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MYOCYTE INJURY AND ALTERED VASCULAR FUNCTION IN DEVELOPING MYOCARDIAL INFARCTS

By
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A thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy in the field of Pathology

University of Auckland
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Volume 1
The cicada's song:
Sentinel insentient
Of imminent death.

Matsuo Basho (1644-1694)
ABSTRACT

The temporal and spatial relationships between altered vascular function and cardiac muscle cell injury at the margins of developing myocardial infarcts were investigated, because such knowledge might provide potential for intervention in the evolution of myocardial infarcts and limitation of their size.

Regional myocardial ischaemia was modelled in isolated rabbit hearts subjected to ligation of the ventral interventricular branch of the left coronary artery (0, 30, 60, 120 or 240 minutes duration), the remainder of the heart being continuously perfused with oxygenated Krebs-Henseleit buffer solution.

After the experimental ischaemic period, the whole heart was fixed by perfusion with phosphate-buffered 2.5% glutaraldehyde which, in preliminary studies, was shown not only to preserve the morphological appearance of cardiac muscle cells, but also to stabilise the distribution of flow through the myocardial blood vessels in the pattern pertaining immediately prior to fixation.

Polymerising acrylic resin (L.R. White) was then injected into the vessels of the ischaemic and non-ischaemic regions simultaneously at identical pressures. Resin injected into the ischaemic region contained lead dioxide whilst that injected into the other vessels contained Fast Red 7B dye to allow identification of the source of supply.
of blood vessels within the heart.

Cryofracture, freeze-drying and imaging by scanning electron microscopy (SEM) with a backscattered electron detector and low vacuum specimen chamber conditions were used. This made possible examination of transmural segments of myocardium spanning the margins of the ischaemic and control ventricular myocardium containing blood vessels filled by resin.

SEM showed severe injury of cardiac muscle cells after 60 minutes of ischaemia, as characterised by separation and swelling of some organelles. Earlier ischaemic changes in some cells (focal increase in prominence of t-tubules and sarcoplasmic reticulum) were seen after 30 minutes. There was a transmural progression in development of irreversible injury from the subendocardium to the epicardium between 60 and 120 minutes corresponding to the "wavefront phenomenon". The lateral margins did not show such marked progression and were typically sharply demarcated on a cell-to-cell basis after 60 minutes.

An increase in the proportion of functional capillary pathways (from 55% to 85%) in early (30 minutes) ischaemia was succeeded by a profound perfusion defect, corresponding to the "no-reflow" phenomenon, which had a very close temporal and spatial association with severe injury of cardiac muscle cells. Loss of patency was associated with increased proportions of collapsed, compressed capillaries and swollen myocardial cells.
This study demonstrated that there is a significant region of myocardium which for a period shows intermediate degrees of myocyte injury (and is thus potentially salvageable) in the subepicardial portion of the developing infarct. Contrary to the claims of various authors similar potentially salvageable lateral "border zones" were neither large nor non-existent. Within 150 microns of the typically abrupt boundary, small discontinuous areas (<20% of this region) showed intermediate degrees of injury, and there was also an increased proportion of non-functional capillaries which were not collapsed or compressed, resulting in a 'low-flow' zone.

This narrow lateral zone requires further investigation to determine whether it is static, and thus of negligible size, or whether it moves in advance of infarction and is thus pathogenetically significant.
# TABLE OF CONTENTS

## Volume 1

<table>
<thead>
<tr>
<th>Section</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>5</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>6</td>
</tr>
<tr>
<td>Introduction</td>
<td>9</td>
</tr>
<tr>
<td>Review of Literature</td>
<td></td>
</tr>
<tr>
<td>Natural evolution of myocardial infarction</td>
<td>15</td>
</tr>
<tr>
<td>Myocardial infarct border zones</td>
<td>41</td>
</tr>
<tr>
<td>Purposes of this study</td>
<td>75</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>78</td>
</tr>
<tr>
<td>Results</td>
<td>93</td>
</tr>
<tr>
<td>Discussion</td>
<td>134</td>
</tr>
<tr>
<td>Conclusions</td>
<td>183</td>
</tr>
<tr>
<td>Appendices</td>
<td></td>
</tr>
<tr>
<td>I The use of acrylic resins as indicators of microvascular function</td>
<td>186</td>
</tr>
<tr>
<td>II Tissue preparation for scanning electron microscopy</td>
<td>195</td>
</tr>
<tr>
<td>III Morphological identification of functional capillaries in the myocardium</td>
<td>206</td>
</tr>
<tr>
<td>IV Labelling of injected resin for the identification of arterial supply of capillaries</td>
<td>215</td>
</tr>
<tr>
<td>References</td>
<td>227</td>
</tr>
<tr>
<td>Curriculum-Vitae</td>
<td>258</td>
</tr>
</tbody>
</table>

## Volume 2

<table>
<thead>
<tr>
<th>Section</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tables 1-9</td>
<td>2</td>
</tr>
<tr>
<td>Figures 1-72</td>
<td>11</td>
</tr>
</tbody>
</table>
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From the conference of the New Zealand Society for Electron Microscopy

INTRODUCTION

The New Zealand population is similar to those of other industrialised nations of predominantly European stock in that about one-third of all deaths are attributable to heart disease. In New Zealand this proportion has fluctuated between 33 and 36% in recent years, and in 1977 (the latest year for which figures are available) was 34% (New Zealand Health Statistics Report, 1980). By comparison deaths from neoplasia, cerebrovascular disease and trauma contributed only 20, 12 and 8% respectively. Acute myocardial infarction was responsible for 4707 of the 8804 deaths from heart disease in that year, and thus is the most common cause of death in this country.

The clinical course of myocardial infarction is similar around the world and is well documented. The widely quoted Framingham study showed 38% of males and 47% of females died within 30 days of their first acute coronary event (Kannel, Sorlie and McNamara, 1979). Studies from many centres show that of those who die in this early period about one-third do so in the first five minutes, one-third within the next 2-6 hours and the remaining third between six hours and 30 days. Subsequent mortality rates of 5% per annum represent a four-fold increase when compared to the general population (Norris, 1982).

There has been a wide appreciation of the central role of coronary artery occlusion by atherosclerosis, thrombosis or spasm since Herrick's classic description of its
relationship to myocardial infarction in 1912. Obviously intervention in this aetiological mechanism could lead to a reduction in mortality from myocardial infarct, indeed the Framingham study investigators concluded it is the only road to a substantial reduction in mortality due to this disease (Gordon and Kannel, 1971). However, despite intensive research this does not appear to be a realisable goal in the near future, so that one is obliged to consider interventions in the pathogenesis of myocardial infarction and its complications as a means of reducing mortality and morbidity.

There is mounting evidence provided by findings of paramedical rescue squads (Libertson, Nagel, Hirschman, Nussenfeld, Blackbourne and Davis, 1974) that the leading mechanism of sudden death is cardiac arrhythmia, particularly ventricular fibrillation (72% of attended cases of cardiopulmonary resuscitation). The development of "coronary care units" in hospitals has been directed primarily at the treatment of such arrhythmias, and has led to a pronounced reduction in the proportion of deaths due to arrhythmias in those reaching hospital. Norris and Sammel (1980) showed a reduction in this proportion from 52% in 1966-7 to 12% in 1977-9. However, the same study showed a concomitant rise (relative, not absolute) in the proportion of deaths arising from contractile dysfunction and output loss from 41% to 63%. This group includes both cardiogenic shock (syn: pump failure) and congestive cardiac failure. The remainder of deaths are attributable predominantly to rupture of the myocardial infarct.
In the early 1970's it was recognised that there is a strong correlation between fatal cardiogenic shock, congestive failure and infarct mass at autopsy (Harnarayan, Bennet, Pentecost and Brewer, 1970; Walstron, Hackel and Estes, 1970; Page, Caulfield, Kastor, de Sanctis and Sanders, 1971; Alonso, Schiedt, Post and Killip, 1973). Further it has been noted in clinical studies that infarct mass (estimated by enzyme release methods) correlates well with prognosis - particularly mortality from cardiogenic shock and congestive cardiac failure (Sobel, Bresnahan, Shell and Yoder, 1972; Norris, Whitlock, Barratt-Boyes and Small, 1975; Nordlander and Nyquist, 1979). Autopsy studies, theoretical models of experimental infarcts (Bogen, Rabinowitz, Needleman, McMahon and Abelmann, 1980) and pathophysiological investigations in experimental animals (Pfeffer, Pfeffer, Fishbein, Fletcher, Spadaro, Kloner and Braunwald, 1979) indicate that a critical infarct mass of greater than 40% of the left ventricle precipitates cardiogenic shock. Such massive infarction results in loss of output in three ways (Geddes, Agey, Pantridge, 1980): the non-contractile infarcted portion no longer contributes to systolic pressure development, but may show paradoxical bulging during systole with consequent substantial reduction of the ejection fraction, systemic hypotension, and inadequate perfusion of essential organs. Paradoxical end-diastolic contraction of the ischaemic region may result in decreased diastolic filling of the ventricle, reduced cardiac output and "backward failure" manifest as congestive cardiac failure
with pulmonary and peripheral oedema. Stiffening of the ischaemic region may have the same effect. Because of their common basis in large infarct mass, these complications have proven very difficult to treat (Norris, 1982), with mortality of up to 80% of hospitalised patients developing cardiogenic shock (Lorente, de Labre, Masquet and Gourgan, 1975).

In 1970, Pantridge proposed that therapeutic interventions designed to limit the size of regional infarcts should be developed and applied to the treatment of cardiogenic shock. At this time there was experimental demonstration of the importance of the oxygen supply and demand balance in the survival of cardiac muscle (Maroko, Braunwald, Covell and Ross, 1969; Maroko and Braunwald, 1973) and extensive investigation of a variety of metabolic and pharmacologic agents and haemodynamic manipulations in experimental animals and patients to improve the oxygen supply-demand relationship and so delay or avoid myocardial cell death. These studies, which have been reviewed in detail (Corday, 1976; Maroko, MacLean and Braunwald 1977; Opie, 1980; Reimer, 1980) demonstrated that the infarct develops as an evolving region, the dimensions of which are not irrevocably determined at the outset.

It has been established in experimental studies in dogs (Reimer, Lowe, Rasmussen and Jennings, 1977; Reimer and Jennings, 1979) that the subendocardial portion of an ischaemic region undergoes irreversible injury within 40-90 minutes of profound ischaemia, but the overlying tissue
takes longer to die, with the zone of necrosis reaching the mid-portion of the wall after 6 hours and the epicardial surface at 24 hours. This transmural spatial evolution with time has been termed the "wave-front phenomenon". These studies showed also that restoration of blood flow in experimental animals during this period of evolution can prevent the death of an otherwise condemned epicardial zone of cardiac muscle. These findings are consistent with the long-recognised variation in transmural extent of human myocardial infarcts, and great interest has been directed at the potential of limitation of infarct size by modifying the "wave-front" progression. The region of potentially salvageable myocardium has been termed the "border zone".

The existence of a similar zone of 'jeopardised' myocardium at the lateral margins of myocardial infarcts is argued, though according to Hearse and Yellon (1981), "much of the controversy arises from inappropriate use of terminology and the misquotation or misinterpretation of previously published studies". Potentially, salvage of such border zones could contribute to a substantial reduction in infarct mass and consequent reduction of mortality and morbidity from pump failure (Reimer, 1980).

The most obvious method of improving myocardial oxygen supply-demand deficit in an ischaemic region is restoration of blood flow. In 1974, a possible major impediment to limitation of infarct size by this means was defined (Kloner, Ganote, Jennings, 1974a). These authors showed
that, in dog hearts, severely ischaemic subendocardium could not be reperfused because of an extreme increase in vascular resistance in that area. This was termed the "no-reflow phenomenon" (NRF), and offers a potential major difficulty in interventions using intravascular pharmacologic or metabolic agents. The possible mechanisms, and experimental studies investigating NRF have recently been reviewed in detail by Gavin, Humphrey and Herdson (1983a). They concluded that careful study of the functional state of blood vessels of the borders of evolving myocardial infarcts is needed to determine whether NRF is pathogenetically significant, or only an interesting but inconsequential late result of myocardial ischaemia.

This thesis describes investigations undertaken to define relationship of alterations in microvascular morphology and function to myocardial cell injury at the border zones of experimental myocardial infarcts. The relevant literature is reviewed in two parts, a general consideration of the natural evolution of myocardial infarction, (p. 15) followed by a critical review of studies of myocardial injury and microvascular function in myocardial infarct border zones (p. 41). The purposes of the present study (p. 75), the material and methods used (p. 78) and results obtained (p. 93) are then presented, followed by a critical discussion of the findings (p. 134) and a list of conclusions (p. 183). Brief descriptions of four preliminary studies are also included as appendices (p. 186).
REVIEW OF LITERATURE

This review is composed of two sections. The first is a general consideration of the natural evolution of myocardial infarction, and the second (p. 41) is a critical review of studies investigating myocardial cell injury and microvascular structure and function at the margins of myocardial infarcts.

The Natural Evolution of Myocardial Infarction

This first section of the review of literature discusses the major categories of experimental models used to study myocardial infarction. It then briefly defines the present consensus on the ultrastructural and biochemical changes in severely ischaemic myocardium with special reference to the concepts of reversible and irreversible ischaemic injury. Spatial and temporal evolution of regional infarcts and the changes peculiar to ischaemic myocardium whose blood supply has been restored are then reviewed with particular emphasis on one of these changes, the no-reflow phenomenon.

Experimental models

There are many limitations to the investigation of the evolution of myocardial infarction in human hearts. These include limited availability of post-mortem material and obvious ethical proscriptions to experimental protocols. Furthermore, post-mortem studies of human hearts have three
inherent disadvantages. First, autolytic changes, which are inevitable because of the medico-legal mechanics of any necropsy service, will confound the very similar changes which characterise ischaemia or infarction (Baroldi, 1975; Armiger, Seelye, Carnell, Smith, Gavin and Herdson, 1976). Second, pre-existing coronary artery disease or other underlying cardiac or non-cardiac disease may introduce uncontrolled variables, and third, the precise interval from onset of coronary blood vessel occlusion to death of the patient is seldom known, a factor which is of great significance in the study of changes which occur within the first few hours of ischaemia (Armiger, 1982). For these reasons, there has been a longstanding interest in models of myocardial ischaemia and infarction in experimental animals.

Investigation of the effects of coronary artery ligation in the hearts of experimental animals such as dogs was commenced one hundred years ago (Cohnheim and von Schulthess - Rechberg, 1881) and the pathogenesis of ischaemic heart disease discussed as early as 1899 (Baumgarten). Surprisingly the causal connection between coronary artery thrombosis and the clinical features of a "heart attack" was not made until later (Herrick, 1912), and it was not until the advent and clinical application of electrocardiography in the 1920's that the relationship between myocardial ischaemia and its clinical effects was well understood. (Baldrey, 1971).
Since then, many different types of experimental models have been used to investigate the aetiology and pathogenesis of myocardial ischaemia, including studies of the associated morphological, biochemical and haemodynamic changes. Five major types of experimental model have been used:

1. Isolated organelles and subcellular components, such as mitochondria and sarcoplasmic reticulum.

2. Cell culture preparations of cardiac myocytes

3. Dissected tissue, such as slices of ventricular myocardium, or isolated perfused interventricular septum or papillary muscle

4. Isolated intact organ preparations of hearts

5. Whole animal preparations with surgical interventions

As this thesis concerns relationships between cardiac myocytes and their associated microvasculature, most emphasis will be laid on the latter two categories, which have been used predominantly for morphological and haemodynamic studies, as opposed to cellular and biochemical studies performed predominantly in those of the three former categories (Armiger, 1973).

Surgically induced coronary artery occlusion in experimental animals has been undertaken by several diverse methods (Oliver, 1972), ranging from simple ligation of a coronary arterial branch on the surface of the surgically
exposed heart of an anaesthetised animal such as the dog, to electrical coagulation of luminal blood by an intracoronary catheter in the awake animal. A great volume of morphological and biochemical data was gained during the 1960's and 1970's from such studies, particularly the widely used left circumflex artery ligation preparation devised by Jennings and Wartman (1957) which allowed easy identification of a reliably and severely ischaemic region in the heart of the dog.

Isolated organ preparations have also been studied over many years, starting with Langendorff (1895). Simple excision and maintenance of the heart at physiological temperature (autolysis) has been used to demonstrate the close similarity of changes of ischaemia in vivo and autolysis in the heart (Herdson, Kaltenbach and Jennings, 1969; Armiger et al., 1976; Gerlach and Denticke, 1966). In other preparations, the heart is perfused to act as a functioning organ, sometimes using blood from a donor animal (Baghirzade, Kirsch and Hauschild, 1970; Roy, Gailis, Morin and Cote, 1971). More commonly however, blood-free, buffered aqueous nutrient perfusates are used in studies of myocardial metabolism and pharmacodynamics, pioneered in its present form by Bleehen and Fisher (1954). More recently Neely, Leibermeister, Battersby, and Morgan (1967) adapted the Langendorff-type preparation, in which the coronary arteries are perfused via the aortic stump so that the left ventricle beats but does not function as a pump, to a 'working' preparation in which the isolated heart pumps its
own recirculating perfusate against a pressure head and supplies its own coronary arteries.

Myocardial ischaemia has been created in isolated hearts in two ways. As well as in in vivo studies, ligation of a coronary artery has been used to create regional ischaemia in Langendorff-type preparations (for instance in rabbits, Barlow, Harken and Chance, 1977). More commonly isolated hearts have been used to assess global rather than regional effects, and these have been brought about by reducing flow rate of perfusion via the aorta (Rovetto, Whitmer and Neely, 1973, Rovetto, 1977; Hearse, Garlick and Humphrey, 1977) or reduction in oxygen tension of the perfusate (so called "anoxic" perfusion, Hearse, Humphrey and Chain, 1973).

Important advantages of isolated heart preparations compared to tissue and cell preparations are the avoidance of the trauma of dissecting into the heart itself, the potential to observe the heart functioning as a pump at physiological temperatures in a rapidly prepared, highly reproducible form which allows accurate control of many experimental parameters (Humphrey, 1983) in a stable form for durations of several hours (Fallen, Elliot and Gorlin, 1967; Neely et al., 1967).

In many in vivo experiments, the influences of collateral flow from extracardiac sites (mediastinal and bronchial arterial anastomoses) and systemically circulating blood-bourne factors such as catecholamines are uncontrolled variables. Preparation of hearts as isolated organs clearly
eliminates this source of variability. However, direct comparison of results from isolated heart preparations rendered globally ischaemic and regional myocardial infarction in man must be made with caution (Schaper, 1980). Regional infarction in isolated hearts is more closely allied, but the use of crystalloid perfusion fluid (lacking cellular and serum blood factors) results in documented functional and structural differences between isolated heart preparations and their in vivo counterparts (Neely et al., 1967; Manning, Hearse, Dennis, Bullock and Coltart, 1980). Isolated heart preparations obviate potential problems of prolonged anaesthesia in the donor animal in experiments using long ischaemic intervals, but obviously cannot be used in recovery-type experiments where the late effects of ischaemia or an intervention are to be assessed.

Schaper (1980) has discussed the importance of choice of animal species in the experimental model. He indicates that, while higher primates such as baboon show greatest similarity to human hearts, they are difficult to procure except in South Africa and are very expensive to maintain, which precludes their use by all but a few centres (Bruyneel and Opie, 1973; Geary, Smith and McNamara, 1981). Pigs show some superficial similarities in cardiovascular anatomy, but Schaper (1980) suggests these have recently been overemphasised, and concludes that the difficulties in achieving satisfactory anaesthesia in pigs make them less useful than the widely used dog model which has dominated physiological, pharmacological and pathological experimental
cardiac studies for twenty-five years.

Recently there has been increased interest in use of smaller mammals, such as rats, rabbits and guinea pigs, not only because of greatly enhanced availability and reduced cost compared to dogs, but because of reduction of variation between animals inherent in using inbred strains which means smaller numbers of animals may result in statistically significant differences between treatment groups. Some studies have shown many features in common between different mammalian hearts both in patterns of circulation (Geary et al., 1981; Harken, Simson, Hazelgrove, Wetstein, Harden and Barlow, 1981) and of ultrastructural changes (Heggtveit, 1969), although differences in rate of biochemical and ultrastructural changes are well recognised (Hearse, Humphrey and Garlick, 1976; Schaper, 1980). Within these limitations investigation of pathological events common to all mammalian hearts can be justifiably undertaken in isolated heart preparations of small mammals such as the rabbit or rat (Humphrey, 1983).

Ultrastructural and biochemical changes in severe ischaemia

The ultrastructural changes which occur in permanent severe ischaemia of dog hearts in vivo (Jennings, Baum and Herdson, 1965; Jennings, Sommers, Herdson and Kaltenbach, 1969), in isolated dog hearts rendered globally ischaemic (Schaper, Mulch, Winkler and Schaper, 1979) and in autolysing dog heart (Herdson et al., 1969) are closely
similar and have been summarised by Heggtveit (1969) and
Jennings and Ganote (1972).

Briefly, the myofibrils show relaxed sarcomeres within
the first five minutes of ischaemia, glycogen granules
progressively disappear so that they are almost absent after
40 minutes, and the nuclear chromatin begins to clump and be
aggregated at the nuclear margin after 5-15 minutes.
Mitochondria begin to swell within the first 10 minutes,
show a decrease in density of their matrix by 20 minutes and
disorganisation and disruption of the cristae by 40 minutes
along with the formation of amorphous electron-dense
inclusions within the mitochondria. Sarcoplasmic reticulum
appears swollen after 30-60 minutes, and intracellular
oedema is apparent by 60 minutes. By two hours plasma
membrane discontinuities are evident.

A closely similar set of qualitative changes to
organelles has been observed in rat (Kloner, Fishbein, Hare
and Maroko, 1979) and rabbit heart (Decker and Wildenthal,
1978), though important differences in time course of these
changes have been noted between species. Rat myocardium
shows advanced changes at 10 minutes, equivalent to those
seen after 40 minutes in the dog, while rabbit heart shows
changes equivalent to those seen at 40 minutes in the dog by
30-40 minutes.

Parallel biochemical changes also show species
differences in time course, but close similarity in
qualitative change (Hearse et al., 1976). The normal
mammalian myocardium is heavily reliant on aerobic metabolism to produce the prerequisite adenosine 5' triphosphate (ATP) fuel, continuously required for the heart to function as a muscular pump. Johnson (1982) concluded, from his recent review of myocardial metabolism in ischaemia, that this fact makes the myocardium uniquely susceptible to oxygen limitation, particularly to ischaemia in which both oxygen supply and metabolite removal are limited. He described the following changes occurring in ischaemic myocardium:

1. Acceleration of relatively inefficient anaerobic glycogenolysis (producing 2-3 moles ATP/mole glucose compared with 37 moles ATP/mole glucose or 129 moles ATP/mole palmitate in aerobic metabolism) in an attempt to maintain cellular ATP.

2. The rise in metabolites, particularly reduced nicotinamide adenine dinucleotide (NADH), lactate and hydrogen ions (H+), which in high concentrations inhibit anaerobic glycogenolysis.

3. Failure of mitochondrial function (oxidative phosphorylation) in an on/off manner at critical levels of oxygen and ADP supply.

4. Loss of high energy phosphate compounds (ATP, ADP, creatinine phosphate) by catabolism to nucleosides which in low energy states is effectively irreversible.

5. Loss of intracellular potassium and magnesium ions
to the extracellular space and a complementary gain in sodium and calcium ions.

The early loss of muscle contraction (manifest as relaxation of sarcomeres) is attributed to ion shifts \((\text{Na}^+ / \text{K}^+, \text{pH})\) because it occurs earlier than the fall in ATP to critically low levels. Ion shifts may result from loss of function of ATP-dependent membrane-pump mechanisms as ATP levels begin to fall, and is later associated with eventual cell swelling and disruption of organelles and the sarcolemma. The increased intracellular concentration of \(\text{Ca}^{++}\) appears to have a key role, and overwhelming of homeostatic mechanisms results in changes to membrane potential development, actin and myosin interaction, and when combined with increased concentration of inorganic phosphate, can result in crystalline deposition of calcium phosphate in organelles - particularly mitochondria.

Reversible and irreversible injury to myocardium

It has been recognised for many years that the mechanical, ultrastructural and metabolic effects of short durations of ischaemic injury are all reversible, but that after a critical time period, complete loss of function and cell necrosis are inevitable (Tennant and Wiggers, 1935; Bayley, La Due and York, 1944; Jennings, Sommers, Smyth, Flack and Linn, 1960). The concept of reversible and irreversible injury has been reviewed by Jennings, Ganote and Reimer (1975) who have been one of the leading groups
investigating the events which bring about this transition. They have indicated that the appearance of amorphous densities in mitochondria was the earliest reliable ultrastructural indicator of irreversible injury. **In vitro** studies of incubated slices of myocardium by this group have shown that another early functional index of irreversible injury to myocardial cells was the loss of ability to regulate cell volume and ion distribution, manifested morphologically as cell swelling and closely associated with evidence of membrane damage.

The morphological alterations observed in the first 20-30 minutes of severe ischaemia in dog heart (see above (p. 22) : relaxation of myofibrils, loss of glycogen, moderate aggregation of nuclear chromatin, and slight mitochondrial swelling), thus appear to be reversible.

Armiger (1982) has related the ultrastructural changes characteristic of irreversible injury to the parallel biochemical changes. She associated mitochondrial degeneration and lactate and H⁺ accumulation; chromatin margination and falling pH; and formation of amorphous mitochondrial inclusions, which are probably composed of denatured protein and lipid (Buja, Dees, Harling and Willerson, 1976; Jennings, Shen, Hill, Ganote and Herdson, 1978a; Hagler, Sherwin and Buja, 1979), with falling pH.

A central role for the critical depletion of high energy phosphate compounds has been suggested by other authors (Jennings, Hawkins, Lowe, Hill, Klotman and Reimer,
1978b) who showed that severely ischaemic dog myocardium (posterior papillary muscle) developed ultrastructural evidence of irreversible injury between 20 and 40 minutes of ischaemia. This was correlated with a fall in ATP levels below a critical value of about 20% of normal concentration. They added a cautionary rider that many interrelated events may occur simultaneously in myocardial cells, which makes proof of causal relationships difficult to obtain from temporal associations.

In summary, the associations of ultrastructural, biochemical and functional events at the time that myocardium becomes irreversibly injured are well defined, but the causal event or events have yet to be conclusively identified.

**Spatial and temporal spread of irreversible injury**

A further consequence of investigation of the mechanisms of irreversible injury was the demonstration that not all parts of the area at risk (i.e. the myocardium normally supplied by the vascular bed of the occluded artery) showed equal degrees of ischaemic injury. In fact the widely used Jennings and Wartman (1957) experimental protocol for dog hearts, was established to provide anatomically easily located site of reliably severely ischaemic myocardium.

Later studies have demonstrated what is termed the "wavefront phenomenon" of cell death which originates in the subendocardial portion of the area of risk and spreads with
time towards the epicardium (Reimer et al; 1977; Reimer and Jennings, 1979). In dog hearts, the subendocardium was severely ischaemic from the outset and showed irreversible injury by 40 minutes. If reperfusion to the ischaemic area was delayed by a further three hours the area of irreversible injury encompassed much of the midmyocardium as well, and by 24 hours some dogs showed irreversible injury (infarction) to the complete transmural width of the area at risk.

This delay in onset of irreversible changes to the more superficial layers of the ventricular wall has been attributed to variations in the extent of oxygen limitation (i.e. ischaemia) created by flow of blood from the unoccluded arteries into the ischaemic zone via collateral vessels (Schaper and Pasyk, 1976; Reimer, et al., 1977). This natural collateral supply is so abundant that in some, but not all, dog hearts complete transmural infarction is never attained (Schaper and Pasyk, 1976).

Other workers have attributed the wavefront phenomenon to documented transmural gradients of contractile work per unit volume, of blood supply, and of metabolite stores per unit volume within the normal heart (Bell and Fox, 1974; Hoffman, 1978, Sabbah and Stein, 1982). Hoffman (1978) suggested that collateral flow and transmural gradients of cardiac work and metabolism may explain the long recognised increased susceptibility of the subendocardium of human hearts to infarction (Mallory, While and Salcedo-Sagar,
1939) which often results in partial thickness rather than transmural regional infarction.

The existence and possible anatomical and physiological bases of a similar evolving wavefront at lateral margins of regional myocardial infarcts is controversial (Hearse and Yellon, 1981), and is considered in more detail later in this review (p. 41).

Changes peculiar to partial or temporary ischaemia

Although ischaemically injured myocardium reperfused with blood (or oxygenated asanguinous perfusate) before the onset of irreversible injury can show complete recovery of function and appearance, it has been recognised that some cells show severe changes not usually seen in areas of permanent ischaemia (i.e. where restoration of blood flow has not been attempted). Explosive cell swelling exceeding that seen in permanent ischaemia and of particularly rapid onset (within seconds of reperfusion), and the formation of zones of hypercontraction of successive sarcomeres with alternate stretched or torn zones in myocardial muscle cells ("contraction bands") are typical structural features of irreversibly injured and reperfused myocardium (Sommers and Jennings, 1964; Herdson, Sommers and Jennings, 1965; Kloner, Ganote, Whalen and Jennings, 1974b).

Uptake and deposition of calcium ions as dense inclusions in mitochondria has been shown to occur after reperfusion with blood in vivo, or after restoration of
normal calcium concentrations to aqueous perfusate of isolated hearts perfused with low calcium concentration solutions (the so called 'calcium paradox' - Nayler, Poole-Wilson and Williams, 1979).

There has been considerable debate as to whether such reperfusion injury occurs only to cells which are already irreversibly injured, or whether reperfusion may be a means of extension of the area of irreversibly injury cells. The association of haemorrhage into ischaemic zones has received particular attention (Mathur, Guinn and Burris, 1975; Bulkley and Hutchins, 1977; Fukuyama and Roberts, 1979; Kloner, Rude, Carlson, Maroko, de Boer and Braunwald, 1980). The present consensus appears to be that reperfusion-type injury and haemorrhage is limited to areas already irreversibly injured (Fishbein, Y-Rit, Lando, Kanamatsuse, Mercier and Ganz, 1980).

One unexpected finding in reperfused myocardium has been the inability to restore blood flow to some obviously ischaemic areas, despite the more typical physiological hyperaemic response in other ischaemic areas (Kloner et al., 1974a). This ischaemic perfusion defect has been termed the "no reflow phenomenon" and is considered in detail in the following section.

**Ischaemic perfusion defects**

A vascular perfusion defect which prevents the reflow of blood following a period of ischaemia has been observed as a
general pathological phenomenon in a wide variety of well-vascularised tissues including kidney (Sheehan and Davis, 1959; Summers and Jamieson, 1971; Flores, di Bona, Beck and Leaf, 1972), skin (Wilms-Kretschmer and Manjo, 1969), brain (Majno, Ames, Chaing, and Wright, 1967; Ames, Wright, Kowada Thurston and Majno, 1968; Chaing, Kowada, Ames, Wright and Majno, 1968; Fischer and Ames, 1972), adrenal (Kovacs, Carroll and Tapp, 1966) and heart.

Termed the no-reflow phenomenon (NRF) by Sheehan and Davis (1959) it was recognised in ischaemic cat heart in 1966 by Krug, de Rochement and Korb, and later in guinea pig heart (Baghirzade, et al., 1970; Hauschild, Baghirzade and Kirsch, 1970), rat heart (Poche, Arnold and Nier, 1969) and dog heart (Grayson, Davidson, Fitzgerald-Finch and Scott, 1974; Willerson, Watson, Hutton, Templeton and Fixler, 1975; Grayson, Davidson, Fitzgerald-Finch and Scott, 1974), but it was not until 1974 that the term NRF and the association with the phenomenon in other tissues was appreciated and applied to the myocardium (Kloner, et al., 1974a). Since then, NRF has been investigated extensively in experimental preparations of in vivo regional ischaemia (Gavin, Seelye, Nevalainen and Armiger, 1978a; Gavin, Nevalainen, Seelye, Webster and Thomson, 1978b; Nevalainen, Gavin, Seelye, Whitehouse and Donnell, 1978) and in globally ischaemic isolated heart preparations Apstein, Mueller and Hood, 1977; Alanen, Nevalainen and Lipasti, 1980; Humphrey, Gavin and Herdson, 1980, 1982a, Humphrey, Seelye and Gavin, (1982b).
Gavin, et al., (1983a) have recently extensively reviewed the methods and findings of investigations of NRF in the heart, and list six possible explanations for the phenomenon which are discussed sequentially below.

1. Endothelial cell swelling
2. Changes in constituents of the blood
3. Vasoconstriction
4. Myocyte swelling and oedema
5. Myocardial ischaemic conracture
6. Cardiac rigor mortis

Endothelial cell swelling

Endothelial cell swelling has been demonstrated as a prominent early change in small blood vessels of ischaemic dog myocardium (Armiger and Gavin, 1975), rat myocardium (Poche, et al., 1969; Kloner, Rude, Maroko and Braunwald, 1979) and rabbit heart (Backwinkel, Schmitt and Thelman, 1971). Membrane-bound blebs appear in the capillary lumen, limiting its diameter, and may contribute to the perfusion defect (Kloner, et al., 1974a; Armiger and Gavin, 1975), though it seems insufficient as a sole cause of NRF as Gavin et al., (1978a, b) have shown many capillaries manifesting endothelial cell swelling in the margin of in vivo infarcts in dog hearts which nevertheless conducted an injected intravascular tracer (colloidal thorium hydroxide).
Changes in constituents of the blood

Changes in blood constituents such as aggregation of erythrocytes have been noted frequently in regions where NRF develops (Krug et al., 1966; Kloner et al., 1974, Gavin et al., 1978a, b). Plugging of capillaries by erythrocytes may well provide a mechanism for the sharply defined boundary of the NRF region observed (Gavin et al., 1978b). In addition, ischaemic metabolism such as loss of ATP and rise in hydrogen ion concentration can lead to decreased deformability of erythrocytes (Weed, La Celle and Merrill, 1969; La Celle, 1969) and reduced compliance of vessel walls, which may further contribute to vascular plugging by cellular elements of blood.

Intravascular activation of the clotting cascade is known to occur in anoxic microvasculature (Weed, 1970; Webb and Howard, 1975) and during ischaemia when flow is reduced or absent, this would be expected to further reduce reperfusability of the ischaemic vascular bed.

There is recent interest in the endothelial cell-platelet interaction in myocardial ischaemia, and formation of platelet thrombi and release of potent vasoactive compounds such as thromboxane A₂ and leukotrienes during ischaemia (Schneider, 1980; Addonizio, Wetstein, Fisher, Feldman, Strauss and Harken, 1982).

While any or all of these changes to blood constituents may occur, NRF has frequently been demonstrated in
heparinised hearts perfused with blood-free perfusate (i.e. aqueous buffer solutions) suggesting that such changes are not the principal cause of NRF (Nevalainen et al., 1978; Alanen et al., 1980). Similarly, heparinisation has been noted not to prevent NRF in ischaemic brain and kidney (Chiang et al., 1968; Summers and Jamieson, 1971).

Vasoconstriction

Clinically, severe vasoconstriction is now a recognised occurrence in vivo as it can be a cause of myocardial ischaemia in the absence of coronary artery stenosis (coronary artery spasm). Such changes have recently been reviewed by Maseri and Chierchia (1982). Experiments to demonstrate focal vasoconstriction as a cause of NRF are particularly difficult to design (Gavin et al., 1983a), as morphological studies cannot usually sample sufficient tissue to confirm or exclude it (Gavin et al., 1978a). On the other hand most functional studies cannot localise such constrictions within the heart. Where a comparison was made between the patterns of perfusion defect created by selective embolic occlusion of arterioles, precapillaries or capillaries and that observed in NRF in globally ischaemic rat heart (Mawson, Gavin and Herdson, 1982), the wide differences in appearance suggested that selective occlusion of any of these three types of vessels was not responsible for NRF, and hence vasoconstriction under ischaemic conditions affecting these vessels was also, by inference, an unlikely cause. Similarly, scanning electron microscopy
of methylmethacrylate corrosion casts of the lumina of
globally ischaemic rat hearts made before and after the
onset of NRF indicate a generalised compression of the
vasculature, not a constriction of contractile vessels
(Gavin, Thomson, Humphrey and Herdson, 1983b).

The depletion during ischaemia of intrinsic
vasodilators such as adenosine, which are important in
coronary autoregulation (Berne, 1963; Belloni, 1979) may be
responsible for NRF, although the initial vasodilatation
seen in ischaemia is closely correlated with a rise in
tissue levels of adenosine (Rubio, Berne and Katori, 1969;
Scott, Chen and Swindall, Dabney and Haddy, 1979).

Humphrey and Seelye (1982) showed that maintenance of
adenosine levels by blockade of its catabolic pathway, did
not modify the extent of NRF in rat hearts, but
paradoxically accelerated its onset. Subsequently these
authors (Humphrey, et al., 1982b) used the same model to
refute the hypothesis that intrinsic release of
catecholamine vasoconstrictors (such as noradrenaline) was a
cause of NRF.

Myocyte swelling and oedema

Myocyte swelling and oedema is a well recognised but
relatively late (90-120 min) event in permanent severe
ischaemia in dog hearts (Jennings and Ganote, 1974; Farber,
Chien and Mittnacht, 1981, Jennings and Reimer, 1981) but a
universal and early feature of reperfusion of dog heart with
oxygenated perfusate after ischaemia of 40 min or more (Jennings, Sommers, Kaltenbach and West, 1964; Herdson et al., 1965; Ganote, Worstell, Ianotti and Kaltenbach, 1977). Swollen myocardial cells may extrinsically compress capillaries and reduce coronary flow (Kloner, et al., 1974a; Camilleri, Joseph, Amat and Fabiani, 1980) and reflow can be improved following temporary myocardial ischaemia by hyperosmotic solutions of agents such as mannitol (Willerson, Powell, Guiney, Stark, Sanders and Leaf, 1972).

However it is unclear whether in permanent ischaemia the degree of cell swelling occurs sufficiently early to cause the NRF phenomenon (Kloner, et al., 1974b) or whether, in models utilising temporary occlusion and reperfusion, the areas developing NRF receive sufficient transient reflow to bring about vascular occlusion (Kloner et al., 1974a). Furthermore, isolated hearts continuously perfused with aqueous medium containing no oxygen (anoxic perfusion) also develop NRF (Humphrey et al., 1982a, b, Lipasti, Alanen and Nevalainen, 1982) indicating that transient reperfusion with oxygen is not essential for NRF to develop. Lipasti et al., (1982), have also shown that where cell swelling is minimised in anoxically perfused rat hearts by infusion of hyperosmotic mannitol, NRF still develops.

In summary, cell swelling resulting from permanent ischaemia or reoxygenation in temporary ischaemia cannot account for all cases where NRF is observed in myocardium though it may contribute in combination with other events to
the development and maintenance of the perfusion defect.

Ischaemic contracture

The four possible causes of the NRF phenomenon discussed above are common to all organs, but two others, the development of ischaemic contracture and rigor mortis are uniquely associated with the heart and the contractile cardiac muscle.

Contracture, defined by Lewis, Grey and Henderson, (1979) as an increase in resting cardiac muscle tension at constant length (or conversely decrease in resting length at constant tension), has been shown to occur when myocardium is rendered ischaemic or anoxic in a wide variety of situations including isolated papillary muscles (Henderson, Palmley and Sonnenblick, 1971), isolated perfused hearts of rats, rabbits and guinea pigs (Apstein et al., 1977; Hearse, et al., 1977; Gaasch, Bing, Pine, Franklin, Clement, Rhodes, Phear and Weintraub, 1978; Alanen et al., 1980; Ogilby and Apstein, 1980), in dog hearts in vivo (Jones, Attarian, Currie, Olson, Hill and Sink, and Wechsler, 1981), and in human hearts subject to prolonged cardiac arrest during surgery (Cooley, Reul, and Wukasch, 1972; Katz and Tada, 1977).

Alanen et al., (1980) and Humphrey et al., (1980), have shown that the development of the subendocardial perfusion defect of NRF in isolated rat hearts was temporally related to the development of ischaemic contracture, and the extent and rate of onset of NRF could be manipulated by
modification of the development of contracture using iodoacetate or low (0.05mM) calcium concentrations in the perfusate.

These studies have led to the development of the biphasic hypothesis for the pathogenesis of NRF (Humphrey et al., 1980). This proposes that an initial closure of the microvasculature by compressive forces produced by ischaemic contracture is subsequently maintained by the development of rigor mortis in the ischaemic region. Experimental support for this hypothesis has been offered by Humphrey, Thomson and Gavin (1981) who have shown that, if the decrease in resting length (contracture) is prevented from occurring in globally ischaemic rat hearts by insertion of an iso-volumic fluid filled balloon in the left ventricular lumen, the extent of the perfusion defect is minimised. In a similar experiment (Apstein and Ogilby, 1980), using intermittent inflation of a luminal balloon in globally ischaemic rabbit hearts (mimicking diastolic filling of the lumen), the abrupt rise in coronary flow resistance observed during ischaemia (and due to the development of the perfusion defect) was prevented from occurring. This demonstrated that if contracture did not take place the no reflow phenomenon was minimised.

At present it is difficult to related these findings made in globally ischaemic hearts to the events which may occur in regions of ischaemia in hearts beating in vivo, where contracture and rigor may be modified by the diastolic
filling and paradoxical bulging frequently observed in ischaemic regions (Eaton and Bulkley, 1981).

Rigor mortis

Gavin et al., (1983a) have indicated the confusion of terms in the literature, and the difficulty in experimentally and conceptually separating ischaemic contracture and rigor mortis. They define the latter as the physical change in compliance (stiffness) of the affected muscle mass, and associate it with the normal molecular link between actin and myosin filaments (so called 'rigor complexes') which require ATP for release in normal muscle contraction and relaxation (Murray and Weber, 1974). When ATP levels fall to critically low levels in ischaemia, or after death, these bonds persist unmodified and result in stiff, immovable, incompressible muscle.

It has not been widely appreciated that different muscles develop rigor mortis at different rates (Lawrie, 1953) and only recently has it been demonstrated (Seelye, Nevalainen, Gavin and Webster, 1979) that cardiac muscle develops rigor mortis more rapidly (40-90 min at 37°C in dog heart) than is usual in skeletal muscle (Bendall, 1973).

Development of rigor mortis (defined by compliance changes) has been shown to be associated with progressive contraction of cardiac myocytes in slices of myocardium freshly obtained from dog hearts with in vivo regional ischaemia, (Vanderwee, Humphrey, Gavin and Armiger, 1981).
This decrease in compliance is located in regions of the heart which develop NRF after 30–90 minutes of ischaemia (Gavin et al., 1978b) and it was concluded that this irreversible contraction resulted in either compression of small blood vessels and obstruction of flow per se, or of sufficient obstruction to result in plugging of vessels by cellular elements of the blood.

**Significance of no-reflow**

There is as yet no clear indication as to whether the perfusion defect of NRF precedes and therefore contributes to the death of ischaemic myocardial cells, is coincident with myocardial cell death, or is an inconsequential result of death of cardiac muscle cells (Gavin et al., 1983a). Some authors have claimed that, because structural integrity of the ischaemic microvasculature is maintained longer than that of the associated muscle cells (Kloner, et al., 1980; Reimer and Jennings, 1982), the no-reflow phenomenon is secondary (and hence non-contributory) to cell-death. Alternatively others believe that structural integrity alone does not indicate the presence of function (Gavin et al., 1978a). Fabiani (1976) showed that no-reflow may precede and contribute to eventual necrosis in in vivo regional infarcts in rat hearts by modifying both the extent of NRF and tissue necrosis with infusions of hypertonic mannitol, dihydroxyacetone or allopurinol.

Most studies of the pathogenesis of NRF have compared
severely ischaemic myocardium with non-ischaemic control tissue well removed from the borders of an evolving infarct, or else utilised globally ischaemic isolated hearts which may provide a good model for transmural evolution of infarcts, but inherently do not consider the lateral evolution potentially present in regional infarcts \textit{in vivo}. Several authors (Reimer and Jennings, 1979; Hearse and Yellon, 1981; Gavin et al., 1983a) have indicated the need for further elucidation, particularly at an ultrastructural level, of the relationship in time and spatial distribution in the heart of NRF and myocardial cell death, particularly at lateral border zones, because it is in these regions that their significance in terms of potential myocardial salvage must be assessed.
Border Zones

The majority of studies of the pathogenesis of myocardial infarction have compared the morphological, biochemical and functional differences between profoundly ischaemic and essentially normal myocardium, but relatively little attention has been directed at the interface between the two. This part of the review of literature examines what is known of such border zones using a "macroscopic" to "microscopic" order for convenience of presentation. The review is particularly concerned with the methods utilised and the evidence for and against the existence of potentially salvageable myocardium in these regions.

Whole animals and isolated intact heart studies

Four methods have been utilised to define border zones in intact hearts. These are to observe the visible border of epicardial cyanosis in experimental "open-chest" dog preparations, to detect the fluorescence of NADH in epicardial tissue, to record changes in epicardial, precordial or intramyocardial electrocardiograms, and to image infused radionuclides.

The appearance of a sharply demarcated area of cyanosis in the dependent tissue of an occluded epicardial coronary artery was noted by early experimenters (Baumgarten, 1899). Their description does not differ significantly from those of recent authors. This boundary, which appears sharp to
the naked eye has been used as a topographic reference point for border zone metabolite and electro-physiologic gradients (Hearse, Opie, Katzef, Lubbe, van der Werff, Peisach and Boulle, 1977). However, earlier authors (Fischl, Sonnenblick and Kirk, 1974) have shown that epicardial landmarks do not always correspond to the distribution of a vascular bed in the deeper layers of the myocardium.

An elegant method to identify epicardial changes utilising the fluorescence of NADH when excited by wavelengths of 366 nm (Barlow and Chance, 1976) has been described. Chance, Williamson, Jameson and Schoener (1975) had previously demonstrated that epicardial fluorescence in isolated rat hearts correlates highly with increased levels of NADH, and hence discriminates between tissue with aerobic and anaerobic metabolism. Steenbergen, de Leeuw, Barlow, Chance and Williamson (1977) subsequently showed in saline perfused rat hearts that the early changes in epicardial fluorescence during high-flow anoxia and low-flow hypoxia (ischaemia) were similar; and that small discrete heterogenous areas of anaerobic tissue with sharply demarcated borders could be identified. They concluded that oxygen gradients at boundaries are very steep, that ischaemia and hypoxia do not grade progressively into normal, and that mitochondria are either anaerobic or aerobic with no intermediate populations. Furthermore, they suggest that these signs of anoxia correspond to the distribution of the capillary bed of an arteriole, with control of flow occurring at that level. Later studies (Harden, Simson,
Barlow, Soriano, and Marken, 1978; Simson, Harden, Barlow and Harken, 1979) showed similar findings in rabbit and dog hearts which correlated highly with ST segment changes and with ultrastructural changes in tissue obtained by transmural core biopsy.

The most widely used non-invasive method for the assessment of ischaemia has been electrocardiography. This technique was used by Maroko et al., (1969, 1971) in pioneering studies of the effects of therapeutic intervention on infarct size. Close correspondence between ST segment elevation and myocardial tissue oxygen tension (Sayen, Sheldon, Pierce and Kuo, 1958), creatine phosphokinase depletion, subsequent cell necrosis (Kjekshus and Sobel, 1970, Kjekshus, Maroko and Sobel, 1972; Ross, 1976), and tissue metabolism (Reid, Pelides and Shillingford, 1971; Karlsson, Templeton and Willerson, 1973) has led to its use in mapping ischaemic myocardium. Particular emphasis has been placed on the use of ST-segment maps to define the ischaemic area immediately following vessel occlusion and on the use of the difference in this area from the area of eventual necrosis as a measure of effectiveness of pharmacological haemodynamic interventions (Maroko et al., 1977). A further extension has been the use of non-invasive precordial mapping in dogs, as this correlates adequately with epicardial mapping (Maroko et al., 1977). However, both theoretical and practical cautions in the interpretation of epicardial and precordial electrocardiograms have recently been expressed (Lekven,

Particularly notable are the studies of Hearse et al. (1977) which showed a wide (8 - 15 mm) lateral zone of intermediate levels of ST elevation in dogs, and that of Janse, Cinca, Morena, Fiolet, Kleber, de Vries, Becker and Durrer (1979) in pigs, where epicardial and intramyocardial ST segment changes were interpreted as demonstrating sharply demarcated zones of normal and ischaemic tissue which interdigitated along the lateral border of the infarct.

There is considerable clinical interest in the demonstration of infarcts by the non-invasive techniques of radionuclide imaging. These methods depend on selective accumulation or exclusion of a perfused nuclide, which is monitored from outside the chest. Technetium (Tc 99m) pyrophosphate complexes with the calcium which accumulates in infarct margins to give a doughnut-shaped "hot spot" (Buja, Tofe, Kulkarni, Mukherjee, Parkey, Francis, Bonte and Willerson, 1977). This nuclide has been used to estimate infarct size in humans and experimental animals (Willerson, Parkey, Stokely, Bonte and Buja, 1977; Henning, Schelbert, Righetti, Ashburn and O'Rourke, 1977; Wackers, Becker, Samson, Sokole, van der Schoot, Vet, Lie, Durrer and Wellens, 1977), although some reservations have been made about its quantitative relationship to the severity of
ischaemia (Peter, Norris, Heng, and Sammel, 1979) and to the use of visual as opposed to computerised quantitation (Davies, Fakhrai, Morgan and Muir, 1979).

Thallium 201, a potassium analogue, has been used to give exclusion "cold spot" negative imaging (Henning, et al., 1977; Wackers, et al., 1977) and animal studies have shown close correlations with other means of assessing infarct size (Nelson, Khullar, Leighton, Budd, Gohara, Ross, Andrews and Windham, 1979; Bulkley, Silverman, Weisfeldt, Burow, Pond and Becker, 1979).

New techniques utilising positron emitters and computerised multi-axis monitoring are being studied (Weiss, Ahmed, Welch, Williamson, Ter-Pogossian and Sobel, 1977; Keyes, Leonard, Brody, Svetcoff, Rogers and Lucchesi, 1978). While these techniques are currently of research interest, their complexity and expense makes their wide clinical application unlikely in the near future.

Some of the methods described so far in this section have obvious application to human studies, a feature lacking in many experimental methods described below. However, investigations of border zone size, and of the structures responsible for such a zone, are severely limited by those methods which examine only the epicardial surface (which sum transmural changes at the epicardial surface) or which have insufficient sensitivity to detect changes from cell to cell.
Macroscopic dissection methods

The classic descriptions of gross appearances of myocardial infarcts in human autopsy material (Mallory et al., 1939; Lodge-Patch, 1950) and of experimental infarcts in dogs (Karsner and Dwyer, 1916) are still valid. Mallory et al., (1939) identified a border zone consisting of a fine yellow line identifiable at the margin of four day old infarcts which correspond with polymorphonuclear leukocyte infiltration. This gradually changed to a reddish-purple zone over the following few days with the formation of the well-perfused new capillaries of granulation tissue. Phagocytic removal of necrotic tissue led to transmural thinning of the infarct and depression about its periphery after 8 - 10 days. Subsequent collagen formation led to easy discrimination of the infarcted region from normal tissue. Lodge-Patch (1951) noted that after about 15 hours the infarct is pale, swollen and oedematous, and that it acquires a haemorrhagic boundary by 36 hours which increases in clarity on succeeding days.

It is apparent from these descriptions that there are difficulties in gross identification of infarcts less than fifteen hours old. One recent advance has been the use of coloured reagents to identify dehydrogenase enzymes in the normal parts' of the myocardium (Pearse, 1961; Nachlas and Shnitka, 1963). The reduction of colourless nitro-blue tetrazolium (NBT) to cobalt blue deformazan is most commonly used. There were initial hopes that these elegant
techniques might provide rapid and accurate maps of recent infarcts in transverse slices of ventricle, and thus have particular application in forensic autopsies and experimental studies. However, six hours has proved to be the earliest time at which definitive changes can be detected, an interval similar to that which produces the first changes detectable by routine light microscopy (Anderson, Popple, Parker, Sayer, Trickey and Davies, 1979).

Some authors claim that the use of triphenyl-tetrazolium chloride (TTC) in place of NBT gives clearly positive results two hours following infarction (Lie, Pairolero, McCall, Thompson and Titus, 1975).

Most authors have described a sharp, well-defined boundary for macroscopic enzyme mapping but Cox, McLaughlin, Flowers and Horan (1968) obtained evidence of an intermediate zone between normal and infarcted tissue. This was macroscopically visible, but could be better defined microscopically. It is described below (p. 62).

Another technique which may find application is the mapping with microelectrodes of cell resting potentials in tissue slices (McGee, Singer, van Eick, Kloner, Belic, Reimer and Elson, 1978). This technique gives results which correlate closely with those obtained by light, phase and electron microscopy and gives in addition a functional index of cell viability. Though these authors do not explicitly describe border zones in the dog hearts used in their study, they show a viable narrow subendocardial zone, and a
gradation of change from densely affected mid-myocardium to less affected subepicardium, which correlates closely with the morphological changes occurring in these regions.

The methods described in this section, save for the last, do not give sufficient information on early changes to be useful in investigations of the evolution of lateral border zones. Though all give excellent mapping information for large areas of tissue, the sensitivity is not sufficient for the small cell to cell changes. The size of lateral border zones may be of microscopic dimension (p. 60) which means that methods of higher spatial resolution are needed for their investigation.

**Tissue Biopsy studies**

This section describes studies of tissue blocks taken from or adjacent to border zones.

Lie et al., (1975) inferred the existence of an intermediate zone between normal and infarcted tissue from the demonstration of an intermediate rate and extent of fall in sodium to potassium ion concentration ratio in three respective zones of myocardium. One to two gram tissue blocks were used for biochemical analysis, and biopsy sites were determined by visual matching of the transverse ventricular slice to be biopsied with an adjacent slice "mapped" by TTC staining of dehydrogenase activity to show normal, infarcted and border zones. The border zone was defined as tissue which appeared normal by TTC mapping and
light microscopy but which was laterally adjacent to the infarct.

Hearse et al., (1977) demonstrated the presence of tissue metabolite gradients 8 to 15 mm wide, spanning the epicardial cyanotic margin of a left ventricular ischaemic zone (21 - 26 minutes duration) in "open-chest" dogs. Multiple cylindrical transmural biopsies were taken with an instrument which allowed 29 individual samples (spanning the ischaemic field in two dimensions) to be taken simultaneously and rapidly frozen. Tissue biopsies were assayed for ATP, creatinine phosphate, lactate, sodium: potassium ion ratios, and glycogen. High correlations were computed between these metabolites, with ST-segment elevation on multiple epicardial electrocardiograms, and with tissue blood flow (measured by radionuclide labelled microsphere trapping). They considered three possible interpretations of their demonstrated intermediate values at border zones:

1. A homogenous zone of cells, all of which have an intermediate degree of injury.

2. A heterogenous zone, with normal epicardial layers overlying infarcted endocardial layers, giving an 'average' intermediate value.

3. A heterogeneous zone, composed of a complex arrangement of normal and infarcted cells, but no intermediate-injured cells, giving an overall average
value.

These authors favoured the first interpretation on the basis of their previous studies with the limitation of infarct size using infused glucose-insulin-potassium regimes, whose effectiveness infers a zone of intermediate cellular injury.

Other authors showed evidence of intermediate zones of ATP, creatinine and phosphate and lactate concentration after two hours of myocardial ischaemia in open-chest pigs (Janse et al., 1979) and found that they correlated closely with epicardial and intramyocardial electrocardiograms. Concurrent histochemical evidence and unchanging border zone electrocardiograms suggested a sharp, but interdigitated boundary consistent with the third possible interpretation of Hearse et al., (1979).

In an endeavour to correct for the interdigitation of tissues supplied by adjacent coronary beds, and for collateral flow, Hirzel, Sonnenblick and Kirk (1977) marked 'normal' tissue with 15 micron diameter radio-labelled microspheres while separately perfusing the LAD bed in open-chest dogs at the same pressure with a bypass system. The LAD was then ligated, a second batch of microspheres injected to define immediate collateral flow to the ischaemic area, and after a further 24 hours the ligated vascular bed was perfused with Evan's Blue dye. Tissue blocks were obtained by careful dissection for simultaneous creatine phosphokinase (CPK) analysis and flow estimation
These data showed a zone of intermediate values for CPK lateral to the depleted central infarcted zone. However, once corrected for "contaminating" normal tissue inadvertently included in the dissection of infarct or border biopsies, the data showed an abrupt transition from normal to ischaemic with no evidence of a significant intermediate zone after the 24 hour interval used in this experiment.

Furthermore, they showed that a close relationship exists between early collateral flow and the subsequent degree of CPK depletion after 24 hours, with a transmural gradient of both parameters extending from subendocardium to epicardium. These authors concluded that the lateral limits of infarcts in dogs are predetermined by the region of supply of the occluded vessel and do not change, while the extension of necrosis transmurally depends on the status of collateral perfusion within the ischaemic region.

The absence of a zone of intermediate blood flow is supported by Marcus, Kerber, Ehrhardt and Abboud (1975) who divided dog left ventricle into 96 segments following coronary occlusion and infusion of 7 - 10 micron labelled microspheres.

However, other authors present contrary evidence. Jugdutt, Hutchins, Bulkley and Becker (1979a,b) used radiolabelled microspheres, visual photographic and stereo-microradiographic methods to interpret the distribution of infused coloured radio-opaque material in a
study of dog hearts. They demonstrated that wide lateral and epicardial zones of intermediate blood flow within the functional vascular bed of a coronary artery existed, and these zones did not undergo necrosis following ligation of that vessel. Theirs was the most refined of a series of studies using radiolabelled microsphere trapping which reported extensive collateral flow and border zones of intermediate flow (Domenech, Hoffmann, Noble, Sanders, Henson and Subijanto, 1969; Schaper, 1971; Becker, Ferriera and Thomas, 1973; Cobb, Bache and Greenfield, 1974; Bishop, White and Bloom, 1976; Rivas, Cobb, Bache and Greenfield, 1976).

Recently, evidence has been presented which suggests inherent errors in microsphere studies due to real or apparent microsphere loss (Pantely, Walsh, Metcalfe and Kloster, 1978; Consigny, Verier, Payne and Hoffman, 1979; Capurro, Goldstein, Aamodt, Smith and Epstein, 1979; Jugdutt, et al., 1979c; Murdoch and Cobb, 1980). Real microsphere loss would result from elution or phagocytosis into circulating macrophages, while apparent changes would result from the demonstrated initial gains in infarct mass from oedema and inflammatory cell infiltration, followed by subsequent loss due to demolition, organisation and collagenisation of the infarct.

Becker and Jugdutt (1978) combined microsphere techniques with a diffusible radionuclide tracer, Thallium 201, to demonstrate in dog hearts a lateral zone of
intermediate flow values 10.4 mm wide in endocardial areas and 18.8 mm in epicardial areas. Biopsy size was 0.02 - 0.1 g, though duration of coronary occlusion was not stated.

These studies have recently been reviewed by Hearse and Yellon (1981) who also supplied new data (Yellon, Hearse, Crome, Grannell and Wyse, 1981) which utilised a finer spatial resolution than their previous studies (Hearse et al., 1977). They concluded that the apparent size of an intermediate zone between normal and severely ischaemic myocardium is closely related to the spatial resolution of the measurement. They argue that any method can demonstrate only the 'zone of change' in which such a transition occurs. In reviewing previous studies, they concluded that, where the resolution of measurement is high, the size of the zone reported in which the transition occurs is small, and from their own data favour the interpretation that there is very little if any myocardium of an intermediate degree of injury lying between normal and severely injured myocardium at the lateral boundary of an infarct.

While different techniques applied after different intervals of ischaemia make direct comparisons difficult, it is clear that the size of the border zone is an unresolved question.

Vascular Perfusion Studies

There is long-standing controversy concerning whether or not coronary arteries function as end arteries. Though very
early authors (Lower, 1669; Thebesius, 1708) recognised intercoronary connecting vessels, the highly respected anatomists of the nineteenth century declared coronary vessels to be functional and morphological end arteries (Hyrtl, 1873; Henle, 1876; Cohnheim and von Schulthess-Richberg, 1881; Dragneff, 1897). If this is so, occlusion of a coronary vessel would inevitably lead to necrosis of the entire vascular bed it supplies, and the concept of a retrievable border zone cannot be entertained unless that occlusion is relieved before irreversible injury occurs.

However, Spalteholtz (1907) was able to demonstrate communicating vessels between branches of the same and different coronary arteries, and Gross (1921) provided unequivocal evidence in photographs of post-mortem angiograms in dog hearts which led him to pronounce that vascular anastomoses were "universal and abundant".

Nevertheless, the end artery concept again gained ascendency through the studies of Schlesinger and his associates (1938) and dominated opinion until the studies of Fulton (1963a,b; 1965) used post-mortem angiograms of radio-opaque gelatin masses in human hearts and photographs of specimens following tissue clearance with solvents to confirm the work of Spalteholtz and Gross.

The presence of abundant collateral connections between coronary vessels in some species now has wide acceptance. Extensive reviews (Bloor, 1974; Paster, 1977)
have recognised four major groups of collaterals: The majority, both in prevalence and functional importance, are intracardiac and run either between branches of the same (intracoronary) or different (intercoronal) arterial trees. Less important are the arterio-luminal anastomoses (including Thebesian veins) and the extracardiac anastomoses to bronchial or mediastinal vessels, through vasa vasorum of the great vessels (following the pattern of supply in the lower vertebrates - Halpern, 1957), and those vessels which traverse the pericardial space.

Schaper (1971) has shown extensive collateral supply in dogs, predominantly in the subepicardial layers. Functional correlation of this with the transmural 'wave-front' progression seen in experimental infarcts in dogs has been made (Reimer et al., 1977, Reimer and Jennings, 1979). Jugdutt et al., (1979a,b) suggest that although collaterals in the dog are extensive, the functional supply is relatively constant. These authors compared the mass of "tissue at risk" following coronary ligation at various sites with the mass of the eventual infarct, and showed that there was a critical minimum mass of "at-risk tissue" below which no infarct was detectable at 48 hours. For greater masses, the proportion of infarct mass to "at-risk" mass was found to be a constant of about 50%. This suggested that following acute occlusions in dogs, immediate collateral supply provides sufficient flow to obviate infarction in the minimal mass (about 20 g), and then a relatively constant degree of flow prevents infarction of about 50% of the
tissue at risk. It is not clear however, whether the 50% of
tissue which escapes necrosis represent "jeopardised
myocardium" which may be salvaged by therapeutic
intervention as they claim, or if this tissue is never
effectively imperilled because of collateral flow.

Gradual occlusion of coronary vessels by constriction
with hygroscopic rings has been studied by Schaper, (1971).
He demonstrated expansion of small collateral channels to
form morphologically and functionally larger channels, and
also showed species differences. Whereas dogs subjected to
gradual occlusion of a major vessel developed extensive
collaterals and few infarcts, pigs developed few collaterals
and near universal infarcts. Humans appear to occupy an
intermediate position, as enlarged size and increased flow
in collaterals has been reported in hearts with
atherosclerotic coronary disease (Prinzmetal, Simkin,
Bergman and Kruger, 1947; Baroldi, Mantero and Scomazzoni,
1956; Fulton, 1963 a,b, 1965; Estes, Entman, Dixon and
Hackel, 1966; Farrer-Brown, 1968a,b,c,d). Nevertheless, it
is apparent from the prevalence of infarction that even an
extensive collateral supply in humans is not adequate to
compensate for some coronary occlusions, either because of
their magnitude or rate of development. It is this concept
which has led to the term "physiologic end arteries" for
coronary vessels.

This concept has been reinforced by studies of the
microcirculation. Filling of capillary beds (as well as
larger vessels) was not technically feasible in many early studies of collaterals, nor was it desirable because of the density of capillary vessels which would mask larger vessels. However the use of intravascular casts of radio-opaque material (with radiographic preparation), silicone elastomers (with tissue clearing and microscopy), and epoxy resins (with scanning electron microscopy) has enabled the demonstration of normal architecture (Brown, 1965; Farrer-Brown, 1968 a,b; Ludwig, 1971; Rakusan, 1971; Murikami, 1971; Bassingthwaite, Yipintsoi and Harvey, 1974; Phillips, Rosenberg, Meir-Levi and Pappas, 1979; Gavin et al., 1983b).

Though Baroldi and Scomazzoni (1967) cite early workers as demonstrating anastomoses at capillary bed level, Okun, Factor and Kirk (1979) using dog hearts showed end capillary loops and close approximation of adjacent capillary beds supplied by different arteries, with no evidence of anastomoses. This is in contrast to observations in skeletal muscle using the same technique where mixing of the different coloured silicone elastomer perfusates (Microfil) was widespread. Grayson, Davidson, Fitzgerald-Finch, and Scott, (1974) could demonstrate no anastomoses between capillary beds of adjacent large vessels using angiographic media of differing viscosities. This anatomical finding supports recent studies (Fischl et al., 1974; Hirzel, et al., 1977; Factor, Sonnenblick and Kirk, 1978) showing no evidence of a significant biochemical border zone at the lateral boundary of infarcts, once its complex
interdigitated form is allowed for.

More recently, Factor, Okun, Minase and Kirk (1982) have used this technique to demonstrate similar overlapped but not interconnected capillary beds in human hearts examined post-mortem. They have applied this technique to show close correlation of capillary bed filling of occluded and non-occluded coronary vessels with the interdigitated peninsulas of normal and infarcted myocardium at the lateral border of 24 hr old in \textit{vivo} infarcts in dog hearts (Factor, Okun and Kirk, 1981).

Brown (1965) and Ludwig (1971) showed anastomoses between apparently different capillary beds, though their technique does not identify the larger vessels supplying those beds. Brown proposed a model of vessel-to-fibre relationships where, as in a cubic crystal matrix, each fibre and vessel have four adjacent vessels or fibres. He suggests that capillary to capillary anastomoses may lead to individual fibres being supplied by capillaries from one to four contributing beds, thus creating the potential situation for varying degrees of ischaemia. Okun et al., (1979) admit to this possibility, but their technique precluded examination of vessel to fibre relationships.

At a grosser level, coloured dyes have been infused into adjacent vessels at identical pressures, with sharp demarcation of vascular beds and no evidence of intermixing at boundaries (Reimer and Jennings, 1979). Korb (1970) combined immediate post-mortem infusion of rat hearts with
'Light Green' dye and vital staining of viable myocardium by acridine orange. Examination of tissue sections by ultra-violet fluorescence microscopy allowed differentiation of normal perfused, necrotic perfused and necrotic non-perfused regions. However this author did not give account of the relative distribution of these regions at infarct border zones. Ultra-violet fluorescence was used also by Sybers, Ashraf, Braithwaite and Lok (1972) to demonstrate the accumulation of tetracycline in normal myocardium, particularly at the marginal zones of infarcts.

In summary, there is a continued disagreement as to the anatomical interconnection of microvascular beds at the lateral boundary between two adjacent coronary arteries in normal hearts, though recent work favours the view that such connections do not occur. Wide differences in techniques and in the species of animals studied make a consensus conclusion concerning the role of such connections at a capillary level, or at higher generations of vessels during ischaemia difficult to attain, but it appears that collateral flow, in the species and situations in which it occurs, is a function of larger vessels which then contribute to discrete capillary beds resulting in sharply discriminated boundaries dictated by microvascular bed anatomical distribution. The imposition on this anatomical pattern of the no-reflow phenomenon (reviewed above, p. 30), and its consequences in vascular supply at the lateral margins of ischaemic regions have received little attention.
Light microscopy studies

The classic descriptions of the histopathology of infarcts, and the time course of changes, (Mallory et al., 1939; Lodge-Patch 1950) remain widely accepted (W.H.O. 1970). Other changes described since then are focal myocytolysis (Schlesinger and Reiner, 1955; Baroldi, 1975), wavy fibres (Bouchardy and Majno, 1974), and contraction bands (Sommers and Jennings, 1964). Recent reviews of the histologic evolution of infarcts in man (Fishbein, Maclean and Maroko, 1978b) and experimental animals (Fishbein, Maclean and Maroko, 1978a; Spadaro, Fishbein, Hare, Pfeffer and Maroko, 1980) account for these more recently described changes, but do not fundamentally alter the classic descriptions.

While an extensive literature compares infarcted with normal myocardium, few studies are directed at border zones or infarct peripheral changes per se. Contraction bands, which consist of densely staining aggregations of A-bands in damaged fibres, were noted to be widespread throughout reperfused infarcted myocardium, but confined to the border zone of permanently ischaemic regions (Jennings et al., 1960; Sommers and Jennings, 1964; Herdson, et al., 1965). These authors suggested that contraction bands result from the contractile forces of adjacent viable fibres on irreversibly injured cells.

In addition to contraction bands, structurally damaged peripheral cells may show specific staining characteristics.
Armiger, Wheeler, Geraghty and Herdson (1977) demonstrated such changes in the border zone between the narrow viable immediate subendocardial layer and deeper infarcted myocardium in experimental infarcts in dogs, using both traditional stains (haematoxylin and eosin, Masson trichrome, phosphotungstic acid-haematoxylin) and newer techniques (Puchtler's, Connor's, and Lie's stains).

Accumulation of neutral lipid has been described in myocytes of the infarct periphery (Wartman, Jennings, Yokohama, and Clabaugh, 1956; Shnitka and Nachlas, 1963; Cox et al., 1967; Dusek, Rona and Kahn, 1971). Fishbein, Hare, Gissen, Spadaro, Maclean and Maroko (1980) noted that in permanent infarcts in rats the lipid deposition increased from faintly visible by light microscopy at one hour to a maximum at nine to twelve hours post-infarct. By this time it defined a zone of about ten per cent of ventricular mass which lay entirely within the viable myocytes in the "normal" side of the infarct boundary.

Similarly, a peripheral distribution of calcium deposition has been described (Yokohama, Jennings, Wartman and Clabaugh, 1956; Shen and Jennings, 1972; Factor et al., 1978) with small groups or individual cells in the border zone containing intracytoplasmic granular deposits of calcium stainable by von Kossa's method. These granules have been interpreted as calcium taken up into swollen mitochondria.

A border zone of intermediate levels of dehydrogenase
activity has been demonstrated by Cox et al., (1968) both macroscopically and microscopically. A zone of coarse granular diformazan deposition, differentiated at light microscopic magnification from the fine granular staining of normal myocardium, was seen interposed between normal and infarcted tissue in experimental infarcts in dogs. They interpreted these findings as indicating an ischaemic but not irreversibly injured zone, with swollen mitochondria giving the coarse granular staining. Electron microscopy was not performed. Dusek et al., (1971) confirmed these findings, but showed that coarse granules were not limited to mitochondria, and that in later stages some cells showed diffuse cytoplasmic staining. They also reiterated the warning of Shnitka and Nachlas (1963) concerning a false positive staining of perinuclear lipid inclusions (rather than mitochondria) by the NBT method or cytochrome oxidase staining techniques.

Though stains for glycogen are of limited use in post-mortem studies because of autolysis (Armiger et al., 1977), glycogen depletion has been used as an index of anaerobic respiration in many experimental studies (Yokohama, et al., 1955; Klionsky, 1960; Meyer and Purdy, 1961; Shnitka and Nachlas, 1963; Lushnikov, 1963; Vokonas, Malsky, Paul, Robbins and Hood, 1978; Janse et al., 1979; Fishbein et al., 1980). All authors agree that glycogen depletion is time related, initially reversible by reperfusion, and precedes to completion by about one hour at the infarct centre. By that time the peripheral cells were either "full" or "empty"
with regard to stainable glycogen (Janse et al., 1979) though at earlier intervals small groups of intermediate staining cells were noted.

Fishbein et al., (1980) examined the rate and distribution of glycogen depletion in regional infarcts in rats, and also aerobic metabolic enzyme activity (succinic dehydrogenase, NADH-diaphorase). They showed a peripheral infarct zone in which glycogen is depleted more rapidly than the central infarct, though by thirty minutes after occlusion depletion was uniform and severe. They attribute this difference to residual contractile activity at the periphery, as opposed to complete cessation at the infarct centre, and to possible inhibition of glycolysis by the relatively lower pH at the infarct centre. A reversed pattern of depletion was noted for the aerobic metabolic enzymes, with relative preservation in peripheral infarct tissue for up to nine hours, by which time depletion was uniform and severe. They imply from these patterns that there is a peripheral zone in the infarct whose cells remain viable longer than the centre.

Both Factor et al., (1978) and Janse et al., (1979) describe an interdigitated lateral boundary about 5 mm wide where normal and infarcted tissue are adjacent but sharply defined at a cellular level. Factor et al., (1978) have demonstrated by serial section reconstruction that the commonly observed "islands" of normal tissue, occurring in infarcts (surrounded by necrotic tissue but close to normal
tissue) are continuous with normal tissue, forming complex "peninsulas" which have sharply defined margins, with no evidence of a population of cells of "intermediate" degree of injury. They use this evidence, seen 24 hours post-infarction in dogs, to support their contention that lateral border zones are defined by vascular supply from the outset and no retrievable border zone exists.

In contrast, Vokonas, et al., (1978) used microradioautographs to detect infused carbon-14 labelled antipyrine in normal, border zone and ischaemic dog myocardium and compared the tracer distribution with histological changes at 24 hours. These authors interpret their results as showing a lateral border of intermediate blood flow, and hence salvageable tissue, averaging 4.5 mm wide, and constituting about 30% of the total ischaemic area.

In summary, light microscopical studies have indicated changes to ischaemic cells adjacent to the lateral boundary (contraction band necrosis) and also changes to non-necrotic cells adjacent to the border of the developing infarct (changes in NBT staining, uptake of lipids and calcium). The demarcation of normal and ischaemic myocardium after 24 hours has been demonstrated by some but not all studies to be abrupt (cell to cell), though arranged as a complex interdigitation, which could easily appear as a zone of intermediate levels of injury in methods of low resolution.

It can be seen that there is a need for further
demonstration at lateral border zones of the relationship of perfusion boundaries to morphological changes at a cellular level, particularly the evolution of changes within the first twenty-four hours following coronary artery occlusion. This requires the use of techniques to define vessel-to-fibre relationships at electron microscopic levels.

Ultrastructural studies

A large number of electron microscopic studies have been performed on infarcted myocardium in humans and a wide range of experimental animals since the mid-1950's. Most have deliberately avoided border zones so that they could examine changes in severe uniform ischaemia. However, some authors have examined the ultrastructure of contraction bands at borders of permanent infarcts, others have examined border zones at early and late stages, and others reperfused infarcts.

Caulfield and Klionsky (1959) used the term "contraction bands" to describe thickened Z-lines in ischaemic rabbit myocardium, but more recent descriptions (Herdson et al., 1965; Korb and Totovic, 1969) have applied the term to dense condensations of several sarcomeres showing thickened Z-lines and hypercontracted myofibrils. These zones are randomly distributed with respect to intercalated discs and alternate with cleared zones where myofibrils have torn apart (apparently at H-bands), and contain aggregates of
mitochondria. The contraction bands occasionally contain small, dense, apparently 'squashed' mitochondria. Entrapped glycogen granules are present long after granules elsewhere in the cell have been consumed by anaerobic metabolism. Fibres with contraction bands show mitochondrial changes including swelling, dense amorphous inclusions, matrix clearing and crystal disorganisation; nuclear changes such as swelling, margination, occasional rupture of the nuclear envelope; sarcolemmal rupture and disorganisation of sarcoplasmic reticulum and t-tubule systems. These changes are ultrastructural evidence of irreversible injury (Jennings et al., 1975; Schaper, et al., 1979).

Page and Polimeni (1977) studied ultrastructural changes in border zones of permanent regional infarcts in rats. They noted characteristic changes within the infarct at one, four, 12 and 24 hours, and a narrow zone, one to five cells wide, surrounding the infarct which showed irreversible injury with contraction band formation at one hour. Outside this zone was a wider zone of "relaxed" fibres, distinguishable from surrounding normal fibres which show contracted sarcomeres following perfusion fixation with osmium tetroxide. This zone of relaxed fibres showed no ultrastructural abnormality one hour after coronary occlusion apart from the I-bands and lengthened sarcomeres indicative of relaxation. At four hours, some of the fibres in this zone contained intracellular lipid vacuoles. By twenty-four hours the lipid vacuoles were more abundant, and were also seen in cells of adjacent 'normal' myocardium.
(i.e. cells showing contracted fibres). In addition some fibres of the "relaxed" zone showed lysis of Z-lines, appearance of N-lines, absence of the M-band opacity and its replacement by an electron-lucent zone. Longitudinal densities at the lateral margins of fibres extending over several sarcomeres were noted. Some of these fibres showed dense amorphous mitochondrial inclusions and sarcolemmal discontinuity.

These authors inferred that the fibre changes (to M- and Z-lines) were indicative of progression to irreversible injury of fibres subject to mild ischaemia. They based this on the distribution of tissue blackening following perfusion fixation by osmium tetroxide, which gave a clear macroscopic boundary. They related this boundary to the narrow zone showing contraction bands and severe fibre damage at one hour, and inferred that tissue further from the boundary was better perfused because of collateral flow gradients. However they did not show whether or not perfusion by highly diffusible osmium tetroxide correlates to actual blood flow.

Page and Polimeni (1977) also did not make clear what proportion of cells in the relaxed zone showed further changes to irreversible injury by 24 hours, nor what relationship lipid vacuolation had to cells with damaged myofilaments. The spatial distribution of fibres with M- and Z-line changes within the 'relaxed' zone was not described, and no comment was made on the appearance of the
microvasculature. They suggested that the changes described as 'ischaemic' may relate to "neural, biochemical, or electromechanical side effects from adjacent ischemic muscle".

Cuenod, Joris and Majno (1979) described lysis of Z-lines of myofibres of the right ventricle subject to acute "pre-load" stress following experimental pulmonary embolisation with plastic micropheres in rats. They described in addition "shredding" and "myofibrillar degeneration", lesions which were not associated with mitochondrial or sarcolemmal changes characteristic of irreversible ischaemic injury. In 1966 Martin and Hackel described the scattered contractural changes centred about intercalated disks (which they termed "zonal" lesions) produced by hypotensive (haemorrhagic) shock in 'open-chest' dogs. They noted that these lesions did not show mitochondrial or sarcolemmal defects and appeared to be reversible in that they were absent (and not replaced by necrosis or fibrosis) three to seven days after the hypotensive period. These authors comment on the difficulty determining the relative roles for ischaemia, mechanical or other modes of injury in production of these lesions.

Dusek et al., (1971) described similar changes in cells surrounded by connective tissue at the margins of infarcts one to four weeks after coronary occlusion in rats. They noted sub-sarcolemmal condensations extending over several sarcomeres and attached to Z-lines, and observed that these
were present in normal cells or myotendinous junctions and in some elements of the conducting system. They cite Haspar (1964) as demonstrating similar cells in rat heart following repeated global ischaemia. These authors attributed the appearance of these cells at infarct margins to the proximity of developing connective tissue, but Page and Polimeni (1977) described the appearance of these cells well before the collagenisation has commenced in the infarct. Other cells present at the margin of healing infarcts described by Dusek et al., (1971) resembled differentiating embryonic myocytes. They attributed these appearances to degenerate viable cells adapted to an hypoxic environment by sacrifice of contractile function and primitive appearance. Similar cells have been noted at the margins of and within regional infarcts previously, and these have been interpreted as evidence of myocardial cell regeneration (Ring, 1950; Wilcken, Shorey and Eickens, 1970; Zhang, Yang, Cheng, Ling, Tang and Yu, 1980).

In summary, electron microscopic studies have often compared myocardium well removed from the border of the developing myocardial infarct, and those few studies which have been directed primarily at the border zone have mostly described the morphology and pathogenesis of contraction band necrosis in the ischaemic myocardium immediately adjacent to the boundary. Only one study (Page and Polimeni, 1977) has attempted the correlation of myocyte appearance and vascular function in regional infarcts, and these authors demonstrated a very small zone of abnormal
myocardium outside the lateral boundary of the early evolving infarct. This indicates the need for further studies at an ultrastructural level, using non-diffusible tracers as indices of vascular function (particularly in relation to the no reflow phenomenon) to correlate microvascular function and ischaemic injury to associated cardiac muscle cells at the lateral boundary of evolving early regional myocardial infarcts.

**Scanning electron microscopy**

The fifteen years in which scanning electron microscopes (SEM) have been available for biological research have seen relatively little attention paid to cardiac tissue. Published reports are of two types, those describing three-dimensional ultrastructure and comparing it with predicted models from established techniques particularly transmission electron microscopy, and papers applying SEM data to pathological or physiologic investigations.

First in the former group were Buss, Hanrath, Kronert and Schoenmackers (1971). Subsequent improvements in preparation techniques gave better definition of subcellular structures in normal myocardium (Ashraf and Sybers, 1974; 1975) and have led to improved understanding of the t-tubule and sarcoplasmic reticulum (McCallister, Mumaw and Munger, 1974; Ashraf, Livingstone and Bloor, 1976; Ashraf and Bloor, 1976; Sommers and Waugh 1976; Myklebust, Dalen and Saetersdal, 1975; Dalen, Myklebust and Saetersdal 1978).
Similar combined light, transmission electron (TEM) and SEM studies have been carried out on normal skeletal muscle and are a useful comparison (Pachter, Davidowitz, Zimmer and Breinen, 1973; Geissinger, Yamashiro and Ackerley, 1978).

The application of SEM to pathological investigations includes comparison of normal and ischaemic myocardium (Ashraf, et al., 1977; Ashraf and Sybers, 1975), demonstration of the loss of endothelial cells from the ventricular luminal surface of ischaemic dog myocardium (Wheeler, Gavin and Herdson, 1973), and elucidation of the pathogenesis of the no-reflow phenomenon in ischaemic dog heart (Gavin, et al., 1978b). Examination of micro-corrosion casts of cardiac vessels is described below (p. 72). Recent advances in culture of isolated myocytes have allowed the study of developing intercalated disks (Bishop and Drummond, 1978).

Comparison of SEM and TEM appearances of cardiac myocytes has been restricted to longitudinal sections of fibres. There appears to be only one published illustration of freeze fractured fibres in cross-section (Gavin et al., 1978b), though Buss et al., (1971) show a cut surface through a fibre. The differences in appearance by SEM of normal and ischaemic fibres in cross-section do not yet appear to have been published. Examination of the other tissue components of the heart has been even more limited, but Caulfield and Borg (1979) and Borg and Caulfield (1981) have studied the normal collagenous cardiac 'skeleton' in
the hearts of rodents.

Despite the usefulness of SEM in the rapid preparation of large specimens for examination at a wide range of magnifications in a three-dimensional mode, no such studies appear to have investigated the interrelationship of normal and ischaemic myocardium, or the relationship between capillary bed perfusion and the pattern of ischaemic injury to myocytes at the margins of regional infarcts.

Micro-corrosion casting

The technique of filling an internal luminal system with a liquid medium which becomes a solid in situ and subsequent corrosion of the surrounding tissue to leave a replica is called micro-corrosion casting. Such techniques date back to Leonardo da Vinci (1452-1519) and have been extensively reviewed by Hodde and Nowell (1980). By the early twentieth century microvascular anatomists reached the limits of resolution and depths of field obtainable by light microscopy of cleared serial sections of injected tissue. However, the use of scanning electron microscopy on rubber latex casts (Nowell, Pangborn and Tyler, 1970, 1972) and on acrylic plastic casts (Murakami, 1971) allowed the microvascular anatomy of many tissues to be studied in much greater detail.

Rubber latex casts have proved disappointing for scanning electron microscopy preparation, despite their ease
of handling and suitability for examination in solvent cleared histological sections. Recently vinyl polymer compounds have been used which withstand corrosion and drying better (Frasca, Carter and Schaffer, 1978; Phillips, et al., 1979).

Since the introduction of acrylic resin corrosion casting for SEM by Murakami in 1971, there have been several technical improvements, changes to the resin ingredients including addition of plasticisers to improve the dissectability of brittle casts (Murakami, Miyoshi and Fujita, 1971; Murakami, 1975), the rendering of the entire specimen conductive with osmium tetroxide and hydrazine hydrate vapour (Murakami, Unehira, Kawakami and Kubotsu, 1973), and a variety of methods and changes in formula, including commercially produced pre-polymerised methacrylates to give a more consistent result with lower viscosity (Ohtani and Murakami, 1978; Hodde and Nowell, 1980).

Micro-corrosion studies of the microvasculature of the heart have been generally limited to simple descriptions of the complexity of the morphology (Murakami, 1971; Phillips et al., 1979). However micro-corrosion casting has been used in studies of the no-reflow phenomenon in globally ischaemic isolated perfused rat hearts (Gavin et al., 1983b; Humphrey, et al., 1981). In other tissues, for instance cat fish gill vascular beds, micro-corrosion casting has been used to identify perfusion distribution patterns in this
complex microvasculature (Olson, 1980). The range of magnification and extreme depth of field available with acrylic micro-corrosion casts makes it a useful technique for the examination of perfusion patterns in adjacent capillary beds of perfused and non-perfused vessels at the border zones of myocardial infarcts.
PURPOSES OF THIS STUDY

The review of literature established that myocardial infarcts continue to increase in volume for up to 24 hours after occlusion of the coronary artery supplying the ischaemic area. This means that there are areas at the margins of early infarcts which, if blood supply is restored, need not inevitably proceed to necrosis. The extent and spatial distribution of these "border zones" in regional infarcts is controversial, with wide variation in the interpretation and conclusions of published studies.

The distribution of coronary arteries and the microcirculation they supply is a crucial characteristic in any consideration of the pattern of development of irreversible injury to cardiac muscle cells in border zones of myocardial infarcts. Previous studies have failed to definitively determine the relationship between the anatomical distribution of microvascular supply to the border zones of early infarcts, and the structural and functional changes which may arise in the microvasculature either as a response to ischaemia or as a result of it.

Recent studies using isolated hearts have contributed greatly to the understanding of the pathogenesis of the loss of function of the microcirculation resulting from ischaemia, and to the development of irreversible injury to cardiac muscle cells. However, it is not clear from studies of globally ischaemic hearts, what influence the no-reflow phenomenon may have at the border zones of evolving regional
infarcts, where the remainder of the heart continues to perform its metabolic and mechanical functions.

The aim of the present study was to establish a model of regional myocardial ischaemia and use it to determine the spatial and temporal relationships between the development of evidence of irreversible myocardial cell injury and changes in microvascular function at the margins of developing early myocardial infarcts, and thus to elucidate the relationship between the development of myocyte necrosis and the loss of competence of the blood vessels which supply them.

This required utilisation of scanning electron microscopy which allowed the study of topographic detail over large areas while retaining a sufficiently high degree of resolution to detect subcellular changes. Quantitative methods were developed to identify those small vessels which permitted intravascular flow and their source of arterial supply. It was necessary to devise tissue preparation methods which allowed simultaneous assessment of morphological changes in function and structure of the capillaries associated with each muscle cell.

The appendices of this thesis describe preliminary studies which led to the selection of the most appropriate intravascular tracers (Appendix I, p. 186), the necessary tissue preparation methods (Appendix 2, p. 195), the effects of tissue fixation on patterns of microvascular flow (Appendix III, p. 206) and methods for labelling resin to
allow identification of the sources of arterial supply
(Appendix IV, p. 215).

Application of these techniques allowed a definition of
the relationship between cardiac cell death, the changes in
microvascular function, and the extent and distribution of
potential 'border zones' around regional infarcts as they
developed in beating rabbit hearts.
MATERIALS AND METHODS

General Outline

Isolated rabbit hearts were maintained in a beating state as modified Langendorff (1895) preparations by perfusion of their coronary vascular beds via the aorta with an aqueous oxygenated perfusate. Cannulation of the ventral interventricular branch (VIB) of the left coronary artery enabled controlled separate perfusion and continuous vascular access to a discrete region of the left ventricular wall which could be made ischaemic by stopping perfusion through this cannula.

After an initial stabilisation period of full perfusion, regional ischaemia was established in five groups of four hearts for 0, 30, 60, 120 and 240 minutes. Each heart was then fixed by perfusion and the vascular beds supplied by the aortic and VIB cannulae filled with acrylic resin labelled so as to permit discrimination of one bed from the other. Tissue blocks from the lateral margin of the ischaemic region, along with blocks from the central area of ischaemia and normal dorsal left ventricular wall, were then dissected from the heart and processed for examination by scanning electron microscopy.

Particular attention was paid to the relationship of adjacent vascular beds at the boundaries of the ischaemic regions and to the correlation of this vascular supply to the pattern of morphological evidence of ischaemic injury in
The Perfusate

Modified Krebs-Henseleit bicarbonate buffer solution with added colloid was prepared according to the method of Armitage and Pegg (1977) for each heart on the day of use. Its composition was as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
<td>119.10 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>4.75 mM</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>1.26 mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25.00 mM</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>1.19 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.19 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>11.0 mM</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>0.1 mg/ml</td>
</tr>
<tr>
<td>Haemaccel</td>
<td>1.75 ml/100 ml</td>
</tr>
</tbody>
</table>

All chemicals were reagent grade. Carbenicillin is a semi-synthetic penicillin (PYOPEN-Beecham Research Laboratories, U.K.) and HAEMACCEL (Behringwerke A.G. West Germany) a 3.5% colloidal infusion preparation composed of hydrolysed gelatin cross-linked with urea bridges to create standard molecular weight sub-units.

The perfusate was made up in three litre aliquots with deionised water from a Milli-Q filtration plant (Millipore Corporation, U.S.A.) and was filtered through a five micron pore size membrane filter (Gelman Sciences Inc. U.S.A.). Approximately thirty millilitres were further filtered through a Whatman No. 1 filter paper cone containing 0.5 mg of methylene blue dye (G.T. Gurr Ltd., U.K.) to give a perfusate with a deep blue colouration. All perfusate was stored at 4°C until use.
Cannulae

Two plastic cannulae were prepared for each heart. Aortic perfusion was made through a polypropylene cannula (Portex Ltd., U.K.) which has a female 'Luer' mount at the proximal end and a ridged tapering distal end (Fig. 1A). The two terminal ridges were removed from the stock cannula to give an appropriate end diameter for insertion into the rabbit aorta. These large cannulae were re-used after cleaning in a solution of detergent (Pyroneg. Diversey Wallace Ltd., New Zealand) and then rinsing with ethanol.

The fine cannulae (Fig. 1A) were not re-used. For each heart a five centimetre length of intravenous vinyl tubing (Dural Plastics Ltd., Australia) with external diameter 0.80 mm and an internal diameter 0.50 mm, was inserted into the full length of the lumen of an 18 x 3/4 swg disposable hypodermic syringe needle (Kempthorne Prosser and Co., New Zealand) from which the distal 0.5 cm had been ground away to give a flat smooth tip. A drop of epoxy resin adhesive (Rapid Araldite, Selleys Chemicals Ltd., New Zealand) was applied to the junction of the needle shaft and protruding cannula to secure the tubing and provide protection against kinking at the junction. The cannula tip was then bevelled to aid insertion into the vessel.

Before use the large and small cannulae were attached to 25 and 1 ml syringes respectively. These were filled with blue coloured perfusate and care was taken to exclude air bubbles. Cannulae and perfusate were chilled to 0°C before
Preparation of the Heart

Adult New Zealand White rabbits of either sex were obtained from the central animal laboratories of the University of Auckland School of Medicine. Each rabbit was anaesthetised with 18 mg/kg Saffan (alphaxolone and alphadalone acetate: Glaxovet Ltd., U.K.) administered via the marginal ear vein using a winged 25 swg infusion needle (Terumo Ltd., Australia). At the same time 500 IU of heparin injection B.P. (Leo Laboratories Ltd., U.K.) diluted in one millilitre of perfusate was also injected. When deep surgical anaesthesia was established the skin over the sternum was opened, and the chest opened ventrally from diaphragm to sternal notch using scissors. While the heart was held gently forward and upwards, the pericardium was then incised and the heart excised together with the aortic root. It was immersed immediately in 100 ml of ice-cold perfusate until contraction had ceased. The aortic root cannula was inserted in the lumen of the aorta and tied in place with 3-0 surgical silk (Abrasilk: Armour Pharmaceuticaal Co., U.K.). Ten ml of blue coloured perfusate from the attached syringe was then injected to flush the entire coronary bed.

This assembly was transferred to a 200 ml bath of ice-cold perfusate on the stage of an Olympus SZ dissecting microscope fitted with an epiillumination unit. The
ventricles were supported in the bath by a layered foam plastic cradle in which a 2 cm central recess had been cut, so that the heart sat upright immersed in cold perfusate up to the atrio-ventricular junction. The syringes were supported on the microscope column with Bluetak silicone rubber (Bostick NZ Ltd.). Next the pulmonary artery root was dissected free from the aorta to expose the aortic root and the origin of the left coronary artery. Stay sutures were placed in the pulmonary artery and the left atrial appendage and tensioned in opposite directions with small haemostatic forceps.

Further intermittent flushing of the aortic cannula with blue perfusate allowed identification of the left main coronary artery, its bifurcation, circumflex branch, and VIB within the epicardial fat. The VIB was then dissected and underrun with 6-0 cardiovascular silk suture on curved round-bodied atraumatic needles (David-Geck Ltd., USA) at two points 2-4 mm apart immediately distal to the origin of the VIB. A 26 x ½ swg disposable syringe needle (Terumo Australia Ltd.) was then passed through the vessel wall as a stylet, and the fine cannula introduced into the vessel (Fig. 1B). Free flow of perfusate was checked by gentle flushing of the cannula from the attached 1 ml syringe. The distal underrun suture was tied over the cannula in the vessel, to secure it in place and prevent leakage back through the insertion site. The VIB was ligated immediately proximal to the cannulation site using the second suture. The heart with both cannulae was finally transferred to the
perfusion apparatus.

**Perfusion with Buffer**

The perfusion apparatus (Fig. 2) was a modified Langendorff (1895) system, similar to that used extensively in the Department of Pathology for studies of isolated rat hearts (Humphrey, et al., 1980), and utilising a re-circulating pool of perfusate (Armitage and Pegg, 1977). Perfusate was continuously oxygenated in the upper reservoir (Fig. 2) by bubbling 95:5% oxygen:carbon dioxide from a dispersion cannula and maintained at 37°C by a recirculating water jacket system. Reservoir height was adjusted to maintain an hydrostatic perfusion pressure of 85 cm of water with respect to the aortic root of heart. Water-jacketed silicone tubing conducted perfusate down through a bubble trap to the cannulae. Three-way taps in the terminal supply to the cannulae provided control of flow and 'Luer' male fittings for attachment of the cannulae.

The syringes were removed from the cannulae and, taking care to exclude air bubbles, the Luer mounts were joined so that the heart was suspended from the cannulae in an airspace bounded by a water jacket which had a polystyrene foam lid (allowing access) and a drain hole (allowing drainage and measurement of coronary effluent). Effluent was conducted to the lower reservoir then pumped by roller pump through a 5 micron pore size Gelman membrane filter up to the upper reservoir. Flow rate was adjusted to maintain
a constant level in the upper reservoir. The recirculating pool volume was 2.7 litres.

The apparatus was disassembled between uses, soaked in hot detergent (Pyroneg), rinsed in hot tap water, and finally rinsed in deionised water before reassembly. Sodium azide was dissolved in the water jacket recirculating pool to suppress microbial growth. There was no contact between this pool and the perfusate. Coronary flow rate was measured by collection of effluent flow into a graduated cylinder over twenty second periods.

At the end of the experimental procedure, each heart was fixed by perfusion with 2.5% glutaraldehyde in 0.1M phosphate buffer (Karlsson and Schultz, 1965) with pH 7.4, buffer osmolarity 318 mOsm per litre and total osmolarity 524 mOsm per litre (Glauert, 1974). This was pre-warmed to 37°C and infused via the terminal three-way taps from a reservoir at identical height to the perfusate upper reservoir. Following perfusion with 100 ml of fixative the hearts were removed from the apparatus, the cannulae were plugged with plastic Luer mount plugs to prevent entry of air bubbles, and the hearts stored in fresh fixative at 4°C until resin perfusion and preparation for scanning electron microscopy was undertaken.

Resin Perfusion

On the day of use two batches of resin were prepared from L.R. White acrylic resin stock (London Resin Co., U.K.)
which is a proprietary, partially polymerised, non-methacrylate resin. Thirty mg of Fat Red 7B dye (George T. Gurr, Ltd., U.K.) was dissolved in 15 ml of resin stock to make one batch.

The second batch was prepared by suspending 1.0 g of dry analytical grade lead dioxide (Reidel de Haen, A.G., Hanover, West Germany) in 10 ml of resin stock in a 20 ml glass vial. This was stirred on a vortex mixer (5 min) and then sonicated for 45 seconds in an ice-water bath which acted as a heat sink. To separate the fine particles required from large particle aggregates, the mixture was centrifuged for five minutes at 1,000 rpm in a Clements GS400 centrifuge, the supernatant containing the fine particles collected, and the deposit resuspended in 10 ml of fresh resin stock. This was repeated three times to yield about 38 ml of resin containing fine particles. The suspension was split into two 20 ml vials and centrifuged at 1500 rpm for 30 minutes, the supernatant discarded, and the fine particles which had been deposited, resuspended and combined in 5 ml of fresh resin stock. After further sonication (30 seconds) in an ice-water bath, particle size was checked by bright field microscopy of a glass slide smear of a drop of suspension. Particle density was estimated on a Spencer "Neubauer" haemocytometer slide (American Optical Co., U.S.A.), and standardised to $1 \times 10^9$ particles per ml by dilution with fresh resin stock. This suspension was stored at 4°C, further vortex mixed and sonicated (15 seconds) immediately prior to use.
Hearts were taken from fixative at 4°C and immersed in 200 ml of phosphate buffer at 20°C. The pulmonary veins were ligated or oversewn at the junction with the left atrial wall with 6-0 cardiovascular silk. This was done to close any openings, thus eliminating any potential low resistance outlets if retrograde flow through the aortic or mitral valve occurred. The resin perfusion apparatus (Fig. 3A) consisted of a two litre bell jar as pressure 'tank', and anaeroid sphygmomanometer as an air pressure supply, two 20 ml glass vials as perfusate reservoirs and two Luer fit 3-way taps mounted on a perspex jig as terminal cannula mounts. Silicone rubber tubing connected each tap to a 5 cm long 14 swg stainless steel tube which passed through the rubber bung in the top of the glass vial to end about 1 mm from the base of the vial. A second stainless steel tube 2.5 cm long, and ending just below the lower surface of the bung was connected by silicone tubing to a Y-junction, and both "channels" then connected to the bell jar. This allowed perfusion of the liquid at the pressure within the bell jar through cannulae attached to the taps in the glass vial reservoirs. Flow was controlled by the three way taps, the 'open' arms of which were occluded by plastic plugs to prevent air leaks.

The two glass vials were filled with 15 ml of 0.1 M phosphate buffer, the system pressurised to 90 torr and the tubing dead space filled with buffer by manipulating the taps. The aortic and VIB cannulae, with the heart attached were then plugged into the 3-way taps, taking care not to
admit air. After the heart was suspended in air from its cannulae over the effluent collection vessel, both cannulae were perfused with phosphate buffer at identical pressure (90 torr). When 15 ml had flowed through the aortic cannula, the taps were turned off and small haemostats clamped across the silicone tubing between the taps and the vials to exclude back flow.

The bell jar was returned to room pressure, the bungs removed from the vials containing buffer and the vials immediately replaced by other vials containing prepared resin stock. That labelled with Fat Red 7B dye was fitted to the aortic cannula channel and the vial containing lead dioxide labelled resin stock was fitted to the channel supplying the VIB cannula. L.R. White cold accelerator was added in the recommended proportion (one drop to 10 ml) to the red labelled resin (75 microlitres to 15 ml), and 25 microlitres of 66% solution of L.R. White cold accelerator in absolute ethanol, added to the lead dioxide labelled resin as determined in the preliminary study (Appendix 4). Each vial was quickly mixed with a separate Pasteur pipette and the bungs inserted and the system pressurised to 90 torr.

The terminal taps were opened simultaneously and resin plus accelerator allowed to flow into the coronary beds under the applied pressure. When 15 ml had flowed through the aortic cannula, flow to both cannulae was stopped, the heart removed and the cannulae plugged. The heart was then placed in phosphate buffer at 20°C for one hour to aid
polymerisation, and then overnight in buffer at 4°C to ensure complete hardening.

The parts of the perfusion rig in contact with polymerising resin were rapidly removed and washed in ethanol, then de-ionised water and air-dried for reuse.

Dissection and Examination

The heart was divided transversely into 2-5 slices (Fig. 3B) approximately 4 mm thick in planes parallel to the atrioventricular junction, starting 4 mm towards the apex from that landmark. Tissue blocks about 4 x 4 mm were then cut with solvent-cleaned single sided razor blades to include:

1. Control tissue from the dorsal wall of the left ventricle.
2. The central ischaemic area - tissue supplied by the VIB, but macroscopically well clear of its boundaries.
3. The lateral boundary area - a transmural block from the antero-lateral left venricular wall spanning the macroscopically defined interface between aortic perfused (red) and VIB perfused (brown) tissue.
4. The interventricular septum - a transmural block spanning the interface between the coloured perfusion areas on the interventricular septum, where this occurred.

All blocks were thus bounded by endocardial and
epicardial surfaces on opposite faces and cut 'vertical' and 'horizontal' surfaces on the other four faces.

**Preparation for Microscopy**

The blocks were rinsed in fresh phosphate buffer and transferred to vials containing 2.82 M dimethyl sulfoxide (Sigma Chemical Corp. St. Louis, USA) in 0.1 M phosphate buffer. These were gently agitated for 12 hours. The tissue blocks were then removed, rinsed briefly in deionised water and excess fluid blotted off on Whatman filter paper. Each block was then submerged in thawing dichlorodifluoromethane (Freon 12 - New Zealand Industrial Gases Ltd. Melting point - 158°C), which had been cooled in liquid nitrogen. Frozen blocks were then placed in liquid nitrogen and fractured by a solvent-cleaned pre-cooled single sided razor blade. The blade was held in pre-cooled locking pliers. A small brass block was used to induce the fracture and a 50 cm³ brass block, cooled in liquid nitrogen used as an anvil. Tissue blocks were orientated prior to fracture so that the fracture plane was 'horizontal' i.e. orthogonal to the long axis of the ventricle and parallel to the plane of the slices taken originally. Resultant fracture fragments were thus intended to be approximately 2 x 4 x 4 mm with the fracture face having a epicardial, endocardial and two (cut) lateral edges.

Fracture fragments were loaded, fracture face upwards, into 5 mm diameter holes drilled 5 mm into a circular
brass block 5 cm in diameter and 4 cm thick. This block was then transferred to the cold stage of an Edwards Pearse EPD2 tissue drier (Edwards High Vacuum Co. U.K.) with a container of liquid nitrogen placed directly above it as a cold trap. The bell jar was then closed and the pressure reduced to 0.02 torr and maintained for a minimum of 48 hours. Temperature was controlled to a maximum of -65°C. Five grammes of phosphorus pentoxide were included in the vacuum chamber as a drying agent.

The dried tissue fragments were then rewarmed to room temperature and mounted, fracture face up, on ½" diameter aluminium stubs using double-sided tape. The stubs were then coated at 10^{-4} torr with carbon then gold in an evaporative coater with rotary stage (Edwards High Vacuum Co., U.K.).

Coated specimens were stored at 10^{-2} torr in a dessicant chamber until examination on the lower stage of an ISI DS-130 scanning electron microscope (ISI Inc. Santa Clara, U.S.A.) fitted with a Robinson RB 130SE scintillator type wide-angle back-scattered electron detector and an ISI Charge-Free Anticontamination System (both ETP Oxford, Sydney, Australia, Fig. 4) or an ISI-60A scanning electron microscope with similar accessory systems. Examinations were made at accelerating voltages of 30 kV, and typically at zero specimen tilt. Photographs were recorded on Pan F 35 mm film developed in 'Perceptol' (both Ilford Ltd, U.K.).

To determine the number of capillaries present, and
their status in regard to filling with resin, standard photographs at 900 x linear magnification of non-overlapping fields were taken using electronically reversed contrast images, which were developed normally and the resultant positive 'transparency' projected from a Vivitar VI photographic enlarger to give a final image size of 30 x 20 cm.

Capillaries were defined as thin walled vessels of less than or equal to 8 microns maximum cross sectional diameter (Rhodin, 1967) and were scored as resin-filled or empty by the presence or absence of solid contents with a glassy fracture face. Empty capillaries were scored as collapsed if their longest cross-sectional dimension was greater than three times the shortest cross-sectional dimension. Filled capillaries were scored as "lead dioxide labelled" if highly back-scattering irregular particles were observed in the resin within them. Capillaries lying across the lower and left margins of the counted fields were excluded whereas those which spanned the upper and right margins were included.

The status of each capillary was marked at its site on the projected image as seen on blank paper placed on the enlarger baseboard. Subsequently, scores for each field were counted by room light. Means and standard deviations of scores were calculated after counting five fields from each of the four following areas, for each heart:

Continuously perfused myocardium well distant from the
boundary of the separately perfused VIB region (block C, Fig. 3B) termed "distant normal". Separately perfused (VIB region) myocardium, at least 2 mm from the boundary (block B, Fig. 3B) termed "distant ischaemic". Blocks spanning the boundary between continuously perfused (red coloured) and separately perfused (VIB region, brown-coloured) myocardium (blocks A, Fig. 3B) were examined to define the lateral boundaries by scanning electron microscopy. Fields immediately adjacent (within 150 micron) of the boundaries were selected to give fields representative of "adjacent normal" and "adjacent ischaemic" areas. Mean values from samples from the four areas of three hearts in each of the 0, 30, 60, 120 and 240 min groups were compared by one-way analysis of variance (Huntsberger and Leaverton, 1970).
RESULTS

Anaesthesia

Insertion of a winged infusion needle to the marginal ear vein was successful on first attempt in 21 of the 24 rabbits. In the other three the contralateral ear was used. Muscular relaxation was noted within 2-5 seconds of infusion of Saffan, and the animals could then be removed from the transit box to the operating table. Loss of deep reflexes as noted after 30-45 seconds, and loss of reaction to painful stimuli by two minutes. Two minutes after injection breathing was spontaneous with a slow regular rate (10-15 breaths per minute), and a palpable precordial heart rate of 180 - 200 beats per minute was evident. There was no external evidence of cyanosis.

Removal of the Heart

The heart and aortic root could be removed within 30 seconds of commencing the incision. It was found practical to open the skin and pectoral musculature with a single scalpel incision, to insert one blade of a large pair of scissors through the diaphragm at the xiphisternum and advance the blade immediately behind the sternum to the sternal notch. This single cut then laid open the entire chest. With small scissors the pericardium could be opened and the heart, great vessels and some adjacent structures removed from the chest with a second single cut. Prior to
this final incision, bleeding was minimal and no difficulty was experienced in the identification of anatomical structures. On removal, the hearts were beating vigorously at a similar rate to that palpated prior to opening the chest.

Cessation of regular heart beats was complete with 5-10 seconds of placing the heart in the ice-cold perfusate. Occasional single beats, less than three per minute, were noted until perfusion was commenced. No clotting of blood was noted in the perfusate beaker, where initial beats rapidly ejected the luminal blood, or in the chest of the cadaver subsequently.

Parietal pericardium, trachea, major bronchi, oesophagus and fragments of hilar lung were frequently removed with the heart and aorta, but these were easily trimmed away immediately the beating of the heart had stopped. Heart wet weights ranged from 4.6-10.0g (mean ± SD 6.65g ± 1.36).

Aortic Cannulation

The diameter of the terminal bulge of the trimmed aortic cannula was found to be slightly greater than that of the relaxed ascending aorta. Thus, when fitted over the cannula using two pairs of forceps, the elasticity of the aorta held it in place while being secured with suture material. No leakage or displacement of the heart from the cannula was observed. The average time from incision of the chest wall
of the anaesthetised animal to completed insertion of this cannula was two minutes (range 1-2.5).

Gentle hand pressure on the attached 25 ml syringe perfused the entire ventricular and atrial mass producing an abrupt colour change from the fresh reddish appearance to blue-grey of the methylene blue labelled perfusate within 1-2 seconds. Effluent from the pulmonary artery consisted initially of heavily bloodstained fluid but, after 10 mls had been perfused, no residual blood was apparent. Flushing with this ice-cold perfusate produced no contraction of the ventricles.

**VIB Cannulation**

Insertion of the fine cannula to the VIB was the most time-consuming and difficult part of the preparative procedure. Elapsed time from the incision of the chest wall to completion of this cannulation ranged from 19-35 minutes (mean $\pm$ SD 25.2 $\pm$ 4.6). Insertion of the stay sutures and dissection between the aortic and pulmonary arterial roots was uncomplicated. The amount and extent of epicardial fat about the origin of the left coronary artery was highly variable and presented the greatest difficulty. Even with methylene blue labelled perfusate, identification of the left main coronary artery and its major branches beneath the visceral pericardium and within the epicardial fat was time-consuming.

Isolation of a suitable length of artery, placement of
under-running sutures and a longitudinal incision in the arterial wall to allow cannula entry required both time and considerable caution. However, once the artery was cleared of fat and stabilised by underrun sutures, the placement of the cannula presented few difficulties and was achieved in less than five minutes. In all cases the outside diameter of the cannula (0.80 mm) was slightly larger than the recipient artery, so that once in place it was held firmly. Firm tying with the distal underrun suture provided sufficient anchorage to withstand the manipulation and tension on the catheter during fitting to the perfusion apparatus, and subsequently to the resin perfusion rig.

The VIB was successfully cannulated in 20 hearts. In three others which were discarded from the quantitative assessment the left circumflex branch was cannulated by the same method, after damage to the VIB during dissection and attempted cannulation. In these three cases the proximal VIB was ligated. In the remaining animal, the VIB was ligated after dissection injury and no attempt was made to cannulate the left circumflex branch, because of its small size. This heart was discarded subsequently.

Perfusion

Perfusion was commenced within 10 seconds of completion of cannulation in all cases. The mean coronary effluent flow rates for each group of hearts during the equilibration period is shown in Table 1. There was a
clear, but not statistically significant, increase in flow rate (Fig. 5) from the second to tenth minute, but the flow stabilised at the 15 minute value. During the subsequent experimental period, the mean coronary effluent flow rates were closely similar among groups (Table 2) and no statistically significant differences were found. A clear decrease in mean flow rate followed the occlusion of the VIB cannula in all hearts at the start of the experimental perfusion period (Fig. 5). The mean flow rate then remained stable at about 4.0 ml/min/g wet weight until about 150 minutes of perfusion, after which there was a gradual decline to an average of 3.4 ml/min/g wet weight by 240 min. After 120 min of perfusion the observed variation was wide and the number of animals small so no statistically significant differences between these values and the previously stable values were found using analysis of variance at a 95% confidence level.

There was an association between the time elapsed from removal of the heart to completion of cannulation, and the establishment of stable heart rate and coronary effluent flow during the equilibration period. Hearts with shorter lapsed times (19-25 min) established sinus rhythm and a high flow rate (> 4 ml/min/g wet weight) within 1-2 minutes, while hearts with longer cannulation times required 5-10 minutes to do so. Typical stable heart rates obtained were 160-180 beats per minute. No ventricular arrhythmias were observed following closure of the VIB cannula supply at the commencement of the experimental period.
The use of a 2.7 litre perfusate pool meant recirculation was not required for perfusion times of one hour or less, as total coronary effluent flow did not exceed the pool size. The inline membrane filter was changed after each 45 minutes of perfusion, to achieve the required flow to maintain a constant level in the upper perfusate reservoir.

In the groups of hearts in which the VIB cannula was closed, a variable region of the anterior and/or lateral left ventricular epicardium was noted to undergo a slight colour change from the pale grey-green of the rest of the heart (containing residual perfused methylene blue) to a pale pink-grey. This lasted 5-10 minutes after which the VIB area could not be discriminated clearly. However, as perfusion continued, a vague epicardial boundary to the VIB area was established by apparent prominence of surrounding tissue compared to the VIB region. This margin was indistinct and followed a complex line. No differences in contractility between the VIB area and the remainder of the heart could be discerned. Filling of the left ventricular lumen and systolic ejection were not noted, and there was consequently no differences in movement of normal and VIB regions during systole or diastole.

Fixation

The delay, resulting from cannula dead space, between cessation of oxygenated buffer perfusion, and the entry
into the hearts of glutaraldehyde fixative was less than two seconds. On contact with fixative the ventricles ceased beating within 1-2 seconds, and atrial contraction ceased within 5 seconds. All hearts showed apparent cessation of contraction in a mid-systolic state, showing neither profound contraction or relaxation. The flow rate of glutaraldehyde was similar to that of oxygenated buffer in the preceding period. Fixation resulted in a change of the ventricular myocardium to a paler grey-brown colour, and later to a deep grey-brown after 2-3 hours. Also, there was a palpable increase in stiffness of the myocardium. Figure 6 shows a recently fixed heart with cannulae attached.

Resin Perfusion

When preparing hearts for resin perfusion it was found that the pulmonary veins and the vena cavae had been excised with variable amounts of atrial wall. However, the atria were easily closed with continuous sutures to produce an inverted edge or pursestring closure.

About 30 seconds were required for 15 ml of 0.1 M phosphate buffer to be perfused through the aortic cannula and the glutaraldehyde-fixed hearts at 90 torr pressure. In that period, 4-5 ml were perfused through the VIB cannula in hearts subject to 0, 30 and 60 minutes occlusion but much smaller amounts (less than 1 ml) could be perfused in the 120 and 240 minute groups.

Once resin had filled the aortic cannula the red
coloured resin could be seen flowing through the epicardial arteries and within 2-3 seconds most of the epicardial surface was coloured by resin within microscopic vessels (Fig. 7). Resin was noted in the epicardial veins first as globules interspersed among aqueous buffer and then as a continuous stream which eventually discharged from the pulmonary artery root.

Brown-coloured lead dioxide labelled resin from the VIB cannula appeared in the anterior left ventricular epicardium, and a mixing of red and brown resins occurred in epicardial veins. In some cases (Fig. 8) epicardial veins carried resin of one colour across well defined regions of the other colour.

After 20-30 seconds of perfusion at 90 torr, the left atrium occasionally became filled with resin, indicating incompetence of the glutaraldehyde fixed aortic and mitral valves, but leakage of resin from the oversewn pulmonary veins was never more than 2-3 drops.

Perfusion of 15 ml of red coloured resin to the aortic cannula required 60 - 90 seconds in all hearts. In all hearts in which regions were ischaemic for up to 60 minutes, the flow of resin in the VIB cannula was 2-5 ml, but following 120 and 240 minutes of ischaemia this flow was less than 1 ml.

Polymerisation of the red coloured resin remaining in the glass vial reservoir required 5-10 minutes to reach
'gel' point, with the endothermic reaction becoming palpable 2-3 minutes later. The brown resin usually reached gel point half to one minute earlier than the red resin, but always well after the duration of the perfusion period. Providing cleaning was not delayed, residual polymerising resin could be removed by washing in ethanol.

External examination of the hearts after resin polymerisation showed a variable area of the anterior or the anterolateral left ventricular wall coloured yellow-brown in contrast to the red of the remainder of the heart (Figs. 7 and 8). The epicardial boundary between yellow-brown and red was sharp, but showed fine interdigitations along the coarsely linear margin (Fig. 7). The region coloured yellow-brown varied from 4-12mm in width, and extended 5-15mm from the atrioventricular groove.

In addition to yellow-brown and red regions, small discrete areas of grey-brown coloured myocardium were seen in three hearts. These always lay between the yellow-brown and red regions as an isolated area, and not as a continuous zone between the two coloured regions (Fig. 8). They were always considerably less than the yellow-brown area. The demarcation on the epicardial surface between this area and those of the two other colours was abrupt but interdigitated at the macroscopic level of examination.
Dissection

The cannulae and the vessel lumen formed a solid resin rod following polymerisation, and it was found convenient to excise each cannula before proceeding further.

The major variable in dissection was the extent of filling of the left ventricular lumen by polymerised resin. In many cases no resin was present and slices could be cut directly with a single-sided razor blade. Similarly where a nubbin of polymerised resin extended into the left ventricular lumen, but did not "cast" the internal walls, dissection was uncomplicated. In four hearts, polymerised resin had formed a solid cast of the left ventricle, including the interstices of the trabeculae carneae. In these cases slices were dissected from the cast in order from the apex of the heart to the atrioventricular groove, with the papillary muscles being trimmed transversely with fine scissors at their entrance to the luminal cast. When excised in this fashion, there was little disruption of endocardial surfaces on removal from the cast.

All hearts contained a solid resin cast closely associated with the trabeculae carneae and the papillary muscles in the right ventricular lumen. This cast was thin enough to be cut with scissors once the surrounding myocardium had been incised by razor blade. The resin was then dissected out of the right ventricular lumen with fine scissors.
Representative ventricular slices are shown in Figures 9 and 10. Two typical patterns were observed. The first (Fig. 9) showed a discretely demarcated yellow-brown zone occupying a transmural sector of the left ventricular wall, involving the anterior wall, interventricular septal junction, and variable amounts of adjacent anterior free wall, right ventricular wall and interventricular septum. This pattern was characteristic of hearts in which cannulation of the ventral interventricular branch was successful. The homogeneous colouration at the cut surface of the VIB region shown in Fig. 9 was typical of control and 30 min group hearts.

However, where the left main or circumflex branches had been cannulated, a larger sector of free wall was involved (Fig. 10), but sharply demarcated transmural boundaries were still evident. The less dense, and sometimes patchy, colouration seen in Figure 10 was typical of hearts from the 60-240 minute groups.

Transmural blocks of red coloured myocardium with a 2-3 mm endocardial width were obtained without difficulty from each heart. Separate blocks consisting of yellow-brown myocardium only could not be obtained from seven hearts where the circumferential extent of the yellow-brown zone at the endocardium was less than 5 mm. In these cases the yellow-brown zone was divided equally transmurally and included in the adjacent blocks which spanned the boundary between red and yellow-brown tissue.
Because the boundary between red and yellow-brown tissue showed changed in orientation through the 4 mm depth of the tissue slices, blocks cut to span the boundary were in all cases larger than those composed of one coloured tissue only. Sizes ranged from 5-11 mm in circumferential width, and large blocks consequently had significantly curved epicardial surfaces.

Large epicardial and intramyocardial vessels within the yellow-brown regions were occasionally noted macroscopically to be filled by red resin in 0, 30 and 60 minute group hearts (Fig. 11). These were the large red-filled veins seen on the epicardial surface (Fig. 7). However yellow-brown coloured resin could not be discriminated macroscopically in any vessels within the red-coloured regions.

**Freeze-fracture**

Tissue blocks where the circumferential width and transmural width were small relative to the tissue slice depth fractured easily along a single relatively flat plane from endocardium to epicardium. Seating the cooled razor blade edge into excess frozen Freon on the endocardial surface with a few very gentle taps followed by a single firm blow resulted in consistent fracture results in blocks of this shape.

Larger blocks with long curved epicardial surfaces also provided single flat fracture faces in about 50% of cases.
However in other cases the fracture face coursed diagonally towards one of the faces of the block, in effect chipping an edge off the block. This resulted in tangential fractures of the endocardial region, and in about 25% cases the fracture plane did not include the midmyocardium or epicardium. Inversion of the block and fracture from the epicardial surface remedied this in two of these samples, but stability of blocks with chipped edges was low and persistent attempts at fracturing resulted in irregular shattering of the remaining blocks. Cementing of frozen blocks of any size to the precooled brass anvil with an adhesive (O.C.T., American Optical Co. U.S.A.) resulted in irregular shattering of the block. No spontaneous fracturing was noted on freezing tissue blocks in either Freon or liquid nitrogen. Satisfactory transmural blocks of all regions could be obtained for three hearts from each group.

Mounting and Coating

Double sided tape provided a clean, practical mounting method which held samples securely without risk of contamination and provided a homogeneous low atomic number background for scanning electron microscopy in the backscattered electron imaging mode.
SCANNING ELECTRON MICROSCOPICAL OBSERVATIONS

This section first describes observations of the general appearances of continuously perfused myocardium fixed after a 15 minute stabilisation period of perfusion to both cannulae and without any experimental ischaemic period. This is followed by a description (p. 118) of the appearances of control myocardium well removed from the borders of the regionally ischaemic zone during the four experimental time intervals (30, 60, 120, 240 minutes). The changes seen in the separately perfused region are then described (p. 115), with comparison of appearances from no ischaemia to 240 minutes of regional ischaemia. Finally the changes of both control and ischaemic myocardium at the border zones are compared (p. 112).

CONTINUALLY PERFUSED MYOCARDIUM (CONTROL)

No Regional Ischaemia

Fracture Planes

Cryofractured blocks of rabbit myocardium typically showed trapezoidal fracture faces (Fig. 12) of about 5 x 4 mm. The epicardial surface was identified as a smooth convex edge, associated with large vessels easily identified at low magnification. The left ventricular luminal (endocardial) surface which lay opposite, was characterised by trabecular convolutions, and was typically narrower (2-4 mm). The fracture face was grossly either
relatively flat or had one or two angulated planes. Small steps and ridges associated with myocardial fibres or bundles of fibres were obvious at low magnification.

Surfaces cut, but not fractured, appeared macroscopically flat and smooth. At higher magnifications, the surfaces of cardiac myocytes in these areas were composed of irregular chunks and fragments (Fig. 13) which in some areas obscured the intercellular spaces and capillary lumina, limiting interpretation.

Orientation of Myocytes

Even at the lowest magnification a marked transmural variation in the orientation of myocardial cells was discernible (Fig. 12). Three zones could be distinguished: a narrow sub-epicardial zone, a broad mid-myocardial zone and a broad sub-endocardial zone.

The epicardial zone was typically 500-1000 microns wide and was occupied by two consistent and one variable component. The epicardial surface was identified as a rough surface lamina, whose fractured edges showed a fibrous structure (Fig. 14A). In some areas a layer of fat cells up to 150 microns deep was situated immediately below the epicardial surface. This was seen most often in blocks from close to the atrioventricular junction. Fat cells were identified as closely packed, thin walled, apparently empty cells 20-30 microns in diameter. The sub-epicardial myocardium contained cardiac myocytes, capillaries and large
vessels. Cardiac myocytes (Fig. 14B) were seen in transverse section or low oblique fracture as wide (up to 40 microns) plates with irregular lateral foldings which partially surrounded the accompanying capillaries. In oblique views a sarcolemmal periodicity of about 1.5 microns was seen. At moderate magnifications transverse sections of cardiac myocytes appeared as flat plates in which subcellular organelles were not discretely visible. In the intercellular spaces, capillaries were seen either as open, thin walled tubes, or as collapsed thin walled cylinders, or as solid cylinders terminating in a glassy fracture face. In profile these vessels varied from round to irregular.

Separated from the epicardium by a narrow zone of obliquely fractured myocytes, the mid-myocardial zone made up about one half to one-third of the transmural width of the fracture and consisted of cardiac muscle cells fractured longitudinally (Fig. 15). Such cells were 10-30 microns wide and extended for several hundred microns in some fracture planes. Lateral connections were common with frequent bifurcation and union of adjacent cells. Wavy and branched strands of fibrous connective tissue ran between, and often parallel to, the cardiac myocytes. At higher power, the sarcomere periodicity of the sarcolemmal surface of cardiac myocytes was clearly evident and accompanying thin-walled, irregular, tubular capillaries could be identified. Typically the fracture plane ran between cardiac myocytes, exposing their sarcolemmal surface only and not their interior (Figs. 16A,B). However, where the
fracture did pass through cardiac myocytes longitudinally, a sub-cellular pattern of longitudinal broad strands and short narrow ellipsoids was defined by the low steps and angles of the fracture plane (Fig. 16B). A sarcomere periodicity was also seen on the interior surface, emphasised by small irregular holes appearing in register with z bands.

The subendocardial half to third of the fracture face (Fig. 17) was separated from the mid-myocardium by a narrow zone of obliquely fractured cells. Within the subendocardium three subdivisions could be seen (Fig. 17): A compact zone of transversely fractured fibres, a zone of relatively dispersed cells close the endocardial surface, and an intervening trabecular zone where cells were collected into bundles 50-100 microns wide and 200-400 microns long (Fig. 18) with wider intercellular spaces (30-50 microns) between the collections of myocytes.

In the subendocardial zone, where cardiac myocytes were seen exclusively in transverse sections, these cells generally had smaller profiles (10-15 microns in largest dimension) than those in the sub-epicardium, although a few large "plate-like" profiles were evident. In the trabecular areas myocyte profiles tended to be smooth irregular shapes interlocked with their neighbours to give a consistently narrow intercellular space which contained small blood vessels and varying amount of connective tissue. The endocardial surface (Fig. 19A) consisted of a thick lamina of fibrous connective tissue closely applied to the
underlying cardiac myocytes. Vessels communicating with the ventricular lumen directly through this lamina were not seen.

Blood Vessels

All types of vessels could be identified in fracture faces. Large muscular arteries (0.3-1.00 mm diameter) were confined to the epicardial zone. These were characterised by thick (10-20 micron) walls formed by orientated lamina with the media and adventitia clearly discernible (Figs. 20A,B). Elastic laminae and the intima were not clearly identifiable. The acrylic resin filling large vessels showed characteristic glass-like step fracture faces. Dichotomous branches were sometimes observed in the tissue fracture plane.

Smaller muscular arteries and arterioles were seen in all zones of the myocardium. They were characterised by a 1-3 micron thick wall, in which narrow separate layers were discernible. These were typically resin filled and usually ran as transmural penetrating arteries.

Capillaries and small venules (3-10 micron diameter) were the most numerous type of vessel and were readily identified, even at low power, as open tubes or resin-filled cylinders (Figs. 19B, 21A & B). In control subendocardium, where blood vessels and myocardial cells are both seen almost always in transverse sections and are thus directly comparable (Fig. 22), capillaries lie in the intercellular
space between cardiac myocytes. In some areas they are closely invested by one, two or three adjacent myocytes but in other areas there is a narrow (1-3 micron) interstitial space between the capillary wall and the adjacent cardiac myocytes. These variations are demonstrated in Fig. 23. These vessels consisted of thin (100-300 mm) single walled tubes, with occasional focal arcuate thickenings (endothelial cell or pericyte nuclei Fig. 23B). Vessels varied in shape from round to elliptical or indented and there were some relatively flat empty profiles. Junctions of capillaries in H or Y conformations were occasionally seen.

Single layer, thin walled vessels larger than 10 microns in diameter were termed venules or veins. In most of these the wall and luminal acrylic resin were closely applied but in some there was little resin within the lumen despite abundant filling of capillaries in the adjacent tissue. The surface of acrylic resin within vessels frequently showed a fine mosaic pattern (Fig. 27B) which could be observed appearing during examination at high magnifications. In resin filled capillaries seen in longitudinal view (Fig. 16B) this artefact appeared as irregular dark longitudinal lines. It was interpreted as electron beam damage to the gold-coating.

Myocyte Appearances

In all regions myocytes presented uniform, relatively
flat and featureless fracture faces through the cellular interior. At high magnification (Fig. 24) these planar faces showed many low steps and angles, narrow channels (about 15nm diameter) some of which communicated with the sarcolemmal surface, and small (100nm diameter) pits which appeared to penetrate the cardiac muscle surface.

Rarely, isolated areas of 3-10 cardiac muscle cell profiles showed a different appearance (Fig. 25A,B) characterised by swollen cells having an "open" appearance within which round bodies (0.5-1.0 micron diameter) and irregular columns were observed. These areas were always associated with capillaries which contained red blood cells which were usually completely absent from the myocardium. The greatest number of such areas observed in a continuously perfused region was three, and the largest of these is demonstrated in Figure 25.

Other Tissue Components

Connective tissue was frequently identified. Its density and distribution varied within and between hearts, and was generally more abundant in hearts from the heavier (i.e older) rabbits. Large bundles of collagen tended to be orientated parallel to the cardiac muscle fibres (Figs. 15, 26), and less commonly at right angles to the muscle fibres (Fig. 26A). Fine fibrillary perivascular networks, and filamentous networks between myocytes were commonly observed (Fig. 19B). Nerves, cardiac conducting cells and lymph
channels were not separately identified.

Thirty to 240 Minutes of Regional Ischaemia

Morphological appearances

A typical field of transversely fractured control myocardium located distant from the macroscopic boundary after an experimental period of 30 minutes is shown in Figure 30A. No detectable differences were noted between these appearances and those of control myocardium described fully above.

Similarly, control region myocardium distant from the ischaemic region showed no detectable differences at 60 minutes (Fig. 37A), 120 minutes experimental ischaemia (Fig. 43A) and 240 minutes (Fig. 49A). Changes in myocyte fracture face morphology, the appearance and filling status of myocardial capillaries and the extent of the interstitial space were specifically compared, and changes in these parameters could not be defined.

Capillary functional status

A quantitative assessment of capillary functional status in the control region distant from the boundary of the separately perfused (ischaemic zone) was made and the results are summarised graphically in two ways. First, a comparison between values at each of the five experimental intervals (0, 30, 40, 120, 240 minutes of ischaemia) was
made using each of four parameters: The mean total number of capillaries observed per field (Fig. 56A), the mean proportion of those capillaries filled by resin (Fig. 57A), the mean proportion of capillaries unfilled by resin and appearing collapsed (Fig. 58A), and the mean proportion of capillaries unfilled by resin and appearing to have an open profile (Fig. 59A).

The values for the control region are also compared graphically to those of the other three regions examined in each heart at separate time intervals, thereby providing a baseline for comparison of changes in the ischaemic and border zones at the separate time intervals. These data for control hearts (Figs. 28, 29) 30 minute group (Figs. 33, 34), 60 minute group (Figs. 41, 42), 120 minute group (Figs. 47, 48) and 240 minute regional ischaemia group (figs. 54, 55) are discussed in detail with the changes in ischaemic and borderzone myocardium below.

The changes in quantitative values in the distant control region with increasing time of perfusion were few. The mean number of observed capillaries per field (Fig. 56A) was modestly reduced at 120 minutes from the 0 minute value of about 60 capillaries/field (significance p<0.05), but no other differences carried statistical significance. Small and statistically weakly significant (p<0.05) changes in the proportion of capillaries filled by resin (Fig. 57A) were observed with the increase from 55-60% at 30 minutes to about 70% at 60 minutes, and a subsequent fall to 55-60% at
120 and 240 minutes. The proportion of capillaries unfilled by resin and appearing to be collapsed (Fig. 58A) showed an apparent increase from about 20% at 60 minutes to about 35% at 120 minutes (p<0.01), though no statistically significant difference between any of the other time intervals can be demonstrated. There were no statistically significant differences between any of the values for the proportion of capillaries which were unfilled and appeared to have an open profile (Fig. 59A).

SEPARATELY PERFUSED MYOCARDIUM (ISCHAEMIC REGION)

0-240 Minutes Regional Ischaemia

Morphological Appearances

The appearances of myocardium in the separately perfused VIB region, which in the 0 minute group was not subject to experimental ischaemia, was closely similar to those seen in the control region. However the vessels filled with resin in this region contained intensely back-scattering irregular particles 0.1-1.0 micron in diameter (lead dioxide) seen in Figure 27. A minority of cardiac myocytes (<20%) showed increased prominence of pits and channels, and sparsely distributed (< 5%) cells showed occasional larger pits (0.25-0.5 microns) in their fractured surface (Fig. 27B).

Labelled capillaries containing lead dioxide particles were seen only in myocardium which macroscopically appeared brown. Because of the close similarity in appearance of
myocardial cells in both control and separately perfused regions, it was not possible to microscopically define the cell to cell boundary of the lateral margin of the separately perfused region. The appearance of labelled capillaries provided the only easily identified index of the boundary between these two regions.

After thirty minutes of regional ischaemia, the myocardium of the separately perfused area showed differences of degree rather than major changes compared to the control region. Transverse fracture faces appeared compact though, compared to control areas, pits in the fracture face were more numerous and larger in diameter, (Fig. 31) and affected a greater proportion of cells (50-70%). A very high proportion of capillaries were filled with resin, some (20-30%) carrying the lead dioxide label (Fig. 31B). Those few capillaries unfilled mostly appeared to have an open profile. There was no apparent change in the extent of the intercellular space, or the space between bundles of cardiac muscle cells (Fig. 31A), nor to the appearance of longitudinally fractured cells (Fig. 32).

Unlike the minor changes in morphology seen after 30 minutes of ischaemia, the 60 minute group showed marked differences in appearance of cardiac muscle cells between control and ischaemic regions, and corresponding pronounced changes in the capacity of the capillaries to conduct the injected resin marker.

At low magnification (Fig. 35) there were no differences
between the control and ischaemic zone except for the more prominent filling of large vessels (arteries and veins) in the control region. However, higher magnification of the ischaemic regions, showed cardiac muscle cells markedly different to those in the control regions (Figs. 36, 38).

In transverse section, cardiac muscle cells appeared swollen with closely apposed sarcolemmal surfaces and a reduced intercellular space, though there were some local variations in this finding. The intracellular appearances were strikingly different from the control region. Numerous and extensive pits and channels were obvious and these isolated solid subcellular structures (Figs. 38B, 39), which in tangential and longitudinal section could be identified as a elliptical mitochondria (Fig. 38A) and columnar myofibrils. The latter showed a sarcomere periodicity, and overlying raised linear structures corresponding to Z-lines which are probably t-tubular systems. Sarcomeres appeared relatively contracted with an average length of approximately 1.5 microns. This separation of organelles was not seen in control regions.

Associated with myocytes of this appearance was a very low proportion of resin filled capillaries (Fig. 38B), the great majority of those unfilled having a collapsed profile. Only occasional, PbO\textsubscript{2} labelled capillaries were seen (Fig. 39) in these regions.

These appearances were not uniform for the entire ischaemic region however. They were reliably and uniformly
noted in the sub-endocardial third and in most of the mid-myocardial third of the myocardium. The sub-epicardial third in most parts showed more compact cells resembling those of control region, but containing more numerous variably sized pits than control region muscle cells, but considerably less than characterised the ischaemic 'open' pattern of the sub-endocardial portions of the same sample, and there was little separation and exposure of organelles. In the sub-epicardial third there was a high proportion (>90%) of capillaries filled by acrylic resin, and some of them contained the lead dioxide marker (fig. 40B).

In the separately perfused region subject to 120 minutes of ischaemia a more uniform appearance was seen, with all cells showing a swollen appearance (Figs. 44A, 45A, 45B) strikingly different from control myocardium. Within the cells organelles were widely separated and thus easily identified. These appearances closely resembled those seen in the subendocardial and midmyocardial thirds of the ischaemic region after 60 minutes, but at 120 minutes extended from endocardial to epicardial surfaces (Fig. 46B). The sarcolemmal surface of many cardiac myocytes appeared to still be intact. Though many cells showed closely apposed sarcolemmal surfaces, there was no consistent evidence of an overall reduction in the intercellular space. Capillaries were typically unfilled and collapsed (Fig. 45) and irregular distorted profiles were common. In the subepicardial portion of the ischaemic zone, the extent of the collapse and compression of capillaries was typically
less than that seen deeper in the myocardium (Fig. 45). The profound reduction in resin filling of capillaries was a consistent and striking finding.

The appearances of longitudinally fractured cardiac myocytes from the mid-myocardial third showed closely similar appearances at 120 minutes to those seen at 60 minutes, though the distinct sarcomere periodicity of the sarcolemmal surfaces was less prominent (Fig. 46A) than in cells from the control region or from the 60 minute ischaemic region.

Few differences in appearance between ischaemic region appearances at 120 and 240 minutes could be detected. The swollen "open" cell pattern, profound reduction in resin filling of capillaries (to less than 10%), and relative increase in the proportion of collapsed or compressed capillaries was consistently present in the 240 minute group (Figs. 49B-53). The prominent Z-band and t-tubule structures seen in the exposed myofibres allowed estimation of the sarcomere periodicity at 1.5 micron which indicates moderate contraction rather than relaxation and stretching.

Capillary functional status

The results of quantitative assessment of capillary functional status in the ischaemic, separately perfused region are summarised as histograms. The mean number of capillaries observed per field (Fig. 56B), the proportion of them appearing filled with resin (Fig. 57B), and the
proportion of capillaries unfilled with resin and appearing to be either collapsed (Fig. 58B) or open (Fig. 59B) are presented to give direct comparison between each experimental interval for this region of the heart.

There was a slight, and statistically weakly significant (p<0.05) reduction in the number of capillaries observed between 0 and 240 minutes. There were profound changes to the proportion of capillaries filled by resin. The value at 0 minutes, where the hearts were separately perfused, but not subject to ischaemia is not significantly different from that for the control regions (Fig. 28B). By thirty minutes of ischaemia however, this proportion has risen markedly from 50-60% to 80-85% indicating hyperaemia. This is followed by a fall to less than 10% of capillaries filled by resin at 60 minutes, which is sustained at 120 and 240 minutes. These differences are statistically significant at a p level of 0.01.

There are complementary changes in the proportion of capillaries unfilled by resin and appearing collapsed (Fig. 58B). There is a significant (p<0.01) decrease in this population from 20-25% at 0 minutes (which is itself not significantly different from the proportion seen in the control region – Fig. 29A) to 5-10% at 30 minutes, and a massive increase to about 90% at 60 minutes which is sustained at similar levels thereafter.

The differences in proportion of unfilled capillaries appearing to have an open profile was less marked in the
ischaemic region. From a level of 20-25% (not significantly different from control regions - Fig. 29B) at 0 minutes there was a significant reduction (p<0.05) to about 10% at thirty minutes, which reduced further to minimal levels at 60-240 minutes.

Direct comparisons between control and ischaemic regions can be made by comparing histograms of number of capillaries per field (Figs. 56A,B) proportions of resin filled capillaries (Fig. 57A,B), proportions of unfilled collapsed capillaries (Figs. 58A,B), and proportions of unfilled but open capillaries (Figs. 59A,B). Alternatively, direct comparisons between values from the four regions quantitated in the hearts are given for each time interval, as detailed above (p. 144).

Evolution of changes with duration of ischaemia

In summary, the separately perfused region showed few differences from control in the absence of ischaemia. Minor differences in morphology, but a marked potential hyperaemic response were seen after thirty minutes. At sixty minutes of ischaemia, there were major changes in cardiac myocyte morphology, with swollen cells indicating ischaemic injury to the endocardial two-thirds of the ischaemic region and a profound reduction in capillary filling with a complementary rise in unfilled collapsed capillaries. There was apparent sparing of the subepicardial one-third at 60 minutes, which showed minor
morphological change and marked hyperaemia closely similar to that seen at 30 minutes in the entire ischaemic region. At 120 and 240 minutes of ischaemia the entire ischaemic region showed swollen cells, with exposed swollen and sometimes disrupted organelles, and a profound reduction in resin filling of the accompanying capillaries.

**BORDER ZONE MYOCARDIUM**

**Control (0 minute) group**

The appearances of cardiac myocytes at the margin of the separately perfused zone in the control group were not detectably different from those of control myocardium distant from the margin. Because of this finding, the margin was defined for quantitative purposes as the region in which lead dioxide labelled capillaries were first noted when traversing from the control region. This point always corresponded to the macroscopically identified transition from red to brown colouration induced by injected resin.

The mean numbers of capillaries observed and the proportion of these filled with resin at the four locations spanning the lateral boundary of the separately perfused region is shown in Figure 28. There was no statistically significant difference between either the number observed (about 55 per field) or the proportion of filled capillaries (about 60%) in myocardium immediately adjacent to either side of the red-brown boundary, nor was there any statistical difference between constantly perfused tissue
immediately adjacent and far removed from the boundary. Similarly, in the separately perfused region, there was no statistical difference between numbers of capillaries or the proportion of resin-filling in immediately adjacent or far distant samples in this region.

Figure 29 shows the proportion of unfilled capillaries which appeared to be collapsed (Fig. 29A) or open (Fig. 29B). For each region, these groups were almost equal (20-25% of the total). There was no statistically significant differences between samples at each of the four regions for these two parameters.

Thirty minute experimental group

Control myocardium immediately adjacent to the macroscopic boundary between control and ischaemic regions, showed quite similar appearances to that at distant sites. Compact, relatively featureless fracture faces with prominent, but small, pits and channels. A mixture of filled, unfilled open and unfilled collapsed capillaries were noted but, compared to distant fields, a greater proportion were filled with resin and more than half of the remainder appeared open rather than collapsed (Fig. 30B).

Ischaemic myocardium immediately adjacent to the lateral macroscopic boundary did not show any detectable differences from ischaemic myocardium distant from the boundary after 30 minutes of ischaemia, and thus only differences of degree (p. 116) rather than major changes could be detected between
control and ischaemic myocardium at the border of the ischaemic region. A relatively high proportion of resin filled capillaries were seen in myocardium adjacent to both sides of the boundary, those on the ischaemic side distinguished by the presence of the lead dioxide marker within the resin.

The mean numbers of capillaries per field and the proportion of them filled by resin in both border regions and comparable distant control and ischaemic areas are shown in Figure 33. There was no statistically significant differences (Fig. 33A) between samples at the four different loci for the number of capillaries per field (50-60). However, there was a significant difference (p<0.01) in the proportion filled with resin (Fig. 33B) between the distant control region (55%) and the adjacent control region (75%). The further increase, to about 85-90%, seen in both adjacent and distant ischaemic regions was not statistically significant. Obviously, the difference between distant control and both ischaemic regions was also highly significant.

There was a corresponding reduction in the percentage of unfilled capillaries in the adjacent control and ischaemic zones compared to distant control regions (Figs. 34A,B), though none of the changes were statistically significant when adjacent loci were compared. However the decrease in proportion of unfilled collapsed capillaries (Fig. 34B) from 20-30% in distant control to 5-10% in both adjacent and
distant ischaemic regions was significant ($p<0.01$).

**Sixty minute experimental group**

The profound difference in morphological appearance between distant control and distant ischaemic myocardium in the inner two-thirds of the separately perfused zone after 60 minutes ischaemic has been described above (pp. 116-8).

The transition between "compact" control region myocardium and "open" ischaemic region myocardium with their associated patterns of capillary filling occurred at the macroscopically evident lateral boundary of the ischaemic reflow. When this region was imaged (Fig. 36) a very narrow (20-50 micron) zone over which cells changed from compact to open appearance was noted. Typically this was on a cell to cell basis with adjacent transversely fractured cells showing either open or compact forms.

The transition line, though continuous, was not a linear boundary across the left ventricular wall but contained many irregularities. Figure 40A shows a narrow tongue or "peninsula" of compact cells which in the fracture plane obtained extended well into the surrounding ischaemic zone.

For descriptive and quantitative purposes, control and ischaemic regions were defined using this transition of morphology of cardiac muscle cells. On this basis, no differences in appearances between adjacent and distant ischaemic tissue could be distinguished either in morphology
of cardiac muscle cells or in the proportions of unfilled collapsed capillaries.

The control region showed some morphological differences between adjacent and distant regions. In the adjacent region, cardiac muscle cells more frequently showed prominent pit and channel structures but still retained a compact relatively flat fracture face without separated, exposed identifiable organelles (this appearances is shown in Fig. 37B).

In the 150 micron wide zone of myocytes immediately adjacent to the transition from "open" ischaemic to "compact" control regions, many fields showed an increased proportion of unfilled open capillaries (Fig. 36) but this was not a constant finding as other fields (Fig. 37B, 40A) showed resin filling of capillaries in control myocardium very close to this lateral boundary. There was no evidence of any constant intermediate zone of capillaries interposed between the predominantly unfilled collapsed appearance capillaries in the ischaemic region and the 50-60% filling of capillaries in the remote control regions.

Nor was there detected any discrete zone showing intermediate degrees of morphological change between the 'open' pattern of the ischaemic region and the 'compact' zone of the control region. Occasional individual cells showed less prominent open forms of partial open and partial compact forms within 2-5 cells of the more usual cell-cell transition, but these were too few (less than 5% of cells)
and too irregularly distributed to form a recognisable separate zone.

Despite the marked differences in morphological appearances noted in the four regions sampled, no significant differences were seen in the total number of capillaries per field (Fig. 41A). In contrast, a marked and highly significant change was noted in the proportion of capillaries filled by resin (Fig. 41B) between the four regions. There was a decrease in proportion of filling from 65-70% in the distant control regions to 30-40% in the adjacent control regions (p<0.01), and corresponding precisely with the change in morphological appearance, a second more marked reduction to less than 10% (p<0.01) in both ischaemic regions.

Figure 42A shows the very large proportion of unfilled capillaries observed in the ischaemic regions, about 85% of which were collapsed. This was significantly (p<0.01) and obviously different to both control regions which showed 20-30% of capillaries unfilled and collapsed.

There were less pronounced but still significant differences in the proportion of unfilled but open capillaries in several regions (Fig. 42B). This confirmed the qualitative observation of increased numbers of capillaries with this appearance in the adjacent control region (35-40%) compared to distant control region (10-20%) and the two ischaemic regions (5-10%).
One hundred and twenty minute experimental group

There was a close similarity in appearances between control zone myocardium adjacent to the lateral border zone and control zone myocardium distant from it (Figs. 43A,B). Two differences were noted: the more easily perceived pits and channels in the fracture face surfaces of some cardiac myocytes (about 20%) adjacent to the border, and the increase in proportion of open but unfilled capillaries in the adjacent control zone within 150 microns of the transition from control to ischaemic appearances. As was observed at sixty minutes, the numbers of open unfilled capillaries was unevenly distributed, and varied widely from field to field both in frequency and distribution relative to the boundary (Figs. 43B, 44) but overall, their mean proportion was increased, although they did not form a definite zone of only open unfilled capillaries.

The transition from compact control to open ischaemic pattern was abrupt at lateral boundaries, on a cell to cell basis (Fig. 44), and correlated very closely with the dramatic fall in the proportion of filled capillaries and rise in unfilled collapsed which characterised the ischaemic zone.

There were no detectable differences between the appearance of ischaemic myocardium adjacent to or distant from the lateral boundary (Figs. 45A,B), the whole of the ischaemic region showing the "open" cell pattern with swollen cells and organelles.
This group was the only one to show any significant difference in the mean number of capillaries observed per field between any of the four regions (Fig. 47A). The adjacent control zone contained about 70 capillaries per field compared to about 50 in the distant control regions. This difference was statistically significant but all other differences were not.

A striking and highly significant reduction in the proportion of filled capillaries characterised the lateral boundary (Fig. 47B), with the ischaemic regions at less than 10% compared to 30-40% for adjacent control and 50% for distant control regions. The difference between the two control regions was also significant (p<0.01).

This difference was associated with an increased proportion of unfilled but open capillaries in the adjacent control zone (Fig. 48B), which was clearly, but because of wide variance, not significantly increased compared to the distant control regions. However this proportion was significantly increased in comparison to both ischaemic regions.

Both ischaemic regions showed a large and highly significant increase in the proportion of unfilled collapsed capillaries compared to the control regions (Fig. 48A).

Two hundred and forty minute experimental group

Just as the morphological appearances of control and
ischaemic myocardium was very closely similar between 120 minute and 240 minute groups when areas distant from the boundary were examined, so too were the appearances of the areas of myocardium adjacent to the lateral boundary closely similar when 120 minute and 240 minute groups were compared.

The ischaemic zone showed uniform changes from the sharp cell-to-cell transition (Fig. 50) of compact and open cell types throughout the remainder of the separately perfused zone (Figs. 51A,B). This was associated with near universal finding of unfilled collapsed capillaries within the ischaemic zone and extending up to the lateral boundary.

Control region myocardium also showed few differences when areas adjacent to or distant from the border were compared, though there was an apparent increase in the number of open and unfilled capillaries in the adjacent control zone (Figs. 49B, 50). As at 120 minutes, these were unevenly distributed throughout a zone about 150 microns wide.

There were no significant differences between mean numbers of capillaries per field in border zone and distant areas (Figs. 54A). The mean proportion of capillaries filled by resin (Fig. 54B) showed a clear and highly significant \((p<0.01)\) demarcation of control and ischaemic regions with a fall from about 55% in distant control regions, to less than 10% in both ischaemic regions. There was a reduction to about 45% in the adjacent control region, but compared to the distant control region this was not
statistically significant.

Similarly, the demarcation of control and ischaemic regions was indicated in striking (and highly statistically significant) fashion when the proportions of unfilled collapsed capillaries were compared (Fig. 55A), with both control regions containing 20-30% compared to 85-90% in both ischaemic regions.

The proportion of unfilled but open capillaries (Fig. 55B) in the adjacent control region (about 30%) was significantly higher than in both distant control (10-15%) and both ischaemic regions (about 5%).

Evolution of changes with the duration of ischaemia

In summary, no morphological difference was observed between control and ischaemic regions until after 30 minutes of ischaemia. These were differences only in degree, with more prominent pits and channels in some parts of the ischaemic region in the main feature. After 60 minutes however gross changes had occurred in the ischaemic zones (which could not be distinguished from the distant ischaemic regions on morphological appearance). Changes in the control zone compared to distant regions remained variable and limited to increased prominence of pits and channels. These appearances were sustained from 60 to 240 minutes of ischaemia without further change except for full transmural extension of severe ischaemic injury by 120 minutes.
The changes in capillary functional status in the border regions with time are summarised graphically. Figure 60 shows the mean number of capillaries observed per field in the adjacent zones. The only significant difference seen was the increase at 120 minutes (relative to 0) in the adjacent control group (p<0.01). The changes in proportions of filled capillaries (Fig. 61) were significant in both ischaemic and control regions. As in the distant ischaemic regions, the adjacent ischaemic regions showed a highly significant rise in the percentage of filled vessels after 30 minutes and a large and sustained fall to about 10% by 60 minutes (Fig. 61B). The rise in filling percentage in adjacent control regions (Fig. 61A) after 30 minutes was closely similar to that seen in the adjacent ischaemic region (and also significant p<0.01). However, the subsequent fall (to about 40%, p<0.01) was much less than that which occurred in the ischaemic regions. In fact, a statistically significant difference could not be demonstrated when comparing 0 and 60, or 0 and 240 minute groups, although 0 and 120 minute groups were significantly different (p<0.01).

There was a clear difference between adjacent regions in the change in proportion of unfilled collapsed capillaries with time (Fig. 62). Little change was seen in the control region, with the only significant difference (p<0.01) being found when comparing the 30 and 120 minute groups. In contrast, the pattern of change in the ischaemic region closely resembled that of the distant ischaemic region, with
a highly significant fall after 30 minutes and a marked and
sustained increase to about 90% after 60 minutes of
ischaemia.

The adjacent control region was the only region to show
any significant increase in the proportion of open but
unfilled capillaries with time (Fig. 63). While the initial
fall between 0 and 30 minutes of ischaemia was not
significant (Fig. 63A), the increase to about 35% after 60
minutes was significant compared to the 0 (p<0.05) and 30
minute groups (p<0.01). The subsequent decline between 60
and 240 minutes was also significant (p<0.01), and was such
that there was no longer any significant difference when 0
and 240 minute groups were compared. The adjacent ischaemic
region however (Fig. 63B), showed a significant fall in
unfilled capillaries only between 0 and 30 minute groups
(p<0.01) and this fall was sustained through to 240 minutes
of ischaemia.
DISCUSSION

The discussion of this investigation is undertaken in four parts. The first section discusses the materials and methods chosen for the investigation and the validity of interpretations possible using them (pp. 134-58). The following three sections deal with the contribution of this study to knowledge of three important aspects of the pathogenesis of myocardial infarction. These are the progression of cardiac muscle cell injury in early myocardial infarcts, vascular function at the lateral margin of myocardial infarcts, and the role of the no reflow phenomenon in regional infarction.

METHODOLOGICAL CONSIDERATIONS

Anaesthesia

"Saffan" proved to be a suitable anaesthetic for the brief interval required to excise the hearts. It is an ultra-rapid acting intravenous neuroleptanalgesic agent composed of two steroid components (alphaxalone and alphadolone) and a solubiliser. Introduced as CT1341 (Child, Currie, David, Dodds, Pearce and Twissell, 1971) it is now the anaesthetic of choice in veterinary practice, particularly for cats, but also for other small animals (Green, Halsey, Precious and Wardley-Smith, 1978), and is widely used in human clinical practice as "Althesin".

The practical difficulties of induction by inhalation
anaesthesia and the very narrow margin of safety of barbiturates in rabbits, makes these methods unsuitable alternatives (Green, 1975). The present investigation required a rapid-acting agent which could be administered intravenously to give the observed smooth induction, suitable muscle relaxation and analgesia, without dangerous levels of respiratory or cardiovascular depression. As Green et al. (1978) has noted, the degree of analgesia occasionally was paradoxically less than indicated by the degree of reflex loss and muscle relaxation.

The cardiovascular effects of Saffan do not appear to have been investigated in rabbits, but Child et al. (1971) has shown histologically that it has no injurious vascular or extravascular effects on rabbits. Gordh (1972) showed minimal cardiodepressant action at therapeutic levels in cats, and Tammisto, Takki, Tigerstedt and Kauste (1973) have shown that the cardiovascular effects of Althesin in humans were equivalent to the barbiturates in equipotent doses. On these grounds, it is highly likely that Saffan is a practical and safe anaesthetic for rapid removal of hearts in isolated perfused heart experiments. Very recently Flecknell, John, Mitchell, Shurey and Simpkin (1983) have shown that the anaesthetic combination fentanyl-fluanisone with diazepam has some advantages in rabbits as it shows negligible cardiovascular effects and excellent analgesia, though slower induction.
Cannulation of a branch coronary artery

The difficulties of identifying and preparing the VIB were reduced substantially by colouring the ice-cold perfusate with methylene blue. With practice (in six hearts not included in the present study) the time taken and success rate improved considerably. During the time taken to finally place the cannula (19-35 minutes) the hearts were intermittently perfused with ice-cold perfusate and the VIB was occluded in total for less than five minutes. Function was restored rapidly and no evidence was found in the separately perfused regions of cell injury or reperfusion damage, and no detectable differences between this region and the remainder of the heart.

Methylene blue dye was used in the ice-cold perfusate in this study solely for its colour, to make small blood vessels more easily visible amidst epicardial fat. In previous studies (Kloner, Ganote, Reimer and Jennings, 1975) this dye has been used for its acid-base indicator characteristics. When perfused into hypoxic myocardium, which contains high hydrogen ion concentrations, methylene blue is reduced from the blue coloured moiety to colourless leucomethylene blue. In this study it was noted that the washout of methylene blue colouration was surprisingly slow so that even after 15 minutes of perfusion with dye-free perfusate, significant colour was retained in all areas. This allowed the observation later in the experiment of regional methylene blue reduction to give a pale grey-pink
background of buffer perfused myocardium following cessation of flow to the separately perfused area, with consequent tissue hypoxia and hydrogen ion generation. This provided an epicardial marker of the extent of regional ischaemia similar to the cyanosis observed in blood perfused hearts (Hearse and Yellon, 1981).

Preparations using separate cannulation of a distal branch of a coronary artery (with supply of autologous blood from a shunt typically based in the carotid artery) have been frequently made in dog hearts in vivo (Hirzel, Nelson, Sonnenblick and Kirk, 1976; Domenech, 1978; Eaton and Bulkley, 1981; Sakai, Tomoike, Ootsubo, Kikuchi and Nakamura, 1982; Patterson and Kirk, 1980, 1983) to study a wide variety of parameters of regional performance of myocardium. But this approach apparently has not previously been used for small beating mammalian hearts in vitro. However, Spadaro, et al., (1980) have used a 25 swg needle to cannulate the left main coronary artery post mortem in rat hearts removed from the animal as a comparison for in vivo ligation studies. They injected 1% Alcian Blue dye into that artery to define its perfusion bed.

Perfusate

In the present investigation it was desirable to be able to perfuse hearts for relatively long periods comparable to intervals in which clinical intervention could be undertaken, and during which development and migration of
border zones would occur. The perfusate chosen for this investigation did maintain the morphological appearances of cardiac myocytes and blood vessels for the duration of the experiment and only a modest decrease (15-20%) in coronary flow and heart rate was observed, and that only after 150 minutes of perfusion.

Bergmann, Clark and Sobel (1979) observed a coronary flow rate in Langendorff isolated perfused rabbit hearts of 4.5 ± 0.8 ml/min/g wet weight. They used Krebs-Henseleit bicarbonate buffer with added dialysed bovine serum albumin perfused at 60 mm Hg pressure (81.6 cm H₂O) in paced hearts. The stable flow rate of 4.0 ml ± 0.12 ml/min/g at 85 cm H₂O pressure (Fig. 5) observed in the present study is a very similar range. Bergmann et al. (1979) noted the impracticability of maintaining whole blood perfused hearts for prolonged periods and, because of preparative logistics, expressed reluctance to consider times of greater than 90 minutes with the washed red cell Krebs-Henseleit solutions tested in their study. On hearts perfused for 90 minutes they commented that "despite some variation in performance from heart to heart, evident at the onset of perfusion, performance of each heart remained quite constant throughout the experimental period". This observation is equally applicable to the present study.

Armitage and Pegg (1977), whose method was chosen, compared the performance of isolated rabbit hearts perfused as Langendorff preparations using a variety of perfusates to
investigate the effect of added protein or protein substitute. They found that 1.75% Haemaccel in Krebs-Henseleit bicarbonate buffer solution, with adjustment of calcium and magnesium balance (considering both Haemaccel bound and free ions), provided the best result. They attributed this to the reduction of tissue oedema brought about by the colloid oncotic pressure developed by the Haemaccel (13.4 cm H₂O). They noted that other solutions with equal or greater oncotic contribution, e.g. PVP, dextran-70 Pluronic, bovine serum albumin, were not necessarily equivalent, and claimed that the initial consideration is the integrity of the vascular endothelium which was severely damaged by some of the plasma expanders they used, after which oncotic pressure becomes important. Unfortunately these authors do not provide figures for coronary flow or heart rates with time, using only developed systolic tension and presence of beat as their parameters of performance. With Haemaccel solution, they observed that isolated hearts would beat for a mean time of 30 hours, and the time to attain a 25% reduction in developed systolic pressure was 4.6 hours ± 0.83 compared with 2.53 ± 0.68 hours for Krebs-Henseleit buffer without Haemaccel.

It is possible that, with the oncotic pressure supplied by Haemaccel, substrate limitation eventually becomes important (Aronson and Serlick, 1976). Perhaps pyruvate added in addition to glucose (Bunger, Haddy, Querengasser and Gerlach, 1975), or β-hydroxybutyrate (Fisher and Williamson 1961), would have been advantageous.
It is reasonable to conclude however that, in comparison to these other published studies, the myocardial performance of the rabbit hearts in the present study was comparably maintained over the whole experimental period.

**Fixation**

Fixation of the myocardium prior to freezing was shown in a pilot study (Appendix II) to greatly improve structural preservation. This conclusion is in agreement with those of other studies (Sybers and Ashraf, 1973; Nowell and Pawley, 1980) of the preparation of cardiac muscle and similar solid tissues for SEM.

The choice of fixative (2.5% glutaraldehyde in 0.1M phosphate buffer) was based on the wide, but not exclusive, acceptance of this formulation and its close variants for administration by intravascular perfusion (Ashraf and Sybers, 1974; Ganote and Kaltenbach, 1979; Humphrey et al., 1980; Thorball and Tranum-Jensen, 1983) for morphological studies. Early investigators used immersion fixation of small (1 mm³) cut tissue samples and higher concentrations of glutaraldehyde at lower temperatures (for example 6.25% glutaraldehyde in 0.1M phosphate buffer at 4°C. Armiger and Gavin, 1975; Gavin et al., 1978a), but there are many advantages in fixation by perfusion and these have been reviewed by Hayat (1981).

In the present study the fixative solution was perfused at 37°C and identical hydrostatic pressure to the aqueous
buffered perfusate. The selection of physiological temperature, as opposed to "ice-cold", is consistent with recent practice in other organ systems (Thorball and Tranum-Jensen, 1983; Coalson, 1983).

In Appendix III, the effect of this fixative injected at 37°C and identical pressure to that used to perfuse the isolated heart was investigated with regard to the demonstrable competence of myocardial capillary pathways (Sage and Gavin, 1983). We showed in rat hearts where all pathways were rendered accessible by procaine infusion, that perfusion with acrylic resin subsequent to fixation filled virtually all capillary pathways. Similarly, the proportion of competent pathways in hearts which had received no procaine, was very close to the values predicted by other workers using different methods (Vetterlein, dal Ri and Schmidt, 1982). The technique thus demonstrates that proportion of capillaries which are functional at any particular time, and rapidly stabilises the cardiac microvasculature with a flow distribution very similar to that found in vivo. Vascular reactions to perfusion fixation in rabbit intestinal segments and hind limbs (Thorball and Tranum-Jensen, 1983) support these conclusions that no appreciable change occurs in vascular resistance as a result of perfusion fixation by 2.5% glutaraldehyde in 0.1M phosphate buffer.

On these grounds, the proportion of competent pathways demonstrated by post-fixation acrylic resin perfusion is
assumed to be very close to that pertaining immediately prior to introduction of the fixative. The remarkably similar and relatively stable proportion of resin filled capillaries in continuously perfused myocardium shown in the present study (about 55-60%. fig. 57A) to that demonstrated in the preliminary study (62%. Appendix III), and to the anticipated values (50-64%) obtained by others (Vetterlein et al., 1982; Bourdeau-Martini, Odoroff and Honig, 1974), allow values which were significantly higher or significantly lower to be interpreted with confidence as evidence of hyperaemia or defective vascular perfusion.

The choice of fixative (2.5% glutaraldehyde in 0.1M phosphate buffer) may have had an important bearing on the occurrence of cell swelling in the ischaemic regions. Recently Tranum-Jensen, Janse, Fiolet, Krieger, Naumann d'Alnoncourt and Durrer (1981) have shown that, if the tonicity of fixative used for ischaemic tissue is increased, morphological evidence of gross cell swelling is minimised. Conversely, if fixative tonicity is maintained at a level appropriate to normal myocardium (vehicle osmolarity in the region of 315-320 mOsm/litre), then severe swelling occurs in myocytes ischaemic for 60 minutes. Their observation that the osmolarity of ischaemic pig myocardium, which rises by 35-40 mOsm/kg during the first 60 minutes of ischaemia (probably due to the catabolism of complex molecules to smaller moieties), accounts for this difference in appearance. It also most likely accounts for the apparent contradictions in the reported time course of the appearance
of severe cell swelling (Baghirzade et al., 1970; Tranum-Jensen et al., 1981; Gavin et al., 1983a) in studies using fixatives of different tonicity.

It is likely that the fixative used in the present study (vehicle osmolarity 318 mOsm/litre) was isotonic or only mildly hypotonic to normal rabbit myocardium, but definitely hypotonic relative to ischaemic myocardium. Whereas the reliability of severe cell swelling as a marker of irreversible injury to cardiac myocytes may have been masked in some previous investigations by the use of hypertonic fixatives, the selection of a fixative solution near isotonic to normal myocardium for the present study was both fortuitous and theoretically correct, because it emphasised the differences in ability of ischaemically injured and uninjured myocytes to maintain normal ionic and water equilibria across the cell membrane, demonstrated by presence or absence of cell swelling.

Some recent authors have also included a colloid oncotic pressure agent (such as Dextran-70) in their perfused fixative (Tranum-Jensen et al., 1981; Coalson, 1983; Thorball and Tranum-Jensen, 1983). In retrospect, inclusion of a colloid oncotic pressure agent in this study would have been theoretically advantageous, but proof of improved preservation of myocardium using these agents has not yet been published.
Resin injection

The present study required an intravascular marker which could identify the functional state of each capillary, and thus allow correlation between vascular supply and myocyte injury at an individual vessel or cell level. This requirement meant diffusible tracers (such as unconjugated fluorescein, dyes, antipyrine) were unsuitable because of lack of precise intravascular localisation. Methods requiring destructive analysis of tissue samples such as injected radioactive labelled microspheres, which are commonly used for measurement of regional cardiac blood flow, give only an integrated value and lack the ability to interpret events on a cell to vessel basis. The embolic obstruction of small vessels, which is the basis of microsphere measurements of blood flow, also makes that method unsuitable in a study of loss of competence of microvasculature, even though some authors claim that the greatest quantity of microspheres required to measure flow leads to obstruction of less than 10% of capillaries (Yipintsoi, Dobbs, Scanlon, Knopp and Bassingthwaite, 1973).

Polymerised LR White resin proved to be an easily identified intravascular marker. Capillaries appeared either completely filled by the homogeneous polymer, which showed characteristic glass-like fracture faces, or completely empty with the endothelial luminal surface exposed. Additional confirmation of the identity of resin could be obtained through the artefactual cracking of the evaporated gold coating on resin surfaces during prolonged
exposure to the electron beam. This created a characteristic mosaic pattern not seen elsewhere in the myocardium. Despite this coating artefact, the resin polymer was quite stable under the electron beam.

There was a possibility that additional information concerning the three dimensional distribution of functional blood vessels at borders of myocardial infarcts could be obtained if the cryofractured blocks examined were subsequently macerated to remove all the tissue and leave only the intraluminal resin cast. However, preliminary investigation indicates that there are difficulties in obtaining casts completely clean of well fixed tissue, and that such small casts tend to collapse rather than remain as a three dimensional network. Corrosion-cast preparations are typically obtained from whole, unfixed organs (Hodde and Nowell, 1980).

The uncertain effects of perfused polymer resin on vascular structure and diameter have been commented on by reviewers of studies which deduce microvascular function from corrosion casts (Olson, 1980; Hodde and Nowell, 1980). Many of these potential problems were circumvented in the present study by prior, thorough perfusion fixation, which stabilised flow distribution and rate (discussed above p. 141), as well as vascular and myocyte structure. As the L.R. White resin was designed as a tissue embedding agent for light and electron microscopy, it presumably would have been selected to minimise artefactual effects on fixed
tissue. Furthermore, it was confirmed in preliminary studies (Appendix II), that perfusion with L.R. White resin caused no detectable SEM changes in the myocardium.

Polymerisation of large volumes of any acrylic resin is associated with a substantial rise in temperature resulting from the exothermic polymerisation reaction. However, this is unlikely to result in any tissue damage because of the small volume of resin in capillaries relative to the heat-sink of surrounding tissue (Hodde and Nowell, 1980). Unpublished data from this laboratory indicates that the temperature rise measured by a thermistor in rat hearts whose vasculature is filled by polymerising methylmethacrylate is less than 2°C.

Since this study was commenced, two papers using injected resin as an intravascular marker have been published. Hanstede and Gerritts (1982) used a new Araldite resin with a "viscosity similar to blood" for concurrent light microscopic morphometric and corrosion-cast studies of hepatic microvasculature. Piek, Alvarez, Jutzy, Morales and Boucek (1982) used a polyacrylamide resin containing dispersed bismuth salts as a solid intravascular marker for coronary radiographs in post mortem human hearts previously fixed by perfusion with formaldehyde. They attributed the penetration of this particular resin only to arteriole level to early polymerisation.

A disappointing finding of the present study was that, despite determination of optimal particle concentration in a
preliminary study (Appendix IV), it was noted that only 20-30% of capillaries in the VIB region contained identifiable PbO₂ in the resin filling the functional capillaries. Although this potentially limited the interpretation of the source of arterial supply of capillaries at the margins of the ischaemic zone, in practice this limitation proved not to be one of major proportions. Capillaries which showed the label could still definitely be identified as having been supplied from the VIB cannula. Furthermore, the proportion of functional capillaries containing the label in the separately perfused region remained constant at 20-30% despite the substantial reduction in proportion of functional capillaries with increasing time of ischaemia. This suggested that the low labelling proportion was a constant characteristic of the injected resin, and was not due to a variable degree of mixing of VIB and control resins during injections. This view was reinforced by the observation that labelled capillaries were never seen outside the ischaemic region.

The interpretation of collateral flow across the lateral boundary was also not difficult. The reduction in proportion of filled capillaries in the ischaemic zone was so great (> 90%) that it was evident that there was little flow into that region either from the VIB or through collaterals from the control zone. Nevertheless, definite labelling of all capillaries in the ischaemic zone, or better still positive and distinctive labelling of both VIB and control resin would have been advantageous.
It is concluded that the use of L.R. White acrylic resin as an intravascular marker appears to have some advantages over resins previously used for corrosion casting experiments, and that alternative materials used in recently published studies do not indicate any significant improvement. In the context of intact hearts fixed by perfusion, few artefacts can be attributed to the injected resin.

**Tissue Preparation and Imaging**

The choice of L.R. White acrylic resin as the injected intravascular marker of competent myocardial capillary pathways carries many advantages as outlined above. However, the solubility of this resin in ethanol, which makes cleaning apparatus very easy, is disadvantageous when conventional methods of tissue preparation for scanning electron microscopy are considered (Nowell and Pawley, 1980). All of the previously used methods use non-polar solvents as dehydrating agents to avoid the destructive formation of intracellular water-ice crystals during freeze-fracture procedures. Subsequently the solvent is sublimated by either freeze or critical point drying.

Because of the solubility of L.R. White resin in ethanol, other common dehydrating agents were examined in a preliminary study (Appendix IV). From those results it was concluded that the retention of intracapillary resin in solvent-dehydrated blocks may be unpredictable. Therefore a
tissue preparation method was designed which avoided polar solvents, and did not produce sufficient ice-crystal artefact to obscure the pathological changes in tissue (Appendix II). This method used a colligative cryoprotectant (dimethyl sulfoxide - DMSO) which suppresses the formation of intracellular ice crystals by increasing both intra- and extracellular tonicity thus giving an effective depression of freezing point (Meryman, 1974). The resultant freeze-fractured, freeze-dried tissue blocks showed little evidence of ice-crystal artefact at magnifications up to the useful resolution of the back scattered electron detector (15-20,000 times).

Previous reviews (Boyde, 1974, 1976; Nowell and Pawley, 1980) have indicated that colligative cryoprotectants such as glycerol are unsuitable for scanning electron microscopy. The possible advantages of DMSO relative to glycerol have been discussed in Appendix II, but these alone did not overcome the potential problems of specimen charging artefact (due to retained cryoprotectant and water) and possible instrument contamination. In the present study these problems were overcome by the application of several recent developments in scanning electron microscopy.

The majority of biological applications of SEM have used the secondary electron image, which is produced by electrons released from the specimen atoms in response to the rastered primary electron beam. These low energy (3-5 eV) electrons are usually detected by an Everhart-Thornley detector which
utilises a high voltage bias grid (usually + 10,000v) to attract the secondary electrons to a aluminium coated perspex scintillator, thence to a photomultiplier which produces an electronic signal suitable for processing and display on a video display unit.

Recently, high performance detectors of the high energy backscattered electrons, which are reflected primary electrons with high energy (50 - 80% that of the incident beam) have been developed (Robinson, 1980). In this study a wide angle scintillator-photomultiplier type detector was used. This consists of a large solid angle (nearly hemispheric) coated scintillator placed immediately above the specimen, allowing collection of a large number of the back-scattered electrons, and a large light guide (compared to conventional detectors) which improves the efficiency of scintillator-photomultiplier performance (Fig. 4).

As more secondary electrons are released by scattered primary electrons passing out of the specimen than by primary electrons entering the specimen surface (Robinson and George, 1978), the secondary electron image and the backscattered electron image have many common features, provided detectors are of similar efficiency. In this study secondary electron and backscattered images were closely similar save for three important differences:

Most importantly, the phenomenon of specimen charging was largely absent in back-scattered images, but a major problem in secondary images of the specimens used in this
study. Specimen charging is a focal collection of primary electrons in a poorly conductive specimen and is seen as very high signal intensity regions which obscure specimen detail directly, distort the signal from surrounding areas, and sometimes discharge to give characteristic irregular line artefacts in the image. Conventionally, specimens such as dried biological materials are rendered conductive by a coating of carbon and gold (Nowell and Pawley, 1980), though it is a common experience that with specimens of complex surface structure, lack of penetration of the coating to deep crevices can lead to persistent charging artefacts. Furthermore, damp or wet specimens charge much more severely than dry specimens, which is why previous reviews have dismissed the use of colligative cryoprotectants which leave a hygroscopic remnant in the specimen. It was anticipated (Robinson, 1978; 1980) that backscattered electron imaging should greatly decrease the incidence of charging because of the difference in energies of the imaged electrons. Small accumulations of electrons can easily result in charging where secondary electrons of energies 3-5 eV are being imaged, but where backscattered electrons with 50-80% of the energy of the primary electron beam (30,000 eV) are used, only massive accumulation is likely to result in charging artefacts.

The second important advantage in using a high performance backscattered electron detector is that it confers the ability to operate the specimen chamber at relatively high pressures (i.e. not as profound a vacuum.)
Robinson, 1976). This principle was used in this study to further control specimen charging and to eliminate the possibility of significant instrument contamination by residual cryoprotectant, the other major objection which has been raised to its use. By separate pumping of the specimen chamber through an accessory rotary pump (Fig. 4), a pressure differential can be maintained across the final aperture which lies between the microscope column and the specimen chamber. Ionisation of residual gas molecules by the electron beam, provides sufficient positive ions to neutralise negative charge (electron) accumulation that would otherwise occur. This means that even highly insulating specimens can be imaged uncoated without charging. Moncrieff, Barker and Robinson (1979) have shown that only 15% of the electrons in a 25 KeV primary electron beam are scattered at typical residual gas pressure of 0.4 mbar, with no increase in beam diameter, so that resolution loss is very small. Residual gas molecules would however, severely degrade the resolution of an image dependent on low energy secondary electrons. Such residual gas would make the operation of the conventional Everhart-Thornley detector extremely hazardous because of potential arcing from the 10 kV grid bias voltage required to attract the secondary electrons. The separate pumping of the specimen chamber in this commercially available accessory system lowered the risk of contamination to the microscope column and its pumps and also circumvented the problems of outgassing of the specimen which can prove difficult because of the low
pumping capacity of oil diffusion pumps which are required where high vacuums are used.

The third advantage of backscattered electron detectors is their ability to demonstrate atomic number differences in the specimen (Robinson, 1980). The difference in atomic number (Z) between lead dioxide particles (lead Z = 82) and surrounding resin (predominantly carbon, Z = 6) was demonstrated by the high intensity of the backscattered electron image of the lead dioxide particles, and this enabled definite identification of resin perfused blood vessels supplied by the separately cannulated coronary artery. The evaporated gold coating of specimens, conventionally applied to render them conductive, but not required for this purpose here, was still used as it greatly enhanced the topographic surface signal (gold Z = 79) and resulted in images of resolution similar to that obtained conventionally from secondary electrons. The thin gold layer did not significantly obscure the Z differences between the particulate lead dioxide and the resin.

**Interpretation of SEM Observations**

Marovitz, Arenberg and Thalman (1970) have recommended that any method of tissue preparation for SEM be compared with several others to ensure accuracy of interpretation. In a preliminary study (Appendix II), the appearance of cryofractured and freeze-dried tissue infiltrated with DMSO (the method used in this study) was compared with samples
dehydrated in either ethanol or tertiary butanol (2-methyl propan-2-ol) before cryofracture and freeze drying according to the methods of previous studies (Buss et al., 1971; Wheeler, Gavin and Seelye, 1975). Further samples were dehydrated in acetone cryofractured and critical point dried. By each method, transversely sectioned control myocardium showed closely similar "compact" appearances with relatively flat transverse and longitudinal fracture faces within cardiac myocytes, but low steps and angles and small tubular profiles were evident in a pattern consistent with organelles.

However, in longitudinally fractured myocytes prepared by the method of Buss et al., (1971), there was some accentuation of organelles similar to that illustrated by Sybers and Ashraf (1973) who critical point dried myocardium using Freon - 113 as transition agent. McAllister et al., (1974) used a very similar method and drew attention to the influence of specimen tilt angle (relative to the incident electron beam) on topographical detail. They showed that high tilt angles (e.g. 60°) result in increased prominence and apparent separation of subcellular organelles. In the present study a 0° tilt angle was used because it optimised the function of the backscattered electron detector (Robinson, 1980) and was mandatory for morphometric comparisons. This may also explain the small difference in appearance between backscattered and secondary electron image topography observed in the preliminary study (Appendix II).
Cardiac muscle ischaemic for sixty minutes could be readily discriminated from control tissue by the presence of severe cell swelling, obvious separation of organelles which facilitated their identification and swelling of mitochondria to smoothly rounded forms. Ischaemic cardiac muscle appeared closely similar to that described in rat heart (Ashraf and Sybers, 1974; 1975), though the time intervals are not directly comparable. The separation of myofibres, swelling of mitochondria, and swollen disrupted t-tubules are common features. Unfortunately these authors do not illustrate or report the changes in transversely fractured cardiac muscle cells, the plane widely utilised in the present study and most useful for comparison of interrelationships between muscle cells and their adjacent capillaries.

However, Gavin et al., (1978b) have used the method of Sybers and Ashraf (1973) and illustrated transversely fractured cardiac myocytes from the marginal zone of canine myocardium ischaemic for ninety minutes. They showed severe cell swelling and separation of organelles similar to that seen in the present study. On the basis of these common SEM features, the swollen, open appearances seen in the present study was taken to indicate severe ischaemic injury to cardiac muscle cells.

Comparison of SEM Observations with TEM

With increasing durations of ischaemia several
consistent and readily identifiable changes occurred in cardiac myocytes. These were an increased prominence of pits and tubular channels, increased cell size, swelling of mitochondria, the appearance of open spaces between organelles, the appearance of sarcolemmal discontinuities and the disruption of organelles. The sequence of development and appearance of these changes are consistent with the findings of TEM investigations of the fine structural changes associated with ischaemia (reviewed previously p.22) in both dog (Herdson et al., 1965; Jennings et al., 1969) and rabbit (Decker and Wildenthal, 1978) myocardium.

Increased number and size of tubular pits and channels were noted in some myocardium ischaemic for 30 minutes. These appearances have received considerable attention in previous studies of cardiac myocytes by SEM (reviewed on p. 70). Buss et al., (1971), Sybers and Ashraf (1973) and McAllister et al., (1974) have interpreted these structures as t-tubules and sarcoplasmic reticulum (SR). Decker and Wildenthal (1978) observed swelling of t-tubules and SR by TEM and included these changes among the features of relatively late ischaemia (90 - 120 minutes). In contrast, Kloner, et al., (1979) concluded that swelling of SR was an early feature of ischaemic change in rat myocardium. Results of the present study suggest that this is an early change because it was seen only in some parts of the myocardium ischaemic for 30 minutes and within control regions close to the infarct border. In tissue subject to
greater duration or severity of ischaemia this change was
superseded by more pronounced changes. Because these
changes to SR and t-tubules are observed within 30 minutes
of ischaemia and thus prior to myocardial cell necrosis
(45-60 minutes) in the rabbit heart (Decker and Wildenthal,
1978) they should not be interpreted as evidence of
irreversible myocyte injury.

However, between 30 and 60 minutes of ischaemia, three
distinct changes developed and these were used as indicators
of irreversible cell injury. They were the appearance of
open spaces between organelles, the swelling of mitochondria
and the commonly associated swelling (by up to 50%) of the
myocytes with a consequent reduction in intercellular space.
It was the appearance of spaces between organelles which was
the most striking change, and the one responsible for the
"open" pattern characteristic of severe ischaemic injury.

Spaces within cardiac muscle cells indicate regions with
a high water content, which like much of the intercellular
space and the intravascular space, contained insufficient
structural protein to appear solid following sublimation of
the water during freeze-drying. The "open" pattern
therefore indicates an increase in cell water which is
consistent with the associated cell swelling and suggests
the loss of ability of the sarcolemma to maintain normal
water and ion equilibria. TEM appearances in rabbit hearts
at similar time intervals show separation of the organelles
with clearing of the intervening cytoplasm, by which time a
variety of other changes including margination of nuclear chromatin, absence of glycogen, osmiophilic intra-mitochondrial densities and mitochondrial swelling are also apparent (Caulfield and Klionsky, 1959; Decker and Wildenthal, 1978). The observed swelling of mitochondria may also be attributed to loss of ability of membranes and ATP-dependent ion pumps to maintain water and ionic equilibria and; in addition, rising concentrations of ischaemic metabolites (H+, lactate, inorganic phosphate) may also contribute (Armiger, 1982). All of the above changes are widely accepted as markers of irreversible cell injury (Caulfield and Klionsky, 1959; Decker and Wildenthal, 1978; Jennings et al., 1975; Jennings et al., 1978b).

Sarcolemmal discontinuities and disruption of organelles (myofibres and mitochondria) were only observed by SEM following 120 minutes or more of ischaemia. This is consistent with their appearance as relatively late (90-240 minutes) features of severe ischaemia in TEM studies of rabbit myocardium (Decker and Wildenthal, 1978), and these features can confidently be included as SEM appearances of irreversible myocardial cell injury.
PROGRESSION OF CARDIAC MUSCLE CELL INJURY IN EARLY ISCHAEMIA

The present study has demonstrated evolution of cardiac muscle cell injury both spatially and with time during the first four hours after occlusion of a coronary artery supplying a portion of the left ventricular myocardium. An important observation was that the rate and extent of progressive development of irreversible ischaemic injury to cardiac muscle cells in the transmural direction (from endocardium to epicardium) differed from the evolution at the lateral margins of the ischaemic region. This section considers first the significance of observations of transmural evolution of regional infarcts, and this is followed by discussion of the differences seen at the lateral margins, and the implications of these findings.

Development of morphological evidence of irreversible injury to the cardiac muscle cells of the subepicardial one third of the ischaemic region was relatively slow. After sixty minutes of ischaemia, the inner two-thirds of the developing infarct showed both irreversible injury to cardiac muscle cells and a severe perfusion defect, but the subepicardial third showed only prominent dilated SR and t-tubules and profound potential hyperaemia (manifest as filling of about 90% of capillaries). However, by 120 minutes the entire wall showed both irreversible injury to cardiac myocytes and the severe ischaemic perfusion defect. The retention of relatively normal structure in the subepicardium at 60 minutes, which showed changes of early
and probably reversible ischaemic injury (discussed on p. 156-7), indicated that there was an extensive zone of 'jeopardised' myocardium forming a transmural ‘border zone’ after 60 minutes which, in the absence of restoration of oxygen supply became irreversibly injured during the following hour.

This finding is similar to that described in regional infarcts in dog hearts in vivo and termed the 'wave front phenomenon' (Reimer et al., 1977, Reimer and Jennings, 1979) (reviewed pp. 26-8). Since the present study was commenced, the wavefront phenomenon has also been demonstrated in rabbit hearts (by Connelly, Vogel, Hernandez and Apstein, 1982). These authors ligated the left circumflex artery in 'open-chest' rabbits for up to 60 minutes, allowed 23 - 24 hours of coronary reperfusion in vivo, and then assessed the extent of eventual myocardial necrosis by nitro-blue tetrazolium staining. They concluded that the transmural wavefront of eventual necrosis progresses to the epicardial surface in some hearts after 30 minutes, and in all cases by 60 minutes temporary coronary occlusion. This is considerably faster than the 24 hours reported for dog hearts (Reimer and Jennings, 1979).

The present study indicated progression of the transmural wavefront of cell death at a rate similar but slightly slower than that indicated by Connelly et al., (1982). This may be due to several important differences in the experimental methods used to identify myocardial cell
injury. In the present study this was assessed by fixation of the myocardium at the completion of the ischaemic period without significant coronary reperfusion. It is possible that some cells that did not show severe ischaemic injury were of the type referred to by Hearse and Yellon (1981) as 'condemned', that is they would eventually show irreversible injury regardless of restoration of their blood supply. If reperfusion had occurred, they might have shown the explosive cell swelling of irreversibly injured cells whose blood supply is restored (reviewed pp. 28-9). It may also be that working myocytes in vivo undergo more rapid transition to irreversible injury than the myocytes of isolated perfused hearts which through beating, do not perform significant stroke-volume work.

The pathogenetic basis of the wavefront phenomenon is usually attributed to epicardial supply and a subsequent transmural pressure gradient of collateral blood flow (Reimer et al: 1977, Reimer and Jennings 1979). Schaper and Pasyk (1976) have shown marked differences in the rate of progression of eventual myocardial cell necrosis in individual dog hearts with different levels of intrinsic collateral flow. They showed that, where collateral flow was initially high, the progression of cell death was slow and frequently the subepicardial zone did not succumb to ischaemic necrosis. On the other hand, in dogs where initial collateral flow was minimal, both myocardial cell injury and the ischaemic perfusion defect developed rapidly in the epicardium, as well as in the deeper layers,
resulting in a transmural infarct. They also demonstrated that significantly increased collateral flow (induced by the ischaemia) did not occur until about six hours after occlusion, well after much of the ischaemic zone had succumbed to ischaemic injury.

The coincident appearance of the transmural ischaemic perfusion defect and transmural myocardial cell injury in the present study argues against abundant epicardial collateral flow in rabbit hearts. In pig hearts, where collateral flow to regional infarcts has been shown to be non-existent (Harken et al., 1981; Savage, Guth, White, Hagan and Bloor, 1981; White and Bloor, 1981), myocardial necrosis progresses across the ventricular wall during the first 120 minutes of ischaemia (Fujiwara, Ashraf, Sato and Millard, 1982). On this evidence it must be assumed that the wavefront phenomenon is not solely due to gradients of collateral flow from the unoccluded arteries, but that other transmural differences, possibly in wall stress, or metabolite concentration (e.g. glycogen or $\text{H}^+$ and lactate), must be invoked.

The basis of the wavefront phenomenon in rabbit hearts is likely to be the same as that in pigs, as other studies of perfusion boundaries concur with the findings of the present study (Ring, 1950; Harden et al., 1978; Harken, Barlow, Harden and Chance, 1978; Simson et al., 1979; Harken et al., 1981; Bittar and Little, 1981; Connelly et al., 1982) by indirectly indicating that collateral flow, even in
the epicardium, is insignificantly small.

There is however some dissenting evidence which suggests rabbit hearts may have a substantial collateral supply. Briden, Teltser and Weiss, (1979) and Weiss (1982) have measured collateral flow in isolated rabbit hearts with regional ischaemia (produced by ligation of the left anterior descending coronary artery), using a single injection of microspheres. They showed a flow reduction in the ischaemic zone of only 40-50% of control values after 60 minutes ischaemia. It may be that the limitations of single dose microsphere protocols (Hirzel et al., 1977) and the small sample size inherent in using rabbit hearts explain this apparent contradiction.

In summary, the present study confirms the presence of an extensive potentially salvageable transmural 'border zone' of reversibly injured myocardium, most likely due to inherent biochemical and functional differences in epicardial muscle cells relative to their endocardial counterparts. The transmural progression of myocardial cell death occurred within two hours, but direct extrapolation of this finding in isolated rabbit hearts to human hearts in vivo must be made with great caution. However, if therapeutic modifications of the transmural extension of human myocardial infarcts are to be effective in a clinical setting, the time course of the wavefront phenomenon in man would need to be considerably slower.

At the lateral margins of the ischaemic region findings
were markedly different. A distinct boundary between the ischaemic zone, which showed uniform irreversible injury, and adjacent control myocardium was evident after 60 minutes, and showed no further changes in appearance in the following three hours. The transition from irreversibly injured to apparently normal myocardium was typically abrupt, from one cell to the next, and there was no evidence of a zone of intermediate injury of significant dimensions. A variable number of cells (1-20%) which showed evidence of swelling of SR and t-tubules, and rare cells (< 5%) partly affected by changes characteristic of irreversible injury were observed, but these were not uniformly distributed and were restricted to a zone within 150 microns of the characteristic cell to cell transition.

This distinct lateral boundary has not previously been demonstrated over such wide areas in such early infarcts in well fixed tissue relatively free of potential mechanical artefact. Previous investigators have inferred (see review, p. 64) that early myocardial infarcts had abrupt cell to cell boundaries from the light microscopic examination of much later infarcts in rabbit (Ring, 1950), dog (Factor et al., 1981; O'Connor, Hamann and Hanley, 1983) and post-mortem human hearts (Lee, Ideker and Reimer, 1980). Unfortunately the inability of light microscopy to reliably detect early ischaemic injury has prevented extrapolation of these findings to the early period during which infarct evolution might be expected to occur.
However, some histochemical studies (reviewed pp. 62-3), including the widely cited study of Cox et al., (1968), have inferred the presence of an extensive zone of myocardium showing intermediate degrees of injury interposed between normal and severely ischaemic regions. The present study does not provide any morphological evidence to support this view.

More recent histochemical and electrocardiographic studies (Janse et al., 1979; Janse and Wilms-Schopman, 1982) support the later conclusions of Hearse, who has retreated from belief in a jeopardised zone 8-15 mm wide (Hearse et al., 1977) to the view that, when the interdigitation of the boundary is accounted for, the transition in biochemical indicators of injury probably occurs from cell to cell (Yellon et al., 1981; Hearse and Yellon, 1981).

It may be that some of the early reversible fine structural changes to ischaemic cardiac muscle cells cannot be detected by the surface topographic imaging of SEM. However, investigations of lateral border zones of myocardial infarcts in rabbit (Caulfield and Klionsky, 1959), rat (Page and Polimeni, 1977) and dog (Gottlieb, Kubo and Alonso, 1981) by transmission electron microscopy (TEM) have all shown that after 30-60 minutes the distance between normal and severely injured myocardium is very small, with estimates varying from 0.1 mm (Caulfield and Klionsky, 1959) to "within 1-2 mm" (Gottlieb et al., 1981). Some of that uncertainty undoubtedly arose from the loss of spatial
orientation inherent in dissection of tissue blocks small enough for preparation for TEM (hence the "1-2 mm" cited above represents 1 or 2 tissue blocks), an inaccuracy which was overcome by the use of large cryofractured blocks and SEM in the present study.

Despite this, Gottlieb et al., (1981) state categorically that the intermediate scores for cells in the "1-2 mm" between fully normal and fully ischaemic, do represent truly intermediate degrees of injury, and not an admixture of fully normal and severely ischaemic cells. This implies that some of the cells near the infarct did show morphological evidence of reversible injury, although it is not clear how extensively distributed the 'intermediate' injury cells reported by Gottlieb et al., (1981) were. The small but variable number of cells which showed swelling of t-tubules and SR within 150 microns of the border, and the rare cells which showed only focal evidence of irreversible injury (within 80 micron of the boundary) in the present study may constitute a similar population.

An alternative interpretation can be made using the findings of Page and Polimeni (1977), who showed outside infarcts a zone of unspecified width consisting of relaxed myocardial cells in which there was no other morphological evidence of ischaemic injury. The present study cannot exclude the presence of such a population of functionally but not structurally abnormal cells forming a border zone.

From the evidence of the present study, it is likely
that the extent and relative mass of myocardium showing reversible injury (which would implicitly be potentially salvageable) at the lateral margins of regional myocardial infarcts in rabbits is very small, both in absolute terms, and by comparison with the substantial transmural border zone also demonstrated. The lateral border zone observed was very considerably less than that claimed in some studies (up to 15 mm, or 30% of the infarct volume). Indeed, provided the observed boundary is a static one, the border zone is negligibly small. The possibility of a mobile though narrow evolving boundary is considered further below (p. 175).
The results of the present study indicate that substantial changes occur in the functional status of the microvascular supply to the lateral border zone during the first four hours of ischaemia. The most striking changes occur in the uniformly ischaemic region without significant difference between the boundary and the centre of the developing infarct. The entire region undergoes an early hyperaemic response with a dramatic rise in the proportion of potentially functional capillaries to about 90%, which is superseded by a profound reperfusion defect (with less than 10% of capillaries filled by resin) corresponding to the no-reflow phenomenon.

There was a very close spatial correlation between the appearance of morphological signs of severe ischaemic injury to myocytes, the loss of filling by resin and the predominance of collapsed unfilled capillaries, such that the perfusion boundary showed an abrupt lateral margin corresponding to the morphological margin of severe ischaemia. Once evidence of severe myocardial cell injury had appeared, (after 60 but not 30 minutes) there was no evidence of a hyperaemic border zone suggesting a physiological response to ischaemia, rather, a profound perfusion defect occupied the whole of the severely ischaemic region.

The findings of the present study are in close agreement
with many recent studies (reviewed pp. 57-9) using light microscopy and intravascular silicone rubber markers (Okun et al., 1979; Factor et al., 1981; 1982), intravascular fluorescein and NADH fluorescence (Harden et al., 1978; Simson et al., 1979; Harken et al., 1981; Bittar and Little, 1981), radioactive embolic microspheres Hirzel et al., 1977; Patterson and Kirk, 1980, 1983) macroscopic patterns of injected dyes (Kloner et al., 1975; Reimer et al., 1977; Gavin et al., 1978a; Reimer and Jennings, 1979; Connelly et al., 1982), and micro-autoradiograms of the microvasculature (Grayson et al., 1974; Lee et al., 1980; Murdock, Harlan, Morris, Pryor and Cobb, 1983) in rabbit, dog, pig, monkey and post-mortem human hearts. The conclusion drawn from these studies is that the boundaries between microvascular beds supplied by adjacent coronary arteries are abruptly demarcated (though interdigitated) with little evidence of capillary level mixing, or intermediate levels of flow.

This conclusion has been extended by the present study which demonstrated for the first time the cell to vessel relationship and thus the microscopic abruptness and coincidence of the interfaces between perfused/unperfused, normal/ischaemically injured myocardium in the important first four hours after coronary occlusion. Such a situation was surmised to exist, in rabbit hearts at least, as long ago as 1959 (Caulfield and Klionsky, 1959) but its confirmation awaited the development of suitable techniques to demonstrate it conclusively.
Not all previous investigators have shared this view. In studies using injected microspheres or radiographic tracers (Becker et al., 1973; Rivas, et al., 1976; Schaper and Pasyk, 1976; Vokonas et al., 1978; Jugdutt et al., 1979a, b) zones with intermediate levels of blood flow interposed between normal and ischaemic regions have been detected. Recent reviewers (Hearse and Yellon, 1981; Murdock et al., 1983) have suggested that some of these data indicating intermediate levels of zonal blood flow have resulted from summation of an admixture of interdigitated but abruptly demarcated normal and ischaemic tissue at the border zone which would be expected to yield an intermediate value.

The results of the present study show that the changes at the lateral boundary are more complex than either of these opposing views indicate. While the transition from near complete absence of reperfusion (no reflow) in the ischaemic zone, and presence of microvascular function in the control zone was abrupt, from capillary to capillary, and spatially coincident with the appearance of morphological changes indicating irreversible injury to cardiac myocytes, a modest reduction in the proportion of resin filled capillaries was observed in a 150 micron wide zone of morphologically non-ischaemic myocardium immediately adjacent to the lateral boundary of the ischaemic zone.

This reduction was dissimilar to the ischaemic zone in that it did not represent a profound perfusion defect, nor
was it the result of a greatly increased proportion of unfilled and collapsed or compressed capillaries which was characteristic of the adjacent ischaemic zone. Rather, the moderate reduction (30 - 40% compared to 55 - 60% in distant normal regions) was the result of a significantly increased proportion of unfilled capillaries which were observed to have round or oval open profiles.

This "low-flow" zone was observed after 60 or more minutes of ischaemia, but qualitatively did not form a uniform zone of unfilled, open profile capillaries interposed between normal and ischaemic tissue, rather it consisted of single or clustered open, unfilled capillaries seen with increased prevalence but patchily distributed in a region up to 150 microns from the lateral margin of the infarct. In some areas the pattern and proportion of filling of capillaries characteristic of distant normal myocardium was seen right up to the lateral margin, while in other fields, extensive clusters of open-unfilled capillaries were noted, in both cases the accompanying myocardial muscle cells showed normal "non-ischaemic" appearances. As a result of this variation, the statistical difference in proportion of filled capillaries between normal appearing myocardium adjacent to and distant from the lateral margin varied, as did the significance of the increased proportion of unfilled open capillaries in the normal adjacent zone compared to the normal distant zone. This indicates variance between fields (indicated by large standard deviations) and differences between animals.
(analysis of variance indicated low levels of significance) are wide, so that a contiguous separate zone of reduced flow or evolving changes within this zone of reduced flow were not apparent. The pathogenesis of this patchy phenomenon is unclear, but three possible explanations for its development are presented.

In studies of isolated crystalloid perfused rabbit hearts with regional ischaemia of brief duration (5 minutes), a uniform zone (so called 'oxygen diffusion zone') lying between the lateral margin of the perfusion bed of the unoccluded coronary arteries (as demonstrated by fluorescein injection) and ischaemic tissue in the distribution of the occluded artery (demonstrated by NADH fluorescence) has been described (Simson et al., 1979; Harken, et al., 1981). These authors have shown that this uniform zone between normal and ischaemic regions is unresolvable (at their resolution limit of 50 microns) in vivo blood perfused hearts, and that there was a linear relationship between zone width and cardiac oxygen consumption in isolated crystalloid perfused hearts. The maximum size of zone they demonstrated was 500 microns in perfused hearts arrested in diastole by high potassium concentrations in the perfusate, but a typical value of 150 microns was found in Langendorff-type hearts performing similar work to those used in the present study. Such a uniform zone is not consistent with the evidence of reduced flow observed in the present study. The higher resolution used here may have allowed observation of some evidence of capillary perfusion...
which would have appeared totally absent in the studies which depend on gross fluorescence. Furthermore the present study demonstrated a low-flow zone deep within the myocardium, rather than just at the epicardial surface where it was observed by Simson et al., (1979). Therefore the presence of a reduced flow zone between 60 and 240 minutes in the present study, and an "oxygen diffusion zone" after 5 minutes of ischaemia in the studies of Simson et al., (1979) may be manifestations of different phenomena.

Nonetheless, it is possible that small areas of myocardium adjacent to the lateral margin of the infarct in hearts in this study are maintained by oxygen diffusion from well-oxygenated neighbouring areas, though this leaves unexplained the observed patchy distribution of unfilled open capillaries.

It has been suggested (p. 24) that rapidly rising H+ in ischaemic tissue may be responsible for the rapid (within seconds) loss of normal contraction in regional ischaemia. A zone of such functional but not structurally changed cardiac muscle cells may offer a second explanation for the patchy reduction of capillary function. It is conceivable, though difficult to test, that H+, lactate or other metabolites diffusing from the ischaemic zone might result in loss of contractility in adjacent relatively well-oxygenated tissue. Such a non-contractile zone might be expected to have a reduced oxygen demand, and thus a homeostatically reduced blood supply. Because normal
control of oxygen supply is achieved through alterations in intermittent perfusion of individual capillaries (the "twinkling phenomenon" - Vetterlein, et al., 1982), the reduced blood supply would be evident as a decreased proportion of resin filled capillaries. As the non-contractile zone is producing neither normal systolic tension nor ischaemic contracture tension, the unfilled capillary pathways would also be expected to maintain an open profile.

Confirmation of such a zone would require demonstration that the myocardial muscle cells in the zone adjacent to the infarct are paradoxically relaxed and the proposal of some mechanism responsible for that relaxation. Simultaneous demonstration of the state of relaxation of myofibres (seen in longitudinal section) and the relationships of vascular function to individual myocyte injury (the aim of the present study) was not possible because of the different orientations of tissue required. However some evidence to support this hypothesis has been given by Page and Polimeni, (1977) who have shown by TEM a 'border' zone of myocardial cells which appeared perfused by the unoccluded artery but paradoxically had relaxed myofibrils following fixation by osmium, without any other evidence of ischaemic injury.

Shared venous drainage provides a third possible explanation for the patchy regions of open, non-functional capillaries observed near the infarct boundary. Epicardial venous drainage of resin from regions supplied by unoccluded
vessels through the perfusion bed of the occluded VIB region was observed, and this provides some support for this hypothesis. Similar observations of overlap of venous drainage and arterial supply have been made in studies of in vivo regional infarction in rat hearts (Camilleri, Joseph Fabiani, de Loche, Schlumberger, Relland and Carpentier, 1976) and in normal myocardium of many species (Ludwig, 1971; Brown, 1965).

If the obligatory venous drainage of some of the adjacent control region passed into vessels within the ischaemic zone, no-reflow could also affect a 'downstream obstruction' mechanism, some capillary beds supplied by the unoccluded artery. Such a mechanism would be expected to result in the observed dilated, open profile capillaries, which nevertheless cannot be perfused, and might also show a patchy distribution in a zone adjacent to the sharply demarcated perfusion boundary, depending on the degree of coincidence of arterial supply and venous drainage.

The relationship of the lateral margin of the eventual area of ischaemic necrosis to the original perfusion bed of the occluded artery must also be considered. If this marginal low-flow zone were to arise from venous occlusion in the no-flow zone, it is conceivable that infarcts would evolve by the advance of a narrow zone which gradually 'consumes' significant areas at the lateral border, even though at any one point in time the volume of imperilled myocardium appears insignificantly small (as observed in
the present study).

Connelly et al; (1982) present evidence that the circumferential extent of eventual necrosis in regional infarcts in rabbits does not increase after the first 15 minutes of ischaemia, and which is contrary evidence to this hypothesis. However the situation in other species, particularly in dogs is less clear. Different investigators have variously reported the lateral margin of eventual necrosis as being either 1-2 mm inside the lateral margin of the perfusion bed of the occluded artery (Reimer et al., 1977), identical with the perfusion bed (Geary et al., 1982), or even several millimetres outside the margin of the original perfusion bed (Sakai, et al., 1982). This last conclusion, which supports the hypothesis proposed above, claims that the authors' new method of assessing the perfusion bed of the artery to be occluded is more accurate than previously reported methods.

The recent demonstration by Janse and Wilms-Schopman (1982) that profound reduction in perfusion pressure in isolated perfused hearts causes a lateral shift in the electrophysiological boundary of regionally ischaemic zones may also indicate a mobile, but narrow boundary. The demonstration of the presence of this phenomenon in dog hearts, but not in pig hearts, also indicates that this may be related to important species differences.

In summary, this study has shown an abrupt lateral boundary between perfused and non-perfused regions and a
narrow and discontinuous zone just outside the infarct in which there is a reduced proportion of functional capillaries. Though insignificantly small in volume, this low flow zone could have an important role if it was mobile and not stationary. Most published evidence for such a mobile zone, which might traverse a significant region of "salvageable" myocardium at the lateral margin of myocardial infarcts is not favourable to this view.

However, further studies are required to clarify firstly, the relationship between fine structural appearances and functional characteristics of the cardiac myocytes associated with the open but non-functional capillaries of the 'low-flow' zone detected in this study, and secondly the static or mobile nature of such a zone. In addition, further experiments directed specifically at the transmural border zone might be undertaken to determine the biochemical or functional basis of the delay of irreversible injury to subepicardial cardiac muscle cells, and the relationship of those changes to cardiac muscle cells and their accompanying capillaries in regard to restoration of function through reperfusion.
In the present study, a profound ischaemic reperfusion defect consistent with the no reflow phenomenon was demonstrated to appear in the time interval between thirty and sixty minutes of ischaemia. The perfusion defect resulted in loss of demonstrable function of >90% of capillaries in the entire ischaemic zone, and was microscopically coexistent with morphological evidence of severe ischaemic injury to the closely associated cardiac myocytes. Both no reflow and severe ischaemic injury were seen after sixty minutes, but it is unclear whether, within the thirty minute interval prior to this, no reflow preceded evidence of severe myocardial cell ischaemic injury or not. It can however be definitely stated that no reflow did not appear much later than severe ischaemic injury has occurred to myocardial cells. Thus no reflow was closely linked to severe ischaemic injury, and was not an inconsequential late result of it.

The demonstration of the no reflow phenomenon in regionally ischaemic hearts is by no means unique to this study, though its occurrence in regional infarcts in rabbits does not appear to have been previously reported. The major contribution of the present study is the microscopic demonstration of the cell-to-cell correlation of the abrupt boundary of ischaemic injury at the lateral margin of the early evolving myocardial infarct and the abrupt margin to the ischaemic perfusion defect.
Previous studies have inferred that this might be so. Investigations using injected dyes in dog hearts with regional infarcts have macroscopically demonstrated a sharp lateral boundary to regions of both no-reflow and ischaemic injury (Grayson et al., 1974; Kloner et al., 1974, 1975; Gavin et al., 1978a,b) in the 30-90 minute period; however the close temporal and spatial coincidence of the two changes was not confirmed or studied at a microscopic level.

Darsee and Kloner (1981) have published contrary evidence from studies in in vivo infarcts in dogs, which indicated that, prior to six hours of ischaemia, the macroscopic lateral boundary of the region demonstrating no reflow, the lateral margin of the region at risk and the ischaemic margin (assessed by triphenyl tetrazolium staining) were separated, with the no reflow region always contained well within the ischaemic zone, leaving a lateral border of ischaemic tissue which did not show evidence of no-reflow. More recently these authors have claimed that the administration of the non-steroidal anti-inflammatory agent flurbiprofen, decreased not only the transmural extent of myocardial necrosis, but also the lateral extent of ischaemic necrosis and no-reflow (Darsee, Kloner and Braunwald, 1981). However the validity of the results of these studies is now uncertain (Kloner, 1982) as they have been withdrawn because of scientific fraud.

The results of the present study indicate that the no reflow phenomenon is spatially and temporally associated
with the collapse and apparent compression of capillaries. Alternative explanations suggested to explain no reflow (reviewed pp. 30-40), such as embolic occlusion of vessels or haemostasis, could not apply in these asanguinous preparations and there was no evidence of constriction of large vessels leaving the small vessels potentially reperfusable but without functional supply. This interpretation of vascular compression is in close agreement with those derived from studies of globally ischaemic hearts (Humphrey et al., 1982,a,b; Gavin et al., 1983a,b) and from an investigation (limited to vascular morphology only) of no reflow in regional myocardial infarcts of rat hearts in vivo (Camilleri, et al., 1976).

There have been two hypotheses advanced to explain the pathogenesis of this microvascular collapse or compression. Baghirzade et al., (1970) ascribed the compressed slit-like capillaries of the no-reflow zone of globally ischaemic guinea-pig hearts to the swelling of the adjacent cardiac myocytes (observed by light microscopy after 45 minutes ischaemia), and Fabiani (1976) extended these finding with his own studies in regional infarcts in in vivo rat hearts to reach a two-stage hypothesis. That was that the initial defect resulted from compression of the microvascular bed by ischaemic contracture of the cardiac myocytes (reviewed p. 36) which is subsequently re-established or rendered permanent by myocardial cell swelling and tissue oedema. Humphrey et al., (1980) studied globally ischaemic isolated hearts and proposed a similar two-stage hypothesis in which
ischaemic contracture compresses and closes the capillaries of the ischaemic zone, which are then rendered permanently inaccessible by the development of cardiac rigor mortis which prevents their reopening (reviewed. p. 38).

The observations of the present study of regional ischaemia are consistent with both hypotheses. There was evidence that myocardial cell swelling occurred and was associated with capillary compression, but shortening of ischaemic myocardium by contracture and changes in muscle stiffness could not be measured in this model.

In summary, the present study has shown that the lateral boundary of the no-reflow region in rabbit hearts coincides exactly with that of severe ischaemic injury to cardiac muscle cells. Both events have a closely related time course, so that reperfusion of the cardiac microvasculature to salvage cardiac muscle cells from progression to inevitable necrosis must be undertaken within a relatively short time from the onset of severe ischaemia.

Morphological evidence of significant capillary level collateral flow from the unoccluded vascular bed across the lateral margin of the developing infarct was not found in the present study. Indeed, the reverse situation of a "low-flow" zone was observed. This may represent a potentially mobile though narrow zone of loss of vascular function in the normal myocardium adjacent to the infarct, possibly induced by the no-reflow phenomenon affecting the venous drainage of both ischaemic and adjacent normal
regions. The significance of this zone needs further investigation.

The present study has indicated that the subepicardial region (transmural border zone) is most likely the only portion of the developing infarct which contains significant volumes of injured but potentially salvageable myocardium for an extended period. There is a need for further clarification of the intrinsic differences between sub-epicardial and sub-endocardial cardiac muscle cells, and the degree of intrinsic collateral blood flow in the development of both the wavefront phenomenon of cell death and the no-reflow phenomenon of loss of vascular function in the subepicardial region. Initially, studies of the cell-to-vessel relationships of microvascular function and irreversible injury to mid-myocardial and sub-epicardial cardiac muscle cells (requiring different tissue preparation techniques to those used in the present study), and studies of the effect of varied left ventricular volume load (with consequent diastolic stretching of the ischaemic myocardium) could be contemplated.
CONCLUSIONS

1. Isolated buffer-perfused rabbit hearts can be prepared, by separate cannulation and controlled perfusion of a branch coronary artery, to provide an experimental model which is stable for a period appropriate to investigations of early evolution of regional myocardial infarcts.

2. Fixation of isolated rabbit hearts by perfusion of the vascular bed with buffered glutaraldehyde solution preserves cardiac muscle cell structure for morphological examination and also stabilises the distribution of flow through the myocardial vasculature in a pattern closely similar to that pertaining immediately prior to fixation.

3. Intravascular injection of low viscosity acrylic resin into the glutaraldehyde fixed hearts is a useful method, with a low rate of artefact, for demonstrating functional capillary pathways.

4. The source of supply of individual capillaries can be determined by inclusion of a particulate heavy metal oxide to the resin injected into one coronary artery but not others.

5. Myocardial tissue with vessels containing polymerised acrylic resin can be prepared for scanning electron microscopy by cryofracture and freeze-drying after immersion in the colligative cryoprotectant, dimethyl sulfoxide. Provided a high performance backscattered electron detector and low-vacuum specimen chamber conditions are used, this
produces specimens without loss of morphological detail, charging artefact, or instrument contamination.

6. Scanning electron microscopy allows examination cell-to-cell and cell-to-vessel relationships of cardiac muscle cell ischaemic injury and microvascular function in large contiguous areas of the margins of developing regional myocardial infarcts.

7. There is, in rabbit hearts, an extensive subepicardial portion of the ischaemic region whose progression to cell necrosis is delayed relative to the subendocardial region. This most likely represents an area which, if blood supply was restored, could be 'salvaged' from inevitable necrosis.

8. At the lateral margin of regional infarcts in isolated rabbit hearts, the demarcation between normal and irreversibly injured myocardium is abrupt, typically cell-to-cell. A very narrow discontinuous zone (< 150 microns wide) showed areas with dilated sarco-tubular structures. Provided this zone does not advance with time, the potential demonstrated by this model for salvage of ischaemic myocardium at the lateral margins of infarcts by restoration of blood flow is negligibly small.

9. A profound ischaemic reperfusion defect corresponding to the no-reflow phenomenon develops with a very close spatial and temporal association to irreversible injury to cardiac muscle cells. It is associated with collapse and compression of capillaries and swelling of ischaemically
injured cardiac muscle cells.

10. A discontinuous zone characterised by a reduced proportion of functional myocardial capillaries lying among cardiac muscle cells showing little evidence of ischaemic injury was demonstrated at the lateral margins of developing infarcts in rabbit hearts. This zone may be due to the involvement of venous drainage of non-ischaemic myocardium through the adjacent ischaemic region manifesting the no-reflow phenomenon.
APPENDIX 1

THE USE OF ACRYLIC RESINS AS INDICATORS OF MICROTANGULAR FUNCTION

Introduction

Hodde and Nowell (1980) have indicated that polymerising acrylic resins are the most useful compounds for producing intravascular casts of microvascular beds for subsequent examination by scanning electron microscopy. Previous studies have used formulations composed of methyl methacrylate monomer which is prepolymerised by addition of catalyst and plasticiser under controlled heating (Murakami 1971, 1975; Murakami et al., 1973), or various proprietary formulations of prepolymerised methylmethacrylate of unstated composition, such as "Mercox" (Matsusaka and Fujibashi, 1974), "Batsons No. 17" (Nowell, 1972) and "Technovit 8001" (Keller, Schafer and Lubbers, 1972).

While complete filling of microvascular beds by formulations of relatively high viscosity (> 50 centipoise) has been demonstrated, there are obvious interpretative advantages in using resins with viscosities when injected close to that of blood (2.70 ± 0.12 centipoises. Diem, 1962). Recently, newer methods utilising ultraviolet light polymerisation of methyl methacrylate monomer (Gannon, 1980), and dilution of proprietary formulations with methyl methacrylate monomer (Mercox diluted; Ohtani and Murakami, 1978; Batsons No. 17 diluted - Nopanitaya, Aghajanian, and
Gray, 1979) resulting in base resin viscosities of 2-20 centipoises, have been used.

The aim of this preliminary investigation was to compare the available resins to determine the viscosity, setting time, and handling characteristics of each.

Materials and Methods

A simple capillary viscometer was constructed to test the viscosities of resins. A stand was constructed and checked for verticality with a spirit level. Disposable plastic 3 ml syringe bodies were used as reservoirs and attached 18 swg x 1½ inch stainless steel syringe needles as the capillary tubes. Test solutions were placed in the syringe body and allowed to flow freely through the needle shaft into a waste pot whose fluid level was constantly maintained just above the tip of the needle so that no dripping of solutions occurred. The time taken for the level of the upper reservoir to fall between two standard marks was measured for four samples of each test solution using a stopwatch.

A set of six standards, whose viscosity/density ratios at 20°C were known, were measured and the results plotted to give a standard curve. Aliquots of acrylic resins were warmed to 20°C, and four samples tested from each batch. The time for each sample was converted to a viscosity/density ratio from the standard curve and the value for each batch expressed as the mean ± S.D. of the
four viscosity/density ratios.

Four resin types were tested: prepolymerised methyl methacrylate prepared by a modification of Murakami's (1971) method as previously used in this laboratory (Humphrey et al., 1980, Gavin et al., 1983b); Gannon's method (1980) utilising ultraviolet light prepolymerisation of methyl methacrylate; the Nopanitaya et al., (1979) method of diluted Batson No. 17 base resin; and a new proprietary formulation which is an acrylic, non-methyacrylate resin (L.R. White resin, London Resin Co., Basingstoke, United Kingdom). The methods of preparation are described below.

Murakami (1971) method

Methyl methacrylate monomer (BDH, Analar grade) was redistilled to remove the hydroquinol stabiliser, and stored in dark bottles in the refrigerator. Benzoyl peroxide (0.050g) was dissolved in 10 ml of methacrylate monomer in a glass vial (Part A), then heated for 14 minutes in a 70°C glycerol-water bath. Dibutylphthalate (1g) and 0.1g benzoyl peroxide were mixed with a further 10ml of monomer (Part B), shaken intermittently for 5 minutes in a 70°C glycerol-water bath. At the conclusion of the heating period each vial was rapidly cooled in an ice water bath and stored in the refrigerator. Immediately prior to use, equal quantities of Part A and B were combined, and allowed to warm to room temperature. Three drops of dimethylaniline were added per 2.5 ml of base resin immediately prior to injection. Four
separate batches were prepared according to this method, and four samples of each tested for viscosity and setting (gel) time.

Gannon (1980) method

In this method methylmethacrylate monomer, which was not redistilled (i.e. contained the hydroquinol inhibitor) was exposed to ultraviolet light in glass scintillation vials. As an Oliphant FL8E 8 watt fluorescent tube described in the Gannon method was not available, three alternatives were compared.

A. Exposure to a Philips UV lamp.
B. Exposure to a Shimadzu chart reader UV fluorescent tube (long wave).
C. Exposure to a Shimadzu chart reader UV fluorescent tube (short wave).

Aliquots were measured for viscosity at 15 minute intervals for 0 - 240 minutes. Four batches were examined for viscosity and setting times.

Nopanitaya et al., (1979) method

Batson no. 17 resin was obtained (Polysciences Inc, Warrington, Pennsylvania, USA). Sevriton (Amalgamated Dental Trade Distributors Ltd, London, England) could not be obtained, but as it is described as a "commercial methylmethacrylate monomer", was replaced by redistilled methyl methacrylate monomer (BDH, Analar). In an ice-bath,
25.0 ml Batson's no. 17 base resin, 7.5 ml of catalyst and 0.5 ml of promotor were combined, and 12 ml of methylmethacrylate monomer added, and viscosity and setting times determined on four batches.

L.R. White acrylic resin

L.R. White acrylic resin was obtained in 500 ml units, remitted by airmail. "Medium" grade was used for all studies. The base resin was used unmodified from stock, allowed to warm to 20°C, and 1 drop (50 microlitres) per 10 ml of L.R. White accelerator, added and mixed immediately prior to use. Viscosity and setting times of two batches from each of two different 500 ml units of stock were determined.

Results

Times for transit of known volumes through the capillary viscometer for six standard solutions are given in Table 3. The standard graph constructed from these values shows a clear linear relationship between viscosity/density and transit time and is shown in Figure 64. Viscosity and setting times for the tested resins are given in Table 4.

Consistently high viscosity resin was obtained by Murakami's method. Though well outside the standard graph limits, the viscosity appeared by comparison with the drip-rate from a glass rod in glycerol to be of the order of 50-100 centipoises. Setting times of 2.5 ml aliquots in
Glass vials were in the useful range of 5-20 minutes.

Gannon's method did not produce consistent results. With the Philips UV lamp, no significant increase in viscosity could be detected between 0-45 minutes, then the resin rapidly underwent complete polymerisation to a glassy solid within the next 15 minutes. The temperature of the resin in glass scintillation vials after 15 minutes exposure to this lamp was 35°C. No significant increase in viscosity in any vials could be detected in samples exposed to the Shimadzu long wave length UV source. Samples exposed to the short-wave length Shimadzu source showed inconsistent results. Two batches showed values within the useful viscosity range at 180 minutes, but at that time one other batch had proceeded to a high viscosity (treacle-like consistency) while the remaining batch showed insignificant difference from monomer. The batches with satisfactory viscosity showed setting times in a useful range of 10-14 minutes.

L.R. White resin proved to have highly consistent viscosity on replicate samples and between batches. Setting times were also highly consistent (8-11 minutes) provided accelerator volume was maintained at a constant value. Increased quantities of accelerator could be used to reduce the gel time to as little as 2.5 minutes (3 drops accelerator to 2.5 ml base resin) which would be too short a time for useful application in intravascular perfusion.

The available trade-literature concerning L.R. White
resin emphasised complete solubility of monomer and partial polymers in ethanol and other non-polar solvents. Aliquots of resin containing Fat Red 7B dye (0.05 g/ml) (2.5 ml base resin plus 1 drop accelerator) were polymerised and allowed to harden for 48 hours, then shattered by a sharp blow from a hammer. Three sets of three weighed fragments were immersed in 5 ml each of absolute ethanol, 100% tertiary butanol, and deionised water respectively and maintained at 30°C for 48 hours. The colour of the supernatant was examined, the fragments air dried at 50°C for 48 hours and reweighed. The change in weight and colour of solution is given in Table 5. Weight loss and leaching of red dye was significant with ethanol immersion. Leaching of dye was obvious with t-butanol, but the samples consistently weighed heavier after drying than prior to immersion. Both sets of samples immersed in solvents were subjectively considerably softer than comparable water immersed, or untreated resin fragments, even after prolonged drying. Ethanol immersed fragments could easily be crumbled by slight pressure.

Discussion

The results of this study clearly indicate that L.R. White resin is the most useful of those tested with respect to viscosity and consistent setting time in an experimentally useful range. Additional advantages are the obvious savings of time inherent in a proprietary product which can be used direct from the stock bottle and L.R. White resin is claimed to be non-toxic by the manufacturers.
It certainly lacks the highly volatile, irritant, flammable and possibly mutagenic characteristics of methyl methacrylate monomer which necessitate use of gloves and fume hoods in handling.

These advantages remain even in comparison to Mercox CL resin, widely used in recent studies (Hodde and Nowell, 1980) but not available in New Zealand during the period of this study.

The solubility of L.R. White in ethanol is a definite advantage in terms of cleaning equipment, but is disadvantageous in scanning electron microscopic preparations which most commonly use non-polar solvents as dehydrating agents prior to freeze or critical-point drying. This study indicates that the solubility of relatively large fragments in vitro is significant, and it is probable that tissue blocks where the microvasculature had been filled with such resin would show similar or more severe effects as the surface area to volume ratio of the resin is greatly increased. The mechanism for the increase in weight of the resin in t-butanol is unclear, but the leaching of dye and softening of resin indicate that this solvent is probably not suitable for use in experimental protocols dependent on identification of small volumes of resin in lumina.

It is probable that the differences in viscosity seen in this study compared to that of Nopanitaya et al. (1979) result from changes to the Batsons no. 17 methacrylate base resin, rather than the substitution of methyl methacrylate
monomer for "Sevriton", because the base resin was observed to have a high viscosity, even though it had been stored in darkness at 4°C.

The inconsistency shown in the results of preparations made by Gannon's method may be due to important differences in wavelength and vial heating characteristics of the UV source used in the original method. However, even if a consistent preparation was obtained by this method, the handling characteristics of L.R. White resin are of greater benefit than the probable cost saving of preparing resin base from methacrylate monomer.

In summary, L.R. White resin showed the most consistent characteristics within the useful viscosity and setting times required for intravascular perfusion, and in addition offered some safety features lacking in other preparations.
APPENDIX II

TISSUE PREPARATION FOR SCANNING ELECTRON MICROSCOPY

Introduction

The results of preliminary studies of suitable acrylic resins for intravascular injection (Appendix 1) showed that L.R. White acrylic resin was the resin of choice. However its use made it necessary to develop methods which did not use non-polar solvents as dehydrating agents or transition fluids.

Recent reviews of tissue preparation methods for scanning electron microscopy (Nowell and Pawley, 1980) have indicated that the primary requirement of tissue preparation is stabilisation of highly hydrated biological structures to prevent distortion of the surface in high vacuum conditions. Usually this has been brought about by substitution of water by a solvent (e.g. ethanol, acetone, tertiary butanol), and subsequent freezing and sublimation of the solvent at high vacuum pressure (freeze-drying, FD) or sublimation by passing the tissue through the critical point (a pressure-temperature combination in a fixed volume) of the solvent (critical point drying, CPD). The critical point for water is well beyond that which could be attained with laboratory equipment. Sublimation, as opposed to simple evaporation is necessary to prevent the intense forces of surface tension disrupting small surface detail during drying.
Three possible alternatives to these methods have been used:

a. Freezing of tissue and examination in the scanning electron microscope in a frozen hydrated state
b. Freeze drying without previous solvent substitution
c. Freeze drying of tissue containing cryoprotectants to inhibit ice crystal artefact.

Lacking the sophisticated and extremely expensive equipment necessary for examination of frozen hydrated tissue, the latter two possibilities were investigated.

Materials and Methods

A total of six rabbit and twenty-two rat hearts were used in this study. All hearts were rapidly excised from anaesthetised, heparinised animals, arrested in ice-cold perfusate and attached to an aortic cannula for continuous perfusion for 15 minutes with modified Krebs-Henseleit bicarbonate solution (Armitage and Pegg, 1977). The perfusate was maintained at 37°C and 85 cm hydrostatic pressure, and was oxygenated by continuous bubbling of the reservoir with a 95% oxygen and 5% carbon dioxide gas mixture. Three groups of animals were used in this study.

Fresh frozen tissue

Hearts of two rabbits and six rats were frozen without prior treatment. One rabbit and two rat hearts were placed into 25 ml of thawing Freon-12 (-158°C) for three minutes and
then transferred to liquid nitrogen (-190°C) for five minutes. The hearts were then shattered by a blow from a precooled brass block, using a second pre-cooled block immersed in the liquid nitrogen as an anvil. Tissue fragments which showed transverse fractures across the ventricular wall were transferred to the stage of a tissue drier (Edwards-Pearse model EPD2, Edwards High Vacuum Co., Crawley, U.K.) and maintained at a maximum temperature and pressure of -65°C and 0.02 torr for 48 hours. Phosphorus pentoxide was included in the chamber as desiccant.

One rabbit heart and four rat hearts were dissected with solvent-cleaned single sided razor blades into transmural blocks less than 4 mm in the greatest dimension immediately after removal from the perfusion apparatus and these tissue blocks were then frozen in Freon-12 as described above. These blocks were fractured with a solvent cleaned pre-cooled razor blade under liquid nitrogen.

Dried tissue samples were mounted on aluminium stubs with Solderlene (Lambart and Smyth Ltd., U.K.) adhesive and examined after coating with carbon and gold in a Cambridge Stereoscan 2A (Cambridge Instruments, Cambridge UK) or ISI 60A (International Scientific Instruments Inc, U.S.A.) scanning electron microscope at accelerating voltages of 15-30 kV.
Frozen fixed tissue

One rabbit heart and two rat hearts were fixed by perfusion with 2.5% glutaraldehyde in 0.1M phosphate buffer for five minutes at 85 cm hydrostatic pressure and 4°C at the conclusion of the 15 min stabilisation period of perfusion. These hearts were immersed in 75-100 ml of fresh fixative at 4°C for at least 24 hours, and then transmural tissue blocks dissected and prepared as described above by freezing in Freon then liquid nitrogen, cryofracture and freeze drying, carbon and gold coating, and examination by scanning electron microscopy.

One rabbit heart and two rat hearts were prepared in a closely similar manner except that, following 24 hours of fixation, the heart was perfused with 10 ml 0.1 M phosphate buffer (at 90 mm Hg pressure) followed by 5 ml (rat heart) or 15 ml (rabbit heart) of L.R. White acrylic resin with 50 microlitres of accelerator per 10ml added immediately prior to injection. After 1 hour, to allow complete polymerisation of the resin, the hearts were treated in the same manner as those described above.

Two other rat hearts were injected with 5 ml of L.R. White acrylic resin with added accelerator at the end of the 15 minute stabilisation perfusion, then immediately the whole heart was immersed in 2.5% glutaraldehyde in 0.1M phosphate buffer at 4°C for at least 72 hours. Subsequently the hearts were dissected and scanning electron microscopy samples prepared by freezing, cryofracture, freeze drying
Cryoprotected tissue

Two rabbit hearts and 12 rat hearts were fixed by perfusion with glutaraldehyde after 15 minutes of stabilisation perfusion as described above. The hearts of four rats were then perfused via the aortic cannula with 5 ml of 2.82M dimethyl sulfoxide (DMSO) in 0.1M phosphate buffer, after which 5 ml of polymerising L.R. White acrylic resin was injected to two of the four hearts, and samples prepared for scanning electron microscopy as previously described.

Four other rat hearts were treated similarly, except that 30% glycerol in 0.1M phosphate buffer was perfused in place of DMSO solution. Tissue blocks from a further two rat hearts fixed and perfused with polymerising resin, and two rat hearts fixed but not perfused by resin, were immersed in 2.82M DMSO in 0.1M phosphate buffer for 12 hours on a rotary agitator. Similar tissue blocks from one rabbit heart fixed by perfusion were immersed in 2.82M DMSO and other blocks from the remaining rabbit heart were immersed in 30% glycerol in 0.1M phosphate buffer for 12 hours, then all blocks were prepared for scanning electron microscopy by the previously described cryofracture and freeze drying method.
Comparative preparation with solvent dehydration

Tissue blocks from rat hearts fixed by perfusion (the remaining tissue from those in the "cryoprotected tissue group" above) were dehydrated in an ascending series of ethanol solutions (immersed for 10 minutes each in 30, 40, 50, 60, 70, 75, 80, 85, 90, 95% solutions and three 10 minute immersions in absolute ethanol) prior to freezing in Freon-12 then liquid nitrogen and preparation for scanning electron microscopy by cryofracture and freeze drying. Similar blocks were dehydrated in tertiary butanol, through a series of 30, 40, 50, 60, 65, 70, 75, 80, 85, 90, 95% solutions at 10 minute intervals and three changes of 100% butanol (prewarmed to 28°C) prior to an identical cryofracture freeze-drying procedure. Other similar blocks were dehydrated in the ascending series of ethanols above, transferred through 50:50 then 25:75 ethanol/acetone mixtures in 15 minute steps to pure acetone (three changes of 15 minutes) prior to critical point drying using carbon dioxide as transition fluid in critical point drying apparatus (Polaron Model E 5000, Polaron Equipment Ltd, Watford, U.K.). Dried samples were mounted, coated and examined as previously described.

Shrinkage determination

Transmural tissue blocks of perfusion-fixed rabbit left ventricular myocardium, measuring approximately 4x4x4 mm were dissected and measured using the vernier scale of a light
microscope using the method of Wheeler, Gavin and Seelye, (1974). One set of five blocks was freeze-dried directly without solvent dehydration or cryoprotectant treatment, and remeasured. Another set of eight blocks was immersed in 2.82M DMSO in 0.1M phosphate buffer for 12 hours at 20°C on a rotary agitator and remeasured. Four of those blocks were subsequently freeze-dried (without cryofracture) as described above and then remeasured.

**Results**

The scanning electron micrographic appearances of myocardium frozen directly in a fresh state, either as a whole heart or after dissection into tissue blocks is shown in Fig. 65. Myocardial cells were severely disrupted by large intracellular defects which did not correspond in shape or distribution to any sub-cellular organelles. Capillaries were difficult to identify, and the definition of the intercellular space was poor.

There was a marked improvement in appearances seen in tissue fixed by glutaraldehyde prior to freezing and cryofracture (Fig. 66). Though cardiac muscle cells continued to show a markedly pitted fracture surface, the size of these artefactual defects was considerably smaller than in unfixed tissue. The intercellular space was more clearly defined and capillaries could be identified as thin-walled open tubes in tissue fixed by perfusion. Where acrylic resin was injected into the intravascular bed
following fixation by perfusion it filled some, but not all, capillary lumina with a glassy solid (Fig. 66B).

Immersion fixed tissue blocks of large dimensions (4x4x4 mm) showed different features to tissue fixed by perfusion of the intact heart (Fig. 66C). The defects in the fracture face showed an heterogeneous pattern, resulting in a combination of features resembling ischaemic myocardium and normal myocardium frozen fresh. When L.R. White resin was injected into hearts prior to fixation by immersion, filling of all types levels of vessels from arteries to capillaries and veins was noted (Fig. 66D).

The filling of vessels in hearts fixed by perfusion with glutaraldehyde and subsequently perfused by 2.82M DMSO showed unusual appearances, with the resin often rounded into spheres and sausage-shaped globules (Fig. 67A,B).

Where hearts were subsequently perfused with 30% glycerol, myocardial cells showed relatively compact fracture faces, with little evidence of the gross pitting evident in tissue prepared without cryoprotectant (Fig. 67C). However, the intercellular space often had an irregular fibrillar structure, which was particularly prone to 'charging' artefact. Where DMSO was used instead of glycerol, myocardial cells again showed compact fracture-faces relatively free of artefact, but 'charging' artefact (Fig. 67D), particularly within vessel lumina and intercellular spaces, was universal in secondary electron images.
When myocardium fixed by perfusion and subsequently immersed in DMSO was examined by backscattered electron imaging (Fig. 68) charging artefact was absent and cardiac myocytes showed compact fracture faces in which organelles where indicated by low steps and angles, fine pits and channels, and regularly repeated structures. The intercellular space was relatively clear, and fine filamentous connective tissue contracting myocardial muscle cells and capillaries could be discerned. Capillaries were seen as thin-walled, typically rounded, open tubes.

At zero specimen tilt angle, all these features could be discerned, but they were modestly accentuated by increasing specimen tilt angle, to produce relative shadowing (Fig. 68A,B).

Closely similar features were seen in perfusion-fixed myocardium prepared by dehydration with t-butanol or ethanol and freeze or critical-point dried (Fig. 69). The steps and angles corresponding to organelles were more prominent in dehydrated tissue, but organelles were not any better defined compared to myocardium prepared after fixation followed by cryoprotection in DMSO before freezing.

Fixed tissue blocks showed little change in linear dimensions following immersion in 2.82M DMSO (1.4 ± 1.6%), but subsequent freeze drying resulted in large changes (26.5 ± 9.4%) compared to the changes of fixed tissue blocks freeze-dried directly from water (5.4 ± 4.7%). Comparable figures are given by Wheeler et al., (1975) for freeze
drying from water (6.8 ± 3.2%), freeze-drying from t-butanol (15.4 ± 7.1) and critical point drying following acetone dehydration (22.4 ± 2.6).

Back-scattered electron imaging resulted in more useful photomicrographs than secondary electron imaging, even in dehydrated tissue, because of the elimination of charging artefact and improved contrast control. The edge-enhancement of the secondary electron mode accentuated further the steps and angles corresponding to organelles.

DISCUSSION AND CONCLUSIONS

This study showed that normal myocardial tissue prepared by perfusion fixation of the intact heart with buffered glutaraldehyde and subsequent immersion of dissected tissue blocks in a colligative cryoprotectant (DMSO, 2.82M in phosphate buffer) resulted in appearances which did not differ appreciably, from standard preparation methods using dehydration in non-polar solvents.

Backscattered electron imaging of coated specimens resulted in images of closely comparable resolution to the secondary electron detector over the (10-20,000x) range of magnification. As well, this mode abolished charging artefact which was particularly troublesome in cryoprotected tissue, and probably resulted from residual, highly hygroscopic cryoprotectant within the tissue rendering the specimens damp (Boyde, 1974; 1976).
Intravascular perfusion of cryoprotectants prior to intravascular injection with acrylic resin proved unsatisfactory because of the detergent effect of the cryoprotectant in causing the resin to form spheres and rounded forms within the blood vessels.

Direct freezing of fixed or unfixed tissue resulted in severe disruptive intracellular defects probably due to intracellular ice crystal growth during freezing (Meryman, 1974). The concentration of colligative cryoprotectant, DMSO, chosen to circumvent this ice crystal growth was based on the transmission electron microscopic studies of Shlafer and Karow, which were originally aimed at cryopreservation of myocardium for use in transplantation procedures (Karow, 1969; Shlafer and Karow, 1971a, b, 1972).

It was observed that differences existed between the proportions of capillaries filled by injected acrylic resin in hearts fixed by perfusion with glutaraldehyde prior to resin injection, and hearts fixed by immersion subsequent to acrylic resin injection. This indicated that possible vasoactive roles for fixatives and acrylic resins could not be ignored, and this view has previously been raised in discussion of experimental studies using microcorrosion casts (Hodde and Nowell, 1980; Olson, 1980). For these reasons a further study investigating the relationship between perfusion fixation and the proportion of capillaries able to be filled by resin was undertaken, and is described in Appendix III.
APPENDIX III

MORPHOLOGICAL IDENTIFICATION OF FUNCTIONAL CAPILLARIES IN THE MYOCARDIUM

Introduction

It is difficult to interpret the functional state of the microvasculature of heart muscle prepared for electron microscopy by conventional methods. Reasons for this include the fact that the effects on vessel patency of solutions used for immersion or perfusion fixation are largely unknown (Hollweg and Buss, 1980), and changes in the contractile state of the surrounding cardiac myocytes may also affect vessel diameter. Furthermore, the inference that open capillary profiles in thin tissue sections represent vessels which can be perfused may not be valid, as focal constrictions outside the plane of section at the level of precapillary sphincters or higher may restrict or prevent blood flow in capillaries which do not appear closed (Humphrey et al., 1981).

The present study has demonstrated that capillaries which permit flow can be identified by perfusion fixation followed by resin injection, and that the proportion of functional myocardial vessels so demonstrated is closely similar to that which was present immediately prior to fixation. This appendix has been accepted for publication in the Anatomical Record in an unaltered form.
**MATERIALS AND METHODS**

Eight male Wistar rats weighing between 250 and 275g were anaesthetized with diethyl ether and then heparin (1000 IU) was injected into the external iliac vein. The thorax was opened and the heart with its aortic root rapidly excised and immersed in ice-cold perfusate (see below). A polypropylene cannula was quickly ligated into the aortic lumen and via this cannula each heart was perfused at 37°C and a constant pressure of 85cm H2O for 10 min as a Langendorff (1895) isolated heart preparation. The perfusate was Krebs-Henseleit bicarbonate buffer with added colloid (Armitage and Pegg, 1977) oxygenated by gassing with 95% O2 and 5% CO2. All solutions perfused were filtered through 5 micron membrane filters. Coronary flow was measured after 1, 2, 5 and 10 min of perfusion by collection of effluent into a measuring cylinder for timed intervals of 30 seconds. Mean flow rates and the standard error of these means were calculated for each group and compared by a two-sample t-test (Huntsberger and Leaverton, 1970).

One group of four hearts received only perfusion buffer. In the other group procaine hydrochloride (McGaw Ethicals Ltd., Auckland, New Zealand) was added to the oxygenated perfusion buffer (final concentration 0.2%) for an additional 30 seconds of perfusion. Fixation was commenced for all hearts by perfusing with 2.5% glutaraldehyde in 0.1M phosphate buffer (Karlsson and Schultz, 1965) at 37°C and 85 cm H2O for 4 minutes. All hearts were subsequently
immersed in freshly prepared 2.5% glutaraldehyde at 4°C for 24 hr.

The fixed coronary vessels were flushed (90 mm Hg) with 20 ml of 0.1M phosphate buffer which was followed by 10 ml of L.R. White acrylic resin monomer (London Resin Co., Basingstoke, England) to which 50 ul of accelerator had been added immediately prior to its perfusion (90 mm Hg). After 2 hr at 20°C to allow polymerization of the resin, the atria and right ventricles were excised from each heart and transmural slices cut from the anterior and posterior left ventricular walls. These slices extended from apex to base along the long axis of the ventricle, and had an endocardial width of 3 mm. Following immersion in 2.82M dimethyl sulfoxide (Sigma chemical Co., St. Louis, U.S.A.) in 0.1M phosphate buffer (20°C, 12 hr) the slices were rinsed briefly in distilled water, blotted on filter paper and plunged into thawing Freon 12 (-158°C). After five seconds the frozen slices were transferred to liquid nitrogen (190°C) where they were transversely fractured on a precooled brass block by a precooled, single-sided razor blade. The frozen fractured blocks were then dried in an Edwards-Pearse tissue drier for 48 hr at a maximum temperature and pressure of -65°C and 0.02 torr. The dried blocks were brought to room temperature and pressure, mounted on aluminium stubs and coated with carbon then gold in a vacuum evaporative coater.

The transmural fracture faces were examined in an ISI 60A scanning electron microscope fitted with a Robinson
back-scattered electron detector and ISI
Charge-Free-Anti-Contamination System (ETP SEMRA, Sydney, Australia). Fields showing transverse sections of myocardial cells and accompanying capillaries were photographed (ten fields per block, twenty fields per heart) at 1000x magnification using reversed polarity to give positive images on the developed film.

Fields were projected onto the baseboard of a conventional photographic enlarger at 10x linear magnification. Capillaries were defined as vessels less than 8 microns in diameter with single-layered walls (Rhodin, 1967). The total number of capillaries per field, and the number of resin-filled capillaries per field were counted. Profiles in contact with or only partly included in the left hand and upper margins of these fields were counted, but those at the lower and right hand margins were excluded.

The mean number of capillaries per field and the percentage of capillaries filled with resin were calculated for each heart. Values from all all eight hearts were then compared by a one-way analysis of variance (Huntsberger and Leaverton, 1970).

Results

All the hearts spontaneously commenced to beat within 15 seconds of the initiation of coronary perfusion via the aorta. The heart beat ceased within 3 seconds of commencing
perfusion with either glutaraldehyde or procaine solution. When glutaraldehyde was perfused directly there was no observable contraction or dilatation of the ventricles, but procaine perfused hearts showed early relaxation resembling prolonged diastole.

Myocardial cells in transverse section showed irregular polygonal, relatively flat fracture faces with lines, low steps and angles indicating organelles which were not usually discretely discernable (Figs. 71 A, B). Vessels containing acrylic resin appeared as solid ovoid structures with smooth glassy fracture faces, whereas vessels without resin appeared as thin-walled tubes with open or collapsed lumina.

There was no significant difference in coronary flow rates between the two groups of hearts during perfusion with standard buffer (Fig. 70). However, after one (p<0.01) two and four (p<0.025) minutes of perfusion fixation the flow of fixative through hearts treated with procaine was significantly greater than through those which were not. The decline in rate of flow of fixative was significant in both the procaine treated (p<0.01) and untreated (p<0.025) hearts.

The mean number of capillaries observed per field and the proportion filled with acrylic resin are given in Table 6. Analyses of variance showed that, for the total number of capillaries observed, the difference between groups was not significantly greater than the differences within groups
at the 5% confidence level. However, for the capillaries which were filled with resin the percentages for the procaine treated group were significantly greater than those for the untreated group (p<0.005).

This study has evaluated the effects of perfusion fixation by glutaraldehyde on the pattern of distribution of blood flow through the microvasculature of the heart and has demonstrated that low viscosity resin injected at systolic pressures can be used to differentiate between functional and non-functional capillary pathways.

Cryofracture and freeze drying of intact tissue offers some advantages over previously used corrosion casting techniques (Olson, 1980; Gavin et al., 1983b) in that unfilled vessels are retained for comparison, and the non-vascular architecture is not destroyed by maceration. The ethanol-soluble, low viscosity (8 cps) proprietary resin used in this study has superior viscosity and handling properties compared to the previously used methacrylate-based resins (Hodde and Nowell, 1980), but required avoidance of non-polar solvent dehydrating agents and application of dimethyl sulfoxide cryoprotection, freeze drying and back-scattered electron imaging methods, a combination not previously applied to myocardial tissue. Such techniques can be used to study the microvasculature in whole animals. However, in the present study we used a well defined isolated organ preparation in order to obtain precise measurements of total coronary flow.
Fixation protocols using vasodilator or arresting agents have been developed to "prevent highly excitable heart tissue from contracting on contact with fixative, thereby preventing its equal distribution through the tissue" (Roy et al., 1971). Diastolic arrest induced by perfusion with solutions containing a high concentration of potassium (Loud, Anversa, Giacomelli and Weiner, 1978), or procaine hydrochloride (Odek-Ogunde, 1982), or high potassium followed by procaine (Gerdes, Callas and Kasten, 1979) have been used in light and transmission electron microscopic morphometric studies of the numbers of capillaries in different regions of the heart (Gerdes et al., 1979) and in myocardial hypertrophy (Loud et al., 1978; Gerdes et al., 1979) and hypertension (Odek-Ogunde, 1982).

At the concentrations used in this study, procaine hydrochloride is a profound vasodilator and causes diastolic arrest of cardiac myocytes (Lucchesi, 1977). It was therefore chosen to produce a high degree of vasodilatation and open capillary pathways. That this had occurred was indicated by the high flow of glutaraldehyde observed one minute after procaine treatment, a rate not significantly different from that of buffer immediately following excision and cannulation of the heart, when hypoxic vasodilatation (Belloni, 1979) would have developed. The high proportion (mean 95.2%) of capillaries which allowed the flow of resin in procaine-treated hearts indicated that absence of filling due to uneven polymerisation or embolic occlusion was uncommon.
The interesting and steady decline (about 25% in 4 min. Fig. 70) observed in both groups in the rate of flow of fixative does not appear to be due to loss of functional capillary pathways because 95% of them can still be filled. It seems much more likely that this reduction of flow was due to the effects of glutaraldehyde on muscular arteries which shrink both longitudinally and radially when fixed with this agent (Hollweg and Buss, 1980).

In the present study, the initial flow rate of glutaraldehyde through hearts fixed without procaine treatment was shown to be not significantly different from that of buffer through the beating hearts just prior to the commencement of fixation. Therefore the 62% of capillaries which allowed the entry of resin is likely to be close to the proportion of functional capillary pathways present in the myocardium. Kloner et al. (1974b) have demonstrated by light and transmission electron microscopy that all capillaries are wide open in myocardium fixed by perfusion with glutaraldehyde alone. It is thus not possible to determine the functional state of small vessels from their morphological appearance unless an indicator of flow is used.

Other authors, using different methods, have shown in normoxic hearts a functional reserve of capillaries in which flow is not occurring. Bourdeau-Martini et al. (1974) examined the epicardial surface of beating rat hearts in vivo and observed 2250 capillaries per mm² exhibiting flow
under normoxic conditions, but under profoundly hypoxic conditions this increased to 4400 capillaries per \( \text{mm}^2 \).

Using a double fluorescent tracer method, Vetterlein et al. (1982) studied the heterogeneity of flow in the subendocardial myocardium of Wistar rat hearts, and reported that the proportion of capillaries perfused over short time intervals was \( 64.1 \pm 6.7\% \), a figure remarkably consistent with the \( 62\% \) of subendocardial capillaries filled by the resin marker in the present study. This suggests that perfusion fixation with glutaraldehyde rapidly stabilises the pattern of flow through the microvasculature with a heterogeneity closely similar to that found in vivo.

We observed a low variance in the percentage of filled capillaries per 120 x 80 \( \mu \)m field in both groups of hearts. This indicates that the control of flow through capillary channels was a function of vessels supplying relatively small tissue volumes. Thus, the assertion that fixation without pretreatment may lead to unequal distribution of fixative (Roy et al., 1971) if true, can only apply to very small volumes, and is probably insignificant when the volume of glutaraldehyde presented (>40 ml) and its capacity for diffusion are considered.
APPENDIX IV

LABELLING OF INJECTED RESIN FOR THE IDENTIFICATION OF

ARTERIAL SUPPLY OF CAPILLARIES

Introduction

The use of acrylic resin to identify capillaries which permit flow is useful in investigations of relationships between microvascular function and myocardial cell death in experimental myocardial infarction. The ability to determine the source of arterial supply of individual capillaries within or adjacent to the border zone between perfusion beds of adjacent arteries would add considerably to the value of this method.

Markers containing chromatic dyes have been used in similar studies which have utilised epi-illumination light microscopy (Factor et al., 1978). Because scanning electron microscopy provides an electron-density image not a chromatic image, many of the markers suitable for light microscopy are unsuitable for SEM identification of vessels. Nevertheless, resin polymers used for microcorrosion casting studies have been labelled with chromatic dyes, such as Sudan IV, in previous studies (Murakami, 1971) to allow easier macroscopic identification unlabelled resin is colourless of resin in vascular beds.

Electron-dense substances, such as thorium hydroxide (Gavin et al., 1978 a) have been used as intravascular
markers in transmission electron microscopy studies, and could potentially be detected by back-scattered electron-imaging (BSI) or energy dispersive x-ray analysis (EDAX) techniques of SEM investigation. Preliminary studies (Appendix II) indicated that BSI combined with a simple environmental cell system (CFAS) was a suitable method for examining myocardial tissue containing L.R. White resin as intravascular marker of capillary competence, so it was appropriate to seek markers which could be used simultaneously with this imaging procedure. EDAX equipment was not available in this laboratory, and would be a very costly acquisition.

The aim of this preliminary study was to investigate a range of potential markers for use with BSI in myocardial tissue, and assess their effects on the L.R. White resin. The study is described in two parts: The first (A) describes the investigation of a range of potential markers and the second (B) the evaluation of the effects of one of those markers, lead dioxide, on L.R. White resin.
A. POTENTIAL MARKERS

Materials and Methods

All potential markers were prepared in 5 ml aliquots of L.R. White stock resin, at 20°C and in glass vials. Three parameters were assessed for each marker:

1. Macroscopic colour of each solution or suspension, and its eventual polymer.
2. Electron density of the polymer (BSI signal intensity).
3. Changes to the rate of polymerisation of resin induced by each marker.

The compounds screened were divisible into four categories.

1. High atomic number (Z) compounds soluble in L.R. White resin.
2. Chromatic dyes, soluble in L.R. White resin, but of unknown Z number.
3. Particulate suspensions of high Z number compounds.
4. Particulate suspensions of chromatic pigments, of unknown Z number.

Electron density was assessed by imaging uncoated fragments of polymer mounted on aluminium stubs with double sided tape in an ISI 60A scanning electron microscope (ISI Inc. Santa Clara, USA) with Robinson RB60 wide-angle scintillar-photomultiplier type back-scattered electron
detector (ETP-SEMRA, Sydney, Australia).

Polymerisation rate was assessed by measuring setting time of 5 ml of prepared resin in glass vials at 20°C, compared to similar volumes of unlabelled resin, to which identical volumes of L.R. White accelerator had been added. In one case dimethyl aminoethanol was substituted for L.R. White accelerator was made.

**Results**

The list of compounds tested and the relevant observations are summarised in Table 7. There was only one high atomic number compound easily available which was significantly soluble in non-polar acrylic resin, and that was uranyl nitrate. This formed a pale green solution, but at concentrations of 0-2 mg/ml this salt completely inhibited polymerisation when L.R. White accelerator was used. However prolonged heating at 50°C resulted in polymerisation. Substitution of dimethyl aminoethanol as accelerant resulted in a colour change to purple-black, formation of a coarse precipitate, and eventual, slow polymerisation. Fragments of the heat-polymerised resin showed higher electron density than plain resin when examined by BSI, but the difference in signal intensity was insufficient to reliably exceed that which could be produced by changes in orientation of surface planes of the fragments.

All of the tested histological fat stains proved highly
soluble in the resin stock, and in concentrations resulting in intense colouration (e.g. 2 mg/ml) did not detectably alter the setting time of the resin. However, none of these compounds showed any detectable increase in BSI signal compared to unlabelled resin.

Particulate pigments did not prove useful in the context of the requirements of this study. Although Batson's pigments provided a dense colouration to L.R. White resin, they tended to be excluded into aggregates during polymerisation. They did not alter setting time detectably, but neither did they show evidence of high atomic number constituents.

One particulate suspension of high atomic number was tested. Lead dioxide (PbO₂, lead Z = 82) was known to be produced by an electrostatic method which inherently produces particles of very small dimensions. A suspension of these particles in resin (the salt is insoluble in resin) was dark brown in colour and did not inhibit polymerisation, rather it decreased setting time by about 60% compared to unlabelled resin. After polymerisation the particles appeared evenly dispersed and showed an intense BSI signal intensity (Figs. 27,A,B) with high electron density (Fig. 72) compared to the resin polymer in which the particles were bound (predominantly carbon, Z = 6).

When the lead dioxide was used directly from stock to form the suspension, SEM images of polymer fragments and bright field microscopy of unpolymerised suspension
indicated a range of particle sizes from about 20 microns at the largest to less than 0.1 micron. Most particles appeared to be between 0.5 and 0.1 micron in diameter. Aggregates of small particles were common in both polymer and unpolymerised suspension.

**Discussion**

This survey produced a single suitable marker for positive identification of the source of arterial supply of capillaries containing acrylic resin, that was a suspension of lead dioxide particles. It was apparent that methods to refine stock lead dioxide in order to obtain only the smallest particles, and to eliminate particle aggregates from the suspension was necessary before the suspension could be used for intravascular perfusion without risk of embolic blockade to small blood vessels. Concentration standardisation at a level sufficient to allow few 'false negative' appearances in labelled capillaries was required. Demonstration that no appreciable differences in viscosity or setting characteristics to unlabelled resin with which the suspension was to be compared was also required before the marker could be experimentally utilised.

It was obvious that histological stains could be used to colour plain resin without altering its handling characteristics and thus give clear macroscopic definition of the patterns of supply of separately injected coronary arteries in the hearts of experimental animals. Fat Red 7B
dye provided the best colour contrast to the deep brown of the lead dioxide suspension. In retrospect, further search for a green or blue dye may have provided a better colour contrast for photographic assessment of dissected hearts.

There are many theoretical advantages in having two positive markers for use in adjacent arteries so that individual capillaries could be definitely labelled as either (A) or (B) or (A+B). The choice of only one positive SEM marker, lead dioxide, resulted in a slightly less desirable situation where capillaries were either labelled or unlabelled. But, provided the proportion of false negative identification is low, unlabelled resin can be considered as having one source of supply, and the labelled resin another. However it could potentially be very difficult to identify vessels with dual artery supply in which labelled and unlabelled were mixed.

The BSI detector used is claimed to have the ability to discriminate Z differences as small as 2% (ETP, 1982), but this is limited to adjacent areas of highly polished flat sections such as those used in investigations of the composition of alloys. With the addition of the surface topographic image component of a complex fracture plane such as that of freeze-fractured myocardium, the BSI image cannot be expected to result in such close discrimination of Z differences, which made the potential application of two different high Z particulate or soluble markers impractical. It is theoretically possible to use two soluble markers and
produced elemental ratios for each capillary using EDAX techniques, but this would be extremely time consuming considering the 5-10 minutes required to determine each ratio for each capillary, and the number of capillaries required to be examined for statistically significant results.
B. QUANTITATIVE ASSESSMENT OF LEAD DIOXIDE SUSPENSION

Materials and Methods

A separation method based on Stokes Law (whereby the rate of sedimentation of particles in a fluid of constant viscosity is directly proportional to their radius) was used to produce L.R. White resin containing only fine lead dioxide particles. It is fully described on p. - and consisted of serial disruption of particle aggregates by sonication, and sedimentation (centrifugation) to differentiate particle sizes. Concentration of particles was estimated by counting particles in a Neubauer Haematocytometer (American Optical Co. USA) by bright field light microscopy and three batches of known concentration were prepared: They were $1 \times 10^8$, $3.3 \times 10^7$ and $1.1 \times 10^7$ particles/ml.

Five millilitre aliquots of each of these concentrations were injected into glutaraldehyde perfusion fixed rat hearts and allowed to polymerise. Tissue blocks containing the resin were then dissected and prepared for SEM by cryoprotective (DMSO) immersion, cryofracture, freeze drying and coating with gold and carbon as described previously (p. 200). The proportion of capillaries filled by resin which contained the electron dense lead dioxide marker was estimated. Further aliquots of these three batches of resin were tested to determine their viscosity according to the method given in Appendix I. The setting time of triplicate
2 ml aliquots of resin in glass vials (particle concentration 1 x 10^9/ml) was then determined using L.R. White accelerator undiluted, and diluted in absolute ethanol to 75%, 66%, 50% and 33%. Plain resin aliquots were prepared in parallel as controls.

Myocardium from hearts injected with resin containing the lead dioxide marker was also prepared for transmission electron microscopy by post-fixation in osmium tetroxide, dehydration, embedding in epoxy resin, and thin sectioning, followed by examination a Philips EM300 transmission electron microscope. Several blocks previously examined by BSI were further examined by BSI and energy dispersive X-ray analysis (EDAX) using a Philips 505 scanning electron microscope in the School of Engineering, University of Auckland, to confirm the identity of high intensity BSI particles by elemental analysis for lead.

Results

The proportion of resin-filled capillaries containing the lead dioxide label is given in Table 8. Increasing concentrations of particles showed an increasing proportion of labelled capillaries, up to 80-90% at 1 x 10^8 particles per ml. This concentration was associated with a modest rise in viscosity/density ratio (Table 8) of 1.2 centipoises. Equal setting times between labelled and plain aliquots of resin were obtained when the accelerator for labelled resin was diluted to 66% with ethanol (Table 9).
Transmission electron micrographs demonstrate irregular, small highly electron dense particles within intracapillary resin (Fig. 72). Energy dispersive xray analysis confirmed characteristic spectra for lead in the particles showing high intensity BSI signal.

Discussion

From the results of this experiment it was seen that many, but not all resin filled capillaries demonstrated the lead dioxide label at a particle concentration of $1 \times 10^8$/ml. This was probably due to fracture through areas not containing particles, so it was concluded that a higher concentration ($1 \times 10^9$ particles per ml) should be used in experimental studies of microvascular function in regional myocardial infarcts.

The difference in viscosity between plain and labelled resin is small in absolute terms (1.2 centipoise), and when it is considered that complete filling of capillary beds can be achieved with resins of viscosities as large as 50-100 centipoise (such as Murakami's (1971) polymethyl methacrylate preparation), such a small difference is unlikely to contribute significantly to differences in the proportion of filled capillaries. Subsequently this was demonstrated to be so in control group hearts where the separately perfused region (perfused with labelled resin) showed no significant differences in the proportion of capillaries filled by resin to the remainder of the heart.
which was injected with plain resin (p. 122). That provided confirmation of the conclusion of this preliminary study, that lead dioxide particle suspension \((1 \times 10^9\) per ml) in L.R. White resin, using a 66% solution of accelerator is a suitable labelled resin to use in contrast to plain resin.
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