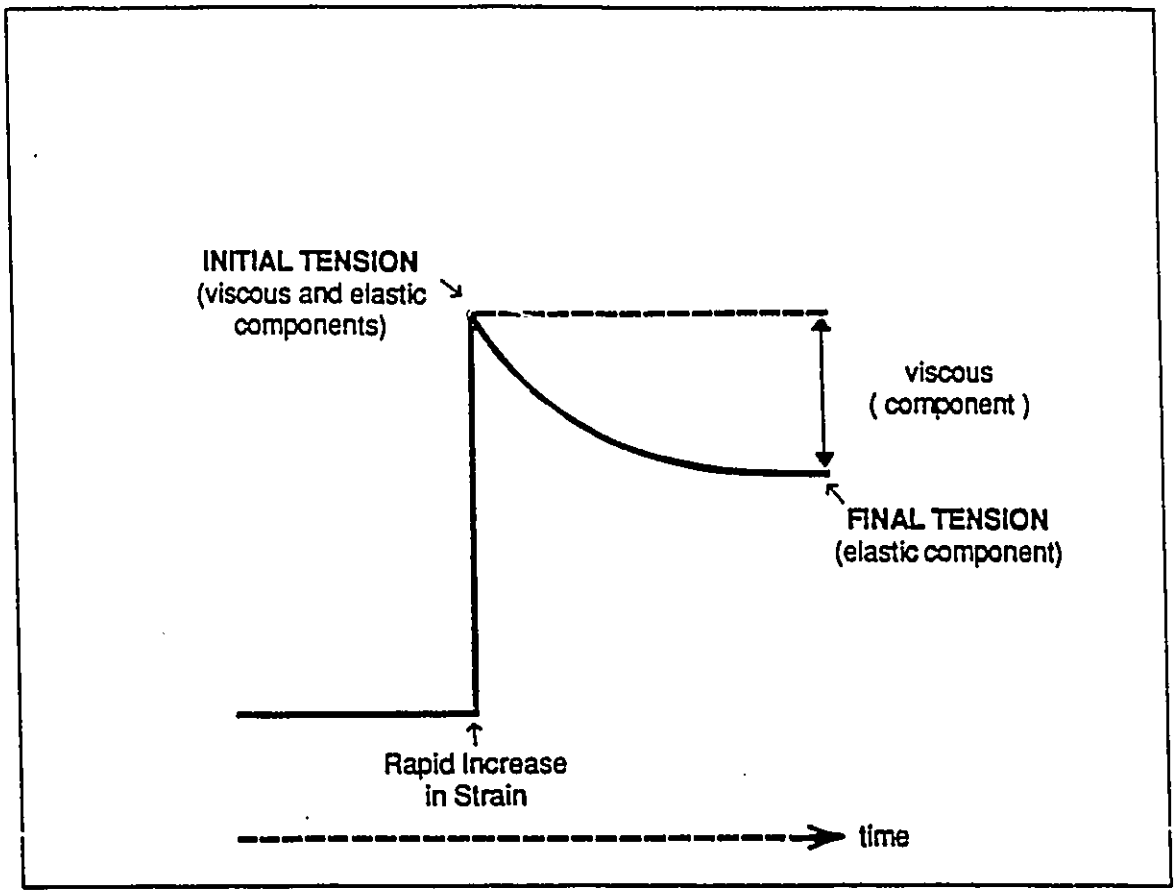


Figure 2.2

Diagrammatic representation of stress relaxation. Two values of tension were obtained for each change in length; an initial tension obtained immediately after rapid elongation, and a final tension after stress-relaxation had occurred. The viscous component is the difference between the initial and final tensions.

Control experiments were initially performed on four hearts to confirm the reproducibility of the technique. Two strips were cut from the free wall and each placed in saline. The first strip was repetitively cycled six times over a set strain range. There was no delay between cycles. The second strip was also cycled six times over the same range, at half hour intervals. The calculated stiffnesses, initial and final, were reproducible in both situations. This indicated that: first, the calculated stiffness of a regional myocardial strip is reproducible over a set strain range, with repetitive cycling; second, that there was no apparent time effect between the harvesting of the strip and mechanical testing of its stiffness. Both of these conclusions are in agreement with other reports of experiments on biological materials (Fung, 1981, Laird and Vallekamp, 1977, Viidek, 1968).

2.7 STATISTICAL ANALYSIS

The group means for each paramater were initially examined. If the variance of a given mean was greater than five times the variance of the control, the experimental group was considered to represent a sample which was different from the control population (personal communication by Dr. R. Nair).

Blood flow measurements were determined from several samples in the myocardium. Mean values were determined for infarcted and noninfarcted regions. A one way analysis of variance (ANOVA) was done on the mean blood flow of each region, by group to determine if flow was altered from the control group. In this, and in all comparisons a "p" value less than 0.05 was considered to be significant.

The results of the dry weight of tissue samples was expressed as a percentage of wet weights. This was determined at multiple sites from the same heart. Two mean values per heart were determined, one for infarcted and one for noninfarcted tissue. A one way ANOVA was performed on the means of each group to determine if the percentage wet weight changed during the experiment.

Sections for biochemical analysis were taken from the infarcted and noninfarcted walls. The results from amino acid analysis were analysed by one way analysis of variance to determine if differences existed between the experimental and control groups.

In the description of the mechanical properties, tissue sections from control anirals were compared from the free and septal wall. A one way ANOVA was done to compare the stiffness of the regions. Four different stiffness slopes were calculated for each strip. A one way ANOVA was performed on the means of the initial stiffness measured over the low and high strain ranges, and the final stiffness, measured after stress-relaxation over both strain ranges.

The final stiffness as a percentage of the total was determined for each strip, at each strain range. A one way ANOVA was performed on the means to assess whether this parameter remained constant in the different regions, over the experimental period. Differences between locations in the same heart were assessed by paired T-Test.

For the above parameters, the ratio between infarct and noninfarct zone tissue was determined for each heart. A one way ANOVA was performed on the means of each group to determine if this ratio was altered during the time course of the experiment.

With all analyses, if differences were present by ANOVA, the location of these was determined using the Scheffe post-hoc test.

3.0 RESULTS

Two regions of the heart were examined; infarcted and noninfarcted regions. Seven experimental groups were studied: control animals, and animals sacrificed at either one, three, seven, fourteen, twenty-one, and twenty-eight days post-occlusion. Each group had approximately six animals. Experimental measurements of blood flow distribution, amino acid concentrations and regional stiffness were made.

In the following discription, and in all tables and figures, results are expressed as mean plus or minus standard error of the mean, unless otherwise stated. All ANOVA tables are reported in Appendix I. The following short forms are used when describing the results:

GROUP 1 • control, noninfarcted animals
2 • animals sacrificed 1 day after infarction
3 • animals sacrificed 3 days after infarction
4 • animals sacrificed 7 days after infarction
5 • animals sacrificed 14 days after infarction
6 • animals sacrificed 21 days after infarction
7 • animals sacrificed 28 days after infarction

OHP • hydroxyproline
3-Mehis • 3-Methylhistidine
Pro • Proline
OPA • o-phthaladehyde amino acid assay
gm • gram
nmole • nano mole
HPLC • high performance liquid chromatography

H_o • null hypothesis
var • variance
S.D. • standard deviation
S.E. • standard error
ANOVA • analysis of variance
df • degrees of freedom

3.1 BLOOD FLOW

3.1.1 CONTROL EXPERIMENTS

Group 1, the control group, received three microsphere injections ten minutes apart, without any other intervention. Blood flow was determined at multiple sites in each heart. The mean flow to each heart was determined by averaging the flow to all samples. This group was used to establish the different sources of variability in blood flow estimation using microspheres. The results are summarized in Table 3.1, Figure 3.1. From these results, the coefficient of variation (standard deviation divided by the mean), and its distribution, can be partitioned. The within-animal temporal variability of serial flow determinations ranged from 4 to 38%; the mean coefficient of variation was 20.3 ±0.1%. Within the same animal, multiple determinations of flow allow estimation of the spatial heterogeneity, yielding a mean coefficient of variation, of 15.6 ±0.02%. Lastly, analysis of the mean flows determined for each animal indicate the magnitude of inter-animal variability. The range of resting flow rates was 0.8 to 1.8 ml/gm/minute with an average of 1.3 ±0.03 ml/gm/minute, yielding a coefficient of variation of 22%. Thus, the inter-animal variability and the within-animal variability of duplicate determinations was roughly comparable at 20 to 22%. Spatial heterogeneity of measurements made at numerous sites in the same heart, at the same time, was somewhat less, at about 15%.

From these measurements, it would appear that the normal range (mean ±2 S.D.) of myocardial blood flow for a group of pigs is 0.7 to 1.9 ml/gm/minute, and flows measured outside this range should be considered aberrant.

3.1.2 FLOW TO THE INFARCT ZONE

Microspheres may become concentrated over several days if tissue shrinks (Murdock and Cobb, 1980); therefore in a longitudinal comparison it is valid to compare: a) the final blood flow determinations (BF3) between groups, b) the second blood flow

determinations as a percentage of the first, ((BF2/BF1) X 100), as these two microspheres would be subject to the same degree of concentration.

Ten minutes after occlusion, blood flow to the infarct zone decreased in all groups to an average of 0.13 ±0.02 ml/min/gm wet weight, (see Table 3.2.1 and Figure 3.2). This represents 6.5% of the mean control group blood flow and 7.3 ±1.6% of the respective preocclusion flows measured in the different groups, in the same zone. While all were significantly lower than the control group flow (see ANOVA Table 6.1.1), no differences could be detected between Groups 2 to 7. This is also evident when infarct zone flow, at ten minutes post-occlusion, is expressed as the percentage of noninfarct zone flow in the same heart (Table 3.4); the infarct zone flows range from 6.7 ±1.1, to 17.8 ±4.5%. These extreme values of 6.7 ±1.1 (Group 2) and 16.7 ±2.1% (Group 4) are the only significant differences detected by Anova (see ANOVA Table 6.1.3). This would indicate that in all experimental groups, regardless of the time of subsequent sacrifice, the infarct zone was greatly deprived of blood flow ten minutes after the occlusion.

At the time of sacrifice (1 to 28 days after occlusion), blood flow to the infarct zone ranged from 0.17 to 0.68 ml/min/gm wet weight; the highest values being observed in the groups sacrificed at one and three days and the lower values seen subsequently (see Figure 3.3). Animals sacrificed at one and three days after occlusion had significantly decreased flows, and those sacrificed at or after seven days exhibited flows significantly lower than both the control group, and groups 2 and 3 (one and three days post - occlusion) (see ANOVA Table 6.1.1). In order to reduce inter - animal variability, the infarct zone flow was expressed as the percentage of non - infarct zone flow, in the same heart (Table 3.4.); these range from 14.3 ±4.2 to 33.6 ±4.7% and ANOVA suggests that Groups 5, 6, and 7 (i.e. those sacrificed at or after 14 days) exhibit flows significantly lower than those sacrificed earlier (see ANOVA Table 6.1.3.). Thus, it would appear that the relatively

higher blood flow to the infarct zone seen during the first week of healing appears to decline somewhat subsequently.

Comparison of the infarct zone flows at the time of sacrifice, shows that all measured flows were significantly lower than control, but on days one and three were significantly higher than subsequently.

The transmural distribution of the blood flow (endocardial - epicardial flow ratio, Table 3.3) in the infarct zone showed a substantial shift away from the endocardium, (ratio ≤ 0.9), both at ten minutes post-occlusion and at the time of sacrifice in all experimental groups. Since the division into endocardial and epicardial halves was don by a section at the midwall, there is no assurance that all microspheres found in these samples had originally lodged in the epicardial or endocardial layers. Differential shrinkage of areas of the wall could introduce an error.

3.1.3 BLOOD FLOW TO THE NONINFARCTED MYOCARDIUM

At ten minutes post-occlusion, blood flow to the normal myocardium ("noninfarct zone") appears to have the same pattern of flows as that measured prior to occlusion. Animals sacrificed between one and fourteen days post-occlusion, (Groups 2, 3, 4 and 5), received flows significantly greater than control (see Table 3.2.2, and Anova Table 6.1.2). The animals sacrificed at twenty-one and twenty-eight days, (Groups 6 and 7), appeared to have flows which were again similar to the control group. The transmural distribution of blood flow (Table 3.4) remained unaltered at all times. Therefore, flow to the noninfarcted myocardium initially increases after infarction, then subsequently normalizes.

3.1.4 PREOCCLUSION BLOOD FLOWS

There appears to be substantial variation in the control blood flow (BF1) measured prior to occlusion in the different groups in the "infarct zone" (Table 3.2.1). In particular, groups 2 and 5 exhibit

flows in excess of what appears to be the normal flow range established in Group 1. This may be the result of microsphere concentration because of tissue shrinkage, or variability due to unknown reasons. Expressing these flows as the ratio of infarct-to-noninfarct zone flows would eliminate some random variability, but not that due to tissue shrinkage. In Groups 2 and 3, these ratios are 0.96 and 0.83 respectively. ANOVA suggests statistically significant differences exist between the groups sacrificed at three days when compared to those sacrificed after fourteen days (see ANOVA Table 6.1.3). In Group 4, the ratio is 1.27 and in Groups 5, 6, and 7 it increases to 1.85, 1.87, and 1.79 respectively. This would suggest that no significant microsphere concentration occurs at one and three days, but significant concentration, probably by virtue of tissue shrinkage, occurs after seven days.

In the noninfarct zone, BF1/BF2 ratios remain constant, but the absolute flows are decreasing with time to sacrifice. In particular, groups 6 and 7 exhibit apparent control flows significantly lower than the others, suggesting that the original microsphere content of these tissues became diluted possibly because of enlargement of the original tissue mass (see Figure 3.6).

TABLE 3.1
SUMMARY OF BLOOD FLOW TO THE HEART IN THE CONTROL GROUP

ANIMAL	VARIABLE	N	MEAN	S.E
1	BF1	18	1.45	0.06
	BF2	18	1.39	0.05
	BF3			
2	BF1	24	1.12	0.03
	BF2	24	1.22	0.03
	BF3	24	1.21	0.04
3	BF1	22	0.76	0.02
	BF2	22	0.95	0.03
	BF3	22	1.05	0.03
4	BF1	20	1.23	0.04
	BF2	20	1.31	0.04
	BF3	20	1.01	0.03
5	BF1	20	1.80	0.05
	BF2	20	1.93	0.05
	BF3	20	1.61	0.04
6	BF1	27	1.28	0.04
	BF2	27	1.22	0.04
	BF3	27	1.48	0.06

† units are ml/min/gm wet weight

Blood flow was determined in multiple tissue samples from heart. Values expressed are the mean blood flows, per heart, at each injection. The variables BF1, BF2 and BF3 are serial blood flow measurements taken at 10 minute intervals.

TABLE 3.2: SUMMARY OF TEMPORAL BLOOD FLOW DATA

TABLE 3.2.1: INFARCT ZONE

GROUP	VARIABLE	N	MEAN	S.E.
2	BF1	32	2.92	0.10
	BF2	32	0.14	0.03
	BF3	32	0.67	0.20
3	BF1	43	1.59	0.11
	BF2	33	0.19	0.04
	BF3	31	0.68	0.08
4	BF1	26	1.81	0.19
	BF2	26	0.17	0.03
	BF3	26	0.31	0.03
5	BF1	32	2.79	0.35
	BF2	24	0.06	0.02
	BF3	32	0.38	0.13
6	BF1	33	1.78	0.24
	BF2	33	0.12	0.02
	BF3	33	0.17	0.02
7	BF1	44	1.22	0.09
	BF2	44	0.11	0.01
	BF3	32	0.30	0.04

† units are ml/min/gm wet weight tissue

TABLE 3.2.2: NONINFARCT ZONE

GROUP	VARIABLE	N	MEAN	S.E.
1	BF1	131	1.25	0.03
	BF2	131	1.32	0.03
	BF3	131	1.28	0.03
2	BF1	68	3.04	0.07
	BF2	68	2.67	0.13
	BF3	68	1.93	0.13
3	BF1	55	1.91	0.10
	BF2	41	1.60	0.19
	BF3	43	2.16	0.18
4	BF1	79	1.42	0.07
	BF2	79	1.43	0.06
	BF3	79	1.69	0.12
5	BF1	103	1.51	0.10
	BF2	95	1.28	0.09
	BF3	87	1.89	0.13
6	BF1	110	0.95	0.05
	BF2	110	0.96	0.03
	BF3	110	1.17	0.04
7	BF1	168	0.68	0.02
	BF2	168	0.78	0.02
	BF3	127	1.51	0.10

Blood flows were determined in many regions of an individual heart. Blood flow measurements presented are the mean flows of each group at each injection time. BF1 equals preocclusion flow, BF2 equals flow 10 minutes post occlusion, and BF3 flow prior to sacrifice.

TABLE 3.3:
ENDOCARDIAL-TO-EPICARDIAL BLOOD FLOW DISTRIBUTION PATTERN

VARIABLE	GROUP						
	1	2	3	4	5	6	7
non-epi BF1	1.15	2.83	1.70	1.19	1.29	0.79	0.58
non-endo BF1	1.38	1.15	2.18	1.58	1.67	1.04	0.79
endo:epi	1.2	1.15	1.28	1.32	1.29	1.31	1.36
non-epi BF2	1.22	2.57	1.41	1.19	1.12	0.82	0.69
non-endo BF2	1.42	2.71	1.75	1.57	1.28	1.05	0.86
endo:epi	1.16	1.05	1.24	1.31	1.14	1.28	1.24
non-epi BF3	1.19	1.57	1.86	1.54	1.69	1.09	1.48
non-endo BF3	1.38	2.03	2.36	1.79	1.99	1.24	1.71
endo:epi	1.16	1.29	1.27	1.16	1.17	1.13	1.15
in-epi BF1	-	2.96	1.33	1.52	2.35	1.54	0.90
in-endo BF1	-	3.03	1.87	2.17	3.24	1.94	1.41
endo:epi	-	1.02	1.40	1.42	1.37	1.25	1.56
in-epi BF2	-	0.18	0.19	0.23	0.09	0.17	0.12
in-endo BF2	-	0.04	0.16	0.11	0.05	0.07	0.11
endo:epi	-	0.22	0.84	0.47	0.55	0.41	0.91
in-epi BF3	-	0.69	0.83	0.42	0.48	0.23	0.34
in-endo BF3	-	0.48	0.54	0.18	0.32	0.12	0.29
endo:epi	-	0.69	0.65	0.42	0.66	0.52	0.85

† units are ml/min/gm wet weight

Multiple blood flow measurements were made in each heart. Total flow was separated into that going to the epicardial and endocardial regions of infarcted and noninfarcted tissue. Values expressed above are the mean regional blood flows per group. The variables used are: "non-epi" equals noninfarcted epicardial tissue, "non-endo" equals noninfarcted endocardial tissue, "in-epi" equals infarcted epicardial tissue, "in-endo" equals infarcted endocardial tissue, BF1 equals preocclusion blood flow, BF2 equals 10 minute post occlusion flow, BF3 equals flow prior to sacrifice and "endo:epi" equals the endocardial-to-epicardial blood flow ratio.

TABLE 3.4:
REGIONAL HETEROGENEITY OF BLOOD FLOW: FLOW TO THE INFARCT ZONE EXPRESSED AS A PERCENTAGE OF FLOW TO THE NONINFARCT ZONE

GROUP	N	MEAN	S.E.	VAR.
PREOCCLUSION FLOW (BF1)				
1	-	-	-	-
2	68	93.02	3.00	615.98
3	51	81.79	4.78	1261.70
4	79	107.74	4.87	1881.01
5	87	166.83	6.87	4114.80
6	110	207.42	6.87	6493.28
7	168	244.68	13.36	30004.74
10 MINUTE POST OCCLUSION FLOW (BF2)				
1	-	-	-	-
2	68	6.73	1.06	77.61
3	41	17.84	4.50	832.65
4	79	16.73	2.09	345.12
5	79	11.66	1.54	187.97
6	110	15.13	1.95	184.07
7	168	12.61	0.98	164.22
FLOW PRIOR TO SACRIFICE (BF3)				
1	-	-	-	-
2	68	33.55	4.67	2281.92
3	43	14.28	4.21	764.67
4	79	32.02	2.49	492.51
5	87	20.79	3.13	854.96
6	110	15.55	1.29	30004.71
7	127	15.46	1.07	147.24

The amount of blood flow to the infarcted tissues was expressed as a percentage of that to noninfarcted tissue in each animal. Values reported are the mean flow values of each group.

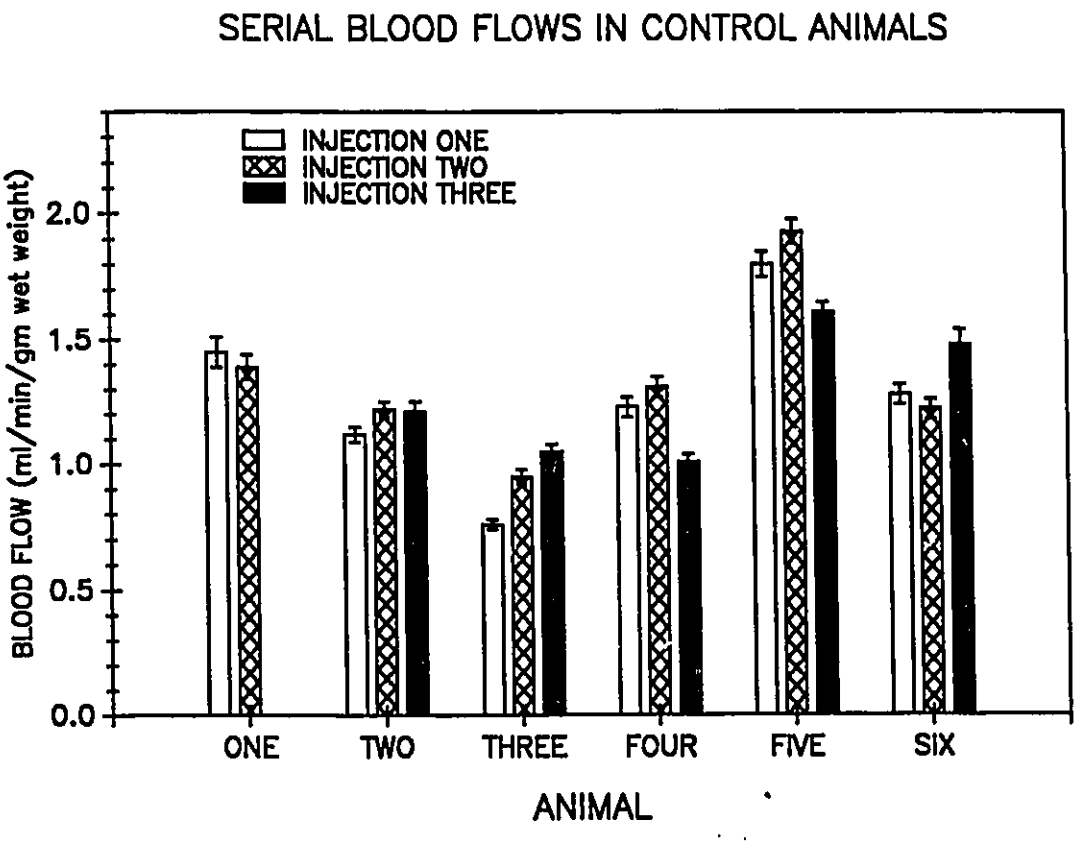


Figure 3.1

Summary of temporal and spatial heterogeneity of blood flow determined in individual pigs comprising the control group by serial injections of radioneuclide-labelled microspheres.

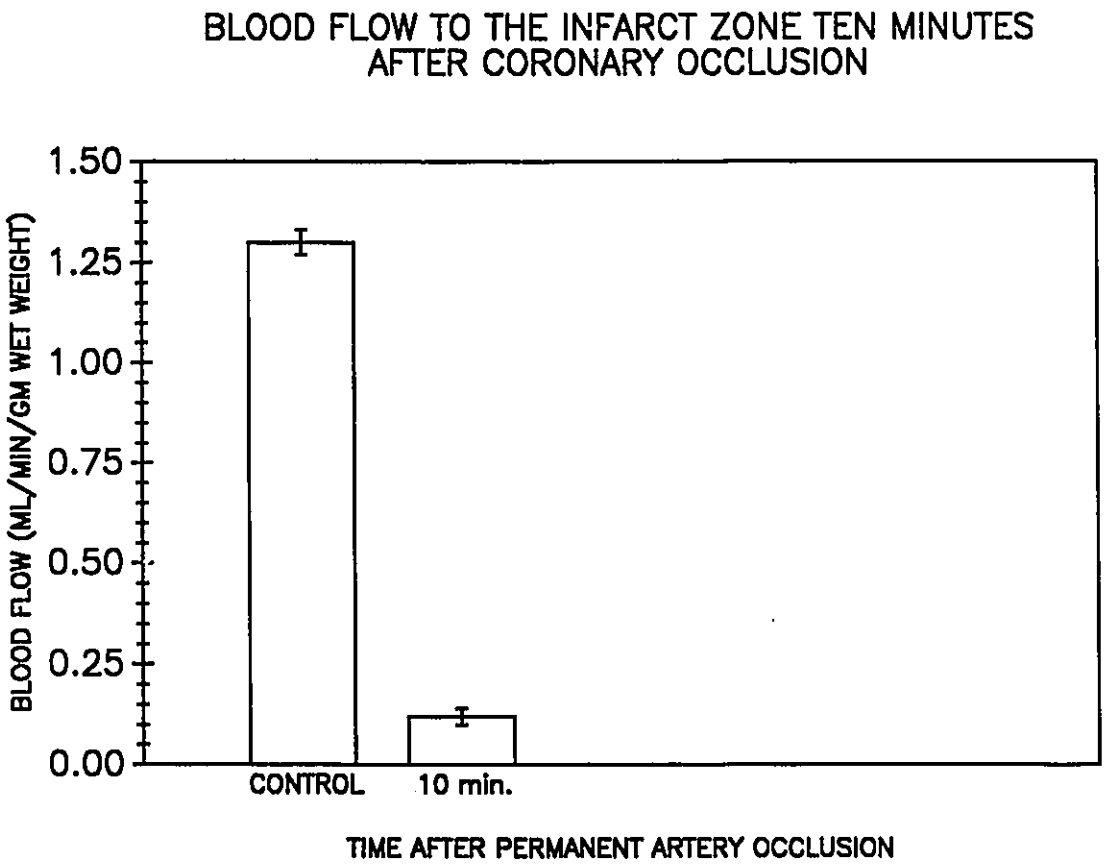


Figure 3.2

Summary of blood flow to the infarct zone ten minutes after permanent occlusion, determined by radionuclide-labelled microsphere injections. All blood flows were pooled for the control and ten minute values; therefore this may overestimate actual post-occlusion values.

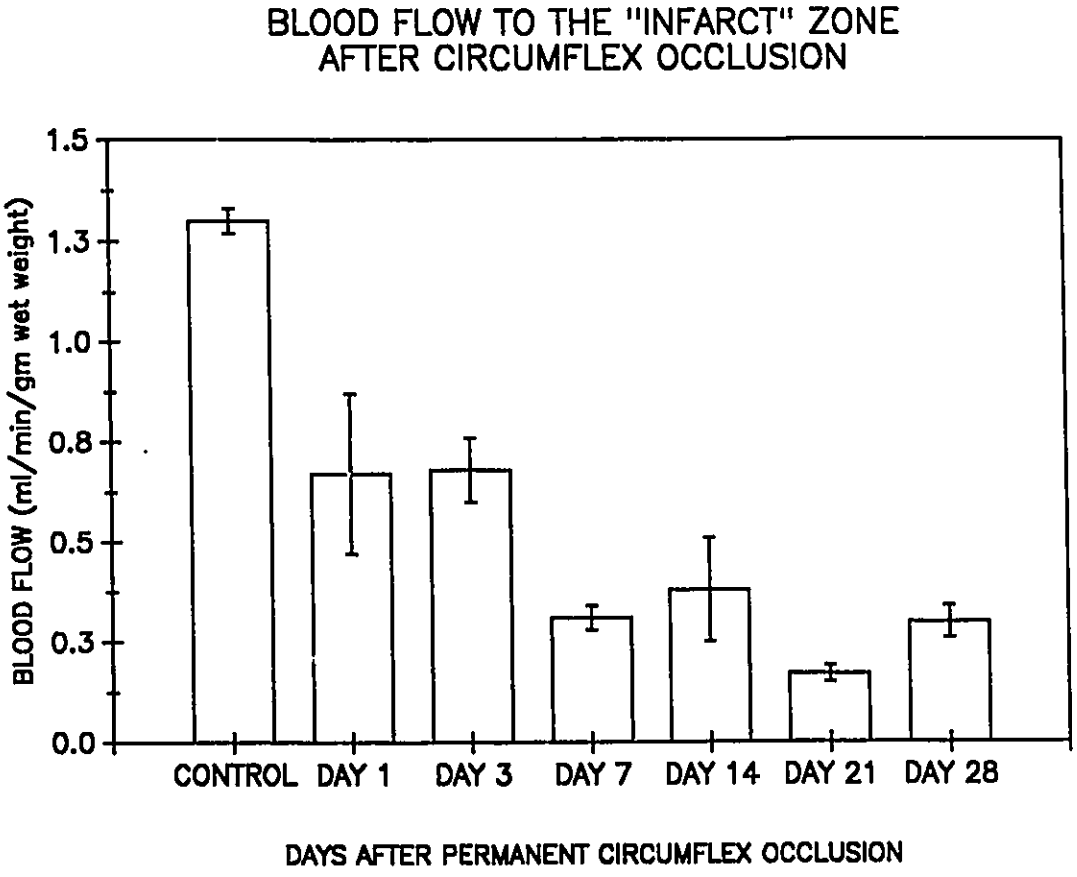


Figure 3.3

Summary of blood flow to the infarct zone in groups of pigs sacrificed at various times after occlusion of the circumflex coronary artery. All are significantly less than the control group. ($p<0.05$)

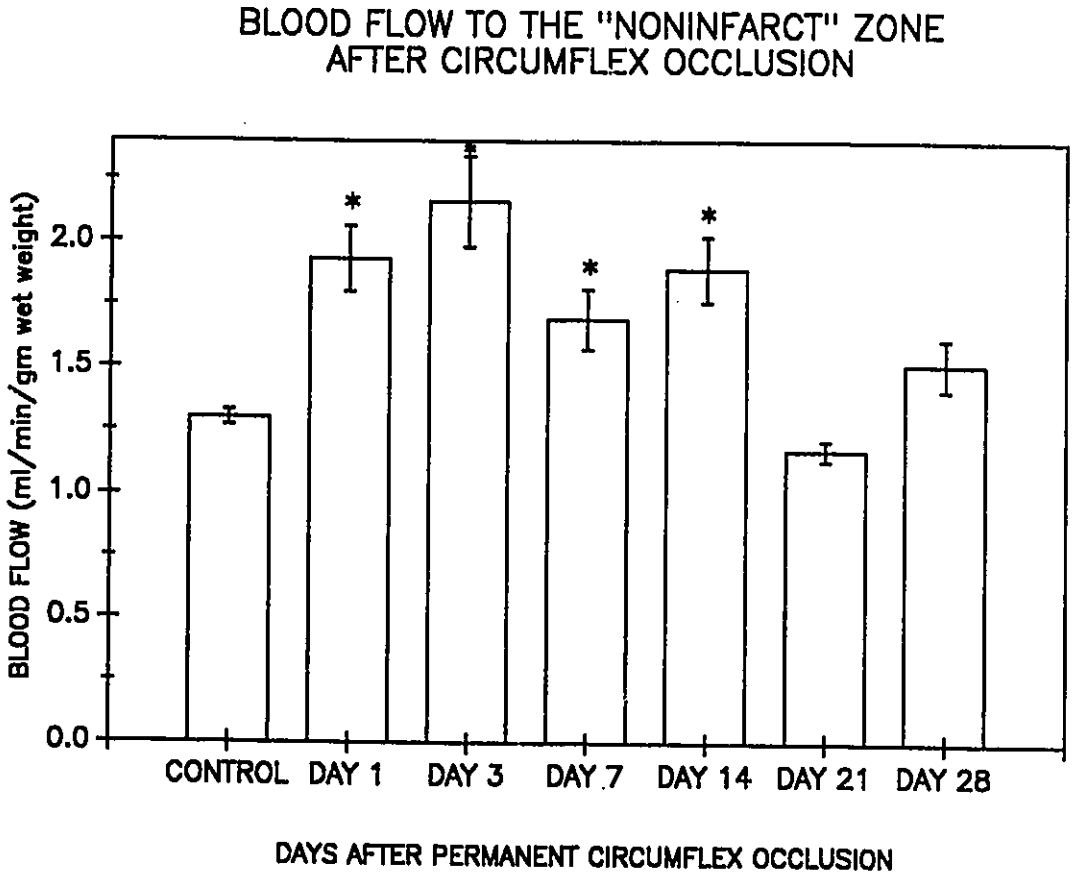


Figure 3.4

Summary of blood flow to the noninfarct zone in groups of pigs sacrificed at various times after permanent occlusion of the circumflex coronary artery. Values marked "*" are significantly different from the control group ($p<0.05$).

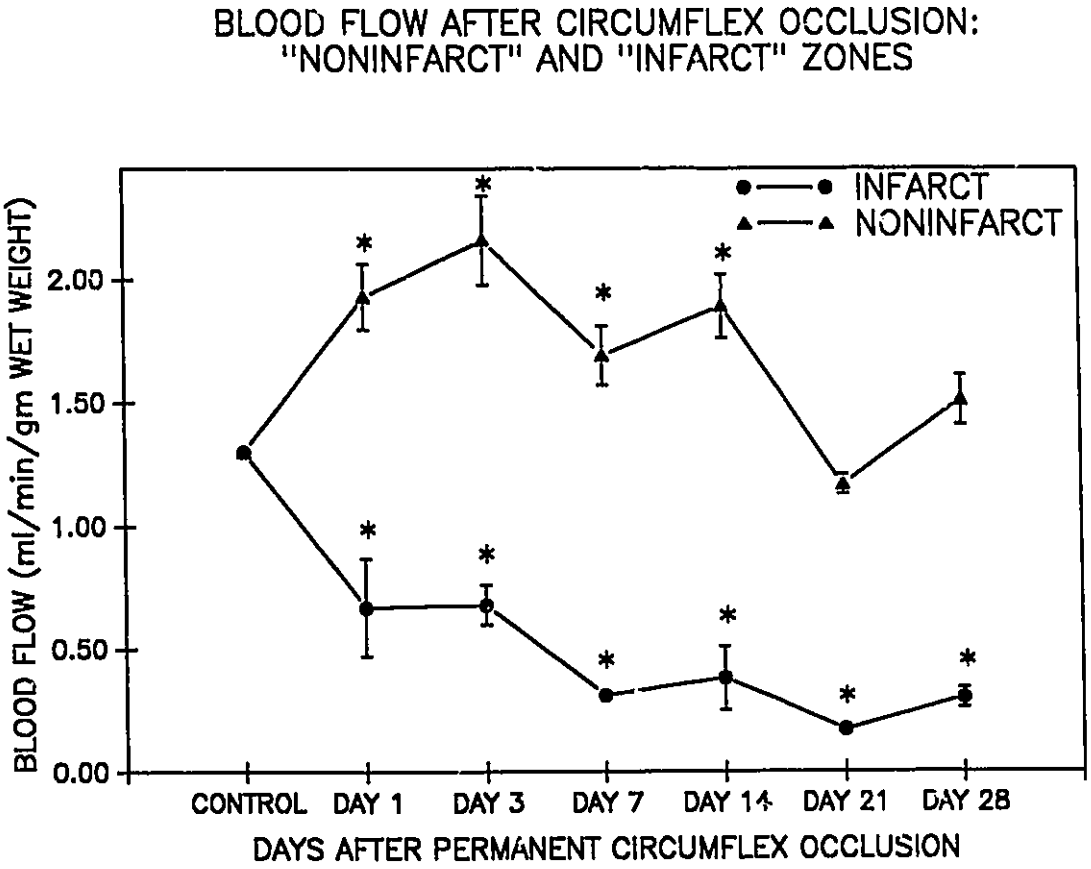


Figure 3.5

Summary of blood flow to the infarct and noninfarct zones in groups of pigs sacrificed at various times after permanent circumflex artery occlusion, determined by serial microsphere injection.

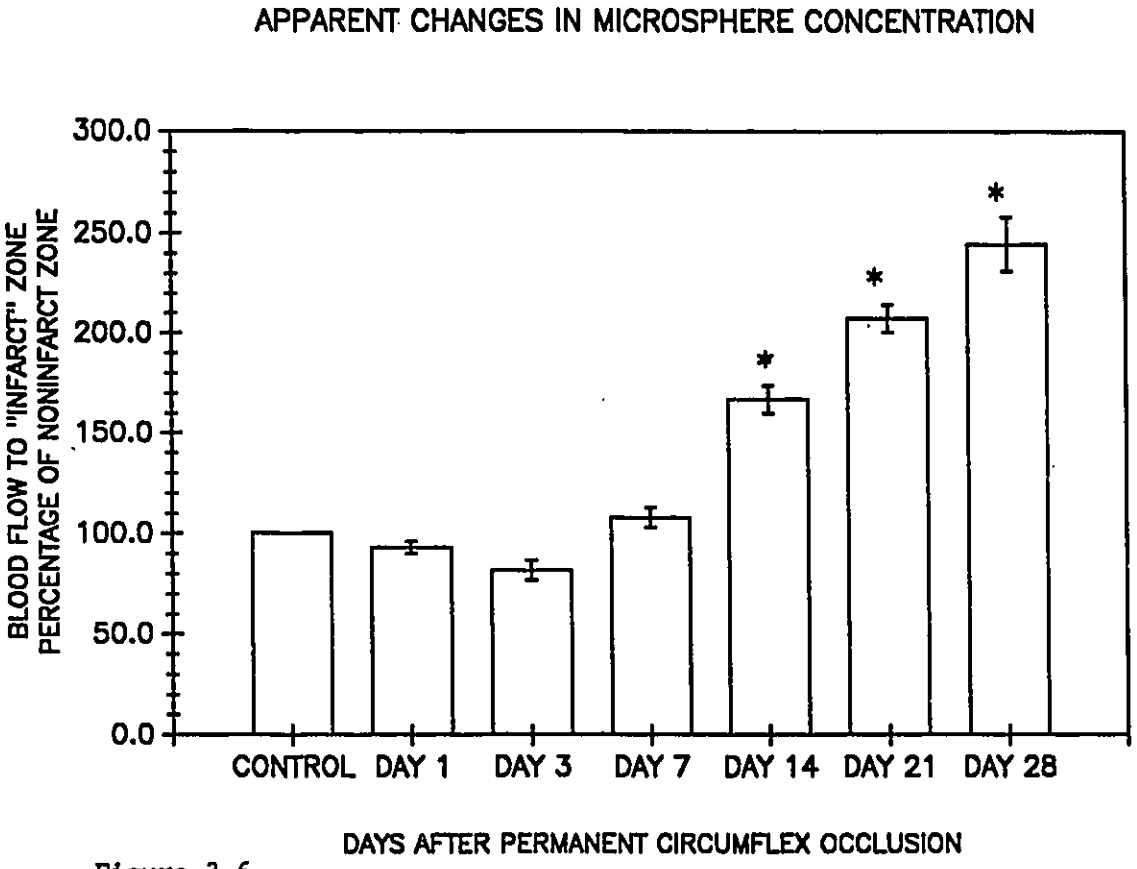


Figure 3.6

Summary of changes in pre-occlusion microsphere content in the infarct zone, during the 28-day period of observation. Preinfarct bloodflow (BF1) to the free wall (zone which was eventually infarcted) is expressed as a percentage of preinfarct flow to the septal wall (zone that was not infarcted). Values marked "*" are significantly different from the control group.

3.2 BIOCHEMISTRY

The amino acid profile of infarcted and noninfarcted regions of the myocardium was determined. Tissue samples were acid hydrolysed prior to the amino acid analysis using HPLC.

The profiles of the free and septal wall were similar in the control group, implying that the left ventricle is homogeneous in this respect. Either wall can be used as the control (see Table 3.5); in particular no differences were seen in the marker amino acids of interest (hydroxyproline, 3-methylhistidine, and proline). Over the twenty-eight day healing period no significant changes in the amino acid profile of the noninfarcted septal wall were found however, many changes were observed in the infarcted wall. By day fourteen, significant increases in hydroxyproline, glycine, and proline were measured. The other amino acids either remained constant or decreased (see Figure 3.6). The changes cannot really be interpreted except for those amino acids which are unique.

3.2.1 HYDROXYPROLINE

3.2.1.1 HYDROXYPROLINE IN THE CONTROL GROUP

The amount of hydroxyproline, an amino acid found primarily in collagen, was determined in infarcted and noninfarcted tissues. It initially was determined using a colourimetric analysis (Berg, 1982), and the Lowry total protein assay (Peterson, 1983). Subsequently the o-phthalaldehyde amino acid determination was performed. Finally the samples were analysed by high performance liquid chromatography (HPLC). The results are detailed in Table 3.6.

The hydroxyproline represents on average 0.5% of the total amino acids in control hearts. The three methods gave comparable results in the control hearts and are summarized in the following Table:

	Berg:Lowry	Berg:OPA	HPLC
Free wall	0.73 ±0.10	0.52 ±0.05	0.43 ±0.03
Septal wall	0.66 ±0.10	0.44 ±0.05	0.42 ±0.07

There were no differences in these measurements between the free and septal wall regardless of the method used (see ANOVA Table 6.1.4).

3.2.1.2 HYDROXYPROLINE IN THE NONINFARCTED REGION

There were small quantitative differences among the three methods used to determinine hydroxyproline content in noninfarcted tissue samples, however, the trends and the relative magnitude of the changes were all similar. The hydroxyproline content initially decreased, then rose slightly from the control values (see Table 3.6 and Figure 3.8). The rise reached significance with two of the three methods utilized, (Berg and Lowry, Berg and OPA).

More specifically, using the Berg and Lowry procedures the hydroxyproline concentration in the noninfarcted region decreased 58% below the control value seven days after the occlusion, then gradually increased 35% above control, to 0.9 ±0.1% of the total protein twenty-eight days after infarction. Day seven (Group 4) was significantly lower than day twenty-eight (Group 7), (see ANOVA Table 6.1.5).

Using the Berg and Opa procedures the number of hydroxyproline residues per 100 amino acids in the noninfarcted zone decreased 36% one day after vessel occlusion. After fourteen days the concentration started to increase reaching 130% of the control value twenty-eight

days after occlusion. The twenty-eight day value was not significantly greater than the control hearts, but was significantly greater than that measured twenty-four hours and seven days after vessel occlusion (see ANOVA Table 6.1.5).

The HPLC technique yielded similar results as the previous two methods. An initial small but nonsignificant decrease in hydroxyproline was measured twenty-four hours after infarction. All subsequent measurements were increased over control. The concentration in the twenty-eight day post infarct group rose 48%, above the control, but ANOVA suggests this rise was not significant (see ANOVA Table 6.1.5).

3.2.1.3 HYDROXYPROLINE IN THE INFARCTED ZONE

Hydroxyproline in the infarct zone increased significantly during the twenty-eight day healing period, becoming significantly greater than control after fourteen or twenty-one days, depending upon the method used (see Table 3.6, and Figure 3.9).

Hydroxyproline in the free wall of the control group was 0.7 ±0.1 residues per 100 amino acids, measured using the Berg and Lowry procedures. It increased in all experimental groups, rising 966% above the control twenty-eight days after coronary vessel occlusion. The concentrations in groups 6 and 7, (twenty-one and twenty-eight days), were significantly greater than the control value, (see ANOVA Table 6.1.6).

A significant increase in the concentration of hydroxyproline was measured using the Berg and Opa techniques. It rose 635% above control at twenty-eight days to 3.30 ±0.67 residues per 100 amino acids, (see Table 3.6). There were no statistically measureable differences among animals sacrificed up to seven days post-occlusion, (groups 1, 2, 3, and 4), but the fourteen, twenty-one, and twenty-eight day post-occlusion groups all had hydroxyproline levels significantly higher than control (see ANOVA Table 6.1.6).

Using HPLC, the concentration of hydroxyproline increased at all times measured after infarction, rising significantly to 760% above control by twenty-eight days, from 0.4 ±0.03, to 3.3 ± 0.5 residues per 100 amino acids, (see ANOVA Table 6.1.6).

3.2.1.4 TIME COURSE OF HYDROXYPROLINE CHANGE IN HEARTS WITH AN INFARCT

To reduce interanimal variability, and better appreciate regional differences in individual hearts, the amounts of hydroxyproline in the infarct and noninfarct zones were compared in each heart. With all methods utilized this ratio increased significantly. The results are presented in Table 3.7 and Figure 3.10.

Using the Berg and Lowry methods a 7.7-fold increase was measured at twenty-one days. It was unchanged at twenty-eight days. This increase was significant by ANOVA (Table 6.1.7), but the Scheffe test was not powerful enough to detect the location of the differences. The variance of groups 3, 5, 6, and 7 was greater than that of the control group; therefore these groups appear to represent populations different from the control group.

With the Berg and Opa techniques the ratio rose almost five fold above control, at twenty-eight days. This increase was significant by ANOVA (see ANOVA Table 6.1.7), but again the Scheffe procedure was not powerful enough to detect the location of these differences. The variance of groups 4, 5, 6, and 7 was greater than that of the control, implying that the distribution of values of the groups were unequal.

The HPLC method gave similar results. The ratio increased 5.8-fold over the twenty-eight days after permanent occlusion. The twenty-one and twenty-eight day groups were significantly greater than control (see ANOVA Table 6.1.7). The variance of groups 3, 4, 5, 6, and 7 were all greater than the control, again implying inequality of sample distribution of the experimental and control groups.

3.2.2 3-METHYLHISTIDINE

3.2.2.1 3-METHYLHISTIDINE IN NONINFARCTED TISSUE

In the porcine myocardium 3-methylhistidine is essentially unique to actin (Kuehl and Adelstein, 1970). Its concentration was determined in two tissue samples from each heart, one from the infarct zone, and one from the noninfarct zone. Using HPLC analysis no measureable differences in the concentration of 3-methylhistidine between the free and septal walls of the control hearts were found (see Table 3.8). The mean concentrations were 0.07 ± 0.02 and 0.03 ± 0.0 per 100 amino acid residues respectively. In the noninfarcted regions of the hearts, the concentration appeared to decrease gradually, over the twenty-eight days after infarction, but was not significant by ANOVA (see Table 6.1.8). This indicated that no statistically significant changes occurred during the time course of the experiment.

3.2.2.2 3-METHYLHISTIDINE IN INFARCTED TISSUE

In the infarcted tissues the concentration of 3-methylhistidine decreased to 50% of its original value the first day after infarction. After twenty-one days the concentration was equal to the control level, then increased 50% above control at twenty-eight days (see Table 3.8). These differences were not significant by ANOVA (see ANOVA Table 6.1.8). Thus, 3-methylhistidine remained at detectable levels at all times in the infarct.

3.2.3 RATIOS OF AMINO ACIDS

3.2.3.1 RATIOS OF AMINO ACIDS IN NONINFARCTED TISSUE

The concentrations of hydroxyproline and 3-methylhistidine were compared to reflect the relative amounts of collagen to actin. These ratios do not indicate the relative abundance of the proteins, because collagen is about one tenth hydroxyproline, whereas one in four-hundred amino acids in actin is 3-methylhistidine. Nevertheless, changes in the ratios with time indicate changes in the relative proportions of

these proteins. The results are detailed in Table 3.10 and Figures 3.11 and 3.12. In control hearts there were no differences in this ratio between the free and septal walls; 12.5 ±1.9, and 8.4 ±2.2 respectively.

In noninfarcted tissue the ratio of hydroxyproline to 3-methylhistidine (OHP:3MeHis) increased more or less steadily. It rose 110% one day after occlusion and rising nearly four-fold twenty-eight days after occlusion. However, this rise was not significant by ANOVA (see Table 6.1.9).

The second ratio examined was proline-to-hydroxyproline (PRO:OHP). In the control group, the ratio was 12.4 ±1.0, and 13.7 ±2.0 in the free and septal walls respectively. In noninfarcted tissue it decreased gradually, falling to 70% of the control level in the twenty-eight day group. This was not statistically lower by ANOVA (see ANOVA Table 6.1.9).

Thus, with substantial variability, the ratios of these amino acids remained relatively stable in the noninfarct zone.

3.2.3.2 RATIOS OF AMINO ACIDS MEASURED IN INFARCTED TISSUE

The ratio of hydroxyproline to 3-methylhistidine in infarcted tissue rose 15-fold above the control group mean of 12.5 ±1.9 by fourteen days after occlusion, then decreased to 8 fold above control by twenty-eight days (see Table 3.10 and Figures 3.11 and 3.12). These changes were significantly different by ANOVA (Table 6.1.9), but the Scheffe test was not powerful enough to detect the location of the differences. However, the variances in the experimental groups were all larger than the control group, implying that all groups were different from control.

In infarcted tissue the ratio of proline to hydroxyproline fell more steeply than in the noninfarcted region. It fell 79% below

control fourteen days after infarction without further change thereafter. Groups 3, 4, 5, 6, and 7 were all significantly lower than the control group (see ANOVA Table 6.1.9). Since the hydroxyproline concentration was rising, the falling ratio suggested the disappearance of the non-collagen, proline-containing proteins.

3.2.4 PROTEIN IDENTIFICATION USING MONOCLONAL ANTIBODIES

3.2.4.1 ACTIN IDENTIFICATION

Acetone powders from control, fourteen and twenty-eight day infarcts were separated on 6% SDS gels. From these it was evident that in all samples a protein was comigrating with the actin standard, 43000 Kilodaltons. C4, the generic antiactin, reacted with all actin standards and with all infarct and noninfarct tissue samples. HUC, the striated actin, did not react with the fibroblast actin standard but reacted with all samples: control, infarct and noninfarct. This suggests that a striated actin was still present in the twenty-eight day infarcted tissue samples.

3.2.4.2 MYOSIN IDENTIFICATION

SDS denaturing gel electrophoresis of samples revealed a protein of approximately 100,000 Kilodalton molecular weight in all lanes, which migrated with the myosin heavy chain standard. Positive staining after reaction with monoclonal antibody was observed in these protein bands from all groups. This implied that myosin was still not completely cleared from the infarct at twenty-eight days after permanent occlusion in the pig.

TABLE 3.5:

THE AMINO ACID PROFILE OF CONTROL HEARTS

AMINO ACID	FREE WALL N	FREE WALL MEAN	FREE WALL S.E.	SEPTAL WALL N	SEPTAL WALL MEAN	SEPTAL WALL S.E.
aspartic acid	5	7.81	0.05	5	8.13	0.22
glutamic acid	5	11.41	0.10	5	11.90	0.49
hydroxyproline	5	0.41	0.06	5	0.42	0.03
serine	5	4.53	0.12	5	4.57	0.11
glycine	5	8.46	0.20	5	8.43	0.14
histidine	5	1.53	0.03	5	1.66	0.09
threonine	5	4.99	0.06	5	4.97	0.02
alanine	5	8.46	0.16	5	8.65	0.05
arginine	5	6.46	0.04	5	6.27	0.14
3-methylhistidine	3	0.07	0.02	2	0.03	0.00
proline	5	5.19	0.10	5	5.19	0.08
tyrosine	5	2.37	0.08	5	2.40	0.07
valine	5	5.35	0.07	4	5.53	0.30
methionine	5	1.54	0.04	4	1.47	0.13
cysteine	5	0.76	0.04	5	0.61	0.12
isoleucine	5	4.48	0.11	4	4.40	0.18
leucine	5	8.36	0.07	4	8.38	0.08
phenylalanine	5	4.26	0.06	5	4.19	0.06
lysine	5	7.08	0.08	5	7.14	0.15

The amino acid profile of the free and septal walls in the control group was determined by HPLC. Values presented are residues per 100 amino acids.

"Aspartic acid" actually represents asparagine and aspartic acid

"Glutamic acid" actually represents glutamine and glutamic acid.

TABLE 3.6:
A SUMMARY OF THREE METHODS USED TO DETERMINE HYDROXYPROLINE CONCENTRATION

		"INFARCT ZONE"			"NONINFARCT ZONE"		
GROUP	VARIABLE	N	MEAN	S.E.	N	MEAN	S.E.
1	Berg-Lowry	5	0.73	0.10	3	0.66	0.10
	Berg-OPA	5	0.52	0.05	4	0.44	0.05
	HPLC	5	0.43	0.03	5	0.42	0.07
2	Berg-Lowry	5	0.97	0.29	4	0.50	0.11
	Berg-OPA	5	0.48	0.09	5	0.29	0.05
	HPLC	5	0.55	0.05	5	0.41	0.05
3	Berg-Lowry	6	0.99	0.17	5	0.44	0.08
	Berg-OPA	6	0.49	0.07	4	0.38	0.02
	HPLC	6	1.26	0.67	6	0.45	0.03
4	Berg-Lowry	6	1.07	0.14	6	0.34	0.03
	Berg-OPA	6	0.90	0.09	6	0.28	0.03
	HPLC	7	1.35	0.18	7	0.43	0.03
5	Berg-Lowry	6	3.74	1.25	6	0.50	0.04
	Berg-OPA	6	2.30	0.60	6	0.39	0.03
	HPLC	7	2.45	0.17	7	0.53	0.02
6	Berg-Lowry	10	6.06	1.86	8	0.60	0.05
	Berg-OPA	9	2.82	0.29	8	0.48	0.05
	HPLC	9	3.28	0.19	8	0.55	0.06
7	Berg-Lowry	8	7.05	1.47	9	0.89	0.11
	Berg-OPA	7	3.30	0.67	8	0.57	0.06
	HPLC	10	3.27	0.48	9	0.62	0.10

Hydroxyproline (OHP) was determined in two locations in each heart, one sample from infarcted and one from noninfarcted tissue, by three methods: Berg and Lowry, Berg and Opa, and HPLC. Values are expressed as a percent of protein which is hydroxyproline (Berg-Lowry), and as a percent of amino acids which is hydroxyproline (Berg-Opa, HPLC).
The "infarct zone" tissue is taken from the free wall, the "noninfarct zone" tissue is taken from the septal wall. For convenience and clarity the results from both the control (Group 1), and the experimental groups have been combined under these headings.

TABLE 3.7:
HYDROXYPROLINE CONCENTRATION: INFARCT REGION EXPRESSED AS A PERCENTAGE OF THE NONINFARCT REGION

BERG-LOWRY

GROUP	N	MEAN	S.E.
1	4	119.91	23.31
2	4	193.17	34.56
3	6	287.20	86.06
4	6	319.71	32.75
5	6	765.36	246.80
6	10	919.78	0.04
7	8	919.67	266.58

BERG-OPA

GROUP	N	MEAN	S.E.
1	4	133.20	25.03
2	5	169.00	14.65
3	6	145.59	64.75
4	7	342.98	52.74
5	7	608.99	177.80
6	9	623.96	77.91
7	7	618.25	120.65

HPLC

GROUP	N	MEAN	S.E.
1	5	111.38	16.41
2	5	137.36	7.95
3	6	267.16	131.94
4	7	322.87	42.43
5	7	465.95	35.56
6	9	620.30	78.12
7	10	648.90	112.88

Hydroxyproline content was determined in the infarcted and noninfarcted regions of each heart using three methods. The above are concentrations in the infarct zone expressed as a percentage of those found in the noninfarct zone. Values expressed are the means per group.

TABLE 3.8:
REGIONAL CONCENTRATION OF 3-METHYLHISTIDINE

	"INFARCT"			"NONINFARCT"		
GROUP	N	MEAN	S.E.	N	MEAN	S.E.
1	2	0.037	0.00	3	0.074	0.02
2	5	0.021	0.009	5	0.044	0.02
3	6	0.018	0.004	6	0.053	0.015
4	3	0.016	0.004	7	0.034	0.006
5	3	0.015	0.001	6	0.046	0.009
6	5	0.035	0.012	5	0.036	0.005
7	4	0.063	0.035	4	0.017	0.002

† units are number of residues per 100 amino acids (by HPLC)

3-Methylhistidine was determined in infarcted and noninfarcted tissues, by HPLC. Values presented are the mean concentrations of each group. The "infarct zone" tissue is from the free wall, the "noninfarct zone" tissue is from the septal wall. For convenience and clarity the results from the control (group 1) and the experimental groups have been combined.

TABLE 3.9:
REGIONAL CONCENTRATION OF PROLINE

	"INFARCT"			"NONINFARCT"		
GROUP	N	MEAN	S.E.	N	MEAN	S.E.
1	5	5.19	0.10	5	5.19	0.08
2	5	5.12	0.10	5	5.06	0.07
3	6	5.46	0.07	6	5.23	0.05
4	7	6.26	0.17	7	5.24	0.14
5	7	7.06	0.18	7	5.23	0.12
6	8	7.64	0.17	8	5.55	0.96
7	9	7.34	0.35	9	5.49	0.14

† units are number of residues per 100 amino acids (by HPLC)

Proline was determined in infarcted and noninfarcted tissues, by HPLC. Values presented are the mean concentrations of each group. The "infarct zone" tissue is from the free wall, the "noninfarct zone"tissue is from the septal wall. For convenience and clarity the results from the control (group 1) and the experimental groups have been combined.

TABLE 3.10:
RELATIVE REGIONAL RATIOS OF HYDROXYPROLINE TO 3-METHYLHISTIDINE AND HYDROXYPROLINE TO PROLINE IN EACH GROUP

		"INFARCT ZONE"			"NONINFARCT ZONE"		
GROUP	VARIABLE	N	MEAN	S.E.	N	MEAN	S.E.
1	OHP:MEHIS	2	12.51	1.87	3	8.42	2.18
	PRO:OHP	5	12.42	1.02	5	13.70	2.05
2	OHP:MEHIS	5	18.23	8.08	5	18.13	6.74
	PRO:OHP	5	9.58	1.07	5	13.12	1.80
3	OHP:MEHIS	6	85.97	42.24	6	14.18	4.81
	PRO:OHP	6	8.05	1.46	6	11.97	0.75
4	OHP:MEHIS	3	85.78	19.22	7	16.27	3.61
	PRO:OHP	7	4.93	0.40	7	12.84	1.33
5	OHP:MEHIS	3	188.44	25.03	6	27.51	17.44
	PRO:OHP	7	2.94	0.17	7	10.49	0.56
6	OHP:MEHIS	5	161.98	58.80	5	15.67	2.85
	PRO:OHP	9	2.37	0.11	8	10.75	0.99
7	OHP:MEHIS	4	103.18	30.40	4	33.71	7.00
	PRO:OHP	10	2.91	0.54	9	10.05	1.04

The concentration of the amino acids hydroxyproline, 3-methylhistidine and proline were determined in infarcted and noninfarcted tissue samples using HPLC. The mean concentration of each amino acid was determined in each group. Results presented are the ratios between hydroxyproline : 3-Methylhistidine (OHP:MEHIS), and proline : hydroxyproline (PRO:OHP). The "infarct zone" tissue is from the free wall, the "noninfarct zone" tissue is from the septal wall. For convenience and clarity the results from the control (group 1) and the experimental groups have been combined.

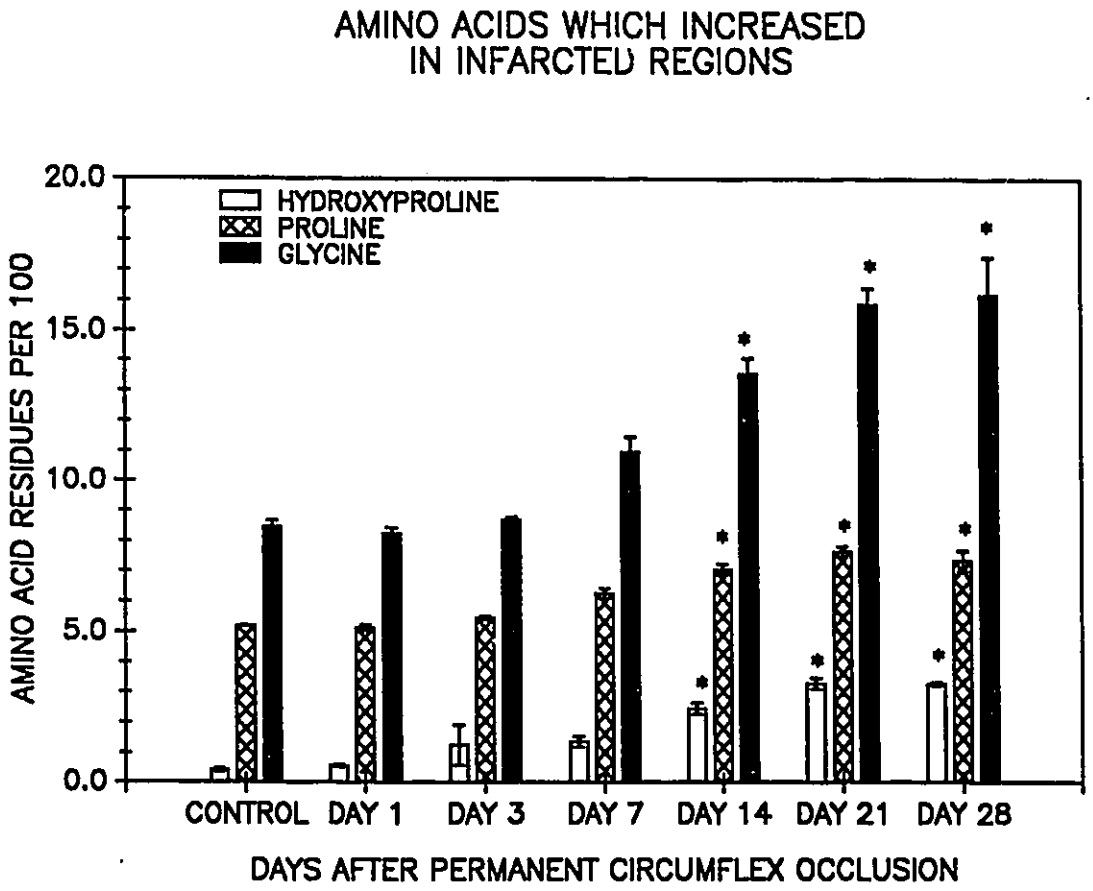


Figure 3.7

Summary of the time-course of changes in those amino acids which at twenty-eight days were significantly greater than the control value.

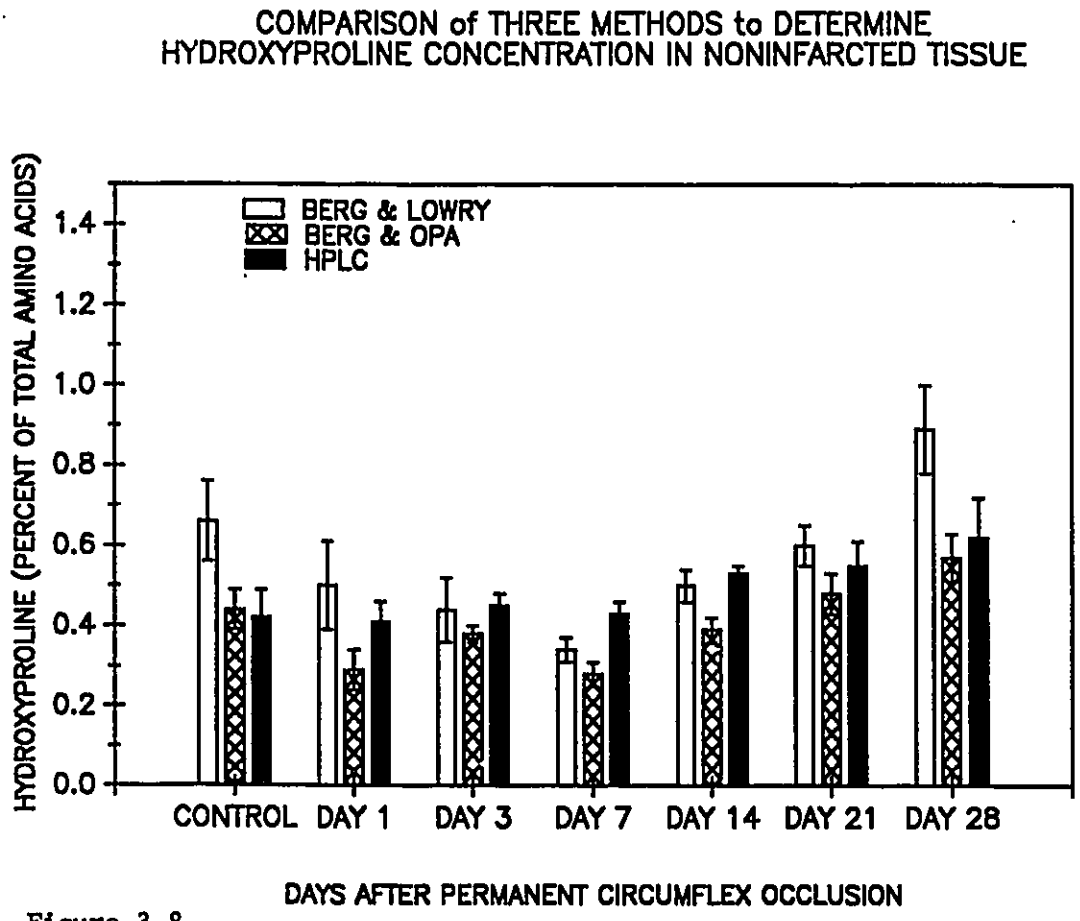


Figure 3.8

Summary of the percentage of hydroxyproline in noninfarcted tissue measured using three different methods. This figure summarizes the results from groups of pigs sacrificed at various times after circumflex artery occlusion. In comparing this to Figure 3.9 on the following page please note the differences in scaling.

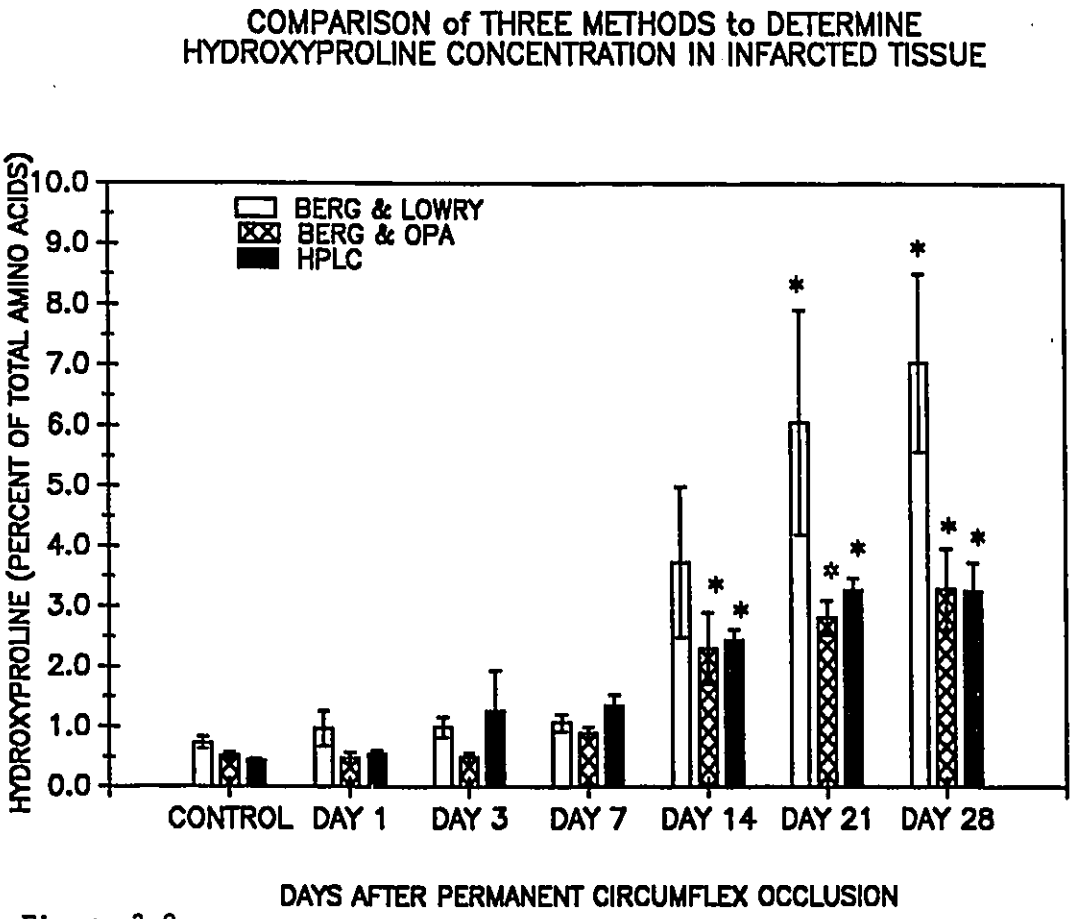


Figure 3.9

Summary of hydroxyproline concentration in infarct tissue determined by three different methods in groups of pigs sacrificed at various times after occlusion of the circumflex coronary artery. Values marked "*" are significantly greater than control ($p>0.05$). In comparing this to Figure 3.8 on the previous page please note the differences in scaling.

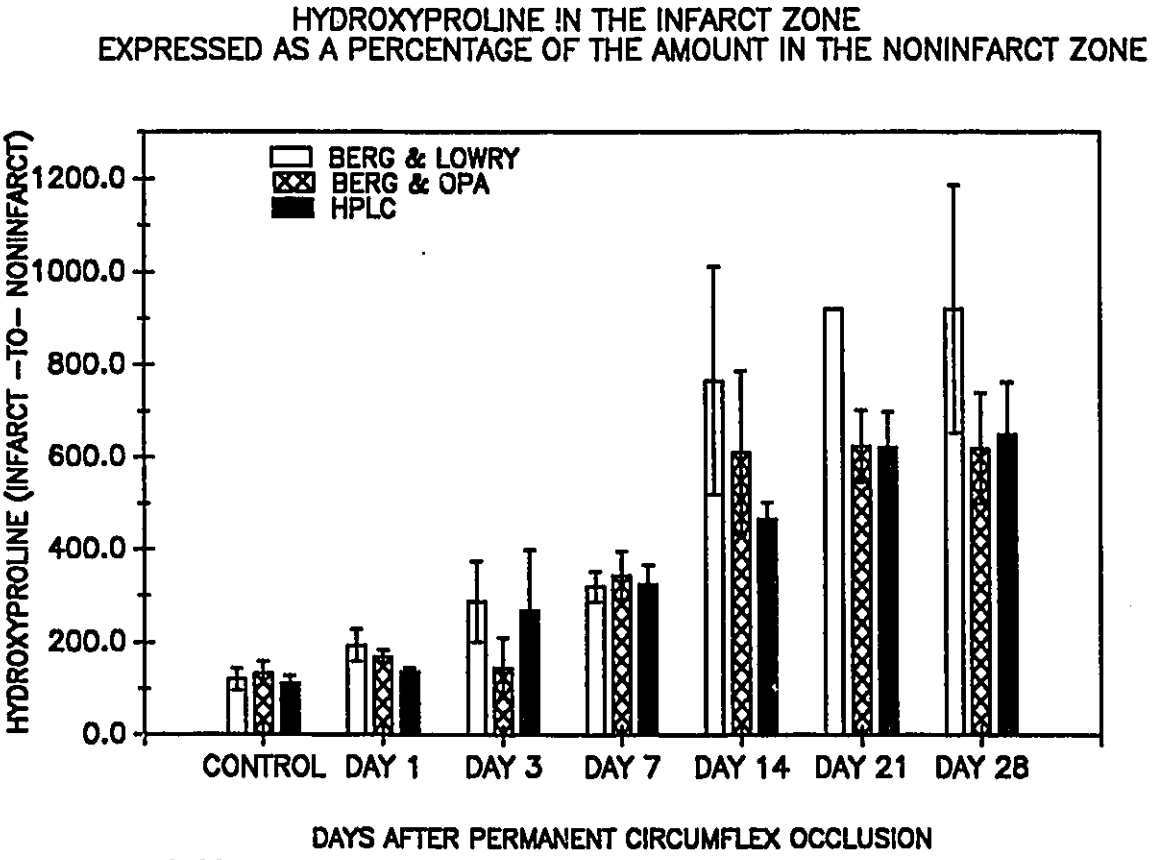


Figure 3.10

Summary of hydroxyproline determined in the infarct and noninfarct zones. The concentration in the infarct zone is expressed as a percentage of that in the noninfarct zone.

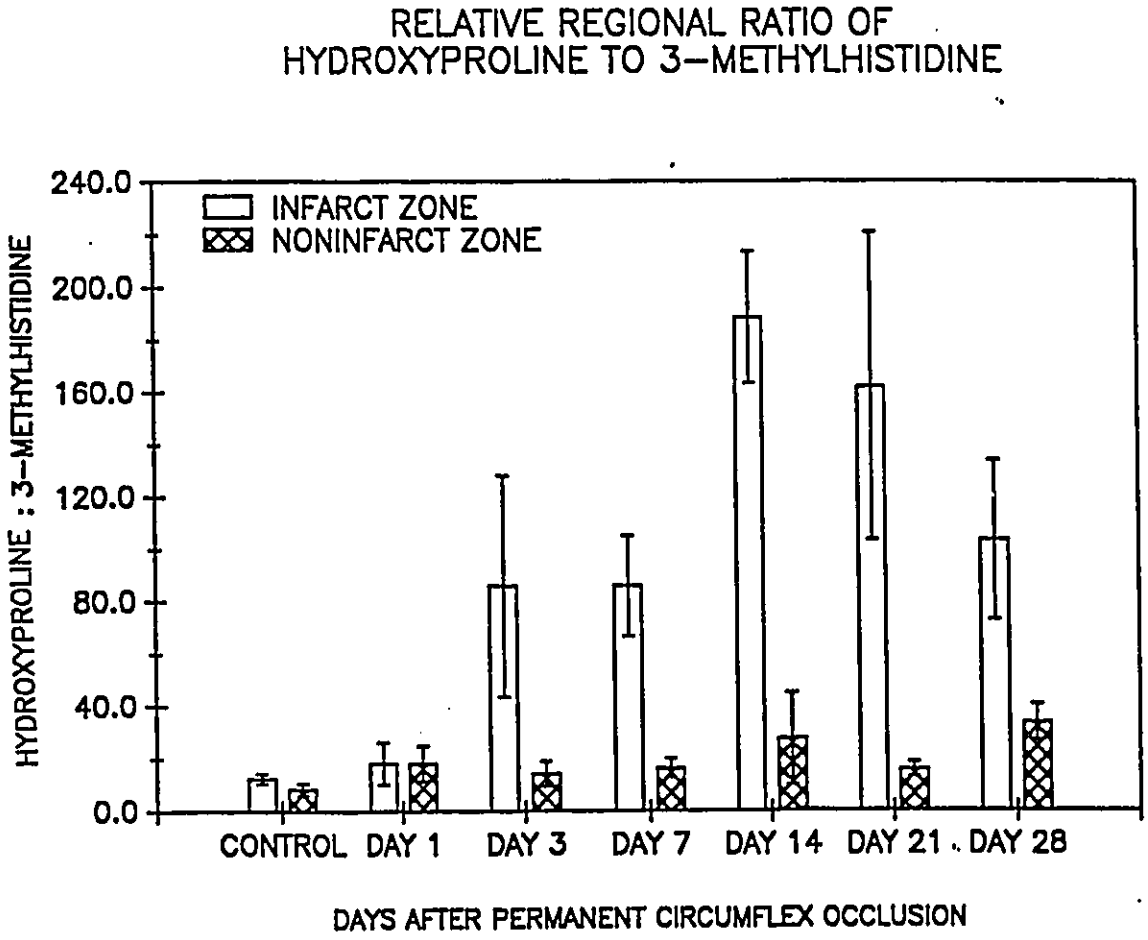


Figure 3.11

Summary of marker amino acids determined in infarct and noninfarct zones of each heart. This summarizes the relative ratio of hydroxyproline (the amino acid marker of collagen) to 3-methylhistidine (the amino acid marker of actin) in groups of pigs sacrificed at various times after permanent circumflex artery occlusion.

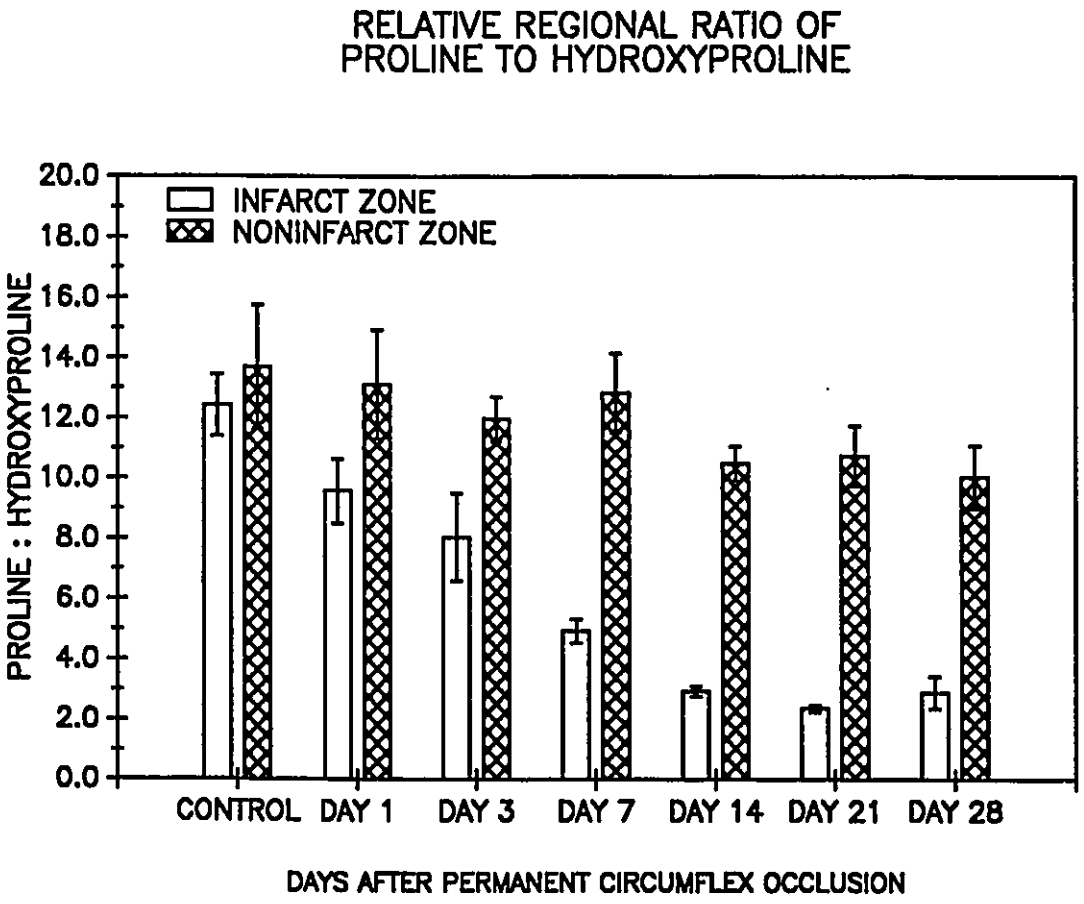


Figure 3.12

Summary of two amino acids determined in infarct and noninfarct zones of each heart. This figure summarizes the relative ratio of proline to hydroxyproline in groups of pigs sacrificed at various times after permanent circumflex artery occlusion.

3.2.5. WATER CONTENT OF THE MYOCARDIUM

The water content of the myocardium is summarized in Table 3.11 and Figure 3.13. The water content of the control group myocardium was 77.5 ± 0.2% of the total wet weight. This remained constant in all noninfarcted tissue from subsequent groups. The water content of the infarcted areas increased significantly one day after occlusion, to 81.2 ± 0.6%, and continued to rise in all subsequent groups (see ANOVA Table 6.10).

TABLE 3.11:
SUMMARY OF WATER CONTENT IN INFARCTED AND NONINFARCTED REGIONS

	"INFARCT ZONE"			"NONINFARCT ZONE"		
GROUP	N	MEAN	S.E.	N	MEAN	S.E.
1				275	77.53	0.19
2	43	81.24	0.56	82	78.03	0.11
3	16	80.27	0.28	26	78.21	0.21
4	15	81.56	1.16	34	77.33	0.30
5	22	83.14	0.90	41	78.76	0.47
6	28	85.09	0.77	84	78.43	0.21
7	33	83.98	0.80	116	77.28	0.16

The dry weight as a percentage of total weight was determined in several tissue samples from each heart. The values presented are the mean water contents, (percentage of total weight) of infarcted and noninfarcted tissue of each group.

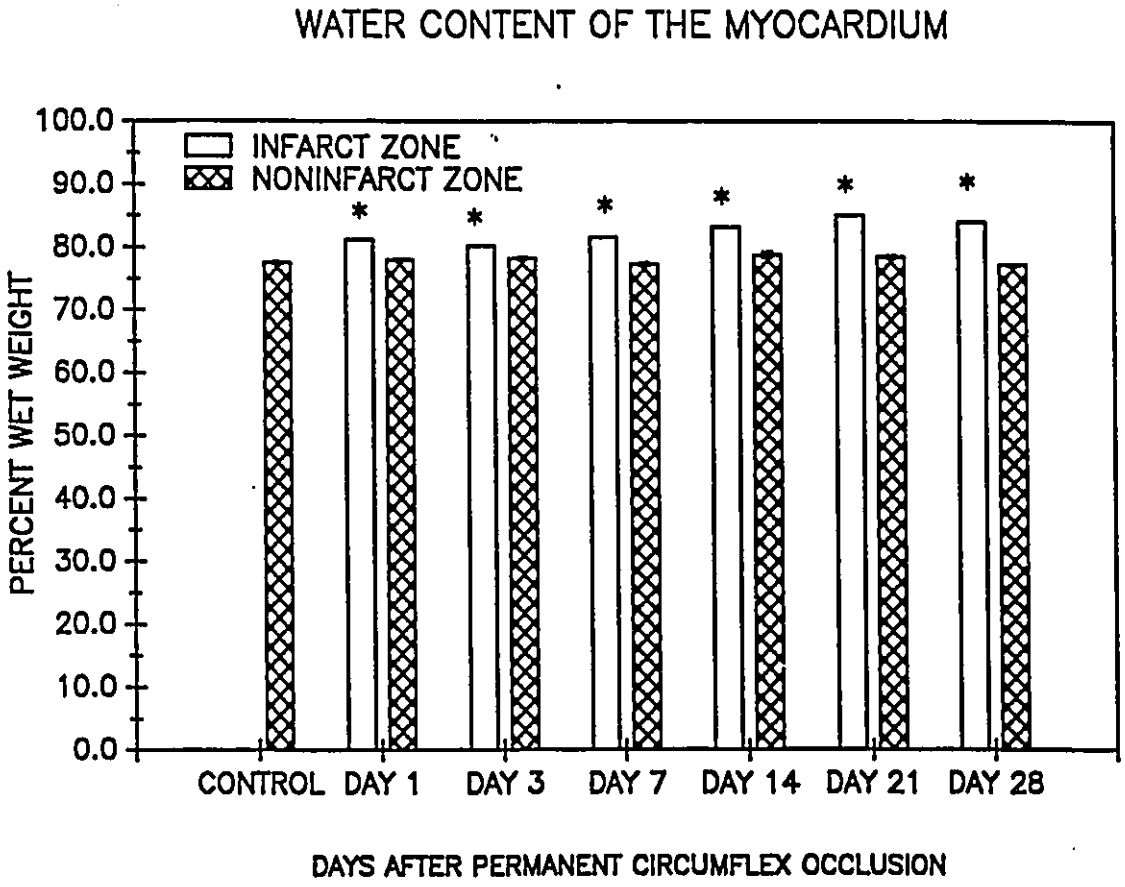


Figure 3.13

Summary of water content in infarct and non-infarct zones, expressed as a percentage of the total wet weight. Values marked "*" are significantly different from control ($p<0.05$).

3.3 HISTOLOGY

The changing location and orientation of collagen in the myocardium, after an infarction are described. Of the successful experiments, (i.e. approximately six in each group) histological slides were taken from three to four hearts in each group. Four histological stains were used, of each at least eight to ten slides were prepared. These were examined under low and high power light micropscopy. Representative areas were photographed.

The stains were specifically chosen to demonstrate different components of tissue, especially the disposition of collagen. With the hematoxylin and eosin stain, nuclei stain blue and cytoplasm stains various shades of pink. The Goldners' Trichrome method stains collagen dark green, nuclei red to brown, muscle red, and cytoplasm red to green. Using the Gomori's modified reticular stain, reticulin fibers stain black, collagen fibers stain pink to purple, and nuclei and cytoplasm stain various shades of grey. With the Herovici polychrome stain nuclei stain black, cytoplasm green to yellow, muscle and red blood cells appear bright yellow, young collagen appears blue and mature collagen red.

3.3.1 HISTOLOGICAL APPEARANCE OF CONTROL TISSUE

Collagen in control hearts was visible in three locations, best appreciated with the Herovici stain. First, a thin border of collagen was present on the epicardial and endocardial surfaces. Second, collagen was located around groups of blood vessels. Third, sparse amounts of collagen was visible between individual myocytes and between groups of myocytes. Few fibroblasts were visible in the tissue (see Figure 3.14.1). Using Gomori's silver stain, the entire cell appeared to stain black, suggesting that the cell was "wrapped" in a "reticulum" fiber mesh. There were small amounts of red staining "rope like" collagen seen between myocytes.

3.3.2 HISTOLOGICAL APPEARANCE OF INFARCTED TISSUE

The changes observed in the infarcted region can be summarized and some aspects illustrated by representative figures. As a general observation, healing progressed unevenly from the epicardial and endocardial borders inwards towards the center of the infarct, healing from the latter progressing faster. The collagen which initially appeared by all stains was thin and randomly oriented. Collagen appearing subsequently seemed to become more organized, running parallel to the circumference of the heart. In all hearts observed at a given time after infarction, different stages of healing were visible. Healing was not completed by twenty-eight days in any heart examined at that time.

3.3.2.1 EARLY INFARCTS

One day after occlusion there was some variability between individual infarcts in the amount of visible damage. Waviness of myocytes, a classical sign of ischemic damage (Bouchardy and Majno, 1974, Fishbein et al., 1978), was present in some areas. Most myocytes in the infarcted zone still appeared to have all their nuclei, although some were changing shape. The infarcted tissue looked intact, but sections stained with hematoxylin and eosin transmurally stained a brighter pink than the control tissues. There were a few locations of heavy concentrations of fibroblasts. At three days changes were intermediate between those observed at one and seven days.

Seven days after occlusion, obvious changes were seen in infarcted tissue. A substantial amount of the necrotic tissue had been cleared. A sharp boundry demarcated the presence of macrophages and fibroblasts from the uncleared necrotic tissue (see Figure 3.14.2). This "sharpness" was maintained for the twenty-eight day duration. There was an extensive infiltration of fibroblasts in all infarcts which was densest at the border where tissue clearing was occurring. A large number of red blood cells were present, also most concentrated near the border of active clearing.

New collagen identified with the Herovici stain, was visible on the epicardial and endocardial borders, and within the wall. The orientation appeared to be completely random (see Figure 3.14.4), being laid down closest to that area being actively cleared. Although the majority of collagen present was new, some histological sections demonstrated "mature" collagen. Both the "mature" collagen and the fibroblasts were oriented around the equatorial circumference of the heart, (see Figure 3.14.5). This orientation was observed regardless of the stain utilized, but was easiest to appreciate with the Herovici and the Gomori stains.

The area of scar which was the oldest contained parallel collagen fibers. The collagen located closest to the area which was being cleared was quite sparse, and there were a great number of fibroblasts present. Fibroblasts were present where there were fine "reticulin" fibers, they were not present where the parallel collagen fibers were (see Figure 3.14.7).

In the seven day infarcts, individual myocytes showed different stages of necrosis. Occasional myocytes had survived the infarct located on the epicardial or endocardial borders, some myocytes had very pale staining and spindle shaped ghost nuclei, and some myocytes had no nuclei left at all. Striations were still apparent in the uncleared myocytes (see Figure 3.14.8).

3.3.2.2 LATE INFARCTS

Prior to fourteen days the infarcted but uncleared myocytes retained an organized appearance. In the fourteen day group this uniform arrangement of necrotic myocytes which had not yet been cleared was disrupted. A great amount of disorientation and breakage of individual, necrotic myocytes, with large gaps in the area was visible (see Figure 3.14.9).

Twenty-one and twenty-eight day infarcts were characterized by: (a) thinning of the wall (a greater degree of thinning at twenty-eight than at twenty-one days); (b) clearing of necrotic areas was not complete; (c) a large amount of mature collagen was present in areas which had been cleared (see Figure 3.14.10). Thus, even at this late stage healing was not yet complete but is characterized by the coexistence of bands of different stages of healing.

Figure 3.14.1

Photomicrographs of noninfarcted porcine myocardium stained with three different stains;

"A" Hemotoxylin and eosin (magnification X 400)

"B" Gomori's Silver Stain (magnification X 400)

"C" Herovici Collagen Stain (magnification X 400)

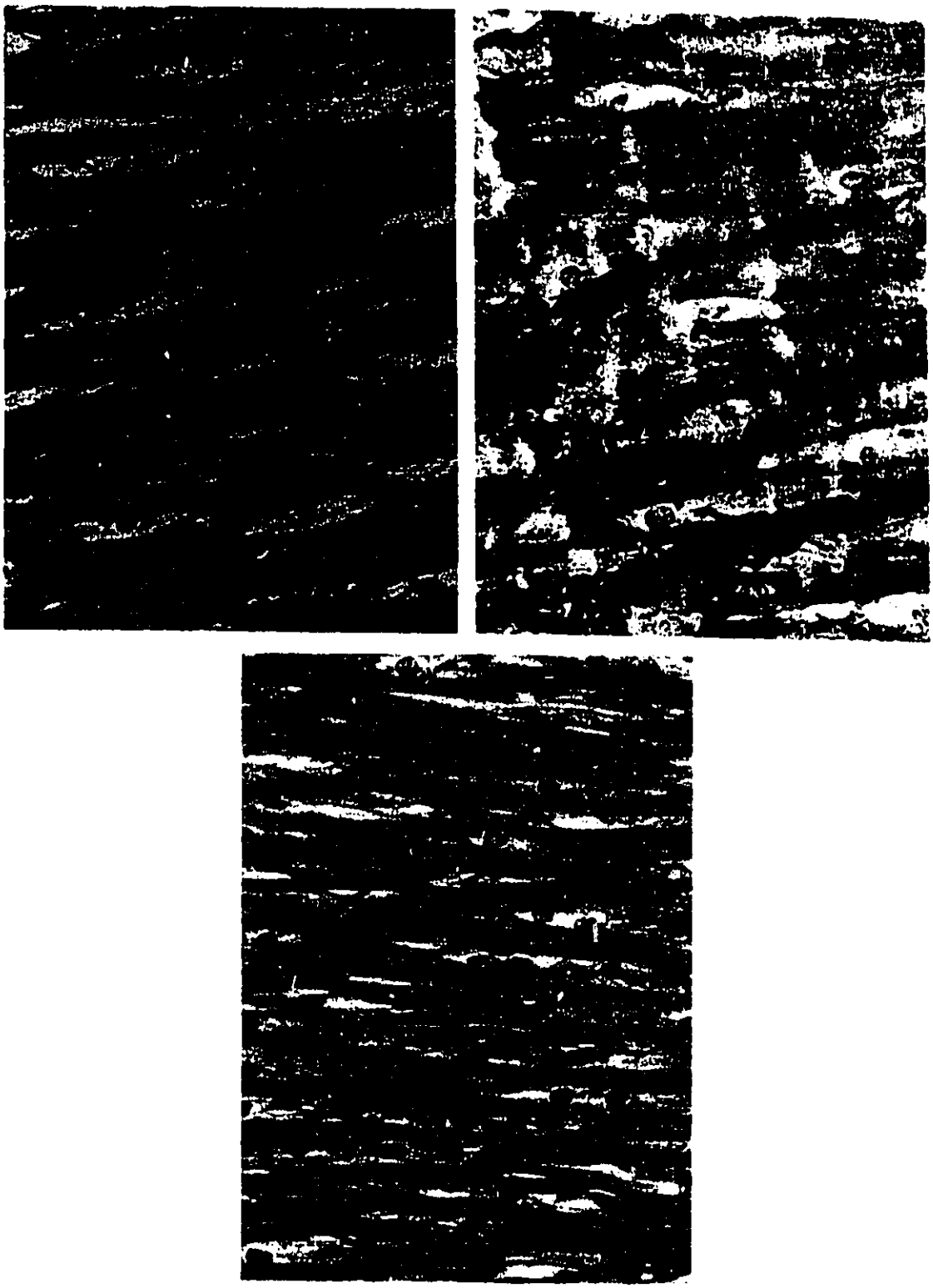
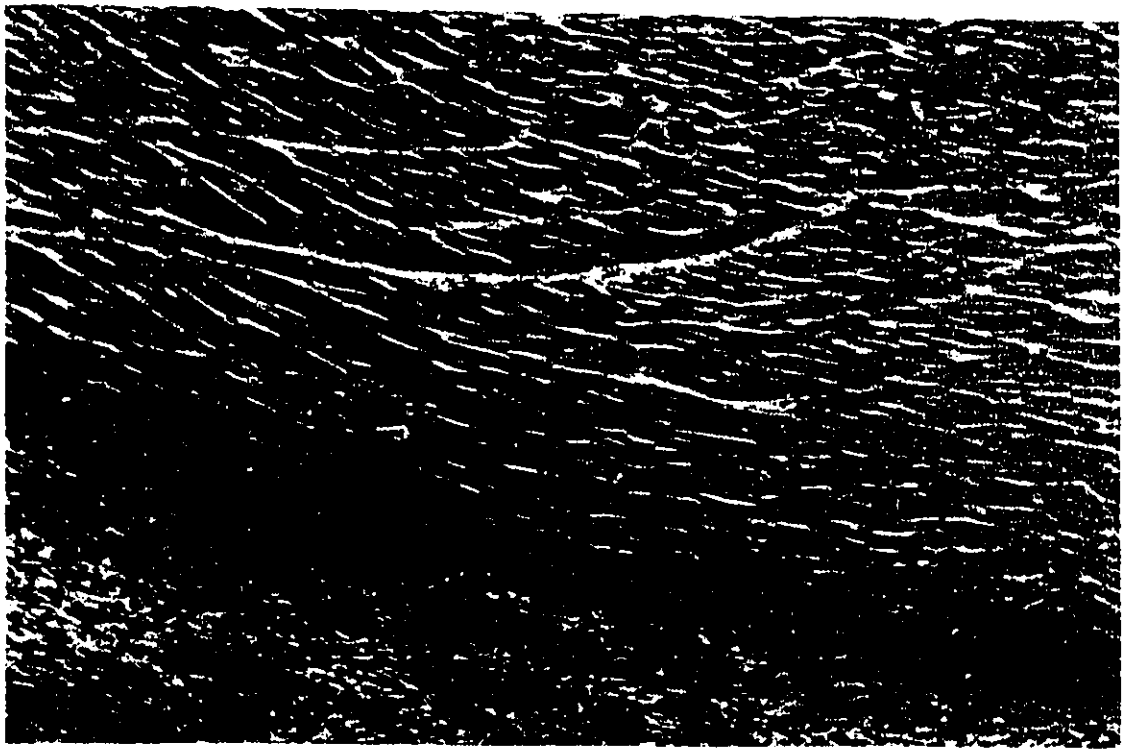


Figure 3.14.2

Photomicrographs illustrating the boundary of the cleared zone of the infarct.

Top photo: hematoxylin-eosin stain. 7-day-old infarct. Note the relatively sharp boundary zone and the abundant infiltration with inflammatory cells.

Bottom photo: Herovici's polychrome stain, 14-day-old infarct. Note the abundance of fibroblasts in the cleared zone adjacent to a zone of myocytes which have not been cleared or infiltrated.



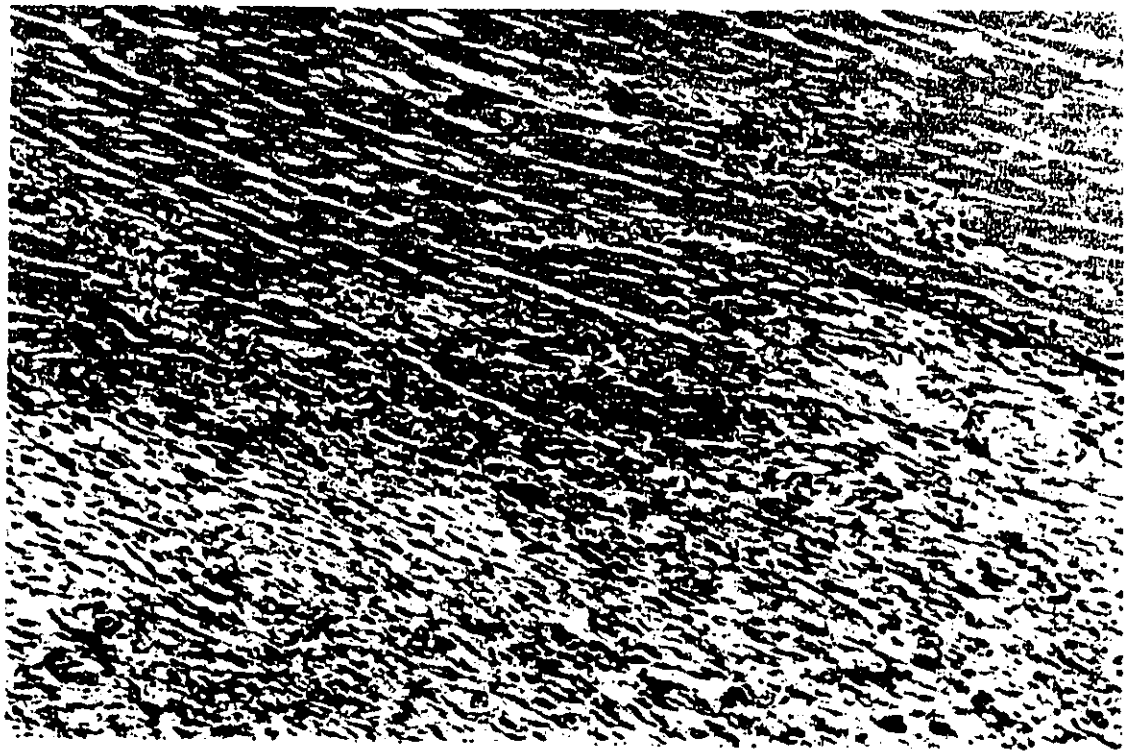


Figure 3.14.3

Photomicrograph illustrating the presence of red blood cells (stained red) in the region where active myocyte clearing and collagen synthesis are occurring. This section was taken from a 14-day infarct stained with Mallory Trichrome (magnification X 200).

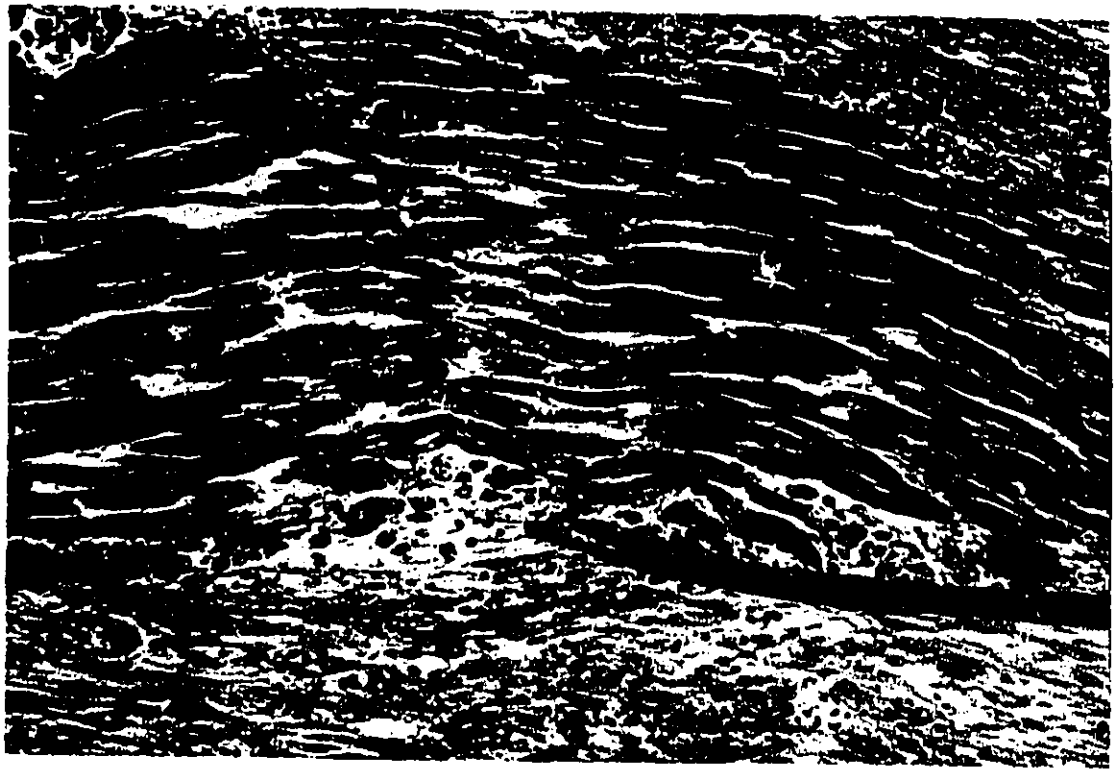


Figure 3.14.4

Photomicrograph illustrating the first "new" collagen to appear in the healing infarct. It is fine and is randomly oriented. New collagen appears blue, fibroblasts black, and red blood cells yellow. The section was taken from a 21-day infarct stained with Herovici Collagen Stain (magnification X 400).

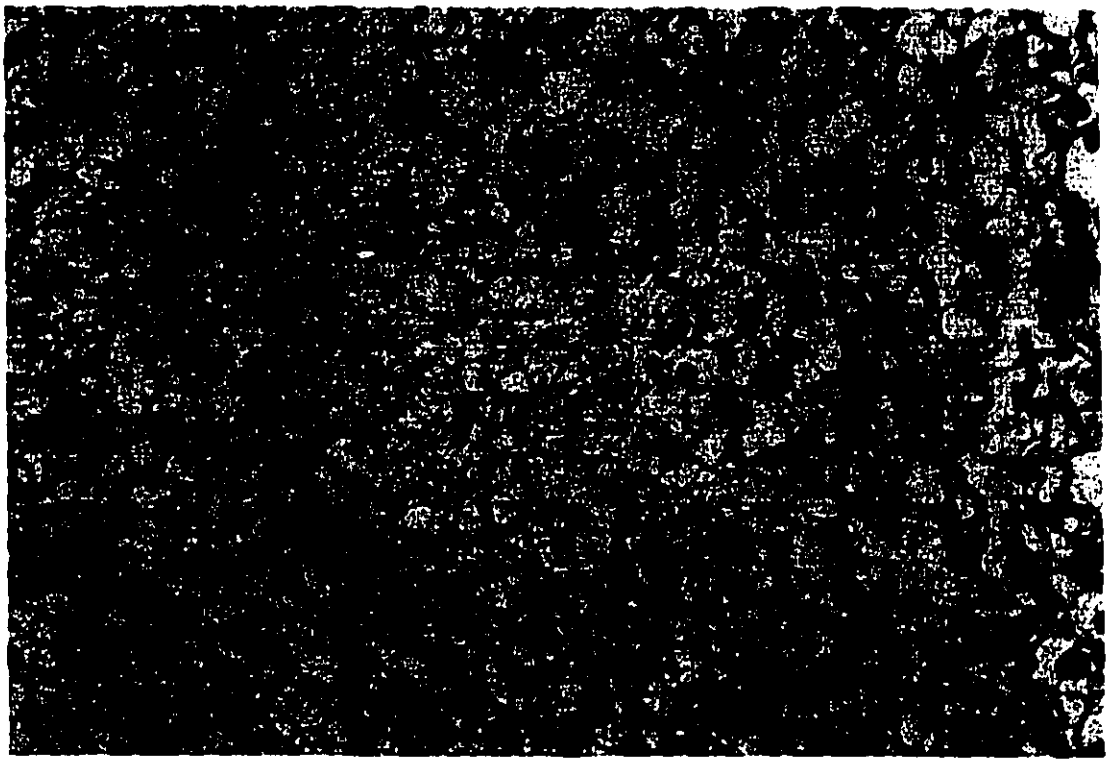


Figure 3.14.5

Photomicrograph illustrating the appearance of "mature" collagen in the latter stages of healing. This collagen is composed of wavy fibers which are oriented parallel to the circumference of the heart. This section was taken from a 7-day infarct stained with Gomori's Silver Stain (magnification X 630).

Figure 3.14.6

Photomicrograph illustrating the subsequent appearance of "mature" collagen. This "mature" (red staining) collagen appears later than the "new" (blue staining) collagen. The density of both fibroblasts (stained black) and red blood cells (stained yellow) are greater in the vicinity of the "new" collagen as compared to the "mature" collagen. This section was taken from a 21-day infarct stained with Herovici Collagen Stain (magnification X 630).

Figure 3.14.7

Photomicrograph illustrating the presence of fibroblasts in the vicinity of black staining reticulin (Type III) collagen fibers. This section was taken from a 28-day infarct stained with Gomori Silver Stain (magnification X 400).

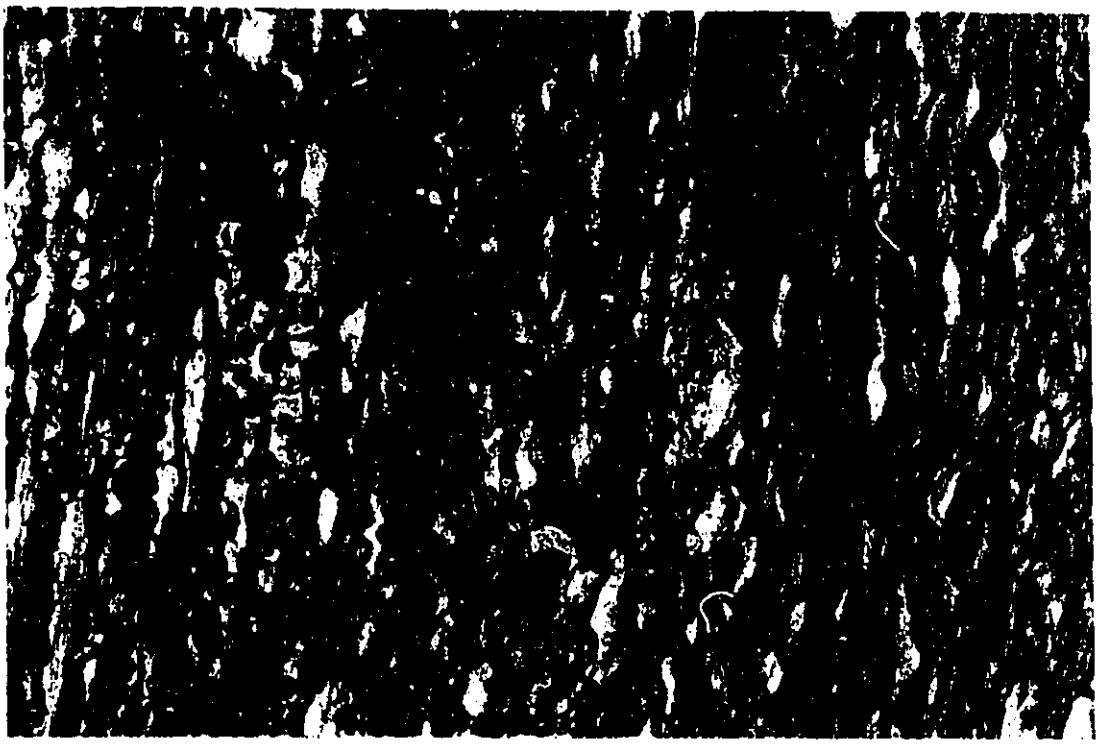


Figure 3.14.8

Photomicrograph illustrating the presence of striations and orderly appearance of uncleared myocytes. This section was taken from a 7-day infarct stained with Herovici Collagen Stain (magnification X 400).

Figure 3.14.9

Photomicrograph illustrating the complete disruption of myocytes in a 14-day old infarct. Compare to Figure 3.14.8. It was stained with Herovici Collagen Stain (magnification X 630).

Figure 3.14.10

Low-magnification photomicrograph illustrating the presence of different stages of healing in a 28-day old infarct. Different collagen types are visible, as well the core has not yet been cleared and replaced with scar tissue. The section was stained with Herovici Collagen Stain (magnification X 25).

3.4 MECHANICS

Regional strips of tissue from infarcted and noninfarcted regions of the heart were tested over two ranges; a low and a high strain range, representing maximum length increases of approximately 20 and 60% respectively. The stiffness was calculated as the slope of the line between stress and strain (r^2 values > 0.90). All strips displayed a stress-relaxation after a rapid length increase. This allowed four stiffness measurements for each strip to be determined. There was an initial and final stiffness at each strain cycle.

The strips were cycled three times over each range, and the stiffness determined at all cycles. The results of the cycles were compared, and of the sixteen groups, in eight there were no significant differences between repetitive cycles, in six groups, cycle 1 was different from 2 and 3, in one group cycles 1 and 2 were different from 3, and in 1 group the location of the difference could not be identified. This would suggest that strips were conditioned after the first cycle, and no further changes occurred with subsequent cycling over the same strain ranges. These results are summarized in ANOVA Tables 6.11.a,b,c,d.

3.4.1 STIFFNESS IN THE CONTROL GROUP

The relationship between stress and strain was not linear, over the entire 0 to 60% strain range. This was apparent as the calculated stiffness determined over the high strain range was greater than that over the low range, (see Table 3.12).

The initial stiffness measured over the low-strain range was similar in both walls, 5.7 ±0.7 in the free wall, and 7.0 ±1.4 in the septal wall. Both relaxed a similar amount, the final stiffnesses being 3.7 ±0.6 and 4.7 ±0.7 respectively. Therefore, the elastic component of stiffness was 64 and 67% of the total stiffness, for the free and septal walls, when calculated over the low strain range. The stiffness

measured over the high-strain range was also similar in both walls, 26.9 ±3.2 in the free wall, and 23.9 ±5.0 in the septal wall. The elastic component of the stiffnesses measured were 63 and 69% in the two locations. There was no measureable difference in the initial or final stiffness of the free and septal walls at either strain range in the control group by ANOVA. The elastic and viscous components were also comparable in the two regions and were similar during low-strain and high-strain tests.

3.4.2 STIFFNESS IN NONINFARCTED REGIONAL STRIPS

A general trend in stiffness of the noninfarcted regional strips was observed over all four ranges, low and high, initial and final (see Tables 3.13 and 3.14). Twenty-four hours after coronary occlusion the stiffness increased above control. This was followed by a small decrease in stiffness between three to seven days, then a subsequent stiffening in later groups, rising above that of the control group. However, these changes were only small and statistically not significant by ANOVA (see Table 6.1.12). The variances of the latter groups, (5, 6, and 7), however, were greater than the control group. This implies that these groups were not similar to the control group.

3.4.3 STIFFNESS IN INFARCTED REGIONAL STRIPS

Although there was no difference in either the absolute strain of any group, or the maximal stresses, there were differences in tensile strength. This was evidenced by the rupture of four of the six full wall strips from three day old infarcts, whereas no ruptures occurred at any other times. However, the strips which fractured, operated within "physiological" strain ranges. They tore during the first high-strain cycle when the strain was increased to values significantly greater than would be seen *in vivo*.

There were no significant changes observed in the stiffness of the full wall infarcted regional strips over either strain range (see

Tables 3.13 and 3.14). The measured stiffness increased slightly but not significantly over both the low and the high strain ranges (see ANOVA Table 6.1.13). The variance of the later groups is greater than the control group.

The general pattern of the infarcted tissue, was not as uniform as that observed with the noninfarcted regional strips. Over the low-strain range both components of stiffness increased slightly but not significantly rising approximately 20% above the control group by twenty-eight days (see Table 6.1.13).

Over the high-strain range, the measured stiffnesses were variable, fluctuating slightly with each group. At twenty-eight days the mean initial and final stiffnesses had increased slightly but not significantly (see ANOVA Table 6.1.13) The variance of the later groups was greater than that of the early ones.

The stiffness of the infarcted epicardial strips increased significantly by approximately two fold, between the control group and the twenty-eight day infarcts, over the low-strain range for both the initial and final stiffnesses (see Tables 3.15 and 3.16). The increased stiffness measured over the high-strain range was approximately 40% above the control group for both the initial and final stiffness but these were not significant by ANOVA (see ANOVA Table 6.1.14).

There was no appreciable change in either component of stiffness of the infarcted endocardial regional strips when tested over the low-strain range (see Tables 3.15 and 3.16). Over the high-strain range, the stiffness increased by approximately 50% from twenty-four hours to twenty-eight days. These changes were not significant (see ANOVA Table 6.1.15).

3.4.4 COMPONENTS OF STIFFNESS

Although there were no significant changes in the total "initial" or "final" stiffness in either the infarct or the noninfarct tissue, there were significant changes in the contribution of the individual components of stiffness. In the control group the elastic component of the total stiffness was comparable in both the free and septal walls. The values for the infarcted and noninfarcted walls gradually diverged, the two regions becoming significantly different from each other three days after the occlusion (see Figure 3.15). By twenty-eight days after the occlusion, the percentage of the elastic (final) stiffness in noninfarcted tissue had decreased 9% over the low strain range, and 6% over the high strain range (See Table 3.17). The opposite trend was observed in infarcted tissue. The contribution of the elastic stiffness rose 4% over the low range and 6% over the high range above the control (see Table 3.18). Although neither of these change were significantly different from the control group (see ANOVA Table 6.1.16), after day three the infarct zone displayed a significantly greater elastic component of stiffness than the noninfarct zone for all time periods.

TABLE 3.12:
SUMMARY OF THE REGIONAL STIFFNESS OF THE CONTROL GROUP

	FREE WALL				SEPTAL WALL			
VARIABLE	N	MEAN	S.E.	VAR	N	MEAN	S.E.	VAR
low initial	4	5.71	0.65	1.71	3	6.96	1.37	5.65
low final	4	3.67	0.61	1.49	3	4.65	0.68	1.41
high initial	4	26.95	3.24	42.24	3	23.93	4.99	74.84
high final	4	17.30	1.99	15.95	3	16.59	3.02	27.29

The units of "stiffness": g/cm²/percent.

Regional strips were stressed over two ranges and the stiffness over each range was calculated. Values presented are the mean stiffness (Δstress/Δstrain) of each group of animals. "Low" and "high" refer to the range of strains applied, 20 and 60% respectively of the initial length. "Initial" refers to the stiffness measured immediately after the extension, "final" refers to the stiffness measured after stress-relaxation was completed.

Low initial - the initial stiffness measured over the low strain range
Low final - the final stiffness measured over the low strain range
High initial - the initial stiffness measured over the high strain range
High final - the final stiffness measured over the high strain range

TABLE 3.13:
STIFFNESS OVER LOW STRAIN RANGE

TABLE 3.13.1: INFARCT ZONE

GROUP	VARIABLE	N	MEAN	S.E.
1	initial	4	5.71	0.65
	final	4	3.67	0.61
2	initial	5	6.40	1.32
	final	5	4.83	0.76
3	initial	6	6.74	0.93
	final	6	4.85	0.67
4	initial	6	6.25	0.78
	final	6	4.61	0.54
5	initial	6	10.13	2.09
	final	6	8.01	1.79
6	initial	6	6.94	1.52
	final	6	4.52	0.97
7	initial	7	6.75	1.40
	final	7	4.83	0.77

TABLE 3.13.2: NONINFARCT ZONE

VARIABLE	N	MEAN	S.E.
initial	3	6.96	1.37
final	3	1.89	0.68
initial	5	11.24	3.87
final	5	6.16	1.92
initial	6	4.98	1.05
final	6	3.19	0.74
initial	6	6.35	1.33
final	6	3.94	0.78
initial	6	8.87	1.24
final	6	5.48	0.72
initial	6	10.36	2.87
final	6	6.26	1.67
initial	6	9.45	2.21
final	6	5.82	1.44

Regional stiffness was determined in infarcted and noninfarcted tissues. Strips were strained with small, equal increments in length from 0 to 20% above resting length. The initial stiffness after extension, and the final stiffness after stress-relaxation, were determined for each strip. Values presented in the table are means.

TABLE 3.14:
STIFFNESS OVER HIGH STRAIN RANGE

		"INFARCT ZONE"			"NONINFARCT"		
GROUP	VARIABLE	N	MEAN	S.E.	N	MEAN	S.E.
1	initial	4	26.95	3.24	3	23.93	4.99
	final	4	17.30	1.99	3	16.59	3.02
2	initial	5	25.80	3.33	5	33.56	13.26
	final	5	18.89	2.21	5	22.83	8.61
3	initial	2	31.73	0.01	6	19.66	4.59
	final	2	25.04	0.14	6	12.54	2.75
4	initial	6	23.87	50.08	6	23.27	3.08
	final	6	18.20	39.20	6	15.36	2.18
5	initial	6	32.27	112.57	6	29.15	5.45
	final	6	25.12	56.21	6	18.06	3.38
6	initial	6	24.75	194.75	5	23.96	3.17
	final	6	18.26	110.64	5	15.05	1.89
7	initial	7	29.73	161.36	5	37.46	10.75
	final	7	21.12	84.33	5	18.11	4.98

Regional stiffness was determined in infarcted and noninfarcted tissues. Strips were strained with small, equal increments in length from 20 to 60% above resting length. The initial stiffness, and the final stiffness were determined for each strip. Values presented in the table are means.

TABLE 3.15:
REGIONAL STIFFNESS OVER THE LOW STRAIN RANGE

		INFARCT-EPICARDIAL REGION			INFARCT-ENDOCARDIAL REGION		
GROUP	VARIABLE	N	MEAN	S.E.	N	MEAN	S.E.
2	initial	5	11.35	2.90	5	13.68	2.59
	final	5	8.18	2.04	5	9.86	1.91
3	initial	2	9.02	0.71	2	11.51	4.64
	final	2	6.54	0.13	2	8.21	3.33
4	initial	5	12.54	1.36	5	19.13	3.95
	final	5	9.01	1.32	5	14.23	2.93
5	initial	5	20.29	5.06	5	14.29	2.33
	final	5	14.81	3.97	5	10.50	1.78
6	initial	6	9.47	1.50	8	10.76	2.49
	final	6	7.20	1.53	8	7.33	1.70
7	initial	6	23.35	3.84	6	12.93	2.56
	final	6	16.30	2.87	6	9.46	2.08

Regional stiffness was determined in epicardial and endocardial infarcted strips. Strips were strained by small equal increments in length, 0-20% above resting length. Values presented are the group means. "Initial" refers to the stiffness measured immediately after the extension, "final" refers to the stiffness measured after stress-relaxation was completed.

TABLE 3.16:
REGIONAL STIFFNESS OVER THE HIGH STRAIN RANGE

		INFARCT-EPICARDIAL REGION			INFARCT-ENDOCARDIAL REGION		
GROUP	VARIABLE	N	MEAN	S.E.	N	MEAN	S.E.
2	initial	5	35.44	10.81	5	35.13	11.07
	final	5	26.10	7.82	5	25.28	7.93
3	initial				1	72.60	
	final				1	57.38	
4	initial	5	52.76	3.96	4	72.04	15.83
	final	5	40.32	3.68	4	51.65	9.66
5	initial	5	46.24	11.12	5	45.82	5.65
	final	5	40.32	13.44	5	32.93	4.25
6	initial	6	36.22	4.48	6	36.45	6.96
	final	6	26.10	3.45	6	31.56	4.57
7	initial	7	49.23	9.24	6	54.24	17.88
	final	7	38.76	10.24	6	39.15	12.74

Regional stiffness was determined in epicardial and endocardial infarcted strips. Strips were strained by small equal increments in length from 20-60% above resting length. Values presented are the group means. "Initial" refers to the stiffness measured immediately after the extension, "final" refers to the stiffness measured after stress-relaxation was completed.

TABLE 3.17:

ELASTIC COMPONENT OF STIFFNESS IN NONINFARCTED TISSUE

GROUP	VARIABLE	N	MEAN	S.E.
1	low	3	68.45	4.44
	high	3	70.62	4.23
2	low	5	58.41	3.35
	high	4	63.66	1.08
3	low	7	63.45	1.69
	high	7	64.44	1.11
4	low	6	62.77	1.11
	high	6	66.22	3.52
5	low	6	62.28	1.59
	high	6	63.21	4.67
6	low	8	59.72	1.38
	high	5	75.29	13.10
7	low	7	59.85	3.66
	high	4	63.82	1.57

All strips displayed stress-relaxation. The amount the tension decreased as a percentage of the initial tension was calculated for each animal. This reflects the elastic component of total stiffness. The values presented are the means per group.

The variables used are: "low", for low strain range, and "high" for high strain range.

TABLE 3.18:
ELASTIC COMPONENT OF STIFFNESS IN INFARCTED TISSUE

GROUP	VARIABLE	N	MEAN	S.E.
1	low	4	64.06	7.13
	high	4	64.45	4.10
2	low	5	79.47	9.41
	high	5	73.99	2.90
3	low	7	70.77	5.71
	high	1	77.78	
4	low	6	74.07	0.63
	high	6	75.72	1.79
5	low	6	78.14	4.90
	high	6	78.57	1.52
6	low	8	64.69	0.88
	high	8	72.84	0.98
7	low	8	67.94	2.00
	high	8	70.29	1.29

All strips displayed stress-relaxation. The amount the tension decreased as a percentage of the initial tension was calculated for each animal. This reflects the elastic component of total stiffness. The values presented are the means per group.

The variables used are: "low", for low strain range, and "high" for high strain range.

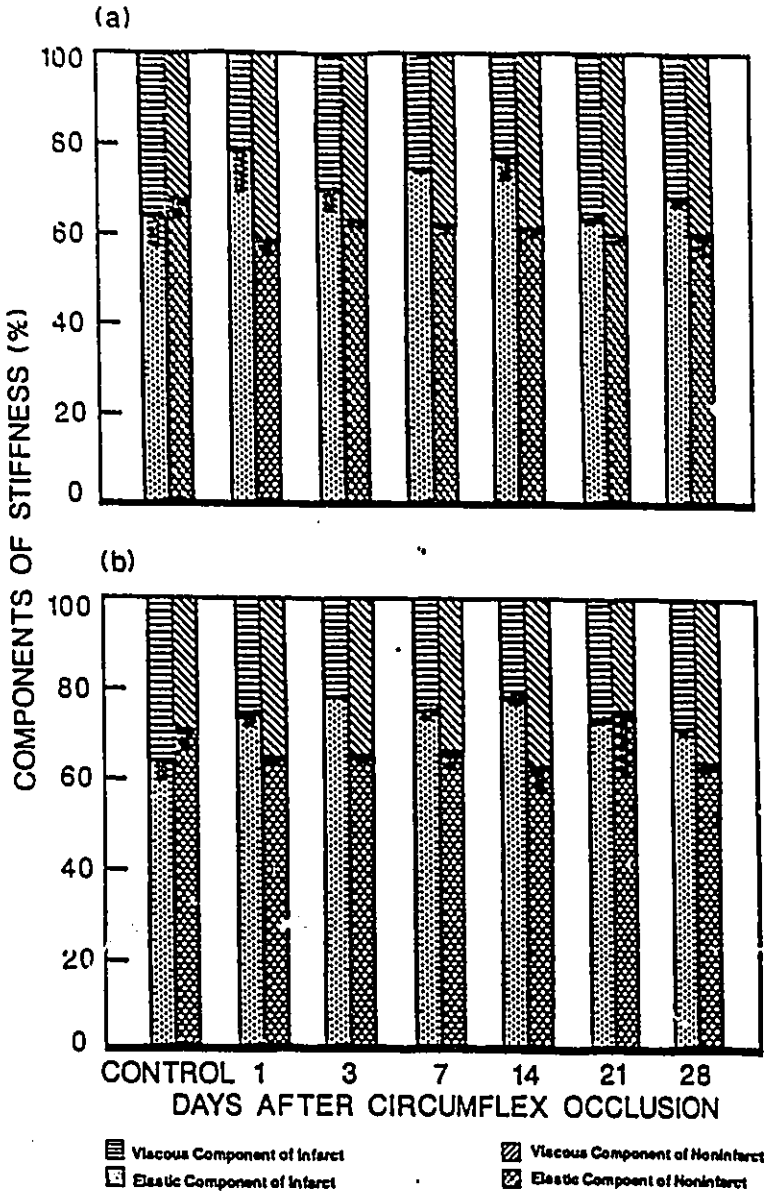


Figure 3.15

Summary of the components of stiffness in groups of pigs sacrificed at various times after myocardial infarction. Figure (a) represents stiffness determined over a 0 - 20% strain range, Figure (b) represents stiffness determined over a 20 - 60% strain range.

3.5. SUMMARY

The following is a brief review of the major findings of this study:

1. There were no significant differences between the free and septal wall of the myocardium in the control group animals, for any parameter measured.

2. Blood flow to the noninfarcted region of the myocardium increased significantly in the early period after permanent occlusion (one, three, seven or fourteen days), then returned to control values by twenty-one days after occlusion.

3. Blood flow to the infarcted region fell to 6.5% of the preocclusion flow ten minutes after occlusion. It rose above this immediate post-occlusion value by twenty-four hours after infarction. It declined slowly thereafter.

4. The tissue concentration of microspheres which had been initially injected in the infarct zone rose above that in the noninfarct zone becoming significantly concentrated fourteen days after infarction.

5. The water content of noninfarcted tissue remained constant throughout the experimental period, that of the infarcted tissue rose significantly at one day after infarction and remained elevated for at least twenty-eight days.

6. Hydroxyproline, an amino acid marker for collagen, increased in both the noninfarcted and infarcted tissue. The increase was significant twenty-eight days after permanent circumflex occlusion in the noninfarcted tissue and after fourteen or twenty-one days in the infarcted region, depending upon the technique used to measure it. A substantially greater rise in hydroxyproline content was observed in the infarct zone than in the non-infarct zone.

7. 3-Methylhistidine, an amino acid marker of cardiac actin, as measured by HPLC, remains detectable in both regions of the heart during the twenty-eight day period after myocardial infarction. There is additional non-quantitative evidence suggestive of the continued presence of contractile protein in the infarct area by antigenically identifiable contractile proteins in twenty-eight day infarcted tissues from the SDS-PAGE and monoclonal antibody experiments, and the histological presence of cross striations in this region.

8. "New" collagen was first visible histologically at seven days after permanent occlusion in the infarcted zone. It appeared fine and was randomly oriented. "Mature" collagen initially appeared at seven days post occlusion, and was present in all samples by twenty-one days. It appeared to be present in an organized fashion, with fibers running parallel to the circumference of the myocardium.

9. The heart displayed a visco-elastic behavior manifested by stress-relaxation following a rapidly applied strain.

10. Only regional strips from three day infarcted zones ruptured, suggesting an initial but transient decrease in tensile strength of the infarcted region.

11. There were no significant changes in regional stiffness of infarcted or noninfarcted myocardium, during the first twenty-eight days after permanent circumflex occlusion in this model.

12. There was an apparent but nonsignificant trend for an increased elastic component of stiffness in infarcted specimens, and an increased viscous component of stiffness in noninfarcted regional strips, which occurred during the first twenty-eight days after myocardial infarction. Although the values were not different from control, the divergence of these parameters between infarct and non-infarct zones was significant after three days post-occlusion.

13. In some variables, at various times, there was a striking increase in the variance about the mean value.

4.0 DISCUSSION

Several changes occur in the myocardium in response to the presence of a large infarction of the left ventricle. This study has attempted to describe the time course of some of these changes in both infarcted and noninfarcted regions of the heart. The following discussion will highlight these changes, suggest some mechanisms and implications and will attempt an integration of some observations.

This study examined the healing which occurred over the first twenty-eight days after occlusion. It became apparent that due to the nature of the changes occurring within the tissue, some techniques used to analyse the control tissues were not valid in the late experimental groups. This was clearly apparent in the sections dealing with the blood flow determinations and biochemical analysis. The issue of a continuously changing reference base has been addressed in the blood flow literature and summarized in Section 1.3.2.1.

4.1 BLOOD FLOW

4.1.1 METHODOLOGIC CONSIDERATIONS

The use of radioactive-labelled microspheres has been the "gold standard" technique for the measurement of regional blood flows for over twenty years. The technique fulfills the requirements of a good tracer (Heymann *et al.*, 1977). Microspheres are small, inert, nontoxic particles, which mix evenly with blood. Their introduction into the left heart does not alter hemodynamics significantly, nor does it alter blood flow distribution patterns. Microspheres recirculate only to a small extent, but are essentially trapped on their first pass. The calculation of regional blood flow measurements is easily accomplished by counting the specific radioactivity in a tissue sample. The only significant technical problems are related to incomplete dispersal of the spheres before their injection, resulting in the trapping of aggregates which may overestimate flow in a specific tissue or organ. A technical problem may be introduced during counting when large

numbers of nuclides with overlapping energy spectra are used. In our experiments the use of only three nuclides with distinctly different energy peaks allowed the efficient correction of spillover effects, in spite of the fact that different specific activities were present in a given tissue sample.

This technique allows for the repeated measurements of regional blood flows. Experimental interventions therefore can be interspersed between microsphere injections and their effect on regional blood flows assessed. Long term measurement of flow depends on the assumption that the tissue concentration of microspheres is unchanged. Changes in the tissue concentration of microspheres, however may occur because of either an absolute loss of microspheres (e.g., by the "milking" action of continual contraction - relaxation cycles resulting in the loss of small sized spheres (less than 9 microns), or by changes in the tissue in which the microspheres were initially trapped, (e.g., edema, hypertrophy, contraction) (Cohen, 1985, Consigny *et al.*, 1982, Jugdutt *et al.*, 1979). Studies where blood flow measurements were calculated eight days after occlusion reported that the ratio of preocclusion infarct to noninfarct zone blood flow was less than 1.0, implying a possible loss of microspheres (Reimer and Jennings, 1979). In contrast, studies lasting longer than this, reported that the relative ratio between the two areas was greater than 1.0, suggesting a gain of spheres (Consigny *et al.*, 1982, Kiewiet de Jonge *et al.*, 1980, Murdock and Cobb, 1980, Reimer and Jennings, 1979). These discrepant findings suggest the likelihood that changes in the tissue where the spheres originally distributed had occurred, rather than a subsequent absolute loss or gain of spheres. Although minimal loss cannot be excluded, there is no substantiation that there is any continual loss of microspheres which are 15 microns in diameter, the size used in this experiment (Cohen, 1985). The edema which is known to occur results in an initial dilution of microspheres, accounting for the infarct to noninfarct ratio of less than unity, and suggests that initial flow measurements underestimate true flow. As an absolute gain is an unlikely event, the subsequent apparent concentration of spheres is due to actual loss of tissue in which they were dispersed.

The findings in this study indicated that mean blood flow in the control pigs was approximately 1.30 ± 0.03 ml/min/gm wet weight tissue. The observed blood flow distribution pattern in the control group was consistent with those reported in the literature for pigs (Cohen, 1985, Fedor *et al.*, 1978, Fujiwara *et al.*, 1982, Sakai *et al.*, 1985, Sjoquist et al., 1984). The presence of both a temporal and spatial regional heterogeneity of flow was also consistent with the literature, as summarized by Duling and Damon (1987). Temporal and spatial distributions between 20 - 40% are considered normal (Bassingthwaighthe *et al.*, 1987). The evenness of the microsphere mixing was assured in these experiments as regional distributions were maintained with serial injections (i.e. endocardial to epicardial blood flow ratios). Our data obtained from the control group agree with the published reports of blood flow distribution patterns in the anaesthesized pig. The measurements made in the control group were not subject to the above dilution or concentration errors, since no time elapsed between injection and subsequent bloodflow determination.

4.1.2 TIME COURSE OF CHANGES IN THE NONINFARCTED REGION

The microspheres injected just prior to sacrifice were used to obtain information on the time course of regional blood flow changes to the infarcted and noninfarcted regions. These flows were compared to those of the control group, to determine if any absolute changes in regional flow occurred over the duration of the experiment. An absolute and significant increase in blood flow above that to the control group was observed in the noninfarcted regions of the hearts from pigs sacrificed one, three, seven or fourteen days after occlusion. After twenty-one days, there were no measurable differences from the control group.

This observation of an increased flow after coronary occlusion to the noninfarcted region agrees with Savage *et al.* (1981), who reported an increased flow two days after permanent coronary artery occlusion. The present study has extended the findings, and it now appears that

flows remain elevated for between two to three weeks after coronary occlusion.

The increased flow to the nonischemic region is probably a reflection of the increased workload this area assumes immediately after occlusion (Theroux *et al.*, 1977, Gilbert and Glantz, 1989). The increased workload likely demands an increased oxygen supply (Guyton and Daggett, 1976, Savage *et al.*, 1981, Yin, 1981). Since increasing oxygen extraction is generally limited in the heart, increased coronary blood flow, is likely the major means of satisfying such augmented demands (Klocke, 1976). Although a slight increase in oxygen extraction of about 2 - 3% after coronary vessel occlusion is reported (Driscol and Eckstein, 1967), this reserve alone does not supply sufficient oxygen to meet an increased demand; therefore, an increased absolute flow to the noninfarcted zone would be the most beneficial method of increasing oxygen supply.

A compensatory hypertrophy of the noninfarcted zone in response to infarction has been documented in several species (Roberts *et al.*, 1984, Rubin *et al.*, 1983). It is likely that the present findings of increased blood flow in the noninfarcted zone are reflecting this process. There would be a need for increased blood flow both during the initial period after the infarction when the workload of the remaining viable myocytes was increased and during the subsequent process of hypertrophy, when the workload per myofibril fell, as new myofibrils were added by the myocyte. When the degree of hypertrophy was sufficient to compensate for the lost tissue mass, (i.e. by about twenty-one days) the work per filament would be normalized and consequently so would the blood flow and oxygen requirements. This process might explain the early increase in flow, followed by subsequent normalization in the noninfarcted region and is in agreement with Thomas *et al.* (1984) and Marchetti *et al.* (1973) who reported no change in the resting blood flows on a per-gram basis in the hypertrophied heart.

4.1.3 TIME COURSE OF CHANGES IN THE INFARCTED REGION

Within ten minutes, flow to the infarcted region had decreased to approximately 6% of its preocclusion level. A modest increased flow to the infarcted region at twenty-four hours above the ten minute post-occlusion level occurred but it was still significantly less than the preocclusion value. This was sustained for three to seven days after the occlusion then declined .

This increased blood flow observed after the infarction is consistent with both the histological and the blood flow literature (Cohen, 1985, Mallory *et al.*, 1939, Savage *et al.*, 1981). The flow is associated histologically with the influx of white cells and fibroblasts to the region. This observed pattern of an increased flow above the immediate post-occlusion levels followed by a gradual decline is also consistant with Cohen (1985), who reported that an eventual regression of vessels was seen histologically. It would appear therefore, that once the necrotic area is cleared and replacement tissue laid down, the flow requirements of the tissue decrease in parallel with this activity. At twenty-eight days the flow to the infarcted tissue was still above that evident at ten minutes. However as none of the infarcts in this experiment were completely healed at twenty-eight days, as observed histologically, it would be reasonable to assume that the damaged area was still undergoing continuing reparative changes and there was still a need for a blood supply to the infarct zone at levels higher than the presumably minimal flows one would expect in a completely healed, metabolically inactive scar. This explanation is consistant with our histological results. In our experiments red blood cells were identified in the infarct regions. They appeared most concentrated near the zone where myocyte clearing and new collagen synthesis were occurring. Very few were seen in "older" parts of the scar (see Figure 3.14.6).

The use of microspheres allows for examination of the regional distribution of blood flow. Reports in the literature describe the immediate transmural decrease in blood flow to the infarcted zone in the pig (Fedor *et al.*, 1978, Fujiwara *et al.*, 1982, Hearse *et al.*, 1986, McDonough *et al.*, 1984, Sakai *et al.*, 1985, Sjoquist *et al.*, 1984). After the coronary artery is occluded, the endocardial to epicardial ratio approaches unity, and the infarction is transmural. This ratio is informative in the control tissues and very early infarctions, yielding information on the regional distribution of flows and on the lack of regional sparing of myocardium. In some species, (i.e. the dog) which have good collateral blood flow, this ratio is more informative. The infarction is usually just endocardial because the collateral flow preserves the epicardial tissues. However, in the pig where the infarction is transmural, and healing occurs at variable rates, this ratio does not actually yield useful information. Changes in the ratio could be artifact due to differential thinning; the epicardial and endocardial layers may shrink at different rates. As well the ratio clearly does not reflect the transmural viability or necrosis of myocytes. This is best exemplified by examination of Table 3.3. An endocardial to epicardial post-infarction blood flow ratio of 0.5 (BF3), implies that the epicardial region is receiving twice the flow of the endocardial. However if the flow to the epicardial region is incompatible with tissue viability, the necrosis will be transmural regardless of the endocardial-to-epicardial ratio. In these experiments transmural infarctions were produced in all pigs. Consequently the relative endocardial:epicardial flow is informative in normal and newly infarcted tissue, however in "older" infarcted tissue still undergoing healing, it is best to examine the absolute values of immediate presacrifice flows.

4.1.4 EVIDENCE OF CHANGING TISSUE REFERENCE

The preocclusion blood flow determinations to noninfarcted regions were not equal in all groups. Flows measured in the early groups (2 and 3), were significantly higher than the control, and flows measured in

the later groups (6 and 7), were significantly lower than control. There is no clearly evident reason for the preocclusion blood flows to be truly different. The decreased preocclusion flow in noninfarcted tissue from groups 6 and 7 is consistent with the dilution of microspheres occurring because of the development of a compensatory hypertrophy of this region (Reimer and Jennings, 1979). Microsphere dilution is also seen with the ten minute post-occlusion flow measurement to the noninfarcted region in the same two groups, as would be expected. Both sets of microspheres were in the tissue for the same amount of time, and would therefore be subject to the same degree of dilution.

Besides the hypertrophy of the noninfarcted tissue, the infarcted area is also changing. It undergoes wall thinning initially due to the disruption of the collagen matrix (Weisman and Healy, 1987), and subsequently by the removal of necrotic tissue (Fishbein *et al.*, 1978). These temporal regional changes in tissue mass and composition increase the concentration of microspheres trapped within it, and consequently the calculated blood flow measurements from BF1 and BF2 may overestimate true flows.

Regional changes are apparent by examining the ratio of initial blood flows (BF1) in "infarcted" and "noninfarcted" tissue in all groups. Generally, over the course of this experiment, microspheres appear to have been diluted in the noninfarcted tissue, and concentrated in the infarcted tissue. The extent of these changes is not the same on a per square unit basis in both regions. The infarction theoretically removed 25-30% of the myocytes in the left ventricle (Weaver, 1986), leaving the remaining 70-75% of the original tissue to accomodate the loss. It is probably valid to expect that on a per gram basis, infarct shrinkage will concentrate microspheres to a greater extent than hypertrophy will dilute them.

The microspheres in theory can be used as a marker of tissue shrinkage as they were placed in the tissue prior to infarction, and

remained there while the healing process was occurring. However, there were significant variabilities in control flows which could not be accounted for, therefore it was not possible to assign the relative contribution of concentration and dilution. Rather we can only report the combined effect. It is also possible that the spheres could be used as a marker if all that had been done was a blood flow analysis of the tissue. However in this experiment mechanics, biochemistry and histology were also examined, and the absolute amount of tissue required for each measurement was relatively constant. Because the infarcted area contracts with healing there was less tissue to work with in older infarcts. It is possible that the tissue samples taken for flow determination in the later groups were taken further from the core than the original and therefore are not concentrated to the same degree.

4.2.1 MEASUREMENT OF MARKER AMINO ACIDS

Hydroxyproline and 3-methylhistidine are unique amino acid markers for collagen (Berg, 1982) and actin (Asatoor and Armstrong, 1967) respectively. Using specific biochemical techniques it was possible to quantitate these amino acids with reasonable accuracy, although those of low abundance, particularly 3-methylhistidine, represented a technical challenge. As each were expressed as the proportion of the total amino acid pool, the choice of the protein standard became an important consideration.

The present "gold standard" to determine the amount of collagen present within a sample is to quantitate the amount of hydroxyproline. Also a protein assay is done, so that the concentration of hydroxyproline can be expressed as a percentage of the total protein. The protein assay most often used is the Lowry procedure. The methods initially chosen in these experiments were the Berg protocol to measure hydroxyproline, and the Lowry assay to measure total protein, and the results obtained, particularly the increased hydroxyproline in the infarcted samples, were exactly comparable with those reported in the literature (Jugdutt *et al.*, 1986). However, close examination of the Lowry protein results showed that the amount of protein being recovered from the acetone powders of infarcted tissues was decreasing as the infarct aged. This therefore introduced some skepticism to the validity of the protein assay results. The initial assumption of the Lowry assay is that all proteins in the tissue sample are soluble. However, mature type I collagen is relatively insoluble, and consequently difficult to work with. As the absolute concentration of collagen in the tissues increased over the twenty-eight day healing period, less of the total protein of the sample was therefore actually measured by this assay. Consequently, as the hydroxyproline was measured accurately with the Berg protocol, and the total protein was underestimated by the Lowry method, the concentration of hydroxyproline was overestimated, especially in infarcted tissue samples.

It was therefore necessary to repeat these measurements using a more appropriate protein assay. The OPA technique was specifically chosen because the measurement of both soluble and insoluble proteins is possible with equal accuracy and reproducibility (Peterson, 1983). Because all protein is hydrolysed to constituent amino acids prior to assay, the method is unaffected by protein solubility. As all the protein in the sample was included in the assay, the expression of hydroxyproline content became more accurate. Consequently, although others have used the Lowry protein assay, the OPA method should be the technique of choice for examination of tissues with a large insoluble protein content, particularily in infarct healing which involves deposition of large amounts of insoluble collagen.

4.2.1.1 HYDROXYPROLINE IN THE NONINFARCTED REGION

Hydroxyproline as a percentage of all amino acids in the control group of animals is comparable to that reported in the literature (Caspari *et al.*, 1978, Chiarello *et al.*, 1986, Fredericksen *et al.*, 1978, Gevers, 1984, Montfort and Perez-Tamayo, 1962). The similarity between the free and septal walls is also consistent (Cannon *et al.*, 1983, Frederiksen *et al.*, 1978).

In the noninfarct region an initial 25% decrease in the percentage of hydroxyproline was observed one day after the occlusion (using the Berg assay). This was followed by a 30% increase over the control group value at twenty-eight days after coronary occlusion. Although not different from the control group, group 7 (twenty-eight days post-occlusion) was significantly greater than the early post-occlusion groups.

Although hydroxyproline changes in the noninfarct zone have not been specifically described in the literature, this observation is not inconsistent with reports of the compensatory hypertrophy of this region after infarction. The hypertrophy is a result of both a volume and a pressure overload of the noninfarct zone. Experiments which

produced ventricular hypertrophy from either a pure pressure or a pure volume overload, describe the changes which occur in the extracellular matrix (Borg *et al.*, 1981, Caulfield and Borg, 1979, Turner *et al.*, 1986, Weber, 1987). The initial pressure overload is associated with the development of an acute inflammatory reaction. Prior to the hypertrophy of myocytes, the extracellular matrix is destroyed (Caulfield *et al.*, 1985, Krane, 1982, Laurent *et al.*, 1985, Turner *et al.*, 1986), the speed and extent of the breakdown of the matrix being proportional to the extent of the inflammatory reaction (Duance and Bailey, 1981). A new matrix develops around the enlarged myocytes. It is not laid down exactly as it was prior to the stress; there is a greater weave network of collagen and the density and diameter of the collagen struts between cells is increased. As well, studies which examine the increase in the individual tissue components in hypertrophy report the turnover rate of collagenous and noncollagenous proteins is not identical (Weber, 1989); the absolute increase in an individual protein being influenced by the nature of the stimulus (Caspari *et al.*, 1977, Turner *et al.*, 1986, Weber, 1989).

Acknowledging the above findings, it is possible to explain the results obtained in these experiments. The initial decrease in hydroxyproline in the noninfarcted zone could represent the initial disruption of the matrix in response to the challenge of the increased workload. The subsequent gradually increasing concentration of hydroxyproline above control is compatible with the development of a compensatory hypertrophy, and the development of the collagenous matrix, in excess of the myocyte and its noncollagenous proteins.

There were slight differences between the results obtained using the Berg and OPA techniques and the HPLC technique; Berg and OPA reached significance, HPLC approached it. Some of the excessive variability could be due to the HPLC technique. The detector for the HPLC measures the absorbance at a specified wavelength. The total number of amino acids eluting from the column were expressed relative to the number of identified peaks. If any samples were not completely dried, or if any

contaminants in the samples eluted from the column and were measured, they could contribute areas of absorbance on the chromatogram and theoretically result in a variable underestimation of the percentage of an individual amino acid (Regnier, 1983).

4.2.1.2 HYDROXYPROLINE IN THE INFARCTED REGION

The same pattern observed in the noninfarcted samples was also seen in the infarcted samples; an initial decrease followed by a subsequent increase in hydroxyproline concentration, becoming significant fourteen days after infarction. Using the Berg and Lowry procedures (duplicating the methods reported in the literature) this study replicated the long term results of Jugdutt *et al.* (1986). However as stated previously, the use of the Lowry technique to measure total protein is a sample with a large amount of insoluble protein is inappropriate. The extent of the rise (966%) measured at twenty-eight days must be considered erroneous, the increase being an overestimation.

More accurate reflections of the increase were obtained using the Berg and OPA technique, and HPLC, 635% and 761% respectively at twenty-eight days. As collagen is approximately 10% hydroxyproline (admittedly this is only an estimate) these results suggest that collagen content increased from the preocclusion value of 4-5% and rose to 33% at twenty-eight days.

It is possible to suggest explanations for these initial differences in the two zones concerning the apparent decrease in hydroxyproline. Collagen degradation and synthesis are occurring in both zones simultaneously; a compensatory hypertrophy with replacement of the matrix in the noninfarct zone, and laying down of scar tissue in the infarct zone. The exact stimuli for both processes have not yet been precisely defined, but it is most probable they are not occurring at the same rate and to the same extent in both zones. It is documented in the general wound healing literature (Anders-Carlstedt, 1987, Mustoe *et al.*, 1987) that a lag phase exists between the initial wounding and

the increase in collagen concentration. It has also been demonstrated in tissue culture that the amount of collagen synthesis is directly influenced by the density of fibroblasts (Bonnet *et al.*, 1986). As tissue destruction is greater and the inflammatory reaction is more intense in the infarct zone, it is possible that the lag between the degradation and the synthesis of new collagen in the infarct zone is less than the lag in the noninfarct zone. This would make the apparent decrease in the hydroxyproline concentration of the infarct zone less. The magnitude of the lag would be hard to identify with statistical methods (i.e. based on a Scheffe test), because of the regional differences in healing in a single wound, and the individual variability in the rate of healing.

There was histological evidence of both extensive damage and fibroblasts in the infarct zone. This observation, coupled with the fact that once proteins are damaged they are replaced, not repaired (Gevers, 1984), strongly suggests that the measured hydroxyproline was from newly formed collagen.

The increased hydroxyproline content in the noninfarcted region although only 50% above control, most likely reflects an absolute increase in its concentration because the total protein content is not likely falling. The increase in the infarcted zone, although 8 fold above control may reflect both an absolute and a relative increase in hydroxyproline. The observed increase in collagen is most likely due to both the removal of the proteins associated with the infarcted myocytes and to the addition of new collagen.

4.2.1.3 TIME COURSE OF CHANGES IN 3-METHYLHISTIDINE CONCENTRATION

3-Methylhistidine is an amino acid marker of actin in the porcine heart. It is present in very small amounts, contributing only one of the four hundred amino acids of actin (Elzinga and Collins, 1977). HPLC, the method chosen to quantitate this amino acid, is capable of resolving picomole quantities of amino acid. However, it is sometimes

difficult to accurately quantitate amino acids having very different concentrations present in the same sample. With particular reference to these experiments, 3-methylhistidine was only 0.03% of total amino acids in the control tissue as compared to glycine which was 8.5% (from Table 3.5). To overcome the potential difficulty of resolution, two runs of each sample were made; one low concentration of about 40 nmoles of total amino acid, the second of about 200 nmoles, to resolve separately the amino acids present in high and low concentrations respectively. The high concentration run resulted in a well resolved, easily identifiable 3-methylhistidine peak.

Technically, quantitation proved to be quite challenging. Because of the low abundance of this amino acid, we were looking for about four amino acids per ten thousand, and because of this difficulty in the measurement, the variability was high. Nevertheless, 3-methylhistidine remained at detectable levels in both the infarct and noninfarct zones for the duration of the experiment, with no significant changes in concentration observed. This implies therefore, that substantial amounts of actin were still likely present. This possibility was enhanced by the finding of both actin and myosin detected with gel electrophoresis and monoclonal antibodies.

4.2.1.4 IMPLICATIONS OF AN UNALTERED 3-METHYLHISTIDINE RATIO IN THE INFARCT ZONE

It was initially expected that 3-methylhistidine would be undetectable in scar tissue in older infarcts. Consequently the continued presence of 3-methylhistidine was not entirely expected. However, the persistence of contractile proteins in the infarct zone is not inconsistent with the literature. Kozlovskis *et al.* (1984) reported finding approximately two thirds the concentration of myosin heavy chain in scar tissue from cat hearts sampled three to twelve months after infarction. They did not address the source of the myosin. Actin is a ubiquitous protein; it could theoretically come from several sources (Garrels and Gibson, 1976). The possibilities include

contamination of the infarcted sample with noninfarcted tissue, myocytes in the infarcted area not yet cleared, or fibroblasts.

Contamination of the infarcted tissue with noninfarcted tissue, although a possibility, is small. Although there were small groups of myocytes which survived the infarct, especially visible in the earlier groups, they were sparse and located just on the epicardial and endocardial borders. Samples chosen for biochemical analysis were carefully cut. They were taken from the center of the infarct, and by inspection, from an area which infarcted transmurally.

The second possibility is that the actin could be from myocytes which had not yet been cleared. There are different mechanisms of myocardial cell death (Baroldi, 1975, Bouchardy and Majno, 1974). The method of cell death observed histologically in these experiments, and best appreciated with the hematoxylin and eosin stain, was coagulative necrosis, which is an ischemic cell death. Both the structural and enzymatic proteins are denatured, leaving the cell looking nearly intact (Robbins, 1984). As the cell membrane still appeared intact, and striations were visible it is possible that the myofibrillar proteins, although denatured, were still present, and therefore represent a probable source of the 3-methylhistidine observed. The presence of these proteins does not however, imply function.

Fibroblasts are a third potential source. Histologically there was a proliferation of fibroblasts in the infarcted area which appeared within days after the occlusion. As 6% of the total protein of fibroblasts is actin (Anderson, 1976), it was possible that these could contribute the 3-methylhistidine. However preliminary results of the actin analysis seem to suggest that the source of the actin in the twenty-eight day infarcts is not only from fibroblasts, because the protein bands reacted positively with the HUC monoclonal antibody suggesting the source was a striated actin.

Thus, it is likely that the apparently undiminished 3-methylhistidine in the infarct may come from at least two or more sources, most probably including dead, but uncleared, myocytes and fibroblasts.

4.2.1.5 IMPLICATIONS OF UNALTERED 3-METHYLHISTIDINE IN THE NONINFARCT ZONE

The concentration of 3-methylhistidine in the noninfarcted zone tended to decrease over the twenty-eight day post-occlusion period. Although not significant, this pattern is not inconsistant with the development of a compensatory hypertrophy, a well documented response to an ischemic challenge (Anversa *et al.*, 1985, 1986, Roberts *et al.*, 1984, Rubin *et al.*, 1983, Weber 1989).

If all components of the wall hypertrophied equally, increasing exactly the same amount, and at exactly the same rate, there would be an absolute increase in all the constituents, but the relative proportions would remain unchanged. If hypertrophy involved only an increase in the myofibers, then the concentration of 3-methylhistidine as a percentage of the total amino acids present should actually increase. If however the other consitituents increased before myofibrillar ones, or if they increase a greater amount then the myofibrillar ones, then the 3-methylhistidine concentration may decrease. Knowing that the turnover rate of collagenous and non collagenous proteins is not similiar, and that the extent of the response is also not similar (Caspari *et al.*, 1977, Weber, 1989), it is most probable that the concentration of 3-methylhistidine (and by inference actin) could decrease relative to the concentration of other amino acids and proteins. These experiments do not contradict the concept that relative differences in the growth of individual components occurs. This concept will be discussed below.

4.2.1.6 RATIO OF HYDROXYPROLINE to 3-METHYLHISTIDINE

In the porcine myocardium hydroxyproline and 3-methylhistidine are unique to collagen and actin respectively, the collagen fiber being approximately one tenth hydroxyproline, and the actin fiber approximately one-four-hundredth 3-methylhistidine. Consequently the relative proportions of each protein can be estimated. A changing ratio of hydroxyproline relative to 3-methylhistidine will reflect relative changes in the concentration of collagenous and contractile proteins. It is useful to examine this ratio because both amino acids were determined from the same tissue, in the same HPLC run.

The calculated values of hydroxyproline and 3-methylhistidine were consistent with expected concentrations in normal myocardium. In the control group, hydroxyproline and 3-methylhistidine content were found to be 0.4 and 0.04% of amino acids, respectively. These yield estimated collagen and actin contents of approximately 4 and 16%. Thus, in normal myocardium we found about four times as much actin as collagen.

The hydroxyproline to 3-methylhistidine ratio is an extremely useful parameter to track the changes in the relative proportions of collagenous and contractile proteins (assuming that myosin content follows that of actin). If the ratio in the experimental groups increases this would imply either an increase in hydroxyproline and/or a decrease in 3-methylhistidine. In the noninfarcted tissue the ratio increased about four fold above control. This most probably reflects the presence of a compensatory hypertrophy consisting predominently of contractile proteins. Both collagenous and contractile proteins could be increasing, but at different rates, and to different values. This concept of a differential growth rate of individual components has been described by Weber (1989), with noncollagenous proteins increasing initially faster than collagen. There is a lag in the synthesis of collagenous protein. When it starts to increase, it increases for a

longer duration and to a greater value than that of the noncollagenous proteins in the heart.

In the infarct zone this ratio was eight fold above control at twenty-eight days. This increase is most probably due primarily to the increased collagen being laid down in the repair process. It is also probable that as myocyte clearing continues, the ratio will continue to increase in this zone, because of preferential loss of contractile protein.

4.2.1.7 RATIO OF PROLINE to HYDROXYPROLINE

Hydroxyproline is unique to collagen, proline is not. Each contribute about 10% of the total amino acids of collagen but proline is also contributed by a variety of other proteins. Therefore, in normal noninfarcted heart muscle the concentration of proline is in excess of that of hydroxyproline. As the ratio of these two amino acids becomes closer to 1.0, this would imply that one of two processes is occurring. Noncollagenous proteins could be disappearing, and the proline present is being increasingly derived from collagen or the absolute amount of collagen could be increasing. In the infarct zone the ratio decreased from 12.4 in the control group to 2.9 in the twenty-eight day group. If it is assumed that all proline-containing non-collagenous proteins are disappearing at the same rate (a tenuous assumption), almost three quarters of these proteins have disappeared, or about five times more collagen added. There is a roughly fivefold differential between the rates of collagen appearance and non-collagenous protein disappearance. This would imply that collagen is still not the only protein comprising the scar tissue, but is becoming the major protein. This is compatible with the literature. In addition to collagen, proteoglycans, elastin and fibroblasts also present in scar tissue (Ross and Reith, 1985), contribute to the proline pool.

4.2.2 TIME COURSE OF WATER CONCENTRATION

In these experiments the water content in the control group was approximately 78%, similar to that reported by others (Lekven and Andersen, 1980). It remained constant in noninfarcted tissues for the twenty-eight day post-occlusion period. However, the water content of infarcted tissues increased with healing, becoming significantly increased twenty-four hours after the permanent occlusion, and continued to increase for the duration of the experiment.

Initially, the increased water in the infarct zone is most probably due to edema which occurs as part of the acute inflammation process; the disappearance of intracellular water being less than the accumulation of extracellular water in the matrix (Fishbein *et al.*, 1978). The most probable explanation for the continuing increase in water content in the infarct zone is that some constituents of the scar tissue can be hydrated. Besides containing collagen, the scar also contains the ground substance, which surrounds the fibers of the connective tissue (Ross and Reith, 1985). This is composed of proteoglycans, the exact composition being unique to the individual scar tissue (Viidek, 1973). These compounds have a protein core with attached side chains of glycosaminoglycans of approximately twenty-five to fifty disaccharide units. Proteoglycans, especially hyaluronic acid, are capable of binding large volumes of water (Nimni, 1983). Hyaluronic acid can bind approximately one thousand times its volume in water (Anders-Carlstedt, 1987), tending to form a "gel- like" matrix (Nimni, 1983). Judd and Wexler (1969) and Shetlar *et al.* (1979) reported the hyaluronic acid concentration in infarcted myocardial tissue samples rose significantly above control. Therefore the increase in the water in the infarcted region may actually be from two different reasons over the entire twenty-eight day period, an initial edema, followed by subsequent laying down of the components of the scar.

4.3 HISTOLOGICAL OBSERVATIONS

The present thought in the literature is that it is not the absolute amount of the collagen which will most affect the mechanical properties of the heart but rather its location and orientation (Carroll *et al.*, 1989, Lee and Boughner, 1981, Przyklenk *et al.*, 1987, Weber, 1989). Histological stains were chosen which would clearly show this orientation.

In these experiments, new collagen identified histologically was visible in the infarcted region seven days after coronary artery occlusion. This collagen was fine and randomly oriented. The next collagen appeared between seven and twenty-one days in different specimens. These fibers were arranged in a more orderly fashion, parallel to the base of the heart. Although not measured, these fibers appeared thicker than the initial collagen.

The observation of two distinct patterns in the collagen is consistant with reports on the healing of other wounds. The accepted theory is that the initial type 111 collagen in a healing wound functions as a template for the laying down of subsequent collagen (Nimni, 1983). The later type 1 collagen is associated with the mechanical properties and tensile strength of the material (Foidart *et al.*, 1981, Nimni, 1983).

This orderly, parallel arrangement of collagen fibers in the infarct has been alluded to in a recent abstract (Carroll *et al.*, 1989). This is the orientation one would expect the collagen fibers to assume, knowing that one of the main functions of the scar is to transmit tension (Fung, 1981). The mechanical factors influencing the organization of collagen were summarized by Nimni (1983). He stated that an organized parallel arrangement of collagen fibers would be laid down if the healing tissue was under continual repetitive strains while the healing process was occurring. Collagen fibers would be oriented along the major stress line. He described the organization of collagen

fibers of injured rabbit ligaments which were immobilized during the healing period. These were laid down in a disorganized fashion, while collagen fibers in ligaments which were not immobilized assumed a parallel arrangement.

Obviously the heart is not immobilized during the healing process. The infarcted myocardium is continually subjected to repetitive stresses while it is healing. These come from three sources: there is an outward force which the compressed blood exerts on the ventricular wall during systole, and there is an adjacent circumferential force from contracting viable myocytes during systole. In addition there may also be a force from the pericardium which acts "inward" to prevent excessive filling. Although the pericardium was cut, fibrous adhesions formed between the pericardium and the epicardium, and may have acted to restrict the contraction. Knowing that collagen is laid down along the lines of the major stresses, it would appear from the histological examination of these tissues that the major stresses exerted upon the developing scar are from the contracting adjacent myocytes.

4.4 MECHANICS

4.4.1 METHODOLOGIC CONSIDERATIONS

The literature suggests that changes occur in the passive infarcted ventricle both globally and regionally. There appears to be two phases of change, an initial period of increased global compliance and decreased regional tensile strength, followed by decreasing global compliance and an increase in regional tensile strength. Both of these later measurements have been correlated with the increasing hydroxyproline content (Lerman *et al.*, 1983, Przyklenk *et al.*, 1987). Properties which remain unaffected, are global bursting pressures and regional stiffness.

Global compliance is a relatively crude measurement, particularly in the presence of a myocardial infarct of variable size. Global compliance is determined by the "stiffness" of all constituents of the ventricle: a stiffness differential between the infarct and noninfarct zone and the relative size of each. Because of substantial inter-individual variability, it requires a rather large infarct zone and a substantial stiffness-differential to demonstrate a statistically significant difference. Moreover, the measurement can be subject to some technical errors (e.g. leakage through the mitral valve, folding of the intraventricular balloon, etc.). Lastly, it is very difficult to standardize for differences in ventricular size between groups and individuals.

It seemed more rigorous to measure unidirectional stiffness in rectangular strips of myocardium taken from the infarct and noninfarct zones, even though alignment along fiber orientation may not have been optimal. By comparing strips taken from the same heart, some inter-animal variability was reduced. By calculating stress (although admittedly the measurement of the cross-sectional area of the strip was subject to some error) the difficulty of "normalizing" for heart size was reduced. By determining mechanical properties of "pure" samples of infarct and noninfarct zones, we could compare different regions of the

same heart and we would also eliminate the problem inherent in the variability of the relative sizes of the infarct and noninfarct zone in the individual ventricle. Lastly, by determining the stress-strain relationship and allowing stress-relaxation to occur, we could estimate the low and high-frequency responses, i.e. the estimation of the viscous and the elastic components of stiffness.

The technique generally described in the literature for testing regional myocardial strips usually involves a slow and steady method of strain application (Lerman, *et al.*, 1983, Mirsky and Parmley, 1973, Przyklenk *et al.*, 1987). This protocol however does not permit a differentiation of the individual stiffness components and disregards the *in vivo* reality that a myocardial infarct is subjected to quite rapidly applied stress, especially during the early phases of ventricular systole. The novel protocol utilized in this set of experiments involved increasing the length of regional strips by rapidly occurring small regular changes in length, a similar protocol to that utilized for examining the viscoelastic properties of the pericardium (Lee and Boughner, 1981, 1985) which was thereby found to have substantial viscous stiffness. The tension within the strip was allowed to stabilize before an additional strain was applied. This method enabled the partitioning of stiffness into its viscous and elastic components, by examining both the initial tension in the strip after the rapid application of strain and the subsequent stress-relaxation which occurred. The initial tension in the strip was used to determine stiffness when it was subjected to a rapid increase in length and included both the viscous and elastic components of stiffness. The final tension in the strip obtained after the strip had relaxed, was a reflection of only the elastic component of stiffness. This final tension, would be analogous to the obtained stress, if the strain had been applied slowly and continously. The difference between the total and elastic stiffnesses was attributed to the viscous component. The experiment was divided into two parts. This stiffness of the strip was determined over a low strain range of approximately 0 to 20%, approximating the length change of the sarcomere *in vivo*. The second

range, 20 to 60% elongation, was in excess of the strains which that portion of the myocardium would have been subjected to *in vivo*.

The accuracy of the results are influenced by the initial measurements of the strip. Small errors in the initial length or cross-sectional area of the strip will introduce error into the stiffness determination. This potential source of error is well acknowledged in the literature (Anders -Carlstedt, 1987, Fung, 1967, Laird and Vallekoop, 1977, Mirsky, 1979). To overcome this source of potential error, some investigators calculate the cross-sectional area from the weight and length of the strip; the weight per unit length being directly proportional to the cross-sectional area. However this is only valid if the density of the strips is constant. Since the density of muscle is substantially less that that of collagen, 1.063 and 1.4 respectively, (Harkness, 1968, Viidek, 1973), and since the percentage of collagen was continuously changing with time, the above approximation seemed to be inappropriate. Instead, it was deemed best to express tension relative to the traditional units of initial cross-sectional area.

The determination of bursting pressure is also a relatively crude approximation of the tensile strength. Admittedly, the former has substantial advantages and relevance. It still allows the "geographic" localization of the rupture and identifies the maximum pressure which the myocardium and the infarct are capable of withstanding. These are clearly of great relevance to the cardiologist treating patients at risk of ventricular rupture. Yet, the experiments showing an average bursting pressure of approximately 650 mmHg (Lerman *et al.*, 1983), are little comfort in the clinical reality in which cardiac rupture does occasionally occur under physiological pressures. Nevertheless, the determination of stiffness under greater - than - physiological loading conditions, seemed preferable to us.

Before discussing the results, it is important to make a clear distinction between stiffness and tensile strength. Stiffness is the

magnitude of the ability to reversibly elongate under an applied load. In constrast, tensile strength is the limit of the ability of the material to withstand irreversible deformation; thus, it defines the failure of attachments or strength of the weakest "links". It is clearly possible that two materials have identical stiffnesses but have different tensile strengths, i.e. while they withstand reversible deformation, they appear identical, but one will fracture under a lesser load. Similarly, two materials may have different stiffnesses but identical tensile strength.

Stiffness, as measured by the stress-strain relationship, describes in a simple quantitative way the apparent behavior of a complex composite material, in a complex "weave" with random orientation of the cc ~nents. In reference to Figure 4.2, under small loads ("A"), only a proportion of the components are aligned in the direction of the loading and are loaded. Thus, a proportion of the components behave as parallel "springs": the load is distributed among these resulting in their elongation. The load per "spring" is relatively large, resulting in large elongation. Under increasing loads ("B"), the alignment of the components changes resulting in an increasing proportion of the "springs" being disposed in parallel and along the axis of loading. This increases the number of components being loaded and will result in the load being distributed among more components: thus the load per "spring" decreases, resulting in less extension of each. The material appears increasingly stiffer as more constituents align. Under further increments in loading ("C"), eventually all available components align, taking all of the load. Thus any further increase in load is distributed among the same number of "springs", the load per "spring" remaining constant, resulting in constant elongation. Apparently "maximal" stiffness represents recruitment by alignment of all available springs. Lastly ("D"), some (weakest) attachments break. This is the reason for the stress-strain relationship being not linear.

Since the stress is dependent upon the degree of applied strain, the rate of applied strain and the history of the strip (Fung 1981), to

obtain meaningful data all strips were initially preconditioned, the strips were strained over two specific ranges, and the stiffness response to both a rapid and a slow strain application was determined.

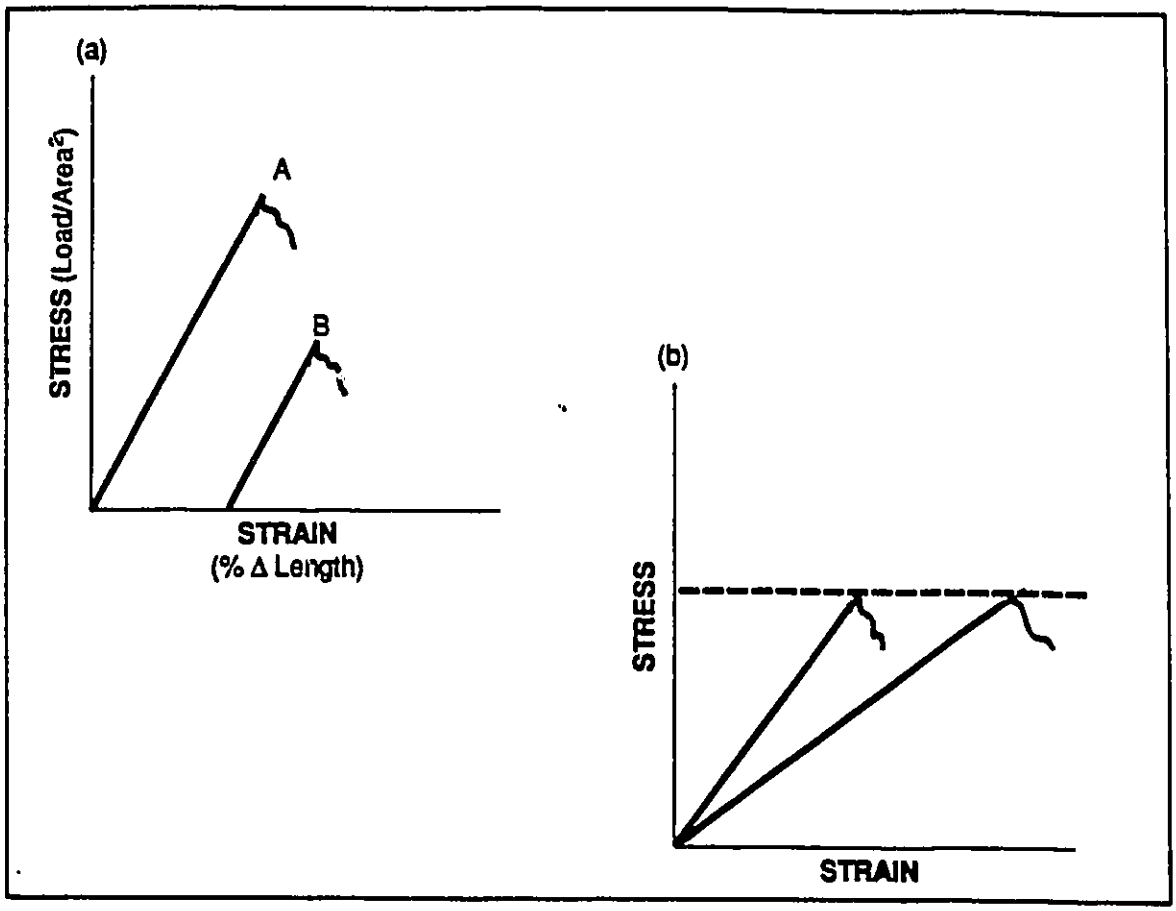


Figure 4.1

Diagrammatic representation of the difference between stiffness and tensile strength. (a) Two materials with the same stiffness but different tensile strength; although both follow the same stress-strain relation, "B" will fracture at a lower stress than "A". (b) Two materials with different stiffness, but with the same tensile strength; both will fracture at the same stress.

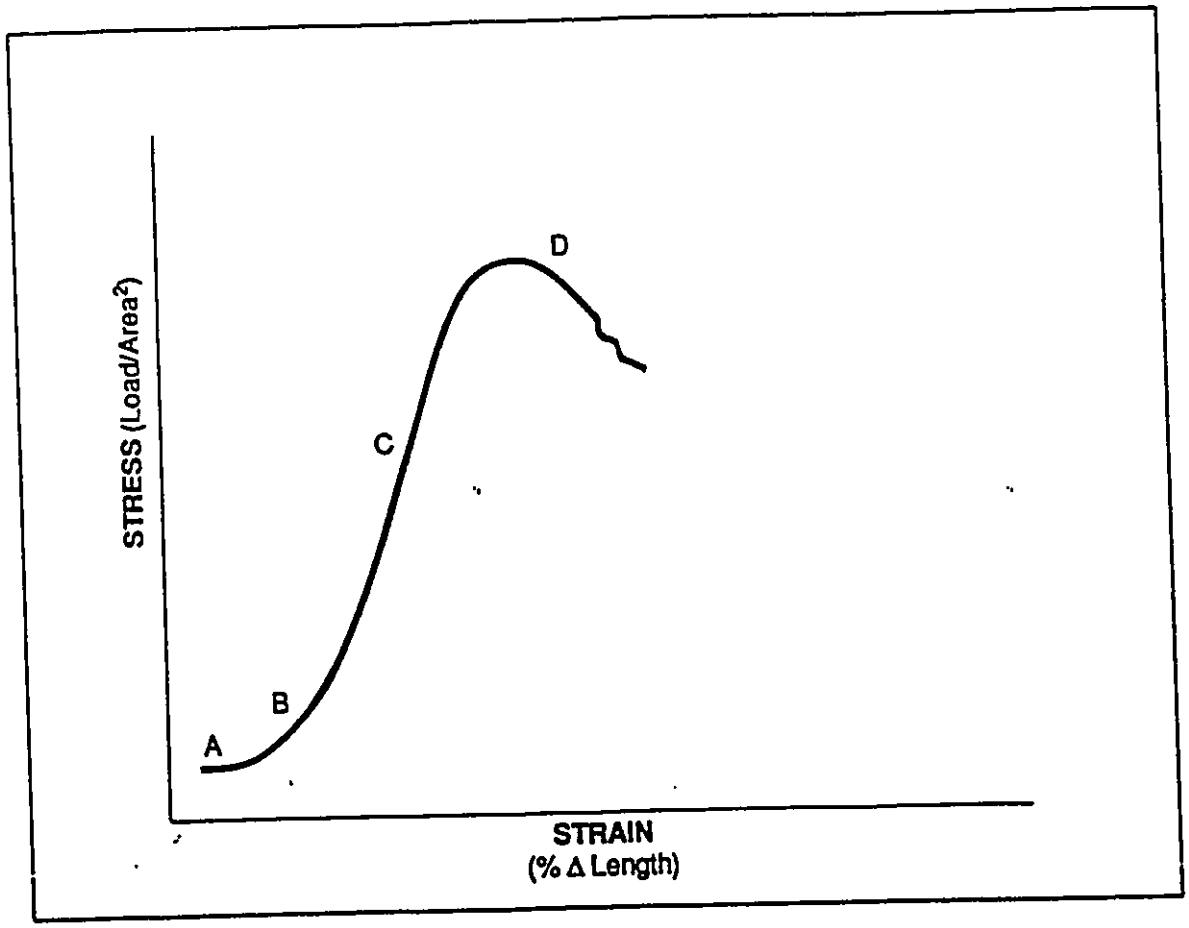


Figure 4.2
Illustration of the relationship between stress (load/area^2) and strain (elongation). For more details please see Section 4.4.1 in the text (taken from Viidek, 1973).

4.4.2 TIME COURSE OF MECHANICAL CHANGE

The regional strips displayed visco-elastic behavior over both strain ranges for the duration of the experiment. This therefore implies that, although histologically the mature collagen fibers appeared parallel, their organization must still be complex and most probably involving a weave pattern. Admittedly other components of scar tissue have been suggested to contribute to its properties including; water, and proteoglycans, and other proteins including elastin, desmin, vimentin, etc. (Lee and Boughner, 1981, Price *et al.*, 1984). Nonetheless, collagen is considered to be the major mechanical component. Consequently, if all fibers were parallel and running the entire length of the strip, and if all stiffness was due only to collagen, there would no longer be a viscous component of stiffness, and the stiffness measured would be uniform at all strains. This would occur because all fibers would be loaded simultaneously, and would not be unloaded as a result of fiber reorientation occurring when the components of the tissue are organized in a weave pattern. The continued presence of a viscous component in regional strips of myocardium from twe ty-eight day-old infarcts implied that fiber reorientation was still occurring, (Harkness, 1968). The increased stiffness at high strains implied the additional recruitment of fibers at high loads. However, as the elastic component of stiffness increased in the infarct zone relative to the noninfarct zone, this suggests the scar tissue was gradually becoming more purely elastic.

4.4.2.1 STIFFNESS

Because Young's modulus is the slope of the linear part of the stress-strain relation, getting to the linear part is not measured. During this phase of exponentially rising stress:strain ratio, a major realignment of the elements occurs, such that more and more elements are taking the load until, on the linear part, all elements are subject to loading. This initial exponential part is also important because it reflects the continuing realignment; however it is not accounted for in

the calculation of Young's modulus. Thus, an unchanged Young's modulus (i.e. same stiffness) tells little about the amount of realignments occurring and the nature of the weave of the material.

There was no significant change from control in the stiffness of the full thickness infarcted or noninfarcted regional strips, measured over either of the two strain ranges. There is to our knowledge no report in the literature demonstrating significant changes in the stiffness of regional myocardial strips after a myocardial infarction. This has been studied in regional strips up to ten days after coronary occlusion (Laird and Vallekoop, 1977), and these experiments extend the finding to four weeks after occlusion.

The infarcted strip was divided in epicardial and endocardial sections. There was an observed increase in the stiffness of the epicardial strip apparent over the low strain range of testings. Although it is possible that healing was occurring in the epicardial region preferentially faster than the endocardial, this is not supported histologically. Therefore it is most probable that the stiffness was due to a fibrous adherance of the pericardium to the epicardium which developed after the infarction as part of the inflammatory process. Although attempts were made to ensure that the pericardium was removed before the mechanical testing was performed, it is possible that some remained. Even normal pericardium is stiffer than cardiac muscle (Gilbert and Glantz, 1989, Przyklenk *et al.*, 1987). As the strain in a composite material is not distributed evenly, but rather is borne by the strongest elements (Lee and Boughner, 1981), the presence of small amounts of adhering pericardium could result in an increased apparent stiffness of the strip.

The uniqueness of the stiffness of an individual heart is documented (Laird and Vallekoop, 1977, Mirsky, 1979). The trend of an increased variance of the later groups (groups 5, 6, and 7), and sometimes of the very early group (2) as compared to the control group is an important observation. Although no significant difference in the stiffness

between control and experimental groups was found, the increased variance suggests that the experimental groups are no longer similar to the control group. It also implies that changes in all hearts at a given time after occlusion are not equal. These differences could be in either the magnitude or the rate of change. This observation is in complete agreement with those made concerning the healing of other tissues. Although the same pattern of events is observed, healing is an individual process and occurs at varying rates in individual animals (Duance and Bailey, 1981).

4.4.2.2 ELASTIC COMPONENT OF STIFFNESS

All strips displayed viscoelastic behavior, exhibiting stress relaxation with loading and creep with unloading. This behavior has previously been described for the myocardium (Gilbert and Glantz, 1989), and for biologic materials in general (Fung, 1981). The pattern exhibited can be modelled most simply with a Maxwell model; a spring in series with a dashpot. However, this model is actually an oversimplification of the observed mechanical behavior over the entire strain range.

Over the twenty-eight day healing period the scarred region gradually became more purely elastic, and the noninfarct area became more viscous, relative to each other. There was an apparent but nonsignificant trend in the data. The elastic component of stiffness (called "final stiffness" in the Results) tended to increase over the twenty-eight day period in infarcted sections, and tended to decrease in noninfarcted strips over the same duration. Although not different from control by ANOVA, the individual components of stiffness in the infarct and noninfarct zones significantly diverged from each other at three days post-occlusion and remained diverged at all subsequent times measured. The trends observed may actually be reflective of differences in the tissue. The increasing elastic component of stiffness of the infarcted regions is compatible with the histological evidence of the parallel arrangement of the collagen fibers. The more

parallel the arrangement the more closely the material is to being purely elastic (Lee and Boughner, 1981, Viidek, 1973, Weber, 1987).

Functionally, an increased viscous stiffness of the noninfarcted region could negate the increased elastic stiffness of the infarcted region. This would allow passive diastolic filling of the intact ventricle to be accomplished without great increases in intraventricular pressures. The infarcted area needs to resist extension in systole, but should not substantially impede filling. Filling (diastole) is a low frequency event, while in systole there is a high frequency length change. It could be expected that filling is less resisted by the infarct than the extension exerted by ventricular contraction when stress increases rapidly. This concept of a changing of the regional properties to maintain global functioning has been repeatedly proposed and documented (Bogen *et al.*, 1984, Gilbert and Glantz, 1989, Swan, 1979, Theroux *et al.*, 1977).

There were clear limitations in our experimental procedure. Length increases averaged 5 -8% of the initial length in about 100 msec. This corresponds to an increase of approximately 3000% per minute. Lee and Boughner (1981, 1985) suggested that the average rate of length change *in vivo* can approach 7000% per minute or greater, easily estimated knowing the heart rate and the diastolic filling time. As the viscous component is dependent upon the rate of the strain application, it is possible that at strain rates greater than applied in this experiment the contribution of the viscous component of stiffness in the noninfarcted region would become even more significant.

4.5 GENERAL CONCLUSIONS

The aim of this project was to document the changes and the time course of healing after a myocardial infarction, using a porcine experimental model.

Histologically no heart examined was completely healed twenty-eight days after permanent occlusion of the circumflex coronary artery. The results of all parameters examined (blood flow, biochemistry, histology, and mechanics) suggested the same processes occur during healing in all hearts, however, the rate and extent of the healing process is individual. This was most apparent when data from infarct and noninfarct zones were compared within the same heart. Significant changes occured in both regions of the heart, the biochemical and blood flow changes preceeding the mechanical ones. This very general observation is consistant with observations others have made concerning wound healing of tissues other than myocardium (Robbins, 1984).

Prior to reviewing the findings from these experiments, it is worthwhile to review the methodologic procedures and their validity on tissues which are constantly changing. First, microspheres can be used to measure serial blood flows. They remain in the tissue for long periods, and consequently those injected prior to the vessel occlusion may be a useful indicator of tissue shrinkage. Second, as insoluble protein (collagen) becomes concentrated in the infarct zone, the method of estimation of total protein becomes important; the use of the Lowry protein assay may substantially underestimate total protein, and result in an overestimation of the relative abundance of a given constituent (i.e. collagen or actin) by the same proportion. Therefore, although the Berg protocol for determining hydroxyproline concentration is valid, the Lowry protein assay, the method of choice of many investigators, is inappropriate: it overestimates hydroxyproline and therefore collagen concentation in scar tissue. More accurate measurements of total amino acid concentration may be obtained using the OPA amino acid assay or HPLC. 3-Methylhistidine can be used as a marker for actin, and can be quantitated using HPLC. Third, it is possible to determine the contribution of the viscous and elastic components of stiffness of regional strips of myocardium using a step-wise stress test and allowing stress-relaxation to occur between length increments.

Measurements performed on control hearts confirmed and extended published reports. No signficant differences were observed between the free and septal walls of the left ventricle with any variable examined. Resting blood flows, the temporal and spatial distributions of blood flow and the uniformity of flow between the free and septal walls all substantiated the literature (Cohen, 1985, Fedor *et al.*, 1978, Fujiwara et al., 1982, Sakai *et al.*, 1985, Sjoquist *et al.*, 1984). The water content of the control tissues (77.5%) also confirmed previous documentation (Levken and Anderson, 1980). Collagen content, as estimated by determination of hydroxyproline content was compatible with published concentrations in normal myocardium. The use of 3-methylhistidine to estimate actin concentration yielded values comparable with reports in the literature (Gevers, 1984). Different components of stiffness were determined on regional strips of myocardium. The elastic component of stiffness was approximately 64-70% of the total stiffness, the remainder being considered the viscous component.

Compensatory hypertrophy of noninfarct tissue secondary to a myocardial infarction is well documented in the literature (Anversa *et al.*, 1985, Roberts *et al.*, 1984, Rubin *et al.*, 1983). This results in increased myocyte size and tends to return systolic wall stress to normal values (Mirsky, 1979, Gilbert and Glantz, 1989). Results from these experiments do not disagree with the development of a compensatory hypertrophy.

In the noninfarct zone bloodflow increased significantly above control one day after occlusion and remained higher until twenty-one days. This finding extends the observations of Savage *et al.*, (1981) who reported an increased blood flow to this region for two days after occlusion. The initial increased flow is compatible with an increased workload, the eventual normalization of flow is compatible with noninfarct zone hypertrophy.

The observed changes in hydroxyproline, 3-methylhistidine and the ratios of specific amino acids are also suggestive of hypertrophy of the noninfarct zone, and of a differential rate of synthesis of collagenous and noncollagenous proteins. The hydroxyproline concentration in this area initially decreased, then gradually increased. By twenty-eight days, although not greater from control, the concentration was greater than measured immediately post-occlusion and the concentration appeared to be continually increasing. The percentage of 3-methylhistidine remained unchanged. The ratio of hydroxyproline to 3-methylhistidine increased nearly four fold above control and the ratio of proline to hydroxyproline decreased 30% below control; both imply a disproportionately greater increase in collagenous than noncollagenous proteins.

No significant changes in stiffness were measured between the control group and the noninfarct zone of any heart examined. However, intra-animal changes in the components of stiffness occurred; the viscous component increased becoming significantly greater than in the infarct zone after three days. The observed mechanical results were consistent with reports in the literature of mechanical changes observed *in vivo*; primarily an altered functioning of the noninfarct zone secondary to an infarct occurs with the result being preservation of ventricular function (Sakai *et al.*, 1985, Tennant and Wiggers, 1935, Theroux *et al.*, 1977). The suggested hypertrophy and increased viscous component of stiffness of the noninfarct zone could compensate for the loss of tissue and the increased elastic stiffness of the infarct region.

A wide range of the variance in measurements made at a given time suggest different rates of progression of healing in individual animals. This makes the demonstration of statistically significant changes more difficult. Had healing been uniform in all hearts, the variance of the different groups would have remained constant. This increase in variance is consistent with both the large interanimal variability, and an individual rate of healing both in individual animals, and in different areas of the same heart.

Blood flow to the infarct zone decreased to 6% of the preocclusion value ten minutes after occlusion. It rose slightly on days one and three post-occlusion, then gradually declined at all subsequent times measured. The healing process involved both clearing of necrotic tissue, and its replacement with collagenous scar tissue. It was apparent that preligation microspheres became concentrated in infarcted tissues. The blood flow in the preligation heart was relatively homogeneous to all areas; however when the infarct to noninfarct ratios of the initial microspheres injected were determined, a significant concentration was observed fourteen days after the occlusion.

Collagen content, appearance and orientation in the infarct zone varied during healing, as revealed by the biochemical and histological investigations. We were able to replicate the increases in hydroxyproline concentration exactly as reported in the literature (Jugdutt *et al.*, 1986). However, we felt the method was not valid for tissues containing large amounts of insoluble protein, and overestimated the actual hydroxyproline concentration. We believe the increases reported in the literature (approximately 1000% by twenty-eight days) are too high. We measured increases of 625%, corresponding to collagen concentrations of approximately 33%; confirmed by two methods: Berg and OPA, and HPLC. Histologically, the collagen which initially appeared in the damaged region was very fine and randomly oriented. Collagen appearing subsequently was thicker, and arranged in an organized parallel pattern. The water content of the infarct zone also increased steadily over the duration of the experiment. This observation is consistent with hydration of individual components of scar tissue (i.e. ground substance).

3-Methylhistidine, a marker for actin remained detectable for the duration of the experiment. The continued presence of contractile proteins was also confirmed by two other methods. First, striations were visible histologically in infarcted but uncleared myocytes in the

infarct zone. Second, the presence of actin and myosin in the infarct zone were identified by SDS-PAGE and monoclonal antibodies. We do not suggest that the presence of these proteins implies they are functional.

The concentration of collagen to actin as estimated by the ratio of hydroxyproline to 3-methylhistidine was increased eight fold above control at twenty-eight days. The concentration of collagen to noncollagen proteins as estimated by the ratio of proline to hydroxyproline decreased 79% below control at twenty-eight days. Examined together these suggest that the concentration of collagenous proteins increased in the infarct zone, above that present in control, noninfarcted hearts.

Only in three day-old infarcts was the tensile strength substantially decreased. This was apparent as only infarcted regional strips from this group ruptured with straining. In this early group there was no obvious increase in the amount of hydroxyproline in the infarct zone. There was also no obvious structural alterations or myocyte clearing. It therefore would seem that during this early period the collagen extracellular matrix was disrupted, but there was as yet no appreciable repair. Despite the increasing collagen concentration, the total stiffness of full-thickness strips from the infarct zone did not change significantly at any time measured after the occlusion. However the stiffness was highly variable. The individual components of stiffness did change; the elastic component of stiffness increased and the viscous component decreased in the infarct zone after three days. These altered components of stiffness are most probably due to a changed orientation of the components of the collagen weave.

Combining the results of the histological appearance of collagen and the observed mechanical properties we hypothesize that the collagen in the scar must be in a weave pattern. This is suggested by the fact that stress-relaxation occurred at all strains, implying the continued

presence of a viscous component. Moreover, the stiffness of the regional strips was not linear but remained exponential; showing an increased stiffness at increasing strains which is indicative of internal reorientation of the constituents of a material.

5.0 REFERENCES

Anders-Carlstedt,C. 1987. Mechanical and chemical factors in tendon healing; effects of indomethacin and surgery in the rabbit. Acta Orthopedaedica Scand. (Suppl 204) 58:7-75.

Anderson,P.J. 1976. Chicken muscle and fibroblast actin structure. Biochem. J. 159:185-187.

Anversa,P. Beghi,C. Olivetti,G. 1985. Myocardial response to infarction in the rat: Morphometric measurement of infarct size and myocyte cellular hypertrophy. Am. J. Pathol. 118:484-492.

Anversa,P. Beghi,C. Kikkawa,Y. Olivetti,G. 1986. Myocardial Infarction in Rats; Infarct size, myocyte hypertrophy, and capillary growth. Circ. Res. 58:26-37.

Asatoor,A.M. and Armstrong,M.D. 1967. 3-Methylhistidine, a component of actin Biochem. Biophys. Res. Comm. 26:168-174.

Babu,A. Sonnenblick,E. and Gulati,J. 1988. Molecular basis for the influence of muscle length on myocardial performance. Science 240:74-76.

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.

Bartrum,R.J. Berkowitz,D.M. and Hollenberg,N.K. 1974. A simple radioactive microsphere method for measuring regional flow and cardiac output. Invest. Radiol. 9:126-132.

Bassingthwaighte,J.B. Malone,M.A. Moffett,T.C. King,R.B. Little,S.E. Link,J.M. and Krohn,K.A. 1987. Validity of microsphere depositions for regional myocardial flows. Am. J. Physiol. 253(Heart Circ. Physiol. 22): H184-H193.

Berg,R.A. 1982. Determination of 3- and 4-hydroxyproline. *In* Methods of Enzymology. Vol.82. *Edited by* S.P. Colowick, and N.O. Kaplan, L.W. Cunningham and D. Frederiksen. Academic Press, New York. pp 372-380.

Bogen, D.K. Needleman, A., and McMahon, T.A. 1984. An analysis of Myocardial infarction; the effect of regional changes in contractility. Circ. Res. 55:805-815.

Bourne,J.A. 1983. *In* Handbook of Immunoperoxidase Staining Methods. Dako Corporation. Santa Barbara,CA.

Bloor,C.M. 1974. Functional significance of the coronary collateral circulation. Am. J. Pathol. 76:562-586.

Bouchardy,B. and Majno,G. 1974. Histopathology of early myocardial infarcts. Am. J. Pathol. 74:301-330.

Borg,T.K. and Caulfield,J.B. 1979. Collagen in the heart. Texas Reports on Biology and Medicine. 39:321-333.

Borg,T.A. Ranson,W.F. Moslehy,F.A. and Caulfield,J.B. 1981. Structural basis of ventricular stiffness. Lab Invest. 44:49-54.

Bray,D. and Thomas,C. 1975. The actin content of fibroblasts. Biochem. J. 147:221-228.

Burton,A.C. 1954. Relation of structure to function of the tissues of the wall of blood vessels. Physiol. Rev. 34:619-642.

Cannon,R.O. Butany,J.W. McManus,B.M. Speir,E. Kravitz,A. Bolli,R. and Ferrans,V.J. 1983. Early degradation of collagen after acute myocardial infarction in the rat. Am. J. Card. 52:390-395.

Capurro,N.L. Goldstein,R.E. Aamodt,R. Smith,H.J. and Epstein,S.E. 1979. Loss of microspheres from ischemic canine cardiac tissue; An important technical limitation. Circ. Res. 44:223-227.

Carroll,E. Janicki,J.S. Pick,R. and Weber,K.T. 1989. Myocardial stiffness and reparative fibrosis following coronary embolization in the rat. Card. Res. 23:655-661.

Caspari,P.G. Newcomb,M. Gibson,K. and Harris,P. 1977. Collagen in the normal and hypertrophied human ventricle. Cardiovas. Res. 11:554-558.

Castor,C.W. 1981. Autocoid regulation of wound healing. *In* Handbook of Inflammation, Vol.3. Tissue repair and regeneration. *Edited by* L.E. Glynn. Elsevier/North-Holland Biomedical Press, Amsterdam. pp 198-203.

Caulfield,J.B. and Borg T.K. 1979. The collagen network of the heart. Lab. Invest. 40:364-372.

Caulfield,J.B. Tao,S.B. and Nachtigal,M. 1985. Ventricular collagen matrix and alterations. *In* Advances in Myocardiology. Vol 4. *Edited by* Peter Harris and Philip A. Poole-Wilson. Plenum Medical Book Company, New York and London. pp. 257-269.

Caulfield,J.B. and Wolkowicz,P. 1988. Inducible collagenolytic activity in isolated perfused rat hearts. Am. J. Pathol. 131:199-205.

Chen,C. Bonzel,T. Hanjoerg,J. Seiffert,G. Zeiher,A. Kasper,W. and Wollschaeger,H. 1986. Quantitative assessment of temporal and spatial ventricular wall motion in normal and infarcted human left ventricles. Am. Heart J. 112:712-721.

Chiariello,M. Ambrosio,G. Cappelli-Bigazzi,M. Perrone-Filardi,P. Brigante,F. and Sifola,C. 1986. A biochemical method for the quantitation of myocardial scarring after experimental coronary artery occlusion. J. Mol. Coll. Cardiol. 18:283-290.

Clore,J.N. Cohen,I.K. and Diegelmann,R.F. 1979. Quantitation of collagen types I and III during wound healing in rat skin (40548). Proc. Soc. Exp. Biol. Med. 161:337-340.

Cohen,M. 1985. *In* Coronary Collaterals: Clinical and Experimental Observations. Futura Publishing Co., New York. pp 200-387.

Consigny,P.M. Verrier,E.D. Payne,B.D. Edelist,G. Jester,J. Baer,R.W. Vlahakes,G.J. and Hoffman,J.I.E. 1982. Acute and chronic microsphere loss from canine left ventricular myocardium. Am J. Physiol. 242 (Heart Circ. Physiol. 11):H392-H404.

Crozatier,B. Ashraf,M. Franklin,D. and Ross,J. 1977. Sarcomere length in experimental myocardial infarction: Evidence for sarcomere overstretch in dyskinetic ventricular regions. J. Mol. Cell. Cardiol. 9:785-797.

Cox,R.H. 1978. Passive mechanics and connective tissue composition of canine arteries. Am. J. Physiol. 234: Heart Circ. Physiol 3(5): H533-H541.

Culling,C.F.A. 1974. Connective Tissue. *In* Handbook of Histopathological Techniques. 3rd Edition. Butterworths, London. pp 425-426.

Daly,C.H. 1982. Biomechanical properties of dermis. J. Invest Derm. 79:17s-20s.

Diamond,G. and Forrester,J. 1972. Effect of coronary artery disease and acute myocardial infarction on left ventricular compliance in man. Circ. XLV: 11-19.

Driscol,T.E. and Eckstein, R.W. 1967. Coronary inflow and outflow responses to coronary artery occlusion. Circ. Res. 20:485-495.

Duance,V.C. Bailey,A.J. 1981. Biosynthesis and degradation of collagen. *In* Handbook of Inflammation. Vol 3; Tissue Repair and Regeneration. *Edited by* L.E. Glynn. Elsevier/North-Holland Biomedical Press, Amsterdam. pp 51-110.

Duling, B.R. and Damon, D.H. 1987. An examination of the measurement of flow heterogeneity in striated muscle. Circ. Res. 60:1-13.

Elzinga,M. and Collins,J.H. 1977. The amino acid sequence of skeletal muscle actin. Cold Springs Harbour Symposia on Quantitative Biology: The Mechanism of Muscle Contraction. 37:1-8.

Eghbali, M. Czaja,M. Zeydel,M. Weiner,F. Zern,M. Seifter,S. and Blumenfeld,O. 1988. Collagen chain mRNAs in isolated heart cells from young and adult rats. J. Mol Cell Cardiol 20:267-276.

Eyre,D.R. 1980. Collagen: Molecular Diversity in the body's protein scaffold. Science. 207:1315-1322.

Factor,S.M. Robinson,T.R. Dominitz,R. and Cho,S. 1986. Alterations of the myocardial skeletal framework in acute myocardial infarction with and without ventricular rupture; a preliminary report. Am. J. Cardiovas. Pathol. 1:91-97.

Fedor,J.M. McIntosh,D.M. Rembert,J.C. and Greenfield,J.C. 1978. Coronary and transmural myocardial blood flow responses in awake domestic pigs. Am. J. Physiol. 235(4):H435-H444.

Fishbein,M.C. MacLean,D. and Maroko,P.R. 1978. The histopathologic evolution of myocardial infarction. Chest 73:843-849.

Fishbein,M.C. Maclean,D. and Maroko,P.R. 1978. Experimental myocardial infarction in the rat: Qualitative and quantitive changes during pathologic evolution. Am. J. Pathol. 90:57-70.

Foidart,M. Foidart,J. and Engel,W.K. 1981. Collagen localization in normal and fibrotic human skeletal muscle. Arch. Neurol. 38:152-157.

Forrester,J.S. Diamond,G. Parmley,W.W. and Swan,J.J.C. 1972. Early increase in left ventricular compliance after myocardial infarction. J.Clin.Invest. 51:598-603.

Frederiksen,D.W. Hoffurg,J.M. Frederiksen,R.T. and Williams,R.B. 1978. The structural proteins of normal and diseased human myocardium. Circ. Res. 42:459-466.

Fujiwara,H. Ashraf,M. Sato,S. and Millard,R.W. 1982. Transmural cellular damage and blood flow distribution in early ischemia in pig hearts. Circ. Res. 51:683-693.

Fung,Y.C.B. :1967.Elasticity of tissues in simple elongation. Am. J. Physiol. 213:1532-1544.

Fung,Y.C.B. 1981. Bio-viscoelastic Solids. *In* Biomechanics: Mechanical Properties of Living Tissues. Springer-Verlag, New York. pp 196-260.

Ganote,C.E. and VanderHeide,R.S. 1987. Cytoskeletal lesions in anoxic myocardial injury: A conventional high-voltage electron-microscopic and immunofluorescence study. Am. J. Pathol. 129:327-344.

Garrels,J.I. and Gibson,W. 1976. Identification and characterization of multiple forms of actin. Cell 9:793-805.

Gevers,W. 1984. Protein metabolism of the heart. J. Mol. Cell. Cardiol. 16:3-32.

Gilbert,J.C. and Glantz,S.A. 1989. Determinants of left ventricular filling and of the diastolic pressure-volume relation. Circ. Res. 64:827-852.

Goto,Y. Igarashi,Y. Yamada,O. Hiramori,K and Suga,H. 1988. Hyperkinesis without the Frank-Starling mechanism in a nonischemic region of acutely ischemic excised canine heart. Circulation 77:468-477.

Guyton,R.A. and Daggett,W.M. 1976. The evolution of Myocardial infarction: Physiological basis for clinical intervention. *In* International Review of Physiology; Cardiovascular Physiology 11, Vol 9. *Edited by* A.C. Guyton and A.W. Cowley. University Park Press, Baltimore. pp 306-334.

Harkness,R.D. 1968. Mechanical properties of collagenous tissues. *In* Treatise on Collagen. *Edited by* B.S. Gould. Academic Press, London. pp 247-299.

Hearse,D.J. Muller,C.A. Fukanami,M. Kudoh,Y. Opie,L. and Yellon,D.M. 1986. Regional myocardial ischemia: Characterization of temporal, transmural and lateral flow interfaces in the procine heart. Can. J. Cardiol. 2:48-61.

Herovici,C. 1963. A polychrome stain for differentiating precollagen from collagen. Stain Tech. 38:204-205.

Heymann,M.A. Payne,B.D. Hoffman,J.I.E. and Rudolph,A.M. 1977. Blood flow measurements with radionuclide-labeled particles. Prog. Cardiovasc. Dis. 20:55-79.

Hood,W.B. Biance,J.A. Kumar,R. and Whiting,R.B. 1970. Experimental Myocardial Infarction:IV. Reduction of left ventricular compliance in the healing phase. J. Clin. Invest. 49:1316-1323.

Hunt,T.K. Knighton,D.R. Thakral,K.K. Goodson,W.H. and Andrews,W.S. 1984. Studies on inflammation and wound healing: angiogenesis and collagen synthesis stimulated in vivo by resident and activated wound macrophages. Surgery 96:48-54.

Hutchins,G.M. and Bulkley,B.H. 1978. Infarct expansion versus extension: Two different complications of acute myocardial infarction. Am. J. Cardiol. 41:1127-1132.

Jalil,J.E. Doering,C.W. Janicki,J.S. Pick,R. Clark,W.A. Abrahams,C. and Weber,K.T. 1988. Structural vs. contractile protein remodeling and myocardial stiffness in hypertrophied rat left ventricle. J. Mol. Cell. Card. 20:1179-1187.

Janse,M.J. Kleber,A.G. Capucci,A. Coronel,R. and Wilms-Schopman,F. 1986. Electrophysiological basis for arrhythmias caused by acute ischemia : Role of the subendocardium. J. Mol. Cell. Cardiol. 18:339-355.

Judd,J.T. and Wexler,B.C. 1969. Myocardial connective tissue metabolism in response to injury: Histological and chemical studies of mucopolysaccharide and collagen in rat hearts after isoproterenol-induced infarction. Circ. Res. 25:201-214.

Jugdutt,B.I. Hutchins,G.M. Bulkley,B.H. and Becker,L.C. 1979. The loss of radioactive microspheres from canine necrotic myocardium. Circ Res. 45:746-756.

Jugdutt,B.I. and Amy,R.W.M. 1986. Healing after myocardial infarction in the dog: changes in infarct hydroxyproline and topography. JACC 7:91-102.

Kane,B.S. McMahon,T.A. Wagner,R.L. and Abelmann,W.H. 1976. Ventricular elastic modulus as a function of age in the syrian golden hamster. Circ. Res. 33:74-80.

Kiewiet De Jonge,M. VanDenBos,G.C. and Elizinga,G. 1980. Changes of microsphere density with time in myocardial infarcts in dogs. Cardiovas. Res. 14:741-744.

Kittleson,M.D. Knowlen,G.G. and Johnson,L.V.T. : Early and late global and regional left ventricular function after experimental transmural myocardial infarction: relationships of regional wall motion, wall thickening, and global performance. Am. Heart J. 114:70-78.

Klein,H.H. Schubothe,M. Nebendahl,K. and Kreuzer,H. 1984. Temporal and spatial development of infarcts in porcine hearts. Basic Res. Cardiol. 79:221-228.

Klocke,F.J. 1976. Coronary blood flow in man. Prog. in Cardiovas. Dis. 19:117-166.

Kozlovskis,P.L. Silver,J.D. Rubin,R.W. Wong,S.S. Gaide,M.S. Bassett,A.L. Cameron,J.S. and Myerburg,R.J. 1984. Regional variation in myosin heavy chain concentration after healing of experimental myocardial infarction in cats. J. Mol. Cell. Card. 16:559-566.

Krane,S.M. 1982. Collagenases and collagen degradation. J. Invest. Derm. 79:83s-86s.

Kuehl,W.M. and Adelstein,R.S. 1970. The absence of 3-methylhistidine in red, cardiac and fetal myosins. Biochem. Biophys. Res. Comm. 39:956-964.

Kumar,R. Hood,W.B. Joison,J. Norman,J.C. and Abelmann,W.H. 1970. Experimental Myocardial Infarction: II. Acute depression and subsequent recovery of left ventricular function: serial measurements in intact conscious dogs. J. Clin. Invest. 49:55-62.

Laemmli,U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Laird,J.D. and Vellekoop,H.P. 1977. Time course of passive elasticity of myocardial tissue following experimental infarction in rabbits and its relation to mechanical dysfunction. Circ. Res. 41:715-721.

Lamas,G.A. and Pfeffer,M.A. 1986. Increased left ventricular volume following myocardial infarction in man. Am. Heart J. 111:30-35.

Laurent,G.J. McAnulty,R.J. and Gibson,J. 1985. Changes in collagen synthesis and degradation during skeletal muscle growth. Am. J. Physiol. 249 (Cell Physiol. 18): C352-C355.

Lautsch,E.V. 1979. Morphological factors of clinical significance in myocardial infarction - a review. Texas Reports Bio. Med. 39:371-386.

Lee,G. 1968. Routine Staining Procedures. *In* Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. McGraw Hill Book Company. New York. pp 38-39.

Lee,J.M. and Boughner,D.R. 1981. Tissue mechanics of canine pericardium in different test environments: Evidence for time-dependent accommodation, absence of plasticity, and new roles for collagen and elastin. Circ. Res. 49:533-544.

Lee,J.M. and Boughner,D.R. 1985. Mechanical properties of human pericardium: Differences in viscoelastic response when compared with canine pericardium. Circ. Res. 55:475-481.

Lehninger,A.L. 1975. Translation: The Biosynthesis of Proteins. *In* Biochemistry. 2nd.Edn. Worth Publishers Inc. New York. pp 935.

Lerman,R.H. Apstein,C.S. Kagan,H.M. Osmers,E.L. Chichester,C.O. Vogel,W.M. and Steffee,W.P. 1983. Myocardial healing and repair after experimental infarction in the rabbit. Circ. Res. 53:378-388.

Levken,J. Anderser,K.S. 1980. Migration of 15 micron micropsheres from infarcted myocardium. Card. Res. 14:280-287.

Lew,W.Y.W. 1987. Influence of ischemic zone size on nonischemic area function in the canine left ventricle. Am. J. Physiol. 252 (Heart Circ.Physiol.21): H990-H997.

Lillie,R.D. Tracy, R.E. Pizzolato,P. Donaldson,P.T. Reynolds,C. 1980. Differential staining of collagen types in Paraffin sections: A colour change in degraded forms. Virchows Arch.A. Path. Anat. and Histol. 386:153-159.

Linsday,B.D. 1989. Cardiac Arrythmias. *In* Manual of Medical Therapeutics, Edn 26. *Edited by* W.C. Dunagan and M.L. Ridner. Little, Brown and Company, Boston. pp 154-170.

Mallory,G.K. White,P.D. and Salcedo-Salgar,J. 1939. The speed of healing of myocardial infarction; A study of the pathologic anatomy in 72 cases. Am. Heart J. 18:647-671.

Marcus,M.L. Harrison,D.G. Chillian,W.M. Koyanagi,S. Inou,T. Tomanek,R.J. Martins,J.B. Eastham,C.L. and Hiratzka,L.F. 1987. Alterations in the coronary circulation in hypertrophied ventricls. Circ.(suppl I) 75:I-19 - I-24.

Maxwell,M.P. Hearse,D.J. Yellon,D.M. 1987. Species variation in the coronary collateral circulation during regional myocarcial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction. Cardiov. Res. 21:737-746.

McClain,P.E. 1974. Characterization of cardiac muscle collagen: Molecular heterogeneity. J. Biol. Chem. 249:2303-2311.

McDonough,K.H. Dunn,R.B. and Griggs,D.M. 1984. Transmural changes in porcine and canine hearts after circumflex artery occlusion. Am. J. Physiol. 246 (Heart Circ. Physiol. 15): H601-H697.

Medugorac,I. 1980. Collagen content in different areas of normal and hypertrophied rat myocardium. Cardio. Res. 14:551-554.

Miller,E.J. and Gay,S. 1982. Collagen: An overview. *In* Methods in Enzymology. Vol 82. Structural and Contractile Proteins, Part A: The Extracellular Matrix. *Edited by* L.V. Cunningham, D.W. Frederiksen. Academic Press, New York. pp 3-32.

Mirsky,I. 1976. Assessment of passive elastic stiffness of cardiac muscle: mathematical concepts, physiologic and clinical consideration, and directions of future research. Prog. in Cardiovas. Dis. 63:277-308.

Mirsky,I. 1979. Elastic properties of the myocardium: a quantitative approach with physiological and clinical applications. *In* Handbook of Physiology, Section 2, The Cardiovascular System. *Edited by* R.E. Berne. Am. Physiolo. Society. Bethesda Maryland. pp 479-531.

Montfort,I. and Perez-Tamayo,R. 1962. The muscle-collagen ratio in normal and hypertrophic human hearts. Lab. Invest. 11:463-470.

Murdock, R.H. and Cobb,F.R. 1980. Effects of infarcted myocardium on regional blood flow measurements to ischemic regions in canine heart. Circ Res. 47:701-709.

Nimni,M.E. 1974. Collagen: Its structure and function in normal and pathological connective tissues. Sem. Arth. Rheum. 4:97-149.

Nimni,M.E. 1983. Collagen: structure, function, and metabolism in normal and fibrotic tissues. Sem. Arth. Rheum. 13:1-70.

Oken,D.E. and Boucek,R.J. 1957. Quantitation of collagen in human myocardium. Circ. Res. 5:357-361.

O'Konski,M.S. White,F.C. Longhurst,J. Roth,D. and Bloor,C. 1986. Ameroid constriction of the proximal left circumflex coronary artery in swine: A model of limited coronary collateral circulation. Am. J. Cardiovas. Path. 1:69-77.

Olsen,E.G.J. 1980. Myocardial infarction. *In* The pathology of the Heart. 2nd Edition. The McMillan Press Ltd, London. pp 111-119.

Orenstein,J. Hogan,D. and Bloom, S. 1980. Surface cables of cardiac myocytes. J. Mol. Cell. Card. 12:771-780.

Patterson,R.E. and Kirk,E.S. 1983. Analysis of coronary collateral structure, function, and ischemic border zones in pigs. Am. J. Physiol. 244 (Heart Circ. Physiol. 13):H23-H31.

Peterson,G.L. 1983. Determinations of total protein. *In* Methods in Enzymology. Vol 91. *Edited by* D.H. Hirs and S.N. Timasheft. Academic Press, New York. pp 95-119.

Pearlman,E.S. Weber,K.T. Janicki,J.S. Pietra,G.G. and Fishman,A.P. 1982. Muscle fiber orientation and connective tissue content in the hypertrophied human heart. Lab Invest. 46:158-164.

Pinnell,S.R. 1982. Regulation of collagen synthesis. J. Invest. Derm. 79:73s-76s.

Price,M. 1984. Molecular analysis of intermediate filament cytoskeleton: A putative load-bearing structure. Am. J. Physiol. 246 (Heart Circ. Physiol. 15): H566-H572.

Prockop,D.J. and Kivirikko,K.I. 1968. Hydroxyproline and the metabolism of collagen. *In* Treatise on Collagen. Vol 2. Biology of collagen. *Edited by* B. Gould. Academic Press, London. pp 215-246.

Prockop,D.J. Kivirikko,K.I. Tuderman,L. and Guzman,N.A. 1979. The biosynthesis of collagen and its disorders. New Engl. J. Med. 301:13-23.

Prockop,D.J. 1986. Genetic defects of collagen. Hosp. Practice. 21(2):125-140.

Przyklenk,K. Connelly,C.M. McLaughlin,R.J. Kloner,R.A. and Apstein,C.S. 1987. Effect of myocyte necrosis on strength, strain, and stiffness of isolated myocardial strips. Am. Heart J. 114:1349-1359.

Reimer,K.A. and Jennings,R.B. 1979. The changing anatomic reference base of evolving myocardial infarction; Underestimation of myocardial collateral blood flow and overestimation of experimental anatomic infarct size due to tissue edema, hemorrhage and acute inflammation. Circ. 60:866-876.

Robbins,S.L. Cotran,R.S. and Kumar,V. 1984. Inflammation and Repair. *In* Pathologic Basis of Disease. 3rd Edition. W.B. Saunders Co., Philadelphia. pp 40-84.

Roberts,C.S. MacLean,D. Maroko,P. and Kloner,R. 1984. Early and late remodeling of the left ventricle after acute myocardial infarction. Am. J. Cardiol. 54:407-410.

Robinson,T.F. Cohen-Gould,L. and Factor,S.M. 1983. Skeletal framework of mammalian heart muscle; arrangement of inter-and pericellular connective tissue structures. Lab. Invest. 40:482-498.

Robinson,T.F. Cohen-Gould,L. Remily,R.M. Capasso,J.M. and Factor,S.M. 1985. Extracellular structures in Heart Muscle. *In* Advances in Myocardiology. Vol 4. *Edited by* Peter Harris and Philip A. Poole-Wilson. Plenum Medical Book Company, New York and London. pp 243-255.

Romsom,J.L. Hook,B.G. Kunkel,S.L. Abrams,G.D. Schork,A. and Lucchesi,B.R. 1983. Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circ. 67:1016-1023.

Ross,M.H. Reith,E. 1985. Connective tissue. *In* Histology: A Text and Atlas. Harper and Row Publ., New York. pp 84-97.

Rossen,R.D. Swain,J.L. Michael,L.H. Weakley,S. Giannini,E. and Entman,M.L. 1985. Selective accumulation of the first component of complement and leukocytes in ischemic canine heart muscle: A possible initiator of an extra myocardial mechanism of ischemic injury. Circ. Res. 57:119-130.

Rubin,S.A. Fishbein,M.C. Swan,H.J.C. and Rabines,A. 1983. Compensatory hypertrophy in the heart after myocardial infarction in the rat. J. Am. Coll. Cardiol. 1:1435-1441.

Sabbah,H.N. Gheorghiade,M. Smith,S.T. Frank,D.M. and Stein,P.D. 1987. Rate and extent of recovery of left ventricular function in patients following acute myocardial infarction. Am. Heart J. 114:516-524.

Sakai,K. Watanabe,K. and Millard,R.W. 1985. Defining the mechanical border zone: A study in the pig heart. Am. J. Physiol. 249 (Heart Circ. Physiol. 18): H88-H94.

Sato,S. Ashraf,M. Millard,R.W. Fujiwara,H. and Schwartz,A. 1983. Connective tissue changes in early ischemia of procine myocardium:an ultrastructural study. J. Mol. Cell. Cardiol. 15:261-275.

Savage,R.M. Guth,B. White,F.C. Hagan,A.D. and Bloor,C.M. 1981. Correlation of regional myocardial blood flow and function with myocardial infarct size during acute myocardial ischemia in the conscious pig. Circ. 64:699-707.

Schaper,J. 1986. Ultrastructural changes of the myocardium in regional ischaemia and infarction. European Heart J. 7(Suppl. B):3-9.

Schaper,W. Jageneau,A. and Xhonneux,R. 1967. The development of collateral circulation in the pig and dog heart. Cardioplegia 51:321-335.

Schaper,W. Schaper,J. Xhonneux,R. and Vandesteene,R. 1969. The morphology of intercoronary anastomoses in chronic coronary artery occlusion. Cardiovasc. Res. 3:315-323.

Shetlar,M.R. Shetlar,C.L. and Kischer,C.W. 1979. Healing of myocardial infarction in animal models. Tex. Rep. Biol. Med. 39:339-355.

Sjoqist,P.O. Duker,G. and Almgren,O. 1984. Distribution of the collateral blood flow at the lateral border of the ischemic myocardium after acute coronary occlusion in the pig and the dog. Basic Res. Cardiol. 79:190-175.

Smalling,R.W. Ekas,R.D. Felli,P.R. Binion,L. and Desmond,J. 1986. Reciprocal functional interaction of adjacent myocardial segments during regional ischemia: an intraventricular loading phenomenon affecting apparent regional contractile function in the intact heart. J.Am. Coll. Cardiol. 7:1335-1346.

Spotnitz,H.M. and Sonnenblick,E.H. 1973. Structural conditions in the hypertrophied and failing heart. Am. J. Cardiol. 32:398-406.

Stowe,D.F. Mathey,D.G. Moores,W.Y. Glantz,S.A. Townsend,R.M. Kabra,P. Chatterjee,K. Parmley,W.W. and Tyberg,J.V. 1978. Segment stroke work and metabolism depend on coronary blood flow in the pig. Am. J. Physiol. 234(5):H597-H607.

Swan,H.J.C. Forrester,J.S. Diamond,G. Chatterjee,K. and Parmley,W.W. 1972. Hemodynamic spectrum of myocardial infarction and cardiogenic shock: A conceptual model. Circ. XLV:1097-1110.

Swan,H.J.C. 1979. Mechanical function of the heart and its alteration during myocardial ischemia and infarction. Circ. 60:1587-1592.

Takemura,R. and Werb,Z. 1984. Secretory products of macrophages and their physiological functions. Am. J. Physiol. 246 (Cell Physiol.15): C1-C9.

Tennant,R. and Wiggers,C.J. 1935. The effect of coronary occlusion on myocardial contraction. Am. J. Physiol. 112:351-361.

Theroux,P. Ross,J.Jr. Franklin,D. Covell,J.W. and Bloor, C.M. 1977. Regional myocardial function and dimensions early and late after myocardial infarction in the unanesthetized dog. Circ.Res. 40:158-165.

Thomas,D.P. Phillips,S.J. and Bove,A.A. 1984. Myocardial morphology and blood flow distribution in chronic volume-overload hypertrophy in dogs. Basic Res. Cardiol. 79:379-388.

Towbin,H. Staehelin,T. and Gordon,J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Proc. Natl. Acad. Sci. USA 76:4350-4354.

Turner,I.E. Oliver,M.H. Guerreiro,D. and Laurent,G.J. 1986. Collagen metabolism during right ventricular hypertrophy following induced lung injury. Am. J. Physiol. 251 (Heart Circ. Physiol. 20):H915-H919.

Tyberg,J.V. Forrester,J.S. and Parmley,W.W. 1974. Altered segmental function and compliance in acute myocardial ischemia. Europ. J. Cardiol. 1/4:307-317.

Uitto,J. Murray,L.W. Blumberg,B. and Shamban,A. 1986. Biochemistry of collagen in diseases. Ann. Int. Med. 105:740-756.

Vandekerckhove,J. and Weber,K. 1981. Actin typing on total cellular extracts; a highly sensitive protein-chemical procedure able to distinguish different actins. Eur. J. Biochem. 113:595-603.

Viidik,A. 1973. Functional properties of collagenous tissues. *In* International Review of Connective Tissue Research. Vol 6. *Edited by* David A. Hall and P.S.Jackson. Academic Press, New York. pp 127-215.

Weaver,M.E. Pantely,G.A. Bristow,J.D. and Ladley,H.D. 1986. A quantitative study of the anatomy and distribution of coronary arteries in swine in comparison with other animals and man. Cardiovasc. Res. 20:907-917.

Weber,K.T. 1989. Cardiac interstitium in health and disease: The fibrillar collagen network. JACC 13:637-652.

Weber,K.T. Clark,W.A. Janicki,J.S. Shroff,S.G. 1987. Physiologic versus pathologic hypertroophy and the pressure-overloaded myocardium. J. Card. Pharm. 10(Suppl.6):S37-S49.

Weber,K.T. Janicki,J.S. Pick,R. Avrahams,C. Shroff,S.G. Bashey,R.I. and Chen,R.M. 1987. Collagen in the hypertrophied, pressure-overloaded myocardium. Circ. 75(Suppl I):I-40 - I-47.

Weber,K.T. 1989. Cardiac interstitium in health and disease: the fibrillar collagen network. JACC 13:1637-1652.

Weisman,H.F. and Healy,B. 1987. Myocardial infarct expansion, infarct extension, and reinfarction: pathophysiologic concepts. Prog. in Cardio. Dis. 30:73-110.

White,F.C. Bloor,C.M. 1981. Coronary collateral circulation in the pig: correlation of collateral flow with coronary bed size. Basic Res. Cardiol. 76:189-196.

Wikman-Coffelt,J. Parmley,W.W. and Mason,D.T. 1979. The cardiac hypertrophy process: analysis of factors determining pathological vs. physiological development. Circ. Res. 45:697-707.

Zhao,M. Zhang,H. Robinson,T.F. Factor,S.M. Sonnenblick,E.H. and Eng,C. 1987. Profound structural alterations of the extracellular collagen matrix in postischemic dysfunctional ("stunned") but viable myocardium. JACC 10:1322-1334.

6.0 APPENDICES

6.1 APPENDIX I ANOVA TABLES

The following are results of detailed statistical analysis performed on obtained data. Data were initially analysed using the SAS statistical package. If significant differences between groups were observed by ANOVA, a Scheffe post-hoc test was performed to locate the differences. The following data are reported in the individual tables:

Source of Variation	This describes the location of the differences; either between groups, or within groups.
df	Degrees of freedom calculated by (k-1) for differences between groups, or k(N-1) for differences within groups; where k= number of groups and N = number of individual measurements.
Sum of Squares	The within group sum of squares is the sum of squared deviations within each sample summed over all ksamples. The between group sum of squares is the sum of squared deviations of sample means from the grand mean (mean of the group means) multiplied by the sample size.
Mean Square	The sum of squares divided by the degrees of freedom.
F Ratio	Ratio of the mean square between groups to the mean square within groups.
Sig. Level	The level of significance; the probability that the 'f' value is significant.
Scheffe	If differences among groups were significant, comparisons were performed using the Scheffe test. Only significant differences are reported. If not reported, then no differences were measureable.

TABLE 6.1.1:
ANOVA TABLES OF TEMPORAL BLOOD FLOW TO INFARCTED TISSUE

PREOCCLUSION BLOODFLOW (BF1)

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	123.37	20.56	23.83	0.0001
Within	334	288.24	0.86		

Scheffe 2 different from all,
5 different from all,
4, 6, 3, 1, and 7 not different from each other

TEN MINUTE POST-OCCLUSION BLOODFLOW (BF2)

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	110.30	18.38	263.87	0.0
Within	316	22.01	0.06		

Scheffe 1 different from 3, 4, 2, 6, 7, 5
2, 3, 4, 5, 6 and 7 not different from each other.

BLOODFLOW AT THE TIME OF SACRIFICE (BF3)

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	59.11	9.85	35.33	0.0001
Within	292	81.42	0.27		

Scheffe 1 different from all,
2 different from 1 and 6,
3 different from 1 and 6,
5, 4, 7, and 6 not different from each other,

TABLE 6.1.2:
ANOVA TABLES OF TEMPORAL BLOOD FLOW TO NONINFARCTED TISSUE

PREOCCLUSION BLOODFLOW (BF1)

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	307.01	51.17	129.70	0.0001
Within	703	278.92	0.39		

Scheffe 2 different from all,
3 different from all,
5, 4, and 1 not different from each other,
6 and 7 not different from each other.

TEN MINUTE POST-OCCLUSION BLOODFLOW (BF2)

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	190.48	31.74	87.28	0.0001
Within	685	249.16	0.36		

Scheffe 2 different from all,
3, 4, 1 and 5 not different from each other,
6 and 7 not different from each other.

BLOODFLOW AT THE TIME OF SACRIFICE (BF3)

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	60.93	10.15	10.70	0.0001
Within	620	588.54	0.94		

Scheffe 3 different from all, 2 different from all,
5 different from all, 4 different from all,
7, 1 and 6 not different from each other.

TABLE 6.1.3:
ANOVA TABLES OF REGIONAL HETEROGENEITY OF BLOOD FLOW MEASUREMENTS

PREOCCLUSION BLOODFLOW (BF1)

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	5	2225661.70	445132.34	39.46	0.0001
Within	561	8554216.91	11280.85		

Scheffe 2 different from 7, 6, 5; 3 different from 7, 6, 5;
4 different from 7, 6, 5; 5 different from 7, 4, 3, 2.

TEN MINUTE POST-OCCLUSION FLOW (BF2)

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	5	5369.04	1073.81	3.76	0.0023
Within	539	153904.82	285.54		

Scheffe 4 different from 2

BLOODFLOW PRIOR TO SACRIFICE (BF3)

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	5	41991.47	8398.29	15.12	0.0001
Within	508	282118.98	555.35		

Scheffe 5 different from 3, 2; 6 different from 3, 2, 4;
7 different from 3, 2, 4.

TABLE 6.1.4:
ANOVA TABLES OF REGIONAL HYDROXYPROLINE CONTENT IN THE CONTROL GROUP

HYDROXYPROLINE CONCENTRATION DETERMINED BY BERG AND LOWRY

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	1	0.009	0.009	0.20	0.6717
Within	6	0.30	0.05		

HYDROXYPROLINE CONCENTRATION DETERMINED BY BERG AND OPA

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	1	0.012	0.012	1.20	0.3092
Within	6	0.072	0.010		

TABLE 6.1.5:
ANOVA TABLES OF HYDROXYPROLINE VALUES
IN NONINFARCTED TISSUE FROM ALL GROUPS

HYDROXYPROLINE CONCENTRATION DETERMINED BY BERG AND LOWRY

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	1.41	0.24	4.87	0.0007
Within	42	2.03	0.05		

Scheffe 7 different from 4

HYDROXYPROLINE CONCENTRATION DETERMINED BY BERG AND OPA

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	0.43	0.07	4.59	0.0012
Within	41	0.64	0.02		

Scheffe 7 different from 2 and 4

HYDROXYPROLINE CONCENTRATION DETERMINED BY HPLC

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	7	0.31	0.04	1.54	0.17
Within	44	1.29	0.02		

TABLE 6.1.6:
ANOVA TABLES OF HYDROXYPROLINE VALUES
IN INFARCTED TISSUE FROM ALL GROUPS

HYDROXYPROLINE CONCENTRATION DETERMINED BY BERG AND LOWRY

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	356.04	59.34	5.50	0.0002
Within	45	485.40	10.79		

Scheffe 1 different from 6 and 7

HYDROXYPROLINE CONCENTRATION DETERMINED BY BERG AND OPA

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	349864.93	58310.82	12.84	0.0001
Within	43	195314.05	4542.19		

Scheffe 1 different from 5, 6, 7; 2 different from 6 and 7;
3 different from 6 and 7; 4 different from 7

HYDROXYPROLINE CONCENTRATION DETERMINED BY HPLC

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	7	73.693	10.53	12.23	0.0001
Within	46	39.61	0.86		

Scheffe 1 different from 5, 6, 7; 2 different from 6 and 7;
3 different from 6 and 7; 4 different from 6 and 7

TABLE 6.1.7:
ANOVA TABLES OF THE HETEROGENEITY OF REGIONAL HYDROXYPROLINE CONCENTRATION: (INFARCT : NONINFARCT)

HYDROXYPROLINE CONCENTRATIONS DETERMINED BY BERG AND LOWRY

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	4415754.59	735959.09	2.60	0.034
Within	36	10200496.43	283347.11		

Scheffe Can reject H_o, however the Scheffe test is not powerful enough to detect which groups are different.

HYDROXYPROLINE CONCENTRATIONS DETERMINED BY BERG AND OPA

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	1919601.35	319933.55	4.80	0.0012
Within	35	2334890.21	66711.14		

Scheffe Can reject H_o, however scheffe is not powerful enough to detect which groups are different.

HYDROXYPROLINE CONCENTRATIONS DETERMINED BY HPLC

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	1981261.10	330210.18	6.18	0.0001
Within	42	2243931.38	03426.94		

Scheffe 1 different from 6 and 7; 2 different from 6 and 7

TABLE 6.1.8:
ANOVA TABLES OF 3-METHYLHISTIDINE CONCENTRATION

3-METHYLHISTIDINE CONCENTRATION IN THE INFARCT ZONE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	0.012	0.0018	1.62	0.1787
Within	23	0.025	0.0011		

3-METHYLHISTIDINE CONCENTRATION IN THE NONINFARCT ZONE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	0.007	0.001	1.37	0.2553
Within	30	0.023	0.001		

TABLE 6.1.9:
ANOVA TABLES OF RATIOS OF AMINO ACIDS

CONCENTRATION OF HYDROXYPROLINE TO 3-METHYLHISTIDINE IN THE NONINFARCT ZONE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	2291.53	381.92	1.04	0.4180
Within	33	12126.35	367.46		

CONCENTRATION OF PROLINE TO HYDROXYPROLINE IN THE NONINFARCT ZONE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	141.34	23.55	1.99	0.085
Within	47	555.30	11.81		

CONCENTRATION OF HYDROXYPROLINE TO 3-METHYLHISTIDINE
IN THE INFARCT ZONE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	126013.77	21002.30	3.53	0.01
Within	26	154610.15	5946.54		

Scheffe — Can reject H_o, however the Scheffe test is not powerful enough to locate the differences.

CONCENTRATION OF PROLINE TO HYDROXYPROLINE IN THE INFARCT ZONE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	1192.10	198.68	26.05	0.0001
Within	49	373.72	7.63		

Scheffe 6 different from 1, 2, 3; 7 different from 1 and 2;
5 different from 1 and 2; 4 different from 1;
3 different from 1.

TABLE 6.1.10:
ANOVA OF WATER CONTENT IN THE INFARCT ZONE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	3064.24	510.71	40.60	0.0001
Within	425	5346.07	12.57		

Scheffe 1 different from 6, 7, 5, 4, 2; 3 different from 6;
2 different from 6 and 1.

TABLE 6.1.11a:
ANOVA TABLES COMPARING STIFFNESS BETWEEN CYCLES:
LOW STRAIN RANGE, INITIAL STIFFNESS

COMPARISON OF INFARCT STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	70.78	35.39	2.93	0.057
Within	129	1558.32	12.08		

COMPARISON OF NONINFARCT STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	72.28	36.14	1.37	0.25
Within	104	2741.44	26.36		

COMPARISON OF EPICARDIAL STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	289.6	144.80	1.61	0.206
Within	85	7661.9	90.14		

COMPARISON OF ENDOCARDIAL STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	111.08	55.54	1.31	0.275
Within	86	3649.84	42.44		

TABLE 6.1.11b:
ANOVA TABLES COMPARING STIFFNESS BETWEEN CYCLES:
LOW STRAIN RANGE, FINAL STIFFNESS

COMPARISON OF INFARCT STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	87.82	43.91	7.98	0.0005
Within	129	709.5	5.50		

Scheffe 1 different from 2 and 3

COMPARISON OF NONINFARCT STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	100.12	50.06	6.09	0.0032
Within	104	855.88	8.22		

Scheffe 1 different from 2 and 3

COMPARISON OF EPICARDIAL STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	361.20	180.60	4.11	0.0197
Within	85	3730.65	43.89		

Scheffe 3 different from 1 and 2

COMPARISON OF ENDOCARDIAL STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	128.82	64.41	2.73	0.07
Within	86	2156.88	25.08		

TABLE 6.1.11c:
ANOVA TABLES COMPARING STIFFNESS BETWEEN CYCLES:
HIGH STRAIN RANGE, INITIAL STIFFNESS

COMPARISON OF INFARCT STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	731.10	386.55	3.09	0.0491
Within	121	15129.84	125.04		

Scheffe Can reject H_o, however the Scheffe test is not powerful enough to locate the differences.

COMPARISON OF NONINFARCT STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	169.42	84.71	0.28	0.75
Within	95	29010.15	305.37		

COMPARISON OF EPICARDIAL STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	6127.38	3063.69	1.41	0.25
Within	80	173659.20	2170.74		

COMPARISON OF ENDOCARDIAL STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	1312.52	656.26	1.07	0.347
Within	79	48371.70	612.30		

TABLE 6.1.11d:
ANOVA TABLES COMPARING STIFFNESS BETWEEN CYCLES:
HIGH STRAIN RANGE, FINAL STIFFNESS

COMPARISON OF INFARCT STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	2367.36	1183.68	20.73	0.0001
Within	121	6907.89	57.09		

Scheffe 1 different from 2 and 3

COMPARISON OF NONINFARCT STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	2875.46	1437.73	18.00	0.0001
Within	95	7587.65	79.87		

Scheffe 1 different from 2 and 3

COMPARISON OF EPICARDIAL STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	12225.52	6112.76	7.03	0.0015
Within	80	69519.20	868.99		

Scheffe 1 different from 2 and 3

COMPARISON OF ENDOCARDIAL STRIPS

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	2	4363.34	2181.67	7.80	0.0008
Within	79	22101.83	279.77		

Scheffe 1 different from 2 and 3

TABLE 6.1.12:
ANOVA TABLES OF STIFFNESS OF THE NONINFARCT REGION

INITIAL STIFFNESS MEASURED OVER LOW STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	173.12	28.85	1.06	0.4043
Within	31	839.94	27.09		

FINAL STIFFNESS MEASURED OVER LOW STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	48.06	8.01	0.91	0.5029
Within	31	273.86	8.83		

INITIAL STIFFNESS MEASURED OVER HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	2218.94	369.82	1.61	0.1802
Within	28	6415.30	229.12		

FINAL STIFFNESS MEASURED OVER HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	696.25	116.04	1.59	0.1885
Within	27	1970.82	72.99		

TABLE 6.1.13:
ANOVA TABLES OF THE STIFFNESS OF THE INFARCTED REGION

INITIAL STIFFNESS MEASURED OVER LOW STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	71.25	11.88	1.06	0.4044
Within	33	368.80	11.18		

FINAL STIFFNESS MEASURED OVER LOW STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	66.63	11.10	1.93	0.1046
Within	33	189.60	5.74		

INITIAL STIFFNESS MEASURED OVER HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	346.72	57.78	0.54	0.7737
Within	29	3104.00	107.03		

FINAL STIFFNESS MEASURED OVER HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	286.57	47.76	0.82	0.5611
Within	29	1682.14	58.00		

TABLE 6.1.14:
ANOVA TABLES OF THE STIFFNESS OF INFARCTED EPICARDIAL STRIPS

INITIAL STIFFNESS MEASURED OVER LOW STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	5	909.99	181.99	3.40	0.0192
Within	23	1231.76	53.55		

Scheffe — Can reject H_o, however the Scheffe test is not powerful enough to locate the differences.

FINAL STIFFNESS MEASURED OVER LOW STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	5	426.36	82.27	2.60	0.0524
Within	23	753.37	32.76		

INITIAL STIFFNESS MEASURED OVER HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	4	1325.78	331.44	0.82	0.5267
Within	23	9316.79	405.08		

FINAL STIFFNESS MEASURED OVER HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	4	1241.81	310.45	0.72	0.5851
Within	23	9875.33	429.36		

TABLE 6.1.15:
ANOVA TABLES OF STIFFNESS OF THE INFARCTED ENDOCARDIAL REGION

INITIAL STIFFNESS MEASURED OVER LOW STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	5	218.47	43.69	0.99	0.4461
Within	24	1062.36	44.26		

FINAL STIFFNESS MEASURED OVER HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	5	141.97	28.39	1.14	0.3681
Within	24	599.32	24.97		

INITIAL STIFFNESS MEASURED OVER HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	5	3896.25	779.25	1.00	0.4449
Within	24	15609.99	780.49		

FINAL STIFFNESS MEASURED OVER HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	5	1627.76	325.55	0.86	0.5256
Within	24	7194.54	378.66		

TABLE 6.1.16:
ANOVA TABLES OF THE ELASTIC COMPONENT OF STIFFNESS

ELASTIC STIFFNESS OF NONINFARCTED STRIPS - LOW STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	276.12	42.02	1.29	0.2872
Within	35	9316.79	405.08		

ELASTIC STIFFNESS OF NONINFARCTED STRIPS - HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	593.00	98.83	0.59	0.7333
Within	28	4667.64	166.70		

ELASTIC STIFFNESS OF INFARCTED STRIPS - LOW STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	1307.89	217.98	1.69	0.1497
Within	37	1231.76	53.55		

ELASTIC STIFFNESS OF INFARCTED STRIPS - HIGH STRAIN RANGE

Source of Variation	df	Sum of Squares	Mean Square	F Ratio	Sign. Level
Between	6	609.78	101.63	4.60	0.0019
Within	31	753.37	32.76		

Scheffe 5 different from 1

6.2 APPENDIX II - RECIPES

The following are detailed procedures of some of the methods which were performed. All are from existing published methods and were used unmodified. Procedures are presented in recipe form.

Short forms used in the recipes:

SDS	sodium dodecyl suphate
M	molar
HCL	hydrochloric acid
mM	millimolar
TEMED	tetramethyl-ethylene diamine
%	percent
gm	gram
BSA	bovine serum
mA	milliamperage
DAB	3-3-diamino benzidine
L	liter
ml	milliliter
DTT	dithiothreitol
µl	microliter
rpm	rotations per minute

6.2.1 GEL ELECTROPHORESIS

SOLUTIONS

Sample buffer

Tris HCl	62.5 mM
SDS	2%
Glycerol	10%
2-mercaptoethanol	5%
Bromophenol blue	0.0001%

Running buffer

Tris	30.0	gm	
Glycine	144.0	gm	dilute 1:10 for use
SDS	10.0	gm	
Water to	1000.0	ml	

Solutions for gels

1. Acrylamide 29.2 gm
   Bisacrylamide 0.8 gm
   Water to 100.0 ml

2. Tris 18.17 gm
   SDS 0.4 gm
   Water to 100.0 ml
   pH to 8.8 using HCl

3. Tris 6.06 gm
   SDS 0.4 gm
   Water to 100.0 ml
   pH to 8.6 with HCl

4. 10% Ammonium Persulphate

Running (Lower) gel 6.0%

water	16.5	ml
1	6.0	ml
2	7.5	ml
4	0.09	ml
TEMED	0.03	ml

Running (Lower) gel 6.0% with Glycerol

water	7.5 ml
1	6.0 ml
2	7.5 ml
4	0.1 ml
glycerol	9.0 ml
TEMED	0.05 ml

Stacking (Upper) gel 4.0%

water	6.17 ml
1	1.33 ml
3	2.50 ml
4	0.06 ml
TEMED	0.02 ml

Silver stain

50% Methanol, 10% Glacial Acetic Acid
5% Methanol, 7% Glacial Acetic Acid
10% Glutaraldehyde
Dithiothreitol (DTT) 5μg/ml
0.1% Silver Nitrate

Developer Solution

50 μl of 37% formaldehyde in 100 ml of 3% sodium carbonate

Place gel in 50% Methanol with 10% Glacial Acetic Acid, then 5% Methanol with 7% Glacial Acetic Acid, then 10% Glutaraldehyde, each for thirty minutes. Rinse with distilled water for two hours. Place in DTT (5μg/ml) for 30 minutes, then Silver Nitrate (0.1%) for thirty minutes. Rinse quickly with water, then two quick rinses with developer solution. Soak in in developer solution. Stop staining with 5 ml Citric Acid (2.3 M) per 100 ml developer. Wash with distilled water, dry.

Coomassie stain

Coomassie Blue-R	1.25 gm
Methanol	227.0 ml
Distilled Water	227.0 ml
Glacial Acetic Acid	46.0 ml

Let gel sit in this for approximately twenty minutes, with gentle shaking. Remove excess stain with destain.

Destain

Acetic Acid	75.0	ml
Methanol	250.0	ml
Distilled Water	675.0	ml

After staining with Coomassie Blue-R stain, pour off coomassie. Rinse gently with water to remove excess dye. Cover with destain and agitate gently. Change destain after 30 and 60 minutes. Takes about 2-3 hours to destain.

6.2.2 WESTERN BLOTTING AND MYOSIN AND ACTIN REACTION WITH ANTIBODY

SOLUTIONS

Blotting Buffer

Tris (pH 8.3)	25 mM
Glycine	192 mM

Store at 4°C, use once only.

Base Solution

Tris	0.5 M
Sodium Chloride	0.85 M
Calcium Chloride	0.02 M

Saturating solution

3% Bovine Serum Albumin (BSA) in base solution

Wash solution

Base solution	250 ml
BSA	0.1 %
NP-40	0.2 %

Stock solution for developing nitrocellose

Tris-HCl (100 mM)	3.03 gm
Imidazole (10 mM)	0.17 gm
Distilled water	250.0 ml

pH to 7.6, then refrigerate. To 20 ml of stock solution add 5 mg 3-3-diamino benzidine tetrahydrochloride (DAB), and 10 μg Hydrogen Peroxide.

Ponceau Red

0.2% Ponceau Red (w/v)
3.0% Trichloroacetic acid (w/v)

METHOD

ELECTROPHORECTIC TRANSFER OF PROTEINS

1. Proteins are initially separated by gel electrophoresis. Cut out area of gel where the proteins of interest are. Mark one corner for identification. Soak in blotting buffer for 15 minutes to allow swelling. Cut piece of nitrocellulose slightly larger than the gel. Soak nitrocellulose briefly in blotting buffer.

2. Place sponge on plexiglass support. Lay piece of wet Watman filter paper on it. Place nitrocellulose on this. Carefully place the gel on top of the nitrocellulose. Mark nitrocellulose to identify. Lay another piece of wet Watman, then lay a sponge on top. Close plexiglass support.

3. Transfer to blotting chamber so that the nitrocellulose paper is closest to the anode.

4. Transfer proteins to nitrocellulose at 250 mA at room temperature. This takes approximately 7 to 8 hours.

5. Remove nitrocellulose. Visualize protein bands with Ponceau Red, then rinse in water to remove the Ponceau Red.

6. Place in saturating solution to equilibrate with gentle shaking (4°C) for overnight or 37°C for one hour.) This will saturate all the membrane with protein (BSA).

EXPOSURE OF PROTEINS TO ANTIBODY

1. After preincubation, place the nitrocellulose paper in saturating solution which contains the appropriate antibody (keep volumes as small as possible). Incubate two hours at room temperature on a gentle shaker.

2. Wash the nitrocellulose three times, 10 minutes each wash, with wash solution.

3. Incubate with second antibody, which is labelled with peroxidase, 1:500, in saturating solution for one hour at room temperature with gentle shaking.

4. Wash three times, 10 minutes each wash, as in step 2.

VISUALIZATION OF THE ANTIBODY

1. To 20 ml of stock solution add 5 mg 3-3-diamino benzidine tetrahydrochloride (DAB), 10 μl 30% hydrogen peroxide (H_2O_2). Place nitrocellulose in this solution.

2. Colour should develop quickly. (DAB is an electron donor; colour product precipitates at site where formed).

3. Stop colour development by transferring to water.

4. Dry on filter paper.

6.2.3 PROTEIN ASSAYS

6.2.3.1 THE PETERSON MODIFIED LOWRY TOTAL PROTEIN ASSAY

SOLUTIONS

2% SDS in 0.2 N NaOH

0.80 N NaOH

5% SDS (w/v)

CTC

0.1 % (w/v) copper sulfate pentahydrate
0.2 % (w/v) Rochelle salt
10.0 % (w/v) sodium carbonate
Stored in glass at 4°C.

Reagent A

Mix 1 part CTC
2 parts 5% SDS
1 part 0.8 N NaOH
Store in amber glass at room temperature.

Reagent B

Mix 1 part Phenol reagent
5 parts H_2O

Store in amber glass at room temperature.

BSA 1 mg/ml prepared in H_2O

PROCEDURE

1. Weigh exactly approximately 5 mg acetone powder samples.
   Add 1.00 ml 2% SDS in 0.2 N NaOH.
   Homogenize to dissolve.

2. Preparation of standard curve, done in duplicate.
   Pipet 1.0 µl stock per µl BSA desired into appropriate tubes and make to 1.00 ml with H_2O.

   eg.

µg BSA	µl H_2O
0.0	1000
10.0	990
20.0	980
50.0	950
80.0	920
100.0	900

3. UNKNOWNS: done in duplicate
   Label tubes.
   Pipet 10.0 µl each sample solution (step #1) into appropriately labelled tubes. Add 990 µl H_2O. Mix.

4. Add 1.000 ml Reagent A. Mix.
   Sit 10 minutes.

5. Add 0.500 ml Reagent B, mixing after each addition.
   Sit 30 minutes.

6. Transfer to cuvettes and read absorbance at 750.
   Calculate. Plot 1/absorbance versus 1/BSA for standard curve. Use the standard curve to calculate the amount of protein in the unknown samples.

6.2.3.2 OPA METHOD FOR DETERMINING TOTAL AMINO ACID CONTENT

SOLUTIONS

Stock OPA Solution

OPA reagent purchased from Pierce Chemical Company, called FLUORALDEHYDE o-Phthalaldehyde Reagent Solution. Add 30 µl 2-mercaptoethanol to 5 ml.

0.5 M NaOH

Pierce Amino Acid Standard

Dilute 40 µl Pierce Amino Acid Standard to 5 ml in 0.01 M HCl.

METHOD

1. Preparation of standards for standard curve done in duplicate.

Amino Acid Diluted Standard	dH_2O
0 µl	100 µl
10 "	90 "
20 "	80 "
40 "	60 "
80 "	20 "
100 "	0 "

2. Preparation of unknown samples, done in duplicate:
   Weigh exactly approximately 1 mg acetone powder. Hydrolyse samples in 6N HCl, then dry down.

3. Dissolve sample in appropriate volume of 0.01 N HCl.

4. Take 100 µl of dissoved sample (will have approximately 10 - 40 nmoles of amino acid) and add 100 µl OPA Reagent.

5. Leave 15 minutes.
   Add 2.0 ml of 0.5 N NaOH.

6. Excite at 340 nm, Read at 450 nm using a spectrofluorometer.

AMINO ACID STANDARD

1 ml Pierce amino acid standard contains 1.25 lmole L-Cystine, 2.50 lmole L-alanine, L-aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine.

6.2.4 HYDROXYPROLINE ASSAY

SOLUTIONS

Concentrated Potassium Hydroxide (KOH)

10 gm KOH bring to 100 ml with distilled water
Store in glass at room temperature

Dilute potassium hydroxide

1:50 concentrated KOH:distilled water
Store in glass at room temperature

Potassium borate buffer

Dissolve 61.8 gm boric acid in 850 ml warm distilled water
Add 225 gm KCl, dissolve
pH to 8.6 using KOH pellets
Bring volume to 1000 ml
Store in glass at 4°C

10% Alanine

Store in glass at 4°C

3.6M Sodium thiosulfate

Store in amber glass at room temperature

Erlick's Reagent

Add 27.4 ml H_2SO_4 to 200 ml cold absolute alcohol
Dissolve 120 gm PABA onto 200 ml absolute alcohol
Gently add the acid/alcohol to the PABA/alcohol mixture. Mix.
Store in amber glass.

1% Phenolphthalein

Store in glass at room temperature

0.2M Chloramine-T MAKE UP FRESH ON DAY OF EXPERIMENT

Dissolve 2.82 gm Chloramine-T in 50 ml cellsolve

Standards

Stock		Hydroxyproline	1.000 mg/ml
Working	A:	Hydroxyproline	10.000 µg/ml
	B:	Hydroxyproline	1.000 µg/ml

PROCEDURE

1. Label screw capped culture tubes. All samples done in duplicate.

2. Add KCl crystals to all tubes.

3. To prepare standard curve:
   Using the hydroxyproline (OHP) standards prepare a standard curve, adding a known amount of standard, and bringing the volume to 4.0 ml with distilled water (dH_2O).

Standards	µg OHP:	ml A or B	ml Distilled Water
	10.0	1.0 ml A	3.0
	5.0	0.5 ml A	3.5
	2.0	2.0 ml A	2.0
	1.0	1.0 ml B	3.0
	0.5	0.5 ml B	3.5
	0.0	0.0 ml	4.0

   To prepare samples:
   Using a known amount of hydrolysed acetone powder, the dried hydrosylate is dissolved in 1 ml 0.1 N HCl. A known aliquot is taken for the assay. The final volume is brought to 4.0 ml with dH_2O.

   Standards and unknown samples are now treated identically.

4. Add one drop phenolphthalein solution to each tube. Adjust colour to pink using KOH.

5. Vortex. Start the H_2O bath, large enough to boil all tubes.

6. Add 0.50 ml 10% alanine. Mix.

7. Add 1.00 ml borate buffer. Vortex.

8. Add 1.00 ml Chloramine-T solution to all tubes doing them three at a time, mixing each tube.

9. Exactly 25 minutes later add 3.00 ml Sodium thiosulfate to all tubes in exactly the same order and at the same rate as the Chloramine-T, mixing at the same intervals.

10. Add 5.00 ml toluene. Cap tightly. Shake tubes horizontally for 4 minutes.

11. Centrifuge 5 minutes at 1500 rpm.

12. Suction off and discard the toluene layer.

13. Cap tubes tightly and place in boiling water bath. When water boiled again, set timer to 30 minutes.

14. Remove tubes from hot water and immerse in cold water to cool.

15. Add 5.00 ml toluene. Shake and centrifuge as in steps 10 and 11.

16. Carefully transfer 2.50 ml of the toluene layer to a clean, dry and labelled test tube.

17. Add 1.0 ml Erlick's reagent. Mix.

18. Sit 30 minutes at room temperature.

19. Read absorbance at 560 nm using matched glass cuvettes, using the Spectrophotometer.

6.2.5 HISTOLOGY STAINS

6.2.5.1 HEMATOXYLIN AND EOSIN

SOLUTIONS

Harris Hematoxylin

Hematoxylin crystals	5.0 gm
Alcohol 100%	50.0 gm
Ammonium Alum	100.0 gm
Distilled water	1000.0 ml
Mercuric Oxide (red)	2.5 gm

Dissolve hemotoxylin in alcohol, alum in water. Mix solutions, boil 1 minute. Remove from heart, add mercuric oxide. Reheat to simmer until becomes purple, remove from heat, place in cold water. Add 2 ml glacial acetic acid per 100 ml solution. Filter.

Acid Alcohol

Alcohol (70%)	1000.0 ml
HCl (conc)	10.0 ml

Saturated Lithium Carbonate

Lithium Carbonate	1.0 gm
Distilled water	100.0 ml

1% Stock Alcoholic Eosin

Eosin Y	1.0 gm
Distilled Water	20.0 ml
Dissolve and add:	
95% Alcohol	80.0 ml

Working Eosin Solution

Eosin Stock Solution	1 part
80% Alcohol	3 parts

METHOD

1. Deparaffinize and hydrate to water
2. Stain with Harris Hematoxylin for 15 minutes
3. Rinse with tap water
4. Differentiate in acid alcohol, 3 - 10 quick dips
5. Wash in tap water, briefly
6. Dip in lithium carbonate water until sections are bright blue (3-5 dips)
7. Wash in running tap water for 10-20 minutes
8. Stain with eosin for 15 seconds
9. Dehydrate with 95% Alcohol until excess eosin is removed, two changes, 2 minutes each.
10. Absolute Alcohol two changes, 3 minutes each.
11. Xylene two changes, 2 minutes each 12. Mount with permount.

RESULTS

nuclei	blue with some metachromasia
cytoplasm	various shades of pink

6.2.5.2 GOLDNER TRICHROME STAIN FOR CONNECTIVE TISSUE

SOLUTIONS

Harris Hematoxylin

Hematoxylin crystals	5.0 gm
Alcohol 100%	50.0 gm
Ammonium Alum	100.0 gm
Distilled water	1000.0 ml
Mercuric Oxide (red)	2.5 gm

Dissolve hemotoxylin in alcohol, alum in water. Mix solutions, boil 1 minute. Remove from heart, add mercuric oxide. Reheat to simmer until becomes purple, remove from heat, place in cold water. Add 2 ml glacial acetic acid per 100 ml solution. Filter.

Axophloxine 0.2%

Thymol	1.0 gm
Axophloxine	2.0 gm
Glacial acetic acid	1000.0 ml

Dissolve axophloxine in 0.2% acetic acid, add thymol, filter.

Orange G 2.0%

Phosphotungstic acid	9.0 gm
Orange G	6.0 gm
Distilled water	1000.0 ml

Dissolve phosphotungstic acid in dH_2O. When dissolved, add Orange G, stir until clear, then filter.

Light Green 0.2%

Light green	1.2 gm
Glacial acetic acid	600.0 ml

Dissolve, then filter.

Glacial Acetic Acid 0.2%

Concentrated acetic acid	4.0 ml
Distilled water	2000.0 ml

METHOD

	Solution	Timing	
1.	toluene	2	minutes
2.	toluene	2	"
3.	abs alcohol	2	"
4.	95% alcohol	2	"
5.	80% alcohol	2	"
6.	50% alcohol	2	"
7.	tap water	2	"
8.	distilled water	10	dips (to rinse)
9.	harris hematoxylin	10	minutes
10.	tap water	10	
11.	axophloxine 0.2%	10	"
12.	acetic acid 0.2%	3	"
13.	orange g	10	dips
14.	acetic acid 0.2%	2	minutes
15.	light green 0.2%	10	dips
16.	acetic acid 0.2%	2	minutes
17.	absolute alcohol	5	"
18.	absolute alcohol	2	"
19.	absolute alcohol	2	"
20.	toluene	2	"
21.	toluene	2	"
22.	toluene	2	"
23.	mount, permount		

RESULTS

Collagen	dark green
Nuclei	red to brown
Muscle	red
Cytoplasm	red to green

6.2.5.3 HEROVICI COLLAGEN STAIN

SOLUTIONS

Nuclear Stains

A. Celestine Blue 0.25 gm
Iron alum 2.50 gm
Distilled water 50.00 ml
Boil for 3 minutes, when cool add 10 ml glycerol.

B. 5% aluminum sulfate in distilled water. Heat to boiling point, then add 50 ml 1% alcoholic solution of hematoxylin. Boil 3 minutes. When cool add 100 ml distilled water, 10 ml 40% aqueous $FeCl_3$, and 1 ml concentrated HCl.

Cytoplasmic stain

C. Metanil yellow 0.25 gm
Distilled water 60.00 ml
Acetic acid 5 drops

D. Distilled water 60.00 ml
Acetic acid 5 drops

E. Distilled water 60.00 ml
Lithium Carbonate 2 drops

Connective tissue stain

F. Picropolychrome mixture

Solution 1:	Methyl blue	0.05 gm
	Distilled water	50.00 ml
Solution 2:	Acid fuschin	0.01 gm
	Picric acid	50.00 ml

Mix solution 1 and 2, then add 10 ml glycerol and 0.5 ml $LiCO_3$.

METHOD

1. Stain in "solution A" for 5 minutes.
2. Wash in running water.
3. Stain in "solution B" for 5 minutes
4. Wash 15 - 20 minutes in running water
5. Stain in "solution C" for 2 minutes
6. Differentiate in "solution D" and rinse in water
7. Place in "solution E" for 2 minutes
8. Flood the slide with "solution F" stain for 2 minutes
9. Place in 1% acetic acid for 2 minutes
10. Dehydrate, clear and apply a cover glass with Canada Balsam in which about 0.1 gm salicylic acid per 100 ml of balsam has been dissolved.

RESULTS

nuclei	black
cytoplasm	green-yellow
muscle	bright yellow
red blood cells	bright yellow
young collagen	blue
mature collagen	red
hyalin	blue-green in nonfibrillary spots

6.2.5.4 GOMORI'S SILVER IMPREGNATION METHOD FOR RETICULIN FIBERS

SOLUTIONS

1% Potassium Permanganate

3% Potassium Metabisulphite

2% Iron Alum

Gold Chloride

3% Sodium Thiosulphate

Silver Solution

To 4 parts 10% aqueous silver nitrate add 1 part KOH. Allow deposit to settle, then remove supernatant and wash deposit twice with distilled water. Make up to original volume with distilled water. Add ammonia (specific gravity 0.88) drop by drop until the deposit is dissolved. Add 10% silver nitrate, drop by drop until the solution takes on a faint sheen. Make solution up to twice the original volume.

METHOD

1. Bring section to water
2. Oxidize with 1% potassium permanganate for 1-2 minutes, then rinse in tap water
3. Decolorize with 3% potassium metabisulphite for 1 minute, then rinse in tap water
4. Sensitize in 2% iron alum for 1 minute
5. Wash in tap water for 2 - 3 minutes, and then rinse in 2 - 3 changes of distilled water
6. Impregnate in silver solution for 3 minutes
7. Rinse quickly in distilled water
8. Reduce in 10% formalin made in tap water for 3 minutes
9. Wash in running water for 2 to 3 minutes, then rinse in distilled water

10. Tone in 1:500 gold chloride (yellow) for 5 - 15 minutes
11. Rinse in distilled water.
12. Reduce toning in 3% potassium metabisulphite for 1 minute
13. Rinse in distilled water
14. Fix in 3% sodium thiosulphate for 1 minute
15. Wash in water
16. Dehydrate, clear, and mount

RESULTS

reticulin fibers	black
collagen fibers	purple
nuclei and cytoplasm	shades of grey