National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

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

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

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

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

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

AVIS

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

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

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

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, tests publiés, etc.) ne sont pas microfilmés.

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

MORPHOMETRIC ANALYSIS OF REGIONAL MYOCARDIAL PERFUSION
IN RATS AS MEASURED BY NON-RADIOACTIVE MICROSPHERES

by

E. Mary Korpan

Submitted in partial fulfillment of the
requirements for the degree of

Master of Science
(Physiology)

to

The School of Graduate Studies
The University of Ottawa
Ottawa, Ontario, Canada

January, 1987

E. Mary Korpan, Ottawa, Canada, 1987.

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-40713-1

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

TABLE OF CONTENTS

	page
Table of Contents.....	i
List of Tables.....	iv
List of Figures.....	vi
Acknowledgements.....	viii
Abstract.....	ix
INTRODUCTION.....	1
Methods of Measuring the Regional Distribution of Myocardial Blood Flow.....	9
The Microsphere Technique.....	12
METHODS AND MATERIALS.....	38
Experimental Design.....	38
Development and Verification.....	38
Animal Model.....	38
Surgical Procedures	
i. Preparation.....	39
ii. Left Ventricular Catheterization.....	39
iii. Left Atrial Catheterization.....	40
Microsphere Suspension.....	41
i. Composition of the Microsphere Injectate	
a. Single Injection.....	41
b. Multiple Injections.....	41
ii. Preparation of the Microsphere Suspension for Injection.....	41
Microsphere Injection	
i. Single Injection.....	42
ii. Multiple Injections.....	42
Morphometric Analysis	
i. Tissue Preparation.....	42
ii. Sampling Technique.....	43
iii. Calculations.....	45
a. Orientations.....	46
b. Deciles.....	47
c. SEN/SEP.....	47

	page
Statistical Analysis.....	49
Pressure-Induced Left Ventricular Hypertrophy	
Animal Model.....	49
Induction of Left Ventricular Hypertrophy	
i. Surgical Preparation.....	50
ii. Surgical Procedure.....	50
Post-Surgical Maintenance.....	51
Regional Microsphere Distribution Study	
i. Animal Model.....	51
ii. Experimental Procedure.....	51
Effect of Injection of a Standard Number of Microspheres on Microsphere Aggregation	
Animal Model.....	53
Composition of the Microsphere Injectate.....	53
Microsphere Injection.....	53
Morphometric Analysis	
i. Tissue Preparation.....	54
ii. Sampling Technique.....	54
iii. Calculations.....	55
Statistical Analysis.....	55
RESULTS	
Preliminary Studies.....	56
Microsphere Size.....	56
i. Coulter Counter-Channelyzer Analysis.....	56
ii. Light Microscopic Examination.....	57
Total Number of Microspheres Injected.....	58
Histological Preparation for Data Analysis	
i. Tissue Preparation.....	60
ii. Sectioning.....	61
iii. Staining.....	61
General Microscopic Observations.....	62
Effect of Injection Site on Myocardial Microsphere Distribution.....	65

	page
Microsphere Distribution in Hypertrophied Hearts.....	76
Effect of Injection of a Standard Number of Microspheres on Microsphere Aggregation.....	87
DISCUSSION.....	91
Regional Myocardial Distribution of Non-radioactive Microspheres Following Left Ventricular and Left Atrial Injection.....	93
Regional Distribution of Non-radioactive Microspheres in Hypertrophied Myocardium.....	100
Advantages and Disadvantages of the Technique.....	103
Technical Recommendations	
i. General Application.....	113
ii. Data Collection.....	113
iii. Calculation of the Subendocardial/Subepicardial Blood Flow Ratio.....	114
Summary.....	117
APPENDIX.....	119
REFERENCES.....	141

LIST OF TABLES

<u>Table</u>	<u>page</u>
1. The average number of microspheres per decile of the left ventricular wall thickness in control animals receiving left ventricular and left atrial microsphere injection.....	67
2. The ratio of subendocardial/subepicardial microsphere distribution (SEN/SEP) in animals receiving left ventricular and left atrial microsphere injection.....	75
3. Basic parameters at the time of microsphere injection for animals with abdominal aortic constriction and their sham-operated controls.....	77
4. The number of subendocardial, midwall, and subepicardial muscle fiber layers in 3 regions of the left ventricular free wall in animals with abdominal aortic constriction and their sham-operated controls.....	78
5. The average number of microspheres per decile of the left ventricular wall thickness in animals with left ventricular hypertrophy and their sham-operated controls.....	80
6. The ratio of subendocardial/subepicardial microsphere distribution (SEN/SEP) in animals with left ventricular hypertrophy and their sham-operated controls.....	86
7. Basic parameters for control animals receiving left atrial injection of a standard number of microspheres.....	89
8. The number of microspheres located singly and as aggregates in 3 zones of the left ventricular wall divided according to muscle fiber orientation.....	90
9. Approximate duration of data collection for the first experimental series, and for a hypothetical series in which the sampling area was adjusted to ensure a minimum of 400 microspheres per unit of tissue.....	110
1A. Summary of data from several selected studies which have used current methods to measure the regional distribution of myocardial blood flow in the left ventricular wall under various experimental conditions.....	119
2A. Fixation and staining procedure for frozen tissue sections. -Periodic Acid Schiff (PAS).....	135

Table

page

3A. The average total number of microspheres counted in each myocardial region sampled per heart as estimated from the mean values shown in Table 1 and Figures 6-8, 9-10 (upper panels) for animals receiving left ventricular and left atrial microsphere injection. The corresponding percentage error of measurement is shown to the right.....	137
4A. The average total number of microspheres counted in each myocardial region sampled per heart as estimated from the mean values shown in Table 5 and Figures 11-14 (upper panels) for animals with left ventricular hypertrophy and their sham-operated controls. The corresponding percentage error of measurement is shown to the right.....	139
5A. The average total number of microspheres counted in each myocardial region sampled per heart as estimated from the mean values shown in Table 8 for animals receiving left atrial microsphere injection. The corresponding percentage error of measurement is shown to the right.....	140

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
1. Schematic representation of the sampling technique.....	44
2. Schematic representation of the measuring device.....	43
3. Parameters recorded for each heart by the orientation method....	46
4. Parameters recorded for each heart by the decile method.....	47
5. Photomicrographs demonstrating microsphere embolization in 16 μ m thick sections of rat left ventricular myocardium.....	63
6. Variation in the distribution of the number of microspheres per decile of the left ventricular wall thickness in control animals receiving left ventricular and left atrial microsphere injection....	66
7. The average number of microspheres per decile of the left ventricular wall thickness in 4 sections from the mid-ventricular level in animals receiving left ventricular and left atrial microsphere injection.....	69
8. The average number of microspheres per decile of the left ventricular wall thickness in 3 regions of the left ventricular wall in animals receiving left ventricular and left atrial microsphere injection.....	70
9. <u>upper panel</u> : The number of microspheres in deciles of the left ventricular wall thickness in animals receiving left ventricular and left atrial microsphere injection. <u>lower panel</u> : Percentage of the total number of <u>microspheres</u> per decile present as aggregates.....	72
10. <u>upper panel</u> : The number of microspheres in 3 zones of the left ventricular wall divided according to muscle fiber orientation in animals receiving left ventricular and left atrial microsphere injection. <u>lower panel</u> : Percentage of the total number of microspheres per orientation present as aggregates.....	73
11. <u>upper panel</u> : The average number of microspheres per decile of the left ventricular wall thickness in 4 sections from the mid-ventricular level in animals with left ventricular hypertrophy and their sham-operated controls. <u>lower panel</u> : Percentage of the total number of microspheres per section present as aggregates.....	81
12. <u>upper panel</u> : The average number of microspheres per decile of the left ventricular wall thickness in 3 regions of the left ventricular free wall in animals with left ventricular hypertrophy and their sham-operated controls. <u>lower panel</u> : Percentage of the total number of microspheres per region present as aggregates.....	82

Figure

page

13. upper panel: The number of microspheres in deciles of the left ventricular wall thickness in animals with left ventricular hypertrophy and their sham-operated controls. lower panel: Percentage of the total number of microspheres per decile present as aggregates.....

84

14. upper panel: The number of microspheres in 3 zones of the left ventricular wall divided according to muscle fiber orientation in animals with left ventricular hypertrophy and their sham-operated controls. lower panel: Percentage of the total number of microspheres per orientation present as aggregates:.....

85

Acknowledgements

I would like to express my sincere gratitude to Dr. K. Rakusan for his supervision, guidance and, above all, his extreme patience throughout this study.

I am also very much indebted for the statistical and computer assistance given by K. Dulberg. The long hours she spent preparing computer programs will never be forgotten.

The technical assistance of C. Kuo, J. Gao and J. Peeke was also greatly appreciated.

Special thanks are extended to A. Bouchard for typing (and retyping) the very lengthy and complicated Table 1A.

To all the members of the Department of Physiology, past and present, thank you for your friendship and encouragement. And a special hello to a very patient listener...Janet MacLean with an "a".

And last, but not least, to my best friend Philip who had to endure all my frustrations throughout the "near-eternal" time it took to complete this thesis. Thank you for your love, understanding, and your Gord Hodanott impressions.

ABSTRACT

We have developed a morphometric technique for measuring regional myocardial blood flow distribution with a high spatial resolution in rats. The technique involves injection of non-radioactive microspheres and subsequent sectioning of the tissue to be studied, allowing for direct visualization of microspheres and muscle fibers. Because microsphere aggregation has been shown to be a significant problem, and may alter both estimates of total myocardial blood flow and its transmural distribution, we evaluated the effect of injection site (left ventricle vs. left atrium), the number of microspheres injected (5×10^6 vs. 1.5×10^5), and left ventricular hypertrophy (abdominal aortic constriction) on microsphere aggregation in the left ventricle of anesthetized rats.

In normal myocardium, aggregation varied around 28% following left ventricular or left atrial injection of the larger microsphere dose, and was more pronounced in the subendocardium, especially following left ventricular injection. With left atrial injection of the smaller microsphere dose, the degree of aggregation was much lower (7%), but the uneven distribution of aggregates persisted. In animals with left ventricular hypertrophy, when compared with their sham-operated controls, the degree of aggregation was significantly higher (31% vs. 25%, $p < 0.03$), and the transmural gradient of aggregation was steeper following left atrial injection of the larger microsphere dose. This was attributed, in part, to an increased coronary blood supply, especially to the innermost subendocardial layers.

These results suggest that the degree of aggregation and the transmural distribution of aggregates must be considered when interpreting data which are based on measurements of tissue radioactivity following injection of radioactive microspheres.

INTRODUCTION

Interest in the investigation of transmural myocardial perfusion in normal and pathological states derives from the well documented phenomenon of the vulnerability of the left ventricular subendocardium to ischemia and infarction (Mallory et al., 1939; Horn et al., 1950; Jennings et al., 1960; Griggs et al., 1972). This could be due to a discrepancy between subendocardial oxygen demand and blood supply.

A transmural gradient of intramyocardial tissue pressure exists in normal hearts, with systolic pressure being equal to or exceeding intracavitary pressure in the subendocardium, and approximating atmospheric pressure in the subepicardium (Johnson and Di Palma, 1939; Kirk and Honig, 1964; Brandi and McGregor, 1969; Baird et al., 1970; Armour and Randall, 1971). Systolic subendocardial blood flow is, therefore, impeded in the presence of a high oxygen demand, while the subepicardium remains adequately perfused (Downey and Kirk, 1974; Downey et al., 1974; Hess and Bache, 1976, 1979). This systolic transmural gradient of coronary blood flow has been described by a vascular waterfall mechanism (Permutt and Riley, 1963; Downey and Kirk, 1974; Hess and Bache, 1976); i.e., flow is determined by vasomotor tone when coronary perfusion pressure exceeds the surrounding tissue pressure, but becomes a function of tissue pressure when tissue pressure exceeds coronary perfusion pressure. The complex pattern of local stresses that occur within the left ventricular wall during contraction (Streeter et al., 1969; Fenton et al., 1978) may also partially affect blood flow gradients.

Although regional differences in myocardial oxygen needs may explain why left ventricular ischemic lesions are more likely to occur in the subendocardium, the majority of studies of transmural myocardial blood flow distribution

indicate adequate or overperfusion of the subendocardium relative to the subepicardium over the entire cardiac cycle under normal, resting conditions. Moir and DeBra (1967) have postulated that local autoregulatory adjustments in vasomotor tone during diastole, the predominant period of subendocardial flow (Buckberg et al., 1972), compensate for the enhanced subendocardial extravascular compression during systole, resulting in adequate subendocardial perfusion as long as normal coronary perfusion pressure and blood flow are maintained. Since the dilatory reserve of subendocardial vessels is decreased, maximal vasodilation in response to ischemia will abolish the transmural gradient in coronary vascular resistance, resulting in relative subendocardial underperfusion. This was confirmed by Griggs and Nakamura (1968), Becker et al. (1971), and Buckberg et al. (1972). However, Downey et al. (1975) failed to observe a redistribution of blood flow following maximal vasodilation in normal hearts, suggesting that autoregulation is not the only mechanism that compensates for the decreased perfusion duration and perhaps higher tissue diastolic pressure (Rouleau et al., 1979) in the subendocardium. A gradient of vascularity favouring the subendocardium was proposed as an additional means for ensuring adequate subendocardial perfusion (Moir, 1972; Downey et al., 1975; Wüsten et al., 1977; Archie, 1978; Rouleau et al., 1979; Eliassen et al., 1982). Transmural coronary blood flow distribution is, therefore, constant over a wide range of coronary perfusion pressures (Rouleau et al., 1979; Haunso, 1981), although coronary vascular resistance may be actively changing (Dole and Bishop, 1982). Only when coronary perfusion pressure is significantly decreased does subendocardial flow become inadequate, while subepicardial flow remains uncompromised until a much lower pressure level is reached (Haunso, 1981).

Clinical evidence of myocardial ischemia in patients with left ventricular hypertrophy in the absence of coronary obstructive disease suggests that myocardial perfusion may be insufficient to satisfy the increased metabolic demand of the hypertrophied heart. Various experimental models of left ventricular hypertrophy have been used to determine the adequacy of myocardial blood flow. Morphological aspects of cardiac hypertrophy are reviewed in detail by Ferrans (1984) and Wikman-Coffelt et al. (1979). Since vascular, biochemical, and ultrastructural differences between models have been observed (Ljungqvist and Unge, 1973; Lund and Tomanek, 1978), which would in part account for any discrepancies in myocardial blood flow determinations, this discussion will be limited to studies relevant to this thesis, namely those of pressure-induced left ventricular hypertrophy.

Studies by Holtz et al. (1977), Marcus et al. (1976), Mittmann et al. (1980), Mueller et al. (1978, 1979), O'Keefe and Hoffman (1976), and O'Keefe et al. (1978) have demonstrated that coronary vascular resistance per unit mass of myocardium in animals with left ventricular hypertrophy is elevated at rest and/or during maximal vasodilation, resulting in a decreased coronary flow reserve that is most pronounced in the subendocardium. It was, therefore, suggested that the cross-sectional area of the coronary vascular bed does not increase proportionately with the degree of hypertrophy. Direct measurements confirm that capillary density is decreased in hypertrophied hearts, especially in the subendocardial layers (Rakusan and Poupa, 1966; Lund and Tomanek, 1978; Rakusan et al., 1980; Tomanek et al., 1986). This may be attributed to absent or negligible capillary proliferation during hypertrophic growth of the heart. Although capillaries do not contribute substantially to resistance, the lower capillary density increases diffusion distance and decreases capillary surface area per unit of tissue, limiting tissue oxygen delivery.

Additional factors have also been proposed to contribute to the increased coronary vascular resistance in left ventricular hypertrophy. These include enhanced extravascular compressive forces (Mueller et al., 1978; Tomanek et al., 1986), an increase in medial thickness of the small coronary arteries (O'Keefe et al., 1978), enhanced development of diastolic tissue pressure (O'Keefe et al., 1978), especially in the subendocardium (Domenech, 1978), and arteriolar rarefaction (Tomanek et al., 1986). Although it is reasonable that all of these factors would have an appreciable effect on the total cross-sectional area of the coronary vascular bed and, thus, on coronary vascular resistance, there is no substantial evidence to designate any one of these factors as the basis for the decreased resistance in left ventricular hypertrophy. Data are presently either unavailable, insufficient, or controversial. For example, Tomanek et al. (1986) did not observe vascular hypertrophy in the early stages of pressure-induced left ventricular hypertrophy, although they did recognize that luminal narrowing of coronary arterioles and arteries due to medial hypertrophy may contribute to the elevated coronary vascular resistance with long-term hypertension.

Anatomical and functional changes may explain the vulnerability of hypertrophied hearts to ischemia. However, left ventricular hypertrophy due to pressure overload is generally not associated with significant limitations of total or regional myocardial blood flow under resting conditions. Only when hypertrophy is severe (Bache et al., 1981; Bishop et al., 1974; Johnson et al., 1978), or the heart is in failure (Zile et al., 1985) does subendocardial flow become inadequate, although this finding is not consistent (Holtz et al., 1977). Coronary blood flow remains autoregulated as in normal hearts because left ventricular function and myocardial oxygen consumption are not altered (Holtz et al., 1977; Malik and Geha, 1977; Marchetti et al., 1973;

Sasayama et al., 1976). The coronary blood supply is not limited even under conditions of increased metabolic demand (Marcus et al., 1976) as long as the flow reserve is adequate. However, maximal coronary vasodilation occurs earlier in all layers of the hypertrophied left ventricle, with subendocardial flow becoming compromised initially, while further vasodilatory capacity remains in normal hearts (Mueller et al., 1979; O'Keefe et al., 1978).

The transmural distribution of myocardial blood flow, and its regulation in normal and hypertrophied myocardium, are presented in several reviews (Hoffman and Buckberg, 1976; Marcus, 1983; Weber et al., 1983; Wicker and Tarazi, 1982). A survey of fifty selected studies which have used current methods (for a brief description, see "Methods of Measuring the Regional Distribution of Myocardial Blood Flow") to determine regional myocardial blood flow distribution under various experimental conditions is also presented in Table 1A in the Appendix. Studies are carried out exclusively on large animals due to technical limitations, and results are generally expressed by the subendocardial/subepicardial blood flow ratio, i.e., the ratio of flow in the inner- and outermost "layers" of the left ventricular wall obtained by multiple tissue sampling. The interested reader is advised to consult these and/or other references for details, but is cautioned that both inter- and intramethod variation may influence the results, as demonstrated by the wide range of variation in the myocardial blood flow measurements presented in this survey.

The radioactive microsphere technique is the most accurate method currently available for measuring the regional distribution of myocardial blood flow in experimental animals. Carbonized, plastic microspheres incorporated with a nuclide emitting a characteristic gamma energy spectrum are injected into the arterial circulation. Microspheres are assumed to be rheologically similar to red blood cells and, therefore, to distribute in proportion to regional myocardial blood flow if the basic criteria of the technique are satisfied (for a detailed discussion, see "The Microsphere Technique"), but are trapped in the microvascular bed. At the end of the experiment, the heart is removed and sectioned, and the blood flow in each myocardial tissue sample is determined by measuring the tissue radioactivity, and converting this activity to a flow rate by comparing it with the activity of an arterial reference sample withdrawn at a known flow rate (Makowski et al., 1968).

The large variation in regional myocardial blood flow measurements obtained using the radioactive microsphere technique, as mentioned above, raises the question as to whether the assumption of uniformity between microsphere and red blood cell flow distribution in the coronary vasculature is accurate. Using vital microscopy, Harell et al. (1977) have shown that microsphere distribution differs from that of red blood cells in the microvascular bed of the hamster cheek pouch. If this difference between the flow behavior of microspheres and red blood cells also exists in the coronary vasculature, estimates of regional myocardial blood flow distribution which are based on measurements of tissue radioactivity may be misleading, as will be demonstrated in the present study.

Although simultaneous, direct visualization of microsphere and red blood cell flow in the coronary vasculature is not possible at present, the flow

behavior of microspheres could be inferred from microscopic studies of the localization of individual microspheres in the myocardium. The few studies that have examined myocardial tissue on a global basis following microsphere injection (Consigny et al., 1982; Eng et al., 1984; Yipintsoi et al., 1973), however, have been inconclusive. Clearly, the myocardial distribution of individual microspheres requires detailed analysis. Unfortunately, such an approach is not feasible in large animal studies because of the extreme time requirement, and the difficulty in obtaining appropriate tissue samples for microscopic analysis. Therefore, it was our intent to develop a morphometric technique that would allow, for the first time, an accurate, detailed investigation of the distribution of individual microspheres in various small regions of the rat myocardium, and to draw reasonable inferences, based on our results, concerning the flow behavior of microspheres in the coronary vasculature and, therefore, the accuracy of the radioactive microsphere technique. Particular consideration was given to the effect of injection site (left ventricle vs. left atrium), the number of microspheres injected (5×10^6 vs. 1.5×10^5), and left ventricular hypertrophy induced by chronic pressure overload on microsphere aggregation.

We have used carbonized non-radioactive polystyrene microspheres in our experiments because a radioactive label was not required. Therefore, we have eliminated several potential problems associated with measurement of regional myocardial blood flow distribution using the radioactive microsphere technique, e.g. radiation hazards, radioactivity leaching into the suspending medium, energy overlap between different labels, and tissue sectioning. Determination of regional microsphere distribution also does not require calculation of absolute flows. Therefore, two factors common to the radioactive microsphere technique for quantitating flow have been omitted, i.e., accurate determination

of the total number of microspheres injected and arterial reference sample withdrawal. By omitting the second factor, we have minimized the potential hemodynamic alterations associated with injection of a large number of microspheres, especially in smaller animals.

METHODS OF MEASURING THE REGIONAL DISTRIBUTION OF MYOCARDIAL BLOOD FLOW

The distribution of blood flow within the myocardium has been intensely investigated. Many contradicting results have been obtained, and some of these appear to depend on the method used.

Only the basic principles of the conventional methods used are described in this section. Detailed descriptions of these methods, including their advantages and disadvantages, are presented in several reviews (e.g. Schaper, 1979; Marcus, 1983).

Diffusible Indicator Technique

i. Intramyocardial Isotope Injection Measurement of the externally monitored clearance of diffusible radioisotopes (e.g. hydrogen, ^{133}Xe) injected into the myocardium indicates inadequate subendocardial perfusion under normal conditions. Of all the methods available for measuring regional myocardial blood flow distribution, this is the least accurate (see review by Moir, 1972).

ii. Intravascular Isotope Injection Following injection of a diffusible indicator into the circulation, regional myocardial perfusion is estimated from the concentration of indicator in different myocardial tissue samples. The indicator (e.g. ^{42}K , ^{86}Rb , ^{133}Xe , radioactive antipyrine) must be physiologically inert, tissue uptake should be independent of metabolic processes, and corrections should be made for recirculation. Furthermore, the inverse proportionality between the rate of blood flow and tissue extraction must be considered.

iii. Inert Gas Clearance Only two methods are currently used for measuring regional myocardial blood flow, neither one of which can measure the transmural distribution of left ventricular perfusion.

a. A non-radioactive inert gas (e.g. helium, hydrogen, argon) is inhaled, and the integrated difference in arterial and coronary sinus gas concentrations, assumed to be proportional to myocardial blood flow per unit weight, is detected with gas chromatography.

b. ^{133}Xe is injected into a coronary artery, and the radioactivity in the myocardium is detected with a multicrystal gamma camera. Two-dimensional images of the heart are obtained, making accurate measurement of regional myocardial perfusion difficult.

Radioactive Microsphere Technique

This technique is based on the principle that labelled microspheres injected into the arterial circulation have rheological characteristics similar to red blood cells, but are trapped in systemic capillary vascular beds. Tissue radioactivity will, therefore, be proportional to tissue blood flow.

Positron Emission Computerized Tomography

This approach is the most recent development in cardiac imaging, and may potentially be an extremely accurate tool, both clinically and in research, for rapid, sequential, noninvasive quantification of regional myocardial blood flow. The method involves intravenous injection of a positron-emitting radionuclide (e.g. ^{13}N -ammonia) and timed arterial blood sample withdrawal. The absolute amount of indicator in multiple regions of the myocardium is

measured with a positron camera gated to the cardiac and respiratory cycles. The positron emissions are then corrected for attenuation, radioactive decay, and myocardial wall thickness.

These methods, with the exception of the one involving isotope injection directly into the myocardium, indicate adequate or overperfusion of the subendocardium relative to the subepicardium over the entire cardiac cycle in normal hearts. Although problems are inherent to all the techniques, they have permitted extensive insight into the regulation of the coronary circulation in normal and pathological states.

The most accurate method currently available for measuring transmural distribution of left ventricular perfusion in experimental animals is the radioactive microsphere technique, which is discussed in detail in the subsequent section.

THE MICROSPHERE TECHNIQUE

The radioactive microsphere - arterial reference sample technique has been used by many investigators for simultaneous determination of cardiac output and regional blood flow in both large and small animal models, and their distribution within specific organs and tissues in large animal models. It has been the preferred method of such studies since its introduction by Rudolph and Heymann in 1967. Most commonly used are insoluble, isotopically labelled, carbonized, plastic microspheres; these have a relative density of 1.30. Even more appealing are Sephadex (dextran) microspheres, since their relative density (1.12) is very close to that of red blood cells (1.10) (Heymann et al., 1977). However, Idvall et al. (1979) found a very good correlation between organ blood flows determined with carbonized and with dextran microspheres ($r = 0.997$).

Since intravascularly injected microspheres are rigid and larger than red blood cells, they are trapped in the microvascular bed where they meet their limiting vascular diameter; spheres have been observed, by cinemicrography, to firmly lodge in either the arteriolar or capillary network (Hales and Cliff, 1977). The distribution pattern of microspheres closely correlates with actual blood flow distribution (Heymann et al., 1977). The precision of the microsphere method primarily depends on the number of microspheres in organs and in the reference sample (Buckberg et al., 1971), and not on the radioactivity as was once believed; radionuclide labelling only allows for accurate calculation of the microsphere number (Heymann et al., 1977).

When correctly applied, the radioactive microsphere method is relatively simple, accurate, and reliable (Heymann et al., 1977). The basic requirements that must be fulfilled are:

1. microspheres must be uniformly distributed in the suspension medium prior to intravascular injection
2. immediately following injection, microspheres must be well mixed with and evenly distributed in the circulation such that the concentration of microspheres reaching all arterial branch sites is equal
3. each sample (organ, tissue specimen, or arterial reference sample) must contain an adequate number of microspheres to assure acceptable errors and confidence limits
4. the total number of microspheres injected must be accurately determined
5. microspheres must be efficiently trapped during their first passage through the microcirculation such that recirculation is insignificant
6. microspheres must remain entrapped until counted
7. microsphere injection, embolization, and reference sample withdrawal should produce insignificant hemodynamic effects
8. the microsphere method must be reproducible and correlate well with others

(Buckberg et al., 1971; Archie et al., 1973; Heymann et al., 1977).

If these criteria are satisfied, microsphere distribution will be proportional to regional blood flow.

(NOTE: the following discussion of the above conditions deals mainly with aspects applicable to both radioactive and non-radioactive microspheres.

Radioactivity aspects are omitted since our study involves the use of unlabelled microspheres.)

1. Microsphere Suspension To disperse microsphere aggregates, and to further ensure that condition 2 (see below) will be met, the microsphere suspension is ultrasonically agitated (times reported vary from 30 sec (Flaim et al., 1984) to at least 1 hr (Neill et al., 1973)) and/or vigorously mixed using a vortex shaker for several minutes prior to injection. The suspension must be injected immediately after mixing to avoid sedimentation of the spheres in the injection syringe (Bartrum et al., 1974). A drop of the suspension is microscopically examined to verify complete dispersion and uniform distribution of the microspheres (Hess and Bache, 1979).

Tween 80 (polyoxyethylene (20) sorbitan mono-oleate) is a surfactant [specific gravity approximately equal to that of microspheres (Wicker and Tarazi, 1982a)] often added to the suspension to prevent microsphere aggregation. However, when used in concentrations of 0.05% or greater it induces small but significant hemodynamic alterations in conscious dogs (Millard et al., 1977) and rats (Flaim et al., 1978). The use of 10% dextran, another microsphere suspending agent, is not recommended since it has severe hypotensive effects (Flaim et al., 1978) and causes anaphylaxis in the Sprague-Dawley rat (Voorhees et al., 1951).

Electron microscopy has shown that occasionally microsphere aggregates are the result of plastic bridges between the spheres and are, therefore, permanent (Heymann et al., 1977). Deterrent observations regarding manufacturers' specifications are not infrequent; other examples include inconsistent sizes and shape irregularities of microspheres (Warren and Ledingham, 1974; Flaim et al., 1984). Bacterial or fungal contamination of the microsphere suspension also increases the probability of aggregation (Heymann et al., 1977). This necessitates careful examination of the microspheres prior to their use.

2. Mixing of Microspheres The three major factors affecting the uniformity of microsphere distribution in the circulatory vessels are the injection site, microsphere size, and the total number of microspheres injected (Heymann et al., 1977). Only random differences in the microsphere concentration between different arteries should exist if mixing of microspheres with the blood is even and a sufficient number of microspheres are injected; regions with the same blood flow are highly unlikely to receive the same number of microspheres due to random variation of microsphere distribution (Foster and Frydman, 1978). Nonrandom distribution, probably due to incomplete mixing, is often seen, but is not a major concern as long as the minimum number of microspheres required for accuracy is present (see condition 3). Heymann et al. (1977) report that only when measuring blood flow within specific organs should microspheres distribute similarly to red blood cells; microsphere size and radial distribution within the cross-section of major blood vessels have no significant influence on measurements of blood flow distribution to major areas.

Controversy exists regarding the most appropriate microsphere injection site. In the first report using radioactive microspheres to study relative blood flow distribution (Rudolph and Heymann, 1967), injection of 50 μ m diameter microspheres suspended in 20% dextran into various venous sites in ~~fetal~~ sheep and goats was considered to result in adequate mixing and even distribution of microspheres within the circulation. This view was based on several observations: bilateral organs received similar blood flows, blood flow measurements obtained by this method closely correlated with those obtained using electromagnetic flowmeters, and microsphere aggregation was absent in thick kidney sections. However, measurements of the percentage of cardiac output delivered to the major vascular areas showed considerable variation, cardiac output values were higher than those generally reported,

and the survival rate was low, probably as a result of inadequate mixing, the inappropriate size of microspheres used, and the hypotensive effects of dextran. Subsequent methodological improvements have substantiated the somewhat ill-founded nature of the view held by Rudolph and Heymann (1967).

An injection site most distant to the first arterial branching theoretically should result in the most adequate mixing. Significant variability in myocardial perfusion following injection by percutaneous left ventricular puncture was reported by Kaihara et al. (1968). Complete mixing of microspheres with the blood was observed with left atrial injection (Kaihara et al., 1968; Domenech et al., 1969; Archie et al., 1973; Stanek et al., 1983).

Wicker and Tarazi (1982a) found that left ventricular microsphere injection in anesthetized rats consistently led to significantly greater variability of coronary blood flow measurements than did left atrial injection, although mean coronary blood flow values from each approach were not significantly different. Mean values and variability of systemic hemodynamic indices were similar following both atrial and ventricular injection. Therefore, they advocate that left atrial injections be used for accurate coronary flow determinations in both anesthetized and conscious (Wicker and Tarazi, 1982b) animals, whereas left ventricular injections are satisfactory only for determination of systemic blood flow and regional flow to relatively distal beds.

Factors interfering with adequate mixing during left ventricular injection, especially in smaller animals, include the proximity of the injection site to the coronary circulation, induction of preferential streaming of microspheres with the catheter placed in the outflow tract or against the myocardium, and blockage of the coronary ostium by the catheter, thereby decreasing coronary flow (Wicker and Tarazi, 1982a).

More recent evidence indicates that the catheter position, rather than the injection site, is the major factor influencing the degree of mixing of microspheres in the anesthetized rat preparation (Kobrin et al., 1984). Variability of coronary flow measurements was significantly greater with aortoventricular (left ventricular injection) than with atrioventricular (left ventricular or left atrial injection) catheterization. Furthermore, this difference in variability was alleviated in the absence of anesthesia, indicating that left ventricular injection provides adequate mixing for accurate coronary blood flow measurement in the conscious rat (Kobrin et al., 1984; Flaim et al., 1984).

Other studies also indicate that microspheres injected into the left ventricle are distributed systemically in proportion to regional blood flow. Although Buckberg et al. (1971) were aware that greater variability can result with left ventricular injections due to inadequate mixing, they found no significant difference in coronary blood flow with left atrial or left ventricular injections. To determine whether left ventricular injection introduces microspheres subendocardially, two different isotopically-labelled microspheres were simultaneously injected in two dogs, one type into the left ventricle and the other type into the ascending aorta just distal to the aortic valve (Neill et al., 1973). Distribution of myocardial blood flow was similar with both types of injections, and not significantly different from that observed with left atrial injection, suggesting that left ventricular injection results in adequate microsphere dispersion.

Foster and Frydman (1978) also found that left ventricular injection resulted in complete mixing of spheres in the aortic root of rats, based on their observation that microsphere distribution from rat to rat and between successive injections was not significantly variable. If mixing was inade-

quate, variation would be significant.

The same conclusion was reached by Idvall et al. (1979) following left ventricular injection in unanesthetized rats. However, they claim that with this injection some microspheres may be deposited on the endocardial surface of the left ventricle, giving unpredictably high values of coronary blood flow. Therefore, to determine coronary flow, excision of the inner lining of the left ventricle prior to measurement was recommended. In contrast, Fortuin et al. (1971) found no evidence of adherence of microspheres to the endocardial surface during their transit through the canine left ventricular cavity.

Although there is less variability with left atrial injection, transmural left ventricular blood flow can be accurately determined with either left atrial or left ventricular injection (Buckberg et al., 1971). This conclusion was supported by the finding that control left ventricular subendocardial/subepicardial blood flow ratios determined with left ventricular injection of 15 μ m diameter microspheres in anesthetized dogs (Einzig et al., 1980) were similar to those reported following left atrial injection of 15 μ m diameter spheres in either conscious or closed-chest anesthetized dogs. Einzig et al. (1980) also found significant heterogeneity of regional left ventricular subendocardial/subepicardial blood flow ratios following left ventricular injection; this is comparable with other studies (Ball et al., 1975; Cobb et al., 1974). If microspheres were not adequately mixed, this reproducibility should not have occurred. Distribution of blood flow to the right and left kidney and to the right and left cerebral cortex was equal, also suggesting uniform mixing of spheres with blood after left ventricular injection. Comparison of flow to symmetric organs or to two arterial catheters distal to the aortic root has been used by many investigators to verify adequate mixing, but even if similar blood flows are found, uniform mixing at the

level of the coronary ostia is not implied (Warren and Ledingham, 1974).

Bartrum et al. (1974) also suggested that left ventricular injection results in accurate blood flow measurements in all major organs of the rabbit, but the myocardial flow values obtained were higher than usually reported by other methods, probably due to inadequate mixing. Left atrial catheterization, however, was not recommended due to the instrumentation difficulties involved when using a small animal model.

If the radial distribution of microspheres within the blood vessel lumen is uneven, the problem of axial streaming has severe consequences. Specifically when studying coronary blood flow, spheres that are oriented toward the centre of streaming blood will be less likely to enter branches of the blood vessel and, therefore, more likely to lodge in the subendocardium, resulting in inaccurate determination of the subendocardial/subepicardial blood flow ratio (Domenech et al., 1969; Neill et al., 1973). Axial streaming is more pronounced with larger microspheres, with a direct proportionality between microsphere diameter and centripetal flow, and much less apparent with 7.5-10 μm diameter spheres, which have a radial distribution more closely approaching that of erythrocytes (Phibbs and Dong, 1970). The greater interaction between microspheres at higher concentrations would also influence their radial distribution within the blood vessel (Phibbs and Dong, 1970). Spatial restriction of 15 μm diameter, but not 10 μm diameter microspheres, may also result in inappropriate subendocardial/subepicardial blood flow ratios; 15 μm diameter spheres may be too large to enter a perfused blood vessel, and that vessel will, therefore, be considered to be unperfused with this technique (Clausen et al., 1981). Alternatively, Fortuin et al. (1971) postulated that both 15 μm and 50 μm diameter microspheres distribute in

proportion to myocardial blood flow in diastole. During systole, however, the larger microspheres tend to become trapped in the subendocardium whereas the 15 μm diameter microspheres more readily distribute to the subepicardial layers as do red blood cells. Therefore, 15 μm diameter microspheres may reflect actual myocardial blood flow whereas 50 μm diameter microspheres may reflect only diastolic flow. The improbability of this hypothesis, however, could be reasoned from the finding that measurements of the transmural distribution of myocardial blood flow using 7-10 μm diameter microspheres are similar whether myocardial flow occurs over the entire cardiac cycle or is limited to an equivalent period in diastole (Hess and Bache, 1976, 1979).

The degree of microsphere aggregation may also indicate the degree of mixing of microspheres with the blood. If serious, microsphere aggregation due to inadequate mixing may cause alterations in hemodynamic indices (see condition 7). Overestimation of organ blood flow is also a serious consequence. However, microsphere aggregation was histologically determined not to be a significant problem in skeletal muscle following aortic injection of 15 μm diameter microspheres in rats. Most spheres were located singly (83-99%), but some existed as pairs (1-12%) or multiples greater than two (0-5%) (Laughlin et al., 1982). Because of the concern of dislodgement of microspheres due to the thinness of the tissue sections, results using both 20 μm and 60 μm thick sections were compared; no significant differences were found between microsphere distribution in the two different sections. The absence of significant microsphere aggregation in skeletal muscle, however, does not imply that mixing of microspheres with the blood is adequate proximal to the site of injection. Nevertheless, similar results of microsphere aggregation were obtained from cleared myocardial specimens following left ventricular injection in dogs (Eng et al., 1984). The grouping of microspheres was suggested to represent

inadequate microsphere dispersion, or to be a consequence of the vascular anatomy; a blood vessel with branches in close proximity may have microspheres embolized at the ostia of the branch points, giving the appearance of an aggregate. Uneven microsphere distribution was also observed by Consigny et al. (1982) in 0.5-1.0 mm thick cleared tissue samples from the posterior left ventricular and septal walls, where microsphere distribution was greatest following sequential injection of two microsphere samples, one containing $0.5-1.5 \times 10^6$ 9 μ m diameter spheres, the other $0.5-1.5 \times 10^5$ 15 μ m diameter spheres, into the left circumflex coronary artery in dogs. The majority of spheres were located singly, although short chains of 2-6 spheres were observed.

In contrast, one study has shown that microsphere aggregation in the myocardium is a significant problem. In vitro microscopic examination of fixed myocardial tissue revealed a large amount of short microsphere chains in capillaries (Yipintsoi et al., 1973). However, this may have been the consequence of injecting a very large amount of microspheres into Langendorff, perfused, nonworking dog hearts. Thus, based on the few studies that have examined myocardial tissue following microsphere injection, it would appear that microsphere aggregation due to inadequate mixing is negligible, and would not affect coronary blood flow measurements provided that a relatively small number of microspheres are injected. Nevertheless, by directly visualizing red blood cell and microsphere flow in the microvasculature of the hamster cheek pouch using vital microscopy, Harell et al. (1977) have also shown that the microsphere chain phenomenon is significant, and occurs in vivo. Although microspheres were occasionally observed to enter blood vessels as aggregates, probably as a result of inadequate mixing, most chains were caused by the interaction of single spheres. In general, a single sphere would impact and protrude into an arterial blood vessel of a larger diameter

than its own. Red blood cell flow was not significantly obstructed, allowing more microspheres to flow into the vessel and collect behind the lodged sphere, thus forming a chain. The relationship between the number of microspheres in the microvascular bed and the percentage of microspheres found in chains was approximately linear. That microspheres are not always completely occlusive to blood cell and/or plasma flow has been reported by others (Hales and Cliff, 1977: in vivo detection of microspheres and red blood cells in the rabbit ear using cinemicrography; Eng et al., 1984: in vitro microscopic detection of microspheres in the canine myocardium following postmortem injection of a corrosion cast material (Microfil) into a coronary artery). Thus, microsphere distribution may differ from red blood cell flow, at least in some microvascular beds. Since a detailed analysis of regional microsphere distribution has never been completed, the view that coronary blood flow measurements are not influenced by microsphere aggregation due to either inadequate mixing and/or microsphere interaction, as mentioned above, remains unresolved.

3. Minimum Number of Microspheres per Sample To have a distribution variability within 10% of the actual flow at the 95% confidence level, each sample must contain 384 microspheres (400 chosen) of each type injected. Nonrandom distribution (probably due to inadequate mixing) is indicated if the 95% confidence limit is exceeded; however, as long as each sample contains at least 400 spheres this nonrandomness does not present much of a problem. Variability between left atrial and left ventricular injection is not significant if at least 400 microspheres are present per sample. Only when less than the required number of spheres are present per sample are errors high (Buckberg et al., 1971).

However, this mathematically derived determination may be overestimated.

Ishise et al: (1980) found that cardiac output in the rat can be accurately determined when more than 200 microspheres are present per sample; with this value, cardiac output determined with the radioactive microsphere technique correlated very well with cardiac output determined with an electromagnetic flow probe ($r = 0.96$). Therefore, for blood flow studies, at least 200 microspheres must be present per sample to ensure that the variability related to random distribution of microspheres is minimal.

Recently, Flaim et al. (1984) proposed that under certain experimental interventions resulting in a large change in magnitude of the measured parameter(s), less than 10% accuracy can be sustained, i.e., fewer than the required amount of microspheres can be injected, without losing the ability to detect the change.

To determine the minimal amount of microspheres to be injected to assure acceptable errors and confidence limits, specifically when measuring perfusion of a given organ, it is important to estimate blood volume, cardiac output, organ blood flow, and the number of tissue samples to be used (Hoffman and Buckberg, 1976). The following equation can then be applied:

$$A \times (\# \text{ of samples}) \times (CO/OBF) = B$$

where A = minimum number of microspheres required per sample

B = minimum number of microspheres to be injected

CO = cardiac output

OBF = organ blood flow

Since $OBF = (\text{organ's fraction of CO}) \times CO$ (Buckberg et al., 1971), the equation can be simplified to:

$$[A \times (\# \text{ of samples})] / [\text{organ's fraction of CO}] = B$$

The radioactive microsphere technique has been successfully applied in the small animal model. While this model has obvious advantages (Flaim et al., 1984), it is not without limitations. In addition to the technical problems involved, a very large microsphere aliquot may have to be injected to assure delivery of an adequate number of microspheres to each sample. The hemodilution and potential altered peripheral vascular resistance associated with such a large injection (see condition 7) places restraints on the number of microspheres that can safely be injected. This necessitates an increased number of experiments to compensate for the larger degree of variability in data associated with injecting less microspheres (Buckberg et al., 1971). However, even with injection of a large number of microspheres, only a small percentage of capillaries will be occluded since capillary density of myocardial tissue is approximately 3,000 capillaries/mm² (i.e., 6,000 capillaries/mm³, assuming a capillary length of approximately 0.5 mm) (Ludwig, 1971; Rakusan, 1971). Finally, for studying transmural myocardial blood flow distribution, the radioactive microsphere technique is restricted to larger animals since relatively large tissue sections from various myocardial regions must be obtained to assure the minimal number of microspheres required for accuracy is present. To date, transmural myocardial blood flow in the rat has not been studied with this technique due to this limitation.

4. Total Number of Microspheres Injected For determination of regional blood flow, cardiac output, and its fractional distribution using the radioactive microsphere technique, and to ensure that condition 3 (see above) is satisfied, especially in organs and tissues with low perfusion rates, the total number of microspheres injected must be carefully calculated (Heymann et al., 1977). The total amount of radioactivity administered, which is

proportional to the total number of microspheres injected, is accurately determined by counting the injection syringe and cannula before and after injection (large animals), or by counting the whole animal in a scintillation detector (small animals) (Idvall et al., 1979). However, such accurate determinations cannot be made when non-radioactive microspheres are used; the total number of microspheres injected can only be approximated based on their concentration in the suspension. Flushing the cannula with isotonic saline immediately after injection to direct the microspheres remaining in the syringe and cannula into the circulation increases the probability that all the microspheres have been injected. Fortunately, when studying transmural distribution of myocardial blood flow with a non-radioactive microsphere technique, knowledge of the total number of spheres injected is not essential since one is interested only in their relative distribution within the coronary microcirculation.

Injection of a very large number of microspheres is beneficial since accuracy of the microsphere technique increases with increasing numbers of microspheres injected (Buckberg et al., 1971). An upper limit to the number of microspheres that may safely be injected without altering hemodynamics has not been firmly established; it will vary with microsphere size, animal size, and probably organ blood flow (Buckberg et al., 1971). Multiple injections of the maximal or submaximal number of microspheres per injection appear to be advantageous over single microsphere injections of the maximal number of microspheres since the accumulation of microspheres is greater, provided that regional blood flow distribution remains constant throughout the duration of the series of injections. For a detailed discussion, see condition 7.

5. Recirculation One principle used to measure cardiac output distribution involves the injection of an appropriate indicator into the circulation, and measurement of its distribution during the first transit within the organ(s) of interest (Sapirstein, 1956). The amount of indicator entering an organ during its first transit is proportional to the organ blood flow. Similarly, to accurately measure regional coronary blood flow with radioactive microspheres, the spheres should be completely trapped in the myocardium during their initial transit so as not to underestimate the flow. Microspheres passing through arterio-venous shunts must be permanently entrapped in the lungs to prevent recirculation.

Downey et al. (1983) estimated that microspheres lodge very quickly in the canine myocardial vasculature following left atrial injection, based on their finding that Evans blue dye injected intra-atrially in dogs completely stained the left ventricular free wall within 2.5 sec. More variable transit times have been reported to hamster cheek pouch (2-20 sec) (Harell et al., 1977), canine femoral arterial sampling sites (to 60 sec) (Buckberg et al., 1971), and rabbit ear (2-120 sec) (Hales and Cliff, 1977) following left ventricular injection. This is possibly attributable to any one or a combination of factors: nature of flow, type of tissue (Hales and Cliff, 1977), injection rate (Buckberg et al., 1971), or transit distance.

Whether microspheres remain entrapped is questionable. Since diameters of capillaries and arterio-venous anastomoses may vary between organs and animal models (Heymann et al., 1977), and may be altered under varying experimental conditions (e.g. anesthesia, Kaihara et al., 1968; Warren and Ledingham, 1974), microsphere size must be carefully considered. In addition, increased sphere dislodgement from myocardium has been suggested to occur when coronary artery pressure exceeds aortic pressure (Consigny et al., 1982).

Nonentrapment of microspheres greater than 25 μm in diameter is virtually negligible (Kaihara et al., 1968; Creasy et al., 1974; Warren and Ledingham, 1974). However, since their rheological properties significantly differ from those of red blood cells, numerous problems are associated with their use (see conditions 2, 7).

With decreasing microsphere diameter, shunting is expected to increase; microspheres recovered from venous reference blood samples or in lungs are smaller in diameter than those collected in arterial reference blood samples (Archie et al., 1973; Consigny et al., 1982). However, findings are not consistent. Whereas substantial losses (1-8%) of 15 μm diameter microspheres from myocardium were found by Neill et al. (1973) in tranquilized dogs, less than 1% of microspheres 15 μm (Fortuin et al., 1971) and 8-10 μm (Buckburg et al., 1971) in diameter entering the myocardial vasculature were recovered from the coronary sinus in anesthetized animals. Archie et al. (1973) also reported negligible shunting of 15 μm diameter microspheres. However, for smaller spheres (4-12 μm diameter), nonentrapment averaged 3.6% in myocardium. This was attributed to microspheres less than 7 μm in diameter. Consigny et al. (1982) reported similar results, noting also that microsphere loss from the myocardium continued for 1 hr following injection.

Microspheres not trapped in peripheral vascular beds are recovered in the lungs. Reported values for the fraction of 15 μm diameter microspheres reaching the lungs following injection into the arterial circulation vary from less than 1% to 30% (Archie et al., 1973; Bartrum et al., 1974; Foster and Frydman, 1978; Idvall et al., 1979; Kaihara et al., 1968; Neill et al., 1973; Warren and Ledingham, 1974), with no correlation between the degree of nonentrapment and animal size.

Although one study reported a 3.7% loss of 9 and 15 μm diameter micro-

spheres from lungs over 2 hr (Consigny et al., 1982), entrapment in the pulmonary vascular bed appears to be virtually complete (Archie et al., 1973; Hales and Cliff, 1977). Fortunately, when studying regional distribution of coronary blood flow, recirculation is not a major concern since recirculating microspheres should distribute within the myocardium as during their first transit, provided that experimental conditions influencing coronary blood flow distribution remain constant over the duration of microsphere circulation.

6. Permanency of Location Carbonized microspheres are composed of a biologically inert plastic matrix and, therefore, should remain entrapped in the microvasculature where they initially lodged almost indefinitely. This view is supported by a study (Kaihara et al., 1968) in which the radioactivity in the heart and kidneys, as measured with an external detector, remained unaltered over a period of 2 weeks following injection in anesthetized dogs of microspheres 50 μm in diameter. In addition, negligible radioactivity appeared in the urine and feces over this period, indicating that the spheres initially injected had not been metabolized.

Hales and Cliff (1977) also reported that microspheres are not metabolized or lost from the tissue in which they were first trapped (following an initial loss within 5 min after injection). However, over the 8 week observation period, movement of microspheres within the tissue was revealed from still pictures of precisely defined fields. Within 2 days following injection, 70% of the microspheres (13.7 μm diameter) lodged in the rabbit ear microvasculature were displaced by a maximum of 150 μm . New microspheres occasionally entered a field, and an average of 20% were lost from fields within 5-25 days. After 3 weeks, most microspheres were extravascular, but there was no noticeable evidence of tissue damage.

In contrast, late microsphere losses from left ventricular myocardium were as high as 40% (average 13.8%), 28% (average 5.7%), and 11% (average 3.3%) for 9, 12, and 15 μm diameter microspheres, respectively, over 5 weeks in conscious dogs (Consigny et al., 1982). Losses of 12 and 15 μm diameter spheres were not significantly different. The majority of spheres leaving the heart, 98% of which became permanently trapped in the lungs, were less than 10.3 μm in diameter.

Long-term microsphere behavior may, therefore, vary under different physiological conditions (e.g. anesthesia), in different tissues, or for microspheres of different diameter.

7. Hemodynamic Effects The use of microspheres yields measurements most closely resembling capillary blood flow (Hales and Cliff, 1977). Whether the assessment of blood flow to small tissue regions is accurate has been questioned (Harell et al., 1977). Transient hemodynamic changes may occur during, or shortly after microsphere injection, thereby influencing both the observed values of total blood flow and the relative distribution of microspheres ~~to and within~~ the individual organs. According to Ishise et al. (1980), the hemodynamic steady state is affected only under conditions of low cardiac output and a high sampling rate. However, the incidence of hemodynamic changes also depends on animal size (cross-sectional area of the microcirculation), microsphere size, the total number of microspheres injected, and the occurrence of large microsphere aggregates (Heymann et al., 1977). Transient hemodynamic changes are more frequent the larger the size and number of microspheres injected.

The temperature of the injectate is also critical. Microspheres have been suspended in warm (37°C) isotonic saline prior to injection (Archie et

al., 1973; Henry et al., 1978), and injection syringes have been prewarmed to 37°C (Warren and Ledingham, 1974). For myocardial blood flow distribution studies, Heymann et al. (1977) recommend that the injectate be warm, especially when injecting into the left ventricular chamber, since a sudden cold flush can produce ectopic ventricular beats which may significantly alter the myocardial blood flow distribution pattern. An electrocardiogram or an arterial pressure pulse should, therefore, be monitored during the injection.

i. Large Animals Left ventricular injection of 25 μ m diameter (3×10^5 microspheres/kg) and 50 μ m diameter (3.6×10^4 microspheres/kg) microspheres in dogs (18-33 kg) resulted in an increased mean aortic pressure (significant in dogs with microsphere-induced cardiac lesions (see below); not significant in the absence of lesions) and left ventricular diastolic pressure, with no significant change in heart rate (Eng et al., 1984). Histological assessment of the left ventricle occasionally revealed discrete necrotic foci surrounding embolized spheres. Microfil (silicone rubber solution) subsequently injected into the coronary circulation generally did not pass around the occlusions. At sites of embolization where micronecrosis was absent, microspheres were not totally occlusive, as Microfil passed around the spheres to fill the distal part of the vessel. Prevention of necrosis produced by embolization of 25 μ m, but not 50 μ m diameter microspheres, by α_1 -blockade implies that an α_1 -adrenergic tonic mechanism is also involved in occluding the vascular lumen, possibly by preventing local metabolic vasodilation, and may persist long enough to produce cellular damage and necrosis. A spastic mechanism occurring at the microcirculatory level, as observed by Wiedeman and Tuma (1979) in the hamster cheek pouch and bat wing circulation following injection of 9 and 15 μ m diameter microspheres, was also

suggested. Necrotic lesions were more frequent and significantly larger in the epicardium and midwall, despite a greater number of microspheres in the endocardium, indicating transmural variability in sympathetic innervation and/or in α_1 -density in the left ventricle. These transmural differences may also be a consequence of the greater collateralization in the outer canine myocardium (Schaper, 1971); i.e., an arteriole in the outer myocardium may have more branches than one located endocardially, so that its occlusion would have more significant consequences.

It is conceivable that occlusion with smaller diameter microspheres would produce similar results at the capillary level since intramural capillaries receive substantial sympathetic innervation (Forbes et al., 1977). However, in a study by Consigny et al. (1982), microscopic examination of fixed canine hearts did not reveal any tissue damage in the left ventricular free wall around microspheres (9, 12, and 15 μ m diameter) embolized 1-5 weeks earlier, although macrophages were occasionally observed surrounding the spheres.

When a dose equal to, or greater than, 4×10^5 15 μ m diameter microspheres was injected into the canine left ventricle, a temporary small decrease in aortic and left ventricular end diastolic pressure was observed, although no detectable effects on the electrocardiogram or coronary venous blood PO_2 were determined 2-3 min following injection (Neill et al., 1973). The transient hypotension was probably mediated by dextran.

Each of three consecutive left atrial injections of 15 μ m diameter microspheres, in doses of 5×10^5 or 4×10^6 per injection, caused an immediate, insignificant, transient increase in canine coronary blood flow (Fortuin et al., 1971). Hemodynamic parameters measured 15 min after injection were unchanged for the lower dose. With injection of $4-12 \times 10^6$ microspheres, the

only significant change was an increase in mean aortic pressure. Since peak reactive hyperemic coronary blood flow following transient coronary artery occlusion was unaltered, the authors claimed that the reactivity of the coronary vascular bed remains unchanged following microsphere embolization. Considering the increase in driving pressure (significant) and coronary blood flow (not significant) associated with successive injections of the larger microsphere dose, peak reactive hyperemic flow in the presence of microsphere emboli was probably overestimated.

Similar observations regarding coronary vascular reactivity were reported by Monroe et al. (1971). Coronary blood flow progressively increased with repeated embolization of the vasculature of isolated dog hearts with 0.5-1.0 ml of a 2% suspension of microspheres averaging 10 μ m in diameter, suggesting effective vasodilator release. However, when a "critical amount" of spheres (value not specified) had accumulated, there was an abrupt decrease in coronary blood flow to control levels concomitant with a sustained, significant increase in left ventricular end-diastolic pressure, indicating decreased ventricular performance; this was attributed to both edema of the left ventricular wall and to focal shortening of sarcomeres where the circulation was compromised.

No significant hemodynamic alterations were observed following simultaneous injection of approximately 3×10^6 15 μ m diameter microspheres in dogs (Einzig et al., 1980), and five sequential injections of more than 1×10^6 15 μ m diameter microspheres/injection in fetal lambs (Heymann et al., 1977). Successive left atrial injections of 15 μ m diameter microspheres in dogs, with a total accumulation of 4.8×10^7 microspheres, also had no significant effect on systemic hemodynamics (Baer et al., 1984). Although approximately 3.5% (in autoregulating hearts) and 14.2% (in maximally vasodilated hearts) of the microspheres lodged in the myocardium, coronary pressure-flow

relationships were not altered. No gross redistribution of blood flow between the right ventricle, the left ventricle, and the interventricular septum was observed. Transmural distribution of blood flow across the left ventricular wall did not significantly change from control with serial injections.

The majority of investigators advocate the use of 15 μm diameter microspheres to study myocardial blood flow and its distribution, with the assumption that they satisfy all the criteria necessary for the microsphere technique, provided that they are not injected in critical doses. However, the in vivo rheology of smaller spheres (7-10 μm diameter) is closest to that of red blood cells (Phibbs and Dong, 1970). Up to 3×10^7 7-10 μm diameter microspheres have been injected in dogs without any apparent hemodynamic effects (Ellis and Klocke, 1979; Falsetti et al., 1975; Hess and Bache, 1979). Since larger values have been reported for 15 μm diameter microspheres (see above), it is reasonable to assume that this value can be exceeded in large animals.

ii. Small Animals Injection of up to 8×10^4 35 μm diameter microspheres did not change pulse rate or contour, blood pressure, or cardiac output distribution in rats (Mendell and Hollenberg, 1971). Similar findings were reported following two consecutive left atrial injections of 15, 25, or 50 μm diameter microspheres (5×10^4 /injection) in conscious rabbits (Warren and Ledingham, 1974). For larger doses (3×10^5 /injection), two consecutive injections of only 15 μm diameter microspheres yielded similar blood flow distribution measurements; significant hemodynamic changes with larger spheres included a transient decrease in blood pressure and cardiac output, transient bradycardia and/or irregular cardiac rhythms, acute hypertension (sometimes associated with convulsions and death with 50 μm diameter micro-

spheres), and occasional transient neurological signs.

Left ventricular injection of $2-4 \times 10^4$ 15 μ m diameter microspheres did not affect arterial pressure or heart rate in anesthetized rats (Nishiyama et al., 1976). However, with the same injection protocol in conscious rats, increased left ventricular systolic pressure associated with tachycardia was sometimes observed following injection.

Tsuchiya et al. (1977) reported that adverse hemodynamic effects, including decreased cardiac output, mean arterial pressure, and myocardial oxygen consumption, and increased heart rate, total peripheral resistance, and myocardial oxygen extraction, resulted when more than 1×10^5 15 μ m diameter microspheres had accumulated in rats. They suggested that a cumulative total not exceeding 1×10^5 microspheres may, therefore, be safely injected in rats, allowing at least 2×10^4 microspheres/injection for accuracy. However, the microspheres were suspended in 10% dextran, and part of the hemodynamic disturbances may have been a direct result of its hypotensive effects (Flaim et al., 1978).

The upper limit to the number of microspheres which may be safely injected in rats as suggested by the previous authors was exceeded by Foster and Frydman (1978) and Idvall et al. (1979); simultaneous injection of up to 4.5×10^5 15 μ m diameter microspheres did not significantly alter systemic hemodynamics in conscious rats. Rarely, the only symptoms observed during injection were minor, transient arrhythmias; cardiac output distribution was not significantly changed with repeated microsphere injections (Idvall et al., 1979).

Flaim et al. (1978) suggested that the upper limit to the number of 15 μ m diameter microspheres that can be administered in a single injection in conscious rats without producing significant changes in heart rate or

mean arterial pressure is 8.5×10^5 . However, measurements were made 10 min following microsphere injection, and acute hemodynamic disturbances may have been missed. More recently, Flaim et al. (1984) claimed successful administration, in a similar rat preparation, of a maximum of four microsphere injections, each containing approximately 1.5×10^5 15 μ m diameter microspheres, without significantly altering hemodynamics.

Ishise et al. (1980) observed that injection of 15 μ m diameter microspheres ($3-3.6 \times 10^5$ microspheres/injection) into the left ventricle of conscious rats resulted in good reproducibility of regional blood flow distribution to all organs. Following three consecutive injections at 10 min intervals, heart rate, mean arterial pressure, cardiac output, and total peripheral resistance remained unaltered; reproducibility of regional distribution of cardiac output was poor only to the liver, spleen (increased organ flow), stomach, and skin (decreased organ flow). It was speculated that central hemodynamics are maintained with multiple microsphere injections and reference sample withdrawals at the expense of altered cardiac output distribution to the splanchnic organs and skin.

Rat hemodynamics were not significantly altered with injection of 3.6×10^5 15 μ m diameter microspheres (Stanek et al., 1983). However, single injections of 7.2×10^5 microspheres significantly, although transiently, lowered cardiac output and heart rate during the injection period. After four serial injections of the smaller microsphere dose, the only significant hemodynamic disturbance was a decreased heart rate; a 10% decrease in cardiac output, and a 20% increase in total peripheral resistance were also observed, but these changes were not statistically significant.

Injection of 3×10^6 non-radioactive microspheres, with an average diameter of 11 μ m, into the aortic arch of anesthetized rats had no signifi-

cant influence on whole kidney GFR. However, when 1×10^6 15 μ m diameter spheres were injected, a 50% reduction in GFR was observed (Poujeol et al., 1975).

Arterial blood pressure transiently increased by 20-30 mmHg, while heart rate remained unchanged, with injection of 7×10^6 15 μ m diameter unlabelled microspheres into the aortic arch of anesthetized rats (Wisnes and Kirkebo, 1976). With a similar injection of smaller diameter spheres, transient hemodynamic disturbances may have been avoided.

Using cinemicrography, Hales and Cliff (1977) have shown that microspheres do not adversely influence the microcirculation in small animals. Approximately 8×10^6 13.7 μ m diameter microspheres were injected into the left ventricle or central ear artery in rabbits. Microsphere embolization resulted in the immediate formation of collaterals in the ear microcirculation. Neither a spastic phenomenon nor necrosis was evident. Adherence of red blood cell ghosts, but not of leukocytes or platelets, was occasionally observed. Intravascular microphage activity was absent up to 2 months following embolization. After 4 months, electromicrographs revealed macrophage accumulation around spheres located extravascularly.

It is apparent from the foregoing review that microspheres 7-15 μ m in diameter can be safely administered in relatively large amounts without significantly altering the hemodynamic steady state of both large and small animals. With few exceptions, central hemodynamic changes were insignificant or transient with injection of up to 4.8×10^7 microspheres in large animals, and up to 7×10^6 microspheres in small animals. However, whether cellular damage results from mechanical blockade of the vasculature is questionable. It is probable that microcirculatory events accompanying microsphere emboli-

zation vary between different organs and animal models, and are affected by the size and number of spheres injected.

8. Reproducibility and Correlation with Other Techniques Since the limits of variability decrease as the number of microspheres per sample increases (see condition 3), greatest reproducibility is obtained when the largest number of microspheres that can be embolized without producing alterations in coronary or systemic hemodynamics is injected.

Techniques currently used to determine regional myocardial perfusion have been discussed elsewhere. Inter- and intramethod discrepancies in regional blood flow determinations (refer to Table 1A in the Appendix) suggest that further evaluation and possible modification of the radioactive microsphere technique is necessary.

METHODS AND MATERIALS

Experimental Design

Three series of experiments were carried out. The purpose of the first series was to develop and verify a practical method to determine regional myocardial blood flow distribution in a small animal model using non-radioactive microspheres. The second series involved the application of the method to regional myocardial perfusion studies in animals with pressure-induced left ventricular hypertrophy. In both series, a large percentage of microsphere aggregates was found, and shown to distort estimates of myocardial blood flow distribution. This may have resulted from injection of a very large number of spheres. Thus, the occurrence of aggregation following injection of a "standard" number of spheres (approximately the median of the values reported for studies on rats) was the basis for the final experimental series.

DEVELOPMENT AND VERIFICATION

The final version of the methodological approach is described here. Details of, and the rationale behind the extensive preliminary studies conducted to determine the appropriate techniques are described in the Results.

Animal Model

Normal, Sprague-Dawley rats unselected for sex were used. Body weights ranged from 230-494 g at the time of the surgical intervention. Animals were housed in plastic cages, with 1-3 animals per cage, and had free access

to food (Standard Purina Rat Chow pellets) and tap water. Housing facilities were maintained on a 12 hr light/12 hr dark cycle, at 22°C, and a relative humidity of 50%.

Surgical Procedures

i. Preparation Prior to surgery, the animals were anesthetized with sodium pentobarbital (Somnotol, 45 mg/kg, i.p.). Anesthesia was maintained throughout the experimental procedure with supplementary doses as required.

Surgery was performed under antiseptic conditions.

ii. Left Ventricular Catheterization With the animal placed supine, limbs extended and restrained, a smooth-tipped polyethylene cannula (PE-10), stabilized by a metal stylet, was introduced into the right carotid artery and secured with a 4-0 silk ligature. The cannula was then advanced into the left ventricular cavity for subsequent injection of microspheres. To ensure proper placement, the cannula was advanced until its tip contacted the endocardial surface of the left ventricular apex. At this point large resistance to further advancement was met. The cannula was then slightly retracted into the cavity and, with the metal stylet removed, was flushed with 0.2 ml of heparinized saline (12.5 mg%). A minute volume of blood was withdrawn to ensure that the cannula tip was not positioned directly against the myocardial wall. The blood was immediately replaced (this process of withdrawing and replacing blood was repeated immediately prior to, and intermittently during, microsphere injection). Proper placement of the cannula was further verified at necropsy.

iii. Left Atrial Catheterization This surgical procedure follows Wicker and Tarazi's (1982a) method of left atrial catheterization for the purpose of microsphere injection in anesthetized rats, with moderate modifications. With the animal placed supine, the limbs extended and restrained, a bevel-tipped 14 gauge radiopaque teflon catheter was inserted into the trachea through a tracheostomy and secured with a 4-0 silk ligature. The animal was connected to a respirator (Type A.C. Small Animal Respirator, C.F. Palmer (London) Ltd.) and ventilated with room air. Next, the position of the left atrium was approximated by palpation, and the left atrium was exposed by small lateral thoracotomy through a left intercostal space. The pericardium was opened, and the left atrium gently supported with forceps. A bevel-tipped, balloon-tipped polyethylene cannula (PE-10), 14.5 cm in length and stabilized by a metal stylet, was inserted into the left atrium by puncture for the purpose of microsphere injection. A small drop of cyanoacrylate glue (Instant Krazy Glue) at the point of insertion further secured the cannula in place. The metal stylet was then removed, and the cannula was flushed with 0.2 ml of heparinized saline (12.5 mg%). The chest wall was closed with a 4-0 silk suture, taking care not to disturb the protruding cannula, as quick turns of the cannula can result in obstruction of its tip, or in complete displacement. The lungs were then expanded by positive pressure, and the animal disconnected from the respirator. If the animal failed to breathe spontaneously, it was reconnected to, and maintained on the respirator for the remainder of the experiment. To ascertain a properly positioned and unobstructed cannula tip, a minute volume of blood was withdrawn and immediately replaced (this verification process was repeated prior to, and intermittently during, microsphere injection). Proper placement of the cannula was further verified at necropsy.

Microsphere Suspension

Non-radioactive, carbonized, plastic microspheres ($9.3 \pm 0.4 \mu\text{m}$ in diameter (Mean $\pm$ SD)) were obtained from the New England Nuclear Corporation (Boston, MA) in a concentration of 1.5×10^6 microspheres/mg, suspended in 1.0 ml of 0.9% saline and 0.01% Tween 80.

To certify the microsphere size specifications, a dilute suspension of microspheres was sized (Coulter Counter Model Zf, 100 μm aperture, Coulter Channelyzer 1000, Coulter Electronics Inc., Hialeah, FL, USA) 1 hr following ultrasonication (Bransonic 220 Ultrasonic Cleaner).

i. Composition of the Microsphere Injectate

a. Single Injection The stock solution (1.0 g/ml) was diluted to 10.0 ml with 0.9% saline. 0.1 ml of this solution was diluted to 3.0 ml with 0.9% saline and 1 drop of Tween 80 to obtain a final solution containing 5×10^6 microspheres/ml.

b. Multiple Injections 0.1 ml of the diluted stock solution (1.5×10^8 microspheres/ml) was further diluted to 6.0 ml with 0.9% saline and 2 drops of Tween 80 to obtain a final solution containing 2.5×10^6 microspheres/ml.

ii. Preparation of the Microsphere Suspension for Injection The final microsphere suspension was vortexed for approximately 5 min to break up microsphere aggregates, and then placed in an ultrasonic bath for 45-60 min of further agitation. Complete microsphere dispersion was verified by light microscopic examination of a drop of the microsphere suspension. The temperature of the suspension was approximately 37°C at the time of injection.

When not in use, the microsphere suspension was refrigerated to prevent

bacterial contamination.

Microsphere Injection

i. Single Injection Immediately after mixing, 1.0 ml of the microsphere suspension (5×10^6 microspheres/ml) was withdrawn into a prewarmed (37°C) 1.0 ml tuberculin syringe connected to a 30 gauge needle. This was vortexed for approximately 30 sec, and immediately injected into either the left ventricular or left atrial cavity, as applicable; over 1 min. The cannula was then flushed over a 1 min period with 1.0 ml of warm (37°C), isotonic saline drawn into the same syringe used for microsphere injection. Care was taken to execute all injections in a smooth and consistent manner.

ii. Multiple Injections Two 1.0 ml volumes of the microsphere suspension (2.5×10^6 microspheres/ml) were carefully injected into the left ventricular cavity by means of a prewarmed (37°C) 1.0 ml tuberculin syringe connected to a 30 gauge needle over a period of 1 min/injection, with a stabilizing period of 2-3 min between consecutive injections. Immediately prior to injection, each microsphere suspension volume was vortexed for approximately 30 sec. The cannula was flushed as above to direct any remaining spheres in the syringe and cannula into the circulation.

Morphometric Analysis

i. Tissue Preparation Approximately 5 min following injection, the hearts were arrested by an intra-arterial injection of saturated KCl and excised. The atria, and the ventricular apex and base were removed. The remaining tissue was gently blotted to remove excess blood, coated with O.C.T. Compound (Tissue Tek II), immersed in liquid nitrogen for 45-60 sec

for quick freezing, and placed in a cryostat (IEC Minotome Microtome-Cryostat) for equilibration at -18° to -20°C for 30-60 min.

ii. Sampling Technique The sampling technique is shown in Figure 1. Four transverse sections of $16\text{ }\mu\text{m}$ thickness were obtained non-sequentially from the midventricular level of each heart (Figure 1i), placed on gelatin-coated slides, and dried at room temperature for 24 hr. The sections were then fixed (5% acetic acid, 95% absolute alcohol) for 1 min, and stained with periodic acid-Schiff (PAS) (see Table 2A in the Appendix).

Tezuka's (1982) method for counting the number of muscle fiber layers across the left ventricular free wall has been modified to examine microsphere distribution at the myocyte level. Data from animals receiving either left ventricular ($n=10$) or left atrial ($n=10$) microsphere injection were collected and subsequently analyzed. Each of the 4 sections sampled per heart was projected (Carl Zeiss Jena Projector) from a distance of 3.3 m onto a vertical surface (mag. 120X). Three regions of the left ventricular free wall (anterior, lateral, and posterior) were sampled per section (Figure 1ii). Papillary muscles were excluded from this study.

A transparent, polyethylene measuring device, 3 mm in thickness, was constructed for the purpose of sampling data. Its dimensions and demarcations are shown in Figure 2.

Figure 2. Schematic representation of the measuring device. The length is slightly greater than the average left ventricular wall thickness at a magnification of 120X. The width (10 cm) corresponds to approximately $850\text{ }\mu\text{m}$ of myocardial tissue.

Fig.1. Schematic representation of the sampling technique.

The measuring device was placed along each sampling site with the central line running perpendicular to the axis of the longitudinal muscle fibers. For ease in counting, the number of microspheres within the 10 cm wide range (including the microspheres intersecting the outer 2 lines), and their position relative to the muscle fibers intersected by the central line were labelled directly onto the measuring device. The mode of occurrence of the microspheres, whether singly or as aggregates, was noted. Starting from the subendocardium, and proceeding to the subepicardium, the number of muscle fibers intersected by the central line was counted. Microspheres were counted concurrently and, therefore, a measure of their distribution relative to the muscle layer number was also obtained. The muscle fibers were then subdivided according to their orientation: subendocardial, transitional, midwall (longitudinal), transitional, and subepicardial. The layers in each transitional zone were divided proportionally among the adjacent zones, thereby obtaining the number of layers in each major orientation (subendocardial, midwall, and subepicardial).

iii. Calculations Distribution of myocardial blood flow across the left ventricular free wall was expressed by:

- a. the number of non-radioactive microspheres in subendocardial, midwall, and subepicardial layers divided according to muscle fiber orientation (Figure liii)
- b. the number of non-radioactive microspheres in deciles of the wall thickness according to the total number of layers, irrespective of muscle fiber orientation (Figure liv)
- c. the subendocardial/subepicardial microsphere distribution ratio (SEN/SEP)

a. Orientations The number of microspheres in each orientation was determined for each sampling site. Two separate data sets were compiled. For one data set all the microspheres counted, whether they occurred singly or as aggregates, were recorded, with the assumption that aggregation was due to random distribution of single spheres within the capillaries. For the second data set each aggregate was counted as 1 microsphere, with the assumption that it entered the capillary as an aggregate due to inadequate mixing either prior to, or following injection.

Therefore, 144 parameters were recorded for each heart, as summarized in Figure 3.

Aggregates Sections Regions Orientations Parameters

	1 base	ant	SEN	MS
all MS counted	2			layers
OR	3	lat	MID	
counted as 1 MS	4 apex	post	SEP	

Figure 3. Parameters recorded for each heart by the orientation method. MS = microsphere; ant = anterior; lat = lateral; post = posterior; SEN = subendocardium; MID = midwall; SEP = subepicardium

Relative microsphere distribution was expressed by the ratio of the number of microspheres to the number of layers (MS/layer) in each orientation. The transformed parameters (72 parameters/heart) were averaged for animals receiving either left ventricular (n=10) or left atrial (n=10) microsphere injection. All data (main effects, 2-5 way interactions) were calculated from these averaged values, and expressed as Mean $\pm$ SEM.

b. Deciles The muscle fiber layers across each sampling site were subdivided into 10 zones, each zone containing an equal number of layers. These zones were referred to as deciles 1-10, with decile 1 comprising the innermost subendocardial layers, and decile 10 the outermost subepicardial layers. The number of microspheres in each decile was then recorded (MS/decile), and taken to be a measure of the relative microsphere distribution. All microspheres were counted, or microsphere aggregates were counted as single spheres as mentioned previously.

For each heart, 240 parameters were recorded (Figure 4).

<u>Aggregates</u>	<u>Sections</u>	<u>Regions</u>	<u>Deciles</u>	<u>Parameters</u>
	1 base	ant	1	SEN
all MS	2		2	
counted			3	
			4	
OR	3	lat	5	
			6	
counted as			7	
1 MS	4 apex	post	8	
			9	
			10	SEP

Figure 4. Parameters recorded for each heart by the decile method. Refer to Figure 3 for abbreviations.

Parameters were averaged for animals receiving either left ventricular (n=10) or left atrial (n=10) microsphere injection. Averaged values were used to calculate all data (main effects, 2-5 way interactions), which were expressed as Mean $\pm$ SEM.

c. SEN/SEP Since values of relative microsphere distribution were obtained both in major orientations of the left ventricular wall and in deciles of the wall thickness, 2 corresponding SEN/SEP ratios were calculated for each sampling site.

orientations From direct observation, the left ventricular wall was divided into 3 zones according to muscle fiber orientation as previously described. The SEN/SEP ratio was determined from the distribution of microspheres in subendocardial and subepicardial layers according to the following equation:

$$\text{SEN/SEP} = \frac{\text{MS/layer in SEN}}{\text{MS/layer in SEP}}$$

where MS = microspheres
SEN = subendocardium
SEP = subepicardium

deciles The left ventricular wall was divided into 2 equal zones according to the total number of muscle layers across it, with the inner half representing the subendocardium and the outer half representing the subepicardium. The SEN/SEP ratio was determined from the distribution of microspheres in the 2 zones according to the following equation:

$$\text{SEN/SEP} = \frac{\sum_{d=1}^5 \text{MS/decile}}{\sum_{d=6}^{10} \text{MS/decile}}$$

where MS = microspheres
d = deciles

Each data set (24 parameters/heart) was treated separately. All data (main effects, 2-4 way interactions) were calculated as above, and expressed as Mean $\pm$ SEM.

Statistical Analysis

Parameters were analyzed using a split-plot factorial analysis of variance (Kirk, 1982). The main plot treatment (grouping factor) was the microsphere injection site (left ventricle vs. left atrium). The subplot factors (within subject factors) common to all the parameters were aggregates (2 levels), sections (4 levels), and regions (3 levels). Additional subplot factors were deciles (10 levels) for parameters recorded by the decile method, and orientations (3 levels) for parameters recorded by the orientation method. The variation between left ventricular and left atrial microsphere injection was observed at all possible combinations of the subplot factors.

The equality of variances was determined by the Levene test (BMDP Statistical Software Manual, 1985), in which an analysis of variance is performed on the absolute deviation of each parameter from its mean. This test is relatively insensitive to the assumption of normality.

A p level of < 0.05 was considered to indicate statistical significance.

PRESSURE-INDUCED LEFT VENTRICULAR HYPERTROPHY

Animal Model

Ten pregnant Sprague-Dawley rats were obtained from Charles River Laboratories (Montreal, PQ) at 16-17 days of gestation. They were housed in individual plastic cages, and maintained on a standard diet of Purina Rat Chow pellets and tap water. Housing facilities were as previously described.

Litters were born at 21-23 days of gestation. To avoid an insufficiency of the milk supply essential for the young, litter size was adjusted to approximately 8 neonates/litter, regardless of the original litter size

(range: 4-12 neonates/litter). Only male rats were used in this study.

Induction of Left Ventricular Hypertrophy

i. Surgical Preparation Forty male rats aged 15-18 days, and weighing 16-36 g at the time of surgery, were arbitrarily assigned to one of two groups: one group (n=28) was subjected to experimental aortic constriction, and the other (n=12) served as their sham-operated controls.

Anesthesia was induced with ether and maintained throughout the surgical procedure as required. Surgery was conducted under semi-sterile conditions.

ii. Surgical Procedure The animal was placed supine, limbs extended and gently restrained, on a warm plate (37°C). A midline abdominal incision extending approximately 3 cm downward from the xiphoid process was made. The intestinal tract was reflected to one side to access the abdominal aorta, covered with sterile gauze, and kept moist with warm (37°C), isotonic saline. The aorta was isolated proximal to the left renal artery. A blunt 26 gauge hypodermic needle, 9 mm in length, was positioned in parallel with the isolated aorta, and the two were secured together with silk, thereby constricting the aorta by approximately 40-50% (Rakusan and Korecky, 1985). The needle was then removed. In the sham-operated control animals, a silk ligature was passed under the isolated abdominal aorta proximal to the left renal artery, and then removed. The intestinal tract was returned and moistened with isotonic saline. The wound was closed by suturing the epidermal layer with a 4-0 silk, cleaned with isotonic saline, and treated with an antibacterial agent (V-Sporin Aerosol).

The animal was allowed to recover on a warm plate (37°C). After regaining consciousness, the animal was returned to its mother.

Post-Surgical Maintenance

Surgery was accompanied by an overall mortality rate of 36%. Ten animals with experimental aortic constriction and 2 sham-operated controls died within 10 days following surgery, presumably due to bleeding, infection and/or cardiac failure. In addition, 2 animals with aortic constriction died 25 and 29 days post-surgery, respectively; both animals exhibited acute weight loss and cyanosis 1-2 days prior to death, indicating cardiac failure.

Body weights were measured at the same time every day to determine and assure a proper rate of growth. At 27-28 days of age, female neonates were killed, and the males (n=28) were weaned. Body weights at the time of weaning ranged from 32-81 g. Animals from the same litter were caged together. They had free access to food (Standard Purina Rat Chow pellets) and tap water. Body weights were monitored daily.

Regional Microsphere Distribution Study

i. Animal Model This study was conducted on animals with experimental aortic constriction (n=16) and their sham-operated controls (n=10) 29-45 days after the initial surgery. Body weights at the time of microsphere injection ranged from 178-267 g.

ii. Experimental Procedure With the animals under sodium pentobarbital anesthesia (45 mg/kg, i.p.), non-radioactive microspheres (5×10^6 microspheres/ml) were injected into the left atrium following the technique previously described. An additional heparinized polyethylene cannula (PE-50) was positioned in the right carotid artery and connected to a Gould pressure transducer. Heart rate (ECG) and mean arterial blood pressure were continuously recorded (Grass Model 7 Polygraph) before, during, and after the microsphere injection

and saline flush. Animals were sacrificed with an intra-arterial injection of a concentrated KCl solution.

Following excision, the hearts were blotted, trimmed of atria, and weighed (Mettler PE 360). Preparation for histological examination was similar to the technique previously described. Modifications were made to improve freezing of the hearts, and thereby minimize tissue distortions. Large ventricular myocardial samples were sliced transversely into two approximately equal portions before freezing. In addition, tissue samples were covered with a fine film of talcum powder prior to being coated with O.C.T. Compound, as this has been shown to greatly accelerate the large tissue freezing rate with liquid nitrogen (Moline and Glenner, 1964).

The methods developed for collecting, calculating, and analyzing data were applied to hearts from selected animals with experimental aortic constriction (n=7) and their sham-operated controls (n=7). The degree of left ventricular hypertrophy in animals with experimental aortic constriction was evaluated by comparing their heart weight to the expected control heart weight for comparable body weight by the heart weight (HW) - body weight (BW) regression equation, which is based on measurements on a large number of animals from our Animal Care Facility:

$$\log HW (mg) = 0.772 \log BW (g) + 0.947 \quad (r = 0.986)$$

Only hearts whose weight was greater than 150% of the expected control heart weight for comparable body weight were considered sufficiently hypertrophic. If this condition was not met, the heart and associated data were rejected.

The average left ventricular muscle fiber diameter in animals with abdominal aortic constriction and their sham-operated controls was estimated

from measurements of wall thickness (MOP-3 Image Analyzer, Carl Zeiss, Inc.) and the total number of muscle layers across the wall.

EFFECT OF INJECTION OF A STANDARD NUMBER OF MICROSPHERES ON MICROSPHERE AGGREGATION

Animal Model

Normal, male Sprague-Dawley rats (175-200 g) were obtained from Charles River Laboratories (Montreal, PQ). They were housed in individual plastic cages in the Animal Care Facility. Food (Standard Purina Rat Chow pellets) and tap water were supplied ad libitum.

Composition of the Microsphere Injectate

0.05 ml of the diluted stock solution (1.5×10^8 microspheres/ml) was further diluted to 25.0 ml with 0.9% saline to obtain a final solution containing 3×10^5 microspheres/ml.

Microsphere Injection

Anesthetized animals (sodium pentobarbital, 45 mg/kg, i.p.) were surgically instrumented for intra-atrial microsphere injection as described previously. Immediately prior to injection, the microsphere suspension was vortexed for 5 sec, and then sonicated for 30 sec. One drop was viewed microscopically to ascertain adequate mixing. 0.5 ml of the suspension was withdrawn into a 1.0 ml tuberculin syringe attached to a 30 gauge needle, carefully injected over a 15 sec interval, and flushed in with 1.0 ml of isotonic saline over the next 15 sec. This protocol for rigorous mixing,

microsphere injection, and saline flush follows the technique developed by Flaim et al. (1984) for injecting radioactive microspheres intraventricularly in conscious rats. Heart rate and mean arterial blood pressure were continuously recorded before, during, and after the microsphere injection and saline flush. Five min following injection, the heart was arrested by injecting a saturated KCl solution via the arterial cannula, and excised.

Morphometric Analysis

i. Tissue Preparation Hearts were weighed, trimmed, and frozen by the usual procedures. The ventricular myocardium was cut transversely (IEC Minotome Microtome-Cryostat) into 20 μ m thick sections. Every fourth section was placed on gelatin-coated slides, and dried at room temperature for 48 hr. Fixation and staining procedures were as previously described, with staining times adjusted for the muscle fibers to be of lower intensity.

ii. Sampling Technique Six hearts were used in this study. Each of 45 serial sections per heart was projected (Carl Zeiss Jena Projector) onto a vertical surface. The left ventricular free wall and interventricular septum were divided into 3 zones according to the muscle fiber orientation (subendocardial, midwall, subepicardial). In the septal portion, the subendocardium, midwall, and subepicardium consisted of left ventricular subendocardial fibers, left ventricular midwall fibers, and right ventricular subendocardial fibers respectively. All microspheres in each zone were counted, and whether they occurred singly or as aggregates was noted. The number of muscle fiber layers in each orientation was not considered.

iii. Calculations The number of microspheres located singly and as aggregates in each orientation of the left ventricle (free wall + septum) was averaged for 6 animals. All data (main effects, 2-3 way interactions), expressed as Mean $\pm$ SEM, were calculated from these averaged values. The percentage of the total number of microspheres counted in each orientation which were present as aggregates was then determined. The percentage of aggregates present as pairs, and groups of 3, 4, or more was also calculated.

Statistical Analysis

A general mixed model analysis of variance (BMDP Statistical Software Manual, 1985) was used to analyze the data. The grouping factor was microspheres (single vs. aggregated). The within subject factors were sections (45 levels) and orientations (3 levels). The variation between single and aggregated microspheres was observed at all possible combinations of the within subject factors. The criterion for statistical significance was $p < 0.05$.

RESULTS

PRELIMINARY STUDIES

To develop a method for assessing regional myocardial blood flow distribution morphometrically in a small animal model, several important factors were considered:

1. microsphere size
2. total number of microspheres injected
3. histological preparation for data analysis

Microsphere Size

Microspheres 7.5-15 μm in diameter are appropriate for use in smaller animals. However, microspheres within the 7.5-10 μm diameter range were desired for our experiments because they are considered to be rheologically similar to red blood cells (Phibbs and Dong, 1970) and, therefore, to more accurately estimate regional myocardial blood flow distribution (Buckberg et al., 1971; Domenech et al., 1969; Utley et al., 1974). The size of the microspheres supplied by the manufacturer was specified as $9.3 \pm 0.4 \mu\text{m}$ in diameter (Mean $\pm$ SD). Subsequent evaluation using a coulter counter-channelyzer showed that the microspheres exceeded their specified size, as described below.

i. Coulter Counter-Channelyzer Analysis Upon receipt, microsphere size was evaluated following 1 hr of ultrasonication of a dilute microsphere suspension. Microsphere diameter averaged 12.2 μm (range: 8.3-16.5 μm). Modal microsphere diameter was 11.4 μm ; while 83% of the total number of

microspheres sized were normally distributed around this value (range: 8.3-12.9 μm), and probably were single, a few were distributed with an approximately equal frequency in the larger diameter range. These abnormally large spheres were probably single as well, since the presence of microsphere pairs due to microsphere interaction and sedimentation in the suspending medium is not likely considering the low microsphere concentration, the long ultrasonication period, and the upper and lower limits of the microsphere size range. It is possible, however, that some of the smallest microspheres could have been permanently coupled (Heymann et al., 1977).

Although the average microsphere diameter differed from the manufacturer's size specifications, it was believed that the microspheres would adequately meet the standards necessary for accurate determination of regional myocardial blood flow distribution. Published results of regional myocardial perfusion following injection of 7.5-15 μm diameter microspheres often are not significantly different (refer to Table 1A in the Appendix). Furthermore, microspheres 15 μm in diameter have been used successfully for measurements of cardiac output and its distribution to various tissues in rats (e.g. Flaim et al., 1984; Stanek et al., 1983; Tuma et al., 1986; Wicker and Tarazi, 1982a, 1982b).

ii. Light Microscopic Examination Immediately after ultrasonication (1 hr), a 1.0 ml volume of a suspension containing 5×10^6 microspheres was withdrawn into a 1.0 ml tuberculin syringe, and a 30 gauge needle was attached. Light microscopic examination of a drop of the microsphere suspension revealed microspheres of uniform, spherical shape, but of varied size. The majority of microspheres were single. However, microsphere pairs, and to a lesser extent, triplets, were occasionally observed. Microsphere interaction and sedimentation in the syringe are unlikely. Aggregates are probably the consequence of

manufacturing defects and/or are artifacts of the wet-mount preparation and, therefore, not representative of the situation with injection into the arterial system. All microspheres existed as large aggregates when the same preparation was viewed 1 hr later.

Identical observations were made from a drop of the microsphere suspension after it had been in the syringe for 1 and 2 min, indicating that microsphere aggregation in the syringe is absent or insignificant, at least during this time period.

To verify the effect of repeated ultrasonication on microsphere integrity, the microsphere suspension was examined after several months of use. No significant changes were noted; microsphere fragmentation was absent.

Total Number of Microspheres Injected

For adequate sampling, a protocol was required to deliver a large number of microspheres to the coronary microvasculature without significantly disturbing the hemodynamic state and regional distribution of myocardial blood flow in the experimental animal. To safely inject a large number of microspheres, a relatively large suspending volume was necessary to prevent obstruction of the syringe/cannula, aggregation, and rapid mass coronary embolization, without causing hemodilution. This necessitates a relatively long injection period. The 3 interdependent factors considered were the composition of the microsphere suspension, and the rate and mode of injection.

For these studies, microspheres were injected intraventricularly; an electrocardiogram was monitored during the microsphere injection and saline flush.

When more than 7×10^6 microspheres were injected, regardless of the concentration of the suspension, or the mode or rate of injection, mortality

was high. Histological examination revealed massive coronary embolization, a large degree of aggregation, and coronary artery blockage, with large areas of the left ventricular wall being void of microspheres. Decreasing the total number of microspheres injected to $1-5 \times 10^6$ increased survival. Therefore, the upper limit to the total number of microspheres which may be injected in anesthetized rats approximates 5×10^6 .

Rapid (15 sec) injection of a 1.0 ml volume of saline plus 1 drop of Tween 80 did not alter the hemodynamic state as evidenced by a stable electrocardiogram and breathing pattern. A single injection (15 sec) of 5×10^6 microspheres suspended in a 1.0 ml volume of saline plus 1 drop of Tween 80 resulted in a transient increase in heart rate, and the rate and depth of breathing. When the injection rate was prolonged to 1 min, no significant hemodynamic alterations were observed. Therefore, a microsphere suspending volume of 1.0 ml, and an injection rate of 1 min were chosen. With this injection protocol, microsphere aggregation in the syringe is insignificant (see above). Unfortunately, a certain degree of hemodilution is unavoidable. Total blood volume in male Sprague-Dawley rats averages 6.0 ml/100 g body weight (Rakusan and Rajhathy, 1973). With injection of a 1.0 ml microsphere suspension and a 1.0 ml saline flush in the first 2 experimental series, and of a 0.5 ml microsphere suspension and a 1.0 ml saline flush in the last series, the estimated total blood volume was temporarily increased, on the average, by 12% (range: 6-19%) (n=40). Although this could have been minimized by omitting the saline flush, since knowledge of the exact number of microspheres injected is not necessary, experimental conditions similar to those of the conventional radioactive microsphere technique were desired for comparison.

There were no significant differences in regional microsphere distribution between animals receiving single microsphere injections (n=7) and those receiving multiple microsphere injections (n=3); therefore, the data were pooled. Single injections were chosen to minimize any potential spatial and temporal fluctuations of regional myocardial blood flow that may occur between consecutive injections (Falsetti et al., 1975; Sestier et al., 1978).

Histological Preparation for Data Analysis

i. Tissue Preparation Glutaraldehyde fixation, although shown to stabilize myocardial structure without causing gross distortion (Yoran et al., 1973), and infiltration and embedding (paraffin, plastic) techniques were not chosen for this study because of the time factor involved. Histological preparation by freezing fresh tissue is a relatively short procedure, and can be used with relative success to maintain myocardial integrity if properly applied.

An optimal freezing rate is one at which cell water is transported across the cell membrane at a rate sufficient to prevent intracellular ice formation, yet not so fast as to make dehydration a problem (Mazur et al., 1972). Coating the tissue with an insulating material has been suggested for better, more homogeneous freezing, with decreased tissue distortion (Luyet, 1961). In the present study, slow freezing in a cryostat (-20°C) for several hours of tissue specimens coated in O.C.T. Compound was inadequate, making sectioning difficult. Very fast freezing in isopentane cooled in liquid nitrogen resulted in extensive tissue fracturing. Adequate sections were obtained with fast freezing in liquid nitrogen for 45-60 sec, depending on tissue sample size. Mild distortion, most common in midmyocardial layers, was occasionally observed, indicating inhomogeneous freezing and intracellular

ice formation. When freezing time was extended for more complete freezing, tissue fracturing was a problem. Therefore, although undistorted sections could not always be obtained, this mode of freezing provided the best sections.

ii. Sectioning To determine whether microsphere distribution in transverse myocardial sections is a reliable measure of regional myocardial perfusion, data from both transverse and sagittal sections were obtained from the same heart (n=3). Transmural microsphere distribution was similar regardless of the sectional plane.

With tissue sectioning, regardless of the thickness, microsphere slicing is inevitable. However, microsphere loss appears to be negligible since microspheres very small in diameter, probably the consequence of sectioning, were also observed. If losses due to cutting are sustained, results are not expected to be significantly affected, as losses would be random. To minimize any possible error, however, sections were cut thicker than the average microsphere diameter. With thicker sections (greater than twice the average microsphere diameter), microspheres deeply embedded may be missed. Therefore, sections of 16 μm thickness were chosen.

iii. Staining Contrast stains for capillaries and muscle fibers, which would not alter microsphere integrity while making them easily visible, were desired. PAS stain modified for frozen sections met these requirements.

GENERAL MICROSCOPIC OBSERVATIONS

Microsphere aggregates were observed following left ventricular and left atrial injection in both control and experimental animals (Figure 5). Whether the occurrence of aggregates was the result of inadequate microsphere dispersion prior to entry into the coronary vasculature, and/or of random microsphere interaction within the vasculature, is not possible to distinguish with the present technique. Single spheres lodged in very close proximity at the ostia of branching vessels may also appear as aggregates in cross-section (Eng et al., 1984).

Occasionally with left ventricular injection, but not with left atrial injection, very large microsphere aggregates were observed blocking a coronary artery. The myocardium supplied by this artery was consequently void of spheres. These hearts were omitted from the study.

It should also be noted that microspheres were never observed on the endocardial surface of the left ventricle, contrary to the postulation by Idvall et al. (1979).

Figure 5. Photomicrographs demonstrating microsphere embolization in 16 μ m thick sections of rat left ventricular myocardium. Although many microspheres are located singly (i), microsphere aggregates are also present, mainly in the subendocardium (ii, iii), and to a lesser extent in the midwall (iv) and subepicardium. Stain: PAS. Counterstain: Fast Green. Magnification: X400.

i.

ii.

iii.

iv.

EFFECT OF INJECTION SITE ON MYOCARDIAL MICROSPHERE DISTRIBUTION

The adequacy of mixing of microspheres injected into the left ventricle or the left atrium may be determined from the data presented in Figure 6 and Table 1. The following were observed:

1. The average number of microspheres per decile of the left ventricular wall thickness was higher following left ventricular injection compared with left atrial injection, although the total number of microspheres injected was similar for both injection sites. Body weights between the 2 groups of animals were also similar (302 ± 55 g (Mean $\pm$ SD), left ventricular injection; 342 ± 86 g (Mean $\pm$ SD), left atrial injection). It is, therefore, assumed that heart weights were comparable.

2. The difference in the number of microspheres was more pronounced when all the microspheres per aggregate were counted. This may be attributed to the significantly greater degree of microsphere aggregation with left ventricular injection (33%) than with left atrial injection (24%) ($p < 0.01$).

3. Variability in the data was significantly greater with left ventricular injection than with left atrial injection ($p < 0.001$). For either site of injection, variability was higher when all the microspheres per aggregate were counted.

Figure 6. Variation in the distribution of the number of microspheres per decile of the left ventricular wall thickness in control animals receiving left ventricular (n=10) and left atrial (n=17) microsphere injection. Data from animals receiving left atrial microsphere injection in the first experimental series (n=10) were combined with data from sham-operated animals in the second series (n=7). Each curve represents the average of 120 observations. LV = left ventricular injection; LA = left atrial injection; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted; MS = microspheres

Table 1. The average number of microspheres per decile of the left ventricular wall thickness in control animals receiving left ventricular (n=10) and left atrial (n=17) microsphere injection. Data from animals receiving left atrial microsphere injection in the first experimental series (n=10) were combined with data from sham-operated animals in the second series (n=7).

	INJECTION SITE	
	Left Ventricle	Left Atrium
AGGREGATES		
all MS counted	12.1 $\pm$ 2.7 (22.3%)	8.0 $\pm$ 1.3 (16.3%)
counted as 1 MS	8.1 $\pm$ 1.5 (18.5%)	6.1 $\pm$ 0.9 (14.8%)

Mean $\pm$ SD. Values in parentheses are SD expressed as the percentage of the mean (coefficient of variation). MS = microspheres

The effect of injection site on microsphere distribution in sections of the left ventricle is presented in Figure 7. When all the microspheres per aggregate were counted, uniform distribution in sections was observed following left atrial injection. However, with left ventricular injection, significantly more microspheres were located in the most basal section compared with the most apical section. When microsphere aggregates were counted as single spheres, uniform microsphere distribution in sections was indicated for both sites of injection.

The effect of injection site on microsphere distribution in regions of the left ventricular wall is presented in Figure 8. When all the microspheres per aggregate were counted, no significant differences in their distribution to the anterior, lateral, and posterior wall were found for either site of injection. When microsphere aggregates were counted as single spheres, uniform distribution in regions was observed following left atrial injection. However, with left ventricular injection, microsphere distribution to the anterior wall was significantly greater than to the lateral and posterior walls, with no difference in the distribution between the latter regions.

Figure 7. The average number of microspheres per decile of the left ventricular wall thickness in 4 sections from the mid-ventricular level in animals receiving left ventricular (n=10) and left atrial (n=10) microsphere injection. Mean $\pm$ SEM. Standard error bars which are not represented are smaller than the symbol corresponding to the mean. LV = left ventricular injection; LA = left atrial injection; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted; MS = microspheres

Figure 8. The average number of microspheres per decile of the left ventricular wall thickness in 3 regions of the left ventricular wall in animals receiving left ventricular (n=10) and left atrial (n=10) microsphere injection. Mean $\pm$ SEM. Standard error bars which are not represented are smaller than the symbol corresponding to the mean. LV = left ventricular injection; LA = left atrial injection; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted; MS = microspheres; Ant = anterior; Lat = lateral; Post = posterior

The effect of injection site on microsphere distribution in deciles of the left ventricular wall thickness is presented in Figure 9 (upper panel). When all the microspheres per aggregate were counted, minor transmural inequalities of microsphere distribution were observed following left atrial injection, but these were not statistically significant. With left ventricular injection, the pattern of microsphere distribution approximated a sine wave; microspheres were predominant in the subendocardium, with a slight decrease in the number of spheres in the deepest subendocardial layer, and were present in the least amount in the subepicardium, with a slight increase in the number of spheres in the outermost subepicardial layer. However, significant differences were not indicated, probably due to the large variability in measurements in the deepest subendocardial layers.

When microsphere aggregates were counted as single spheres, transmural microsphere distribution was uniform following both left ventricular and left atrial injection. Thus, the proportion of microspheres present as aggregates was highest in the subendocardial layers and decreased gradually towards the subepicardium, as shown in Figure 9 (lower panel). The decrease was more pronounced following left ventricular injection ($r = -0.93$; slope of regression line = -1.03) than with left atrial injection ($r = -0.72$; slope of regression line = -0.71).

The effect of injection site on microsphere distribution to the subendocardial, midwall, and subepicardial layers of the left ventricular wall divided according to orientation is presented in Figure 10 (upper panel). Uniform transmural microsphere distribution was observed following both left ventricular and left atrial injection whether all the microspheres per aggregate were counted, or microsphere aggregates were counted as single spheres.

Figure 9. upper panel: The number of microspheres in deciles of the left ventricular wall thickness in animals receiving left ventricular (n=10) and left atrial (n=10) microsphere injection. Mean $\pm$ SEM. Standard error bars which are not represented are smaller than the symbol corresponding to the mean. lower panel: Percentage of the total number of microspheres per decile present as aggregates. Data were calculated from mean values shown in the upper panel. LV = left ventricular injection; LA = left atrial injection; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted; MS = microspheres; SEN = subendocardium; SEP = subepicardium

Figure 10. upper panel: The number of microspheres in 3 zones of the left ventricular wall divided according to muscle fiber orientation in animals receiving left ventricular (n=10) and left atrial (n=10) microsphere injection. Mean $\pm$ SEM. Standard error bars which are not represented are smaller than the symbol corresponding to the mean. lower panel: Percentage of the total number of microspheres per orientation present as aggregates. Data were calculated from mean values shown in the upper panel. LV = left ventricular injection; LA = left atrial injection; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate was counted; MS = microspheres; SEN = subendocardium; MID = midwall; SEP = subepicardium

Subendocardial/subepicardial microsphere distribution ratios are summarized in Table 2. Microspheres were uniformly distributed between subendocardial and subepicardial layers when microsphere aggregates were counted as single spheres, irrespective of injection site. With both left ventricular and left atrial injection, subendocardial/subepicardial ratios were higher when all the microspheres per aggregate were counted, indicating a predominance of aggregates in the subendocardial layers. The difference was statistically significant only with left ventricular injection ($p < 0.01$).

Table 2. The ratio of subendocardial/subepicardial microsphere distribution (SEN/SEP) in animals receiving left ventricular (n=10) and left atrial (n=10) microsphere injection.

	LV		LA	
	(-)	(+)	(-)	(+)
decile data	1.17 $\pm$ 0.05	1.29 $\pm$ 0.06	1.06 $\pm$ 0.07	1.09 $\pm$ 0.04
orientation data	1.19 $\pm$ 0.09	1.49 $\pm$ 0.15	0.98 $\pm$ 0.05	1.06 $\pm$ 0.08

Mean $\pm$ SEM. LV = left ventricular injection; LA = left atrial injection; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted

In summary, microsphere distribution following left atrial injection was uniform in all areas of the left ventricular wall in normal, anesthetized rats. Conversely, less consistent results were obtained following left ventricular microsphere injection. This may be attributed to a higher degree of microsphere aggregation, with preferential location of aggregates in the subendocardial layers, and to the increased variability in the number of microspheres per unit of tissue. Microspheres were, therefore, injected into the left atrium in the remaining series of experiments.

MICROSPHERE DISTRIBUTION IN HYPERTROPHIED HEARTS

Heart weight, heart rate, and mean arterial pressure were significantly elevated in animals with abdominal aortic constriction compared with their sham-operated controls (Table 3). The increase in heart weight was due to significant enlargement of left ventricular muscle fibers; the estimated average fiber diameter was 22.1 μm in animals with aortic constriction and 16.6 μm in the control group. Neither signs of hyperplasia nor a redistribution of muscle fiber layers in sections, regions, or orientations of the left ventricular free wall were seen (Table 4).

Microsphere injection and saline flush had no apparent hemodynamic effects as evidenced by a stable heart rate and mean arterial pressure.

Table 3. Basic parameters at the time of microsphere injection for animals with abdominal aortic constriction (n=7) and their sham-operated controls (n=7).

	Controls	Experimental
Body Weight (g)	214 $\pm$ 9	228 $\pm$ 10
Heart Weight (mg)	559 $\pm$ 54	1040 $\pm$ 73*
(mg/100 g BW)	258 $\pm$ 16	440 $\pm$ 25*
Heart Rate (bpm)	370 $\pm$ 11	430 $\pm$ 6*
Mean Arterial Pressure (mmHg)	110 $\pm$ 4	165 $\pm$ 13*

Mean $\pm$ SEM. * = significantly different from controls (Student's t-test; $p < 0.05$); BW = body weight

Table 4. The number of subendocardial, midwall, and subepicardial muscle fiber layers in 3 regions of the left ventricular free wall in animals with abdominal aortic constriction (n=7) and their sham-operated controls (n=7).

	REGION			
	anterior	lateral	posterior	average
<u>Controls</u>				
SEN	95 ± 6	73 ± 14	87 ± 11	85 ± 8
MID	89 ± 8	94 ± 11	104 ± 11	96 ± 8
SEP	36 ± 10	29 ± 5	28 ± 4	31 ± 4
total	219 ± 4	196 ± 11	218 ± 13	211 ± 9
<u>Experimental</u>				
SEN	99 ± 8	64 ± 10	82 ± 10	81 ± 7
MID	78 ± 11	90 ± 16	100 ± 11	90 ± 12
SEP	33 ± 5	46 ± 6	28 ± 4	36 ± 4
total	210 ± 15	201 ± 18	210 ± 16	207 ± 15

Mean ± SEM. SEN = subendocardium; MID = midwall; SEP = subepicardium

The overall effect of left atrial injection on microsphere distribution in deciles of the left ventricular wall thickness in control and hypertrophied hearts is summarized in Table 5. The observations are:

1. The average number of microspheres per decile was significantly greater in animals with left ventricular hypertrophy compared with their sham-operated controls ($p < 0.05$). This difference was more pronounced when all the microspheres per aggregate were counted.

2. Microsphere aggregation was significantly greater in hypertrophied hearts (31%) than in control hearts (25%) ($p < 0.03$).

3. Variability in the data was significantly greater in hypertrophied hearts than in control hearts ($p < 0.001$). For each heart, variability was higher when all the microspheres per aggregate were counted.

Microsphere distribution in sections (Figure 11, upper panel) and regions (Figure 12, upper panel) was uniform in animals with left ventricular hypertrophy and their sham-operated controls. The pattern of microsphere aggregation in sections (Figure 11, lower panel) and regions (Figure 12, lower panel) had no significant effect on the distribution profiles.

Table 5. The average number of microspheres per decile of the left ventricular wall thickness in animals with left ventricular hypertrophy (n=7) and their sham-operated controls (n=7).

	Controls	Experimental
AGGREGATES		
all MS counted	10.4 $\pm$ 2.4 (23.1%)	17.9 $\pm$ 5.1 (28.5%)
counted as 1 MS	7.8 $\pm$ 1.6 (20.5%)	12.4 $\pm$ 3.0 (24.2%)

Mean $\pm$ SD. Values in parentheses are SD expressed as the percentage of the mean (coefficient of variation). MS = microspheres

Figure 11. upper panel: The average number of microspheres per decile of the left ventricular wall thickness in 4 sections from the mid-ventricular level in animals with left ventricular hypertrophy (n=7) and their sham-operated controls (n=7). Mean $\pm$ SEM. Standard error bars which are not represented are smaller than the symbol corresponding to the mean. lower panel: Percentage of the total number of microspheres per section present as aggregates. Data were calculated from mean values shown in the upper panel. SHAM = sham-operated controls; LVH = left ventricular hypertrophy; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted; MS = microspheres

Figure 12. upper panel: The average number of microspheres per decile of the left ventricular wall thickness in 3 regions of the left ventricular free wall in animals with left ventricular hypertrophy (n=7) and their sham-operated controls (n=7). Mean $\pm$ SEM. Standard error bars which are not represented are smaller than the symbol corresponding to the mean. lower panel: Percentage of the total number of microspheres per region present as aggregates. Data were calculated from mean values shown in the upper panel. SHAM = sham-operated controls; LVH = left ventricular hypertrophy; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted; MS = microspheres; Ant. = anterior; Lat = lateral; Post = posterior

Microsphere distribution in deciles of the left ventricular wall thickness in animals with left ventricular hypertrophy and their sham-operated controls is shown in Figure 13 (upper panel). Whereas transmural microsphere distribution was uniform in the control group, the number of microspheres in the innermost subendocardial layers (decile 1-2) tended to be higher with left ventricular hypertrophy. This difference was more pronounced when all the microspheres per aggregate were counted.

The transmural gradient of microsphere aggregation (Figure 13, lower panel) as previously described in normal hearts (Figure 9, lower panel) was more pronounced in hypertrophied hearts ($r = -0.92$; slope of regression line $= -1.15$) than in control hearts ($r = -0.84$; slope of regression line $= -0.85$).

Similar results were obtained when transmural microsphere distribution was measured in subendocardial, midwall, and subepicardial layers of the left ventricular wall divided according to muscle fiber orientation (Figure 14).

The subendocardial/subepicardial microsphere distribution ratios which are summarized in Table 6 are similar between animals with left ventricular hypertrophy and their sham-operated controls. Although mean values for both groups indicate greater microsphere distribution to the subendocardial layers of the left ventricular wall, this being most evident when all the microspheres per aggregate were counted, significant levels were not reached.

Figure 13. upper panel: The number of microspheres in deciles of the left ventricular wall thickness in animals with left ventricular hypertrophy (n=7) and their sham-operated controls (n=7). Mean $\pm$ SEM. Standard error bars which are not represented are smaller than the symbol corresponding to the mean. lower panel: Percentage of the total number of microspheres per decile present as aggregates. Data were calculated from mean values shown in the upper panel. SHAM = sham-operated controls; LVH = left ventricular hypertrophy; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted; MS = microspheres; SEN = subendocardium; SEP = subepicardium

Figure 14. upper panel: The number of microspheres in 3 zones of the left ventricular wall divided according to muscle fiber orientation in animals with left ventricular hypertrophy (n=7) and their sham-operated controls (n=7). Mean $\pm$ SEM. Standard error bars which are not represented are smaller than the symbol corresponding to the mean. lower panel: Percentage of the total number of microspheres per orientation present as aggregates. Data were calculated from mean values shown in the upper panel. SHAM = sham-operated controls; LVH = left ventricular hypertrophy; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted; MS = microspheres; SEN = subendocardium; MID = midwall; SEP = subepicardium

Table 6. The ratio of subendocardial/subepicardial microsphere distribution (SEN/SEP) in animals with left ventricular hypertrophy (n=7) and their sham-operated controls (n=7).

	SHAM		LVH	
	(-)	(+)	(-)	(+)
decile data	1.21 $\pm$ 0.05	1.30 $\pm$ 0.07	1.27 $\pm$ 0.03	1.36 $\pm$ 0.03
orientation data	1.32 $\pm$ 0.11	1.47 $\pm$ 0.13	1.24 $\pm$ 0.05	1.35 $\pm$ 0.07

Mean $\pm$ SEM. SHAM = sham-operated controls; LVH = left ventricular hypertrophy; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted

EFFECT OF INJECTION OF A STANDARD NUMBER OF MICROSPHERES ON MICROSPHERE AGGREGATION

The preferential location of microsphere aggregates in the subendocardial layers that was found in the previous experiments could have resulted from myocardial blood flow alterations which may occur secondary to injection of a very large number of spheres in rats (Stanek et al., 1983; Tsuchiya et al., 1977; Tuma et al., 1986). The purpose of this experimental series was to determine whether the injection protocol generally used for coronary blood flow determinations in rats with the radioactive microsphere technique also results in uneven distribution of microsphere aggregates. For adequate microsphere sampling, the number of sections was increased from 4 to 45. Due to the time factor involved, the number of muscle fiber layers across the left ventricular wall was not determined as in the previous experiments and, thus, a measure of microsphere distribution in deciles of the wall thickness was not obtained.

The animals supplied for this study unexpectedly had a significantly higher mean arterial pressure (Table 7) compared with the sham-operated animals in the second experimental series (Table 3). Histological examination occasionally revealed left ventricular hypertrophy. These generalized findings imply a mixture of both normal and spontaneously hypertensive rats. However, for the purpose of this study, this condition does not present a problem.

Heart rate and mean arterial pressure were not significantly altered with the microsphere injection and saline flush.

The distribution of microspheres located singly and as aggregates in the subendocardium, midwall, and subepicardium of the left ventricle (free wall +

septum) is summarized in Table 8. Of the total number of microspheres counted, 93.1% were found as individual spheres, and 6.9% (maximum 9.1%) were present as aggregates. Most aggregates consisted of two spheres (83.8%), but aggregates of up to a maximum of 6 spheres were also observed.

There were no statistically significant differences between the distribution of microspheres located singly or as aggregates among the 45 transverse sections. However, the transmural distribution of single spheres was slightly, but significantly, different from that of microsphere aggregates ($p < 0.05$). Of the total number of microspheres present in each muscle fiber orientation, aggregation was highest in the subendocardium (7.2%) as compared to the midwall (6.8%) and subepicardium (6.7%).

Table 7. Basic parameters for control animals (n=6) receiving left atrial injection of a standard number of microspheres.

Body Weight (g)	363 $\pm$ 30
Heart Weight (mg)	893 $\pm$ 62
(mg/100 g BW)	248 $\pm$ 10
Heart Rate (bpm)	335 $\pm$ 9
Mean Arterial Pressure (mmHg)	124 $\pm$ 6

Mean $\pm$ SEM. BW = body weight

Table 8. The number of microspheres located singly and as aggregates in 3 zones of the left ventricular wall divided according to muscle fiber orientation.

	MICROSPHERES	
	Single	Aggregated
ORIENTATION		
subendocardium	23.5 $\pm$ 0.4	1.8 $\pm$ 0.1
midwall	26.2 $\pm$ 0.5	1.9 $\pm$ 0.1
subepicardium	17.6 $\pm$ 0.3	1.3 $\pm$ 0.1
average	22.4 $\pm$ 0.4	1.7 $\pm$ 0.1

Mean $\pm$ SEM.

DISCUSSION

The radioactive microsphere technique has been used to determine cardiac output and its fractional distribution to various organs in the rat (Idvall et al., 1979; Ishise et al., 1980; Malik et al., 1976; McDevitt and Nies, 1976; Mendell and Hollenberg, 1971; Rakusan and Blahitka, 1974; Sasaki and Wagner, 1971). However, the technique has not been applied to studies of blood flow distribution within specific organs of the rat due to its low spatial resolution. This limitation may be overcome with the use of non-radioactive microspheres since their distribution is detected microscopically, eliminating the need for multiple tissue sampling.

A method to determine the regional distribution of non-radioactive microspheres with a high spatial resolution in rat myocardium has been proposed by Vetterlein (1975). This method involves counting and mapping of unlabelled spheres in various planes of 1 mm thick tissue sections made transparent with sodium hydroxide. Unfortunately, the pattern of microsphere distribution cannot be related to the myocardial morphology. This may be limiting, especially when measuring regional myocardial perfusion under conditions in which the myocardial morphology is altered (e.g. hypertrophy, infarction). Furthermore, the tissue thickness necessitates constant focusing and refocussing of microspheres in the various planes. Data collection, may, therefore, be subject to error. Vetterlein (1975) did not report quantitative data on regional microsphere distribution, and we are not aware of any subsequent use of this technique. Therefore, the present morphometric method was developed to accurately measure regional myocardial perfusion in rats by ensuring that the structural integrity of the myocardium was maintained, and that other potential sources for error were eliminated.

To minimize the variability related to random microsphere distribution, it was necessary to inject a very large number of spheres. The greatest amount of spheres reported to be administered in a single injection in rats without significantly altering cardiovascular hemodynamics, apart from a transient increase in mean arterial pressure during the injection period, is 7×10^6 (Wisnes and Kirkebo, 1976). However, the microspheres were injected into the aortic arch. Based on anatomical considerations, the number of spheres which enter the coronary circulation following intra-aortic injection is probably smaller compared with a similar injection into the left atrium or ventricle. The lower mean coronary blood flow values reported for intra-aortic compared with left ventricular injection in rats (Tuma et al., 1986) support this view. Thus, potential hemodynamic disturbances associated with injection of a very large number of microspheres may have been avoided with intra-aortic injection in the above study. It is likely, however, that the relatively low values reported as the upper limit to the number of microspheres that can be administered in a single injection into the left atrium or ventricle of rats (1×10^5 : Tsuchiya et al., 1977; Tuma et al., 1986; 3.6×10^5 : Stanek et al., 1983) are underestimated. With serial left atrial injections, Stanek et al. (1983) found that significant hemodynamic changes were absent until 1.44×10^6 microspheres had accumulated in the rat. The rapid coronary embolization and the pronounced occurrence of aggregation that are expected with the small injection volumes and rapid injection rates used in these studies may account for the hemodynamic changes which are observed when the "upper limit" is exceeded. By increasing the microsphere suspension volume and prolonging the injection period, we found that a maximum of 5×10^6 microspheres may be injected in the anesthetized rat without evidence of dysfunction due to embolization of the coronary vascular bed. The use of

relatively smaller microspheres in the present study may have also increased the probability of maximizing the number of spheres which may be injected (Poujeol et al., 1975). Thus, hemodynamic events accompanying microsphere injection and embolization are affected not only by the size and number of microspheres injected (Heymann, et al., 1977), but also by the microsphere concentration and injection rate.

Accurate determination of myocardial blood flow distribution also requires that microspheres be completely mixed with the blood before entering the coronary circulation. Wicker and Tarazi (1982a, 1982b) have shown that microspheres injected into the left ventricle are inadequately mixed, based on their finding that coronary blood flow determinations in rats are significantly less variable with left atrial injection compared with left ventricular injection. However, this finding has not been universally accepted (e.g. Flaim et al., 1984). Since the above observations were based on measurements of tissue radioactivity, we reassessed the effect of injection site on microsphere distribution.

Regional Myocardial Distribution of Non-radioactive Microspheres Following Left Ventricular and Left Atrial Injection

The results of the first experimental series confirm that microspheres injected into the left atrium are more uniformly distributed in the aortic root, resulting in more accurate determination of regional myocardial blood flow distribution. Inadequate mixing with left ventricular injection is indicated by 1) the greater delivery of microspheres to the coronary circulation, 2) the greater variability in the number of microspheres per unit of tissue, and 3) the greater degree of microsphere aggregation, with preferential location of aggregates in the subendocardium.

Preferential streaming of a large number of microspheres due to inadequate mixing may in part explain why mean values of coronary blood flow are generally higher with left ventricular injection compared with left atrial injection (Kobrin et al., 1984; Wicker and Tarazi, 1982b), especially at fast injection rates (Tuma et al., 1986). The greater interaction between microspheres at higher concentrations (Phibbs and Dong, 1970) would also increase the percentage of the microspheres injected entering the coronary circulation as aggregates. Microsphere aggregates, in turn, would preferentially lodge in the subendocardial layers due to axial streaming, resulting in overestimation of subendocardial flow. Aggregation due to random distribution of single spheres within the coronary vasculature also increases with increasing numbers of microspheres entering the coronary circulation (Harell et al., 1977). This would, at least in part, account for the significantly higher degree of aggregation across the left ventricular wall following left ventricular injection. Single spheres lodged in very close proximity at the ostia of branching vessels may also appear as aggregates in cross-section (Eng et al., 1984). Although it is not possible to distinguish the cause of microsphere aggregation following left ventricular injection with the present technique, it is probable that all of these factors are involved, and result in overestimation of coronary blood flow.

The significance of the "redistribution" of microspheres in sections and regions of the normal left ventricular wall following left ventricular injection is less apparent, and may require further investigation. It is possible that the tissue sampling protocol used in this study was inadequate to compensate for the significant nonrandom component of microsphere distribution associated with left ventricular injection, resulting in misleading distribution measurements. If this was the case, however, distribution

differences would have been more pronounced following left atrial injection due to the presence of significantly fewer microspheres per unit of tissue. Further evidence supporting the adequacy of tissue sampling is presented in a subsequent section (see "Advantages and Disadvantages of the Technique"). The absence of significant hemodynamic disturbances during the injection period suggests that the mechanism by which microspheres are "redistributed" in sections and regions is also not related to significant alterations in total myocardial flow. Central hemodynamics are speculated to be maintained with left ventricular injection of a large number of microspheres ($\sim 1 \times 10^6$) in rats at the expense of blood flow alterations in the splanchnic organs and skin (Ishise et al., 1980). However, this conclusion is based on measurements in conscious rats. It is possible that the lower cardiac output in anesthetized rats may increase the susceptibility of the heart to flow changes following occlusion of the coronary vasculature with a relatively large number of microspheres. This is indicated from a recent study that reported a significant reduction in total myocardial flow following left ventricular injection of 3.75×10^5 microspheres in anesthetized rats (Tuma et al., 1986). However, the rapid injection of a concentrated microsphere suspension in this study probably resulted in occlusion by microsphere aggregates of a large coronary vessel(s), thereby decreasing total flow. In contrast, when a significantly greater number of microspheres (5×10^6) were injected slowly in a similar rat preparation in the present study, coronary occlusion by microspheres did not appear to alter the delivery of additional spheres to the coronary circulation, as suggested from the absence of large unperfused areas in the hearts used for data analysis. Without appropriate tests, however, this possibility cannot be excluded.

An alternative, and the most likely explanation for these distribution

differences, is based on the flow behavior of single and aggregated microspheres in the coronary vasculature, several aspects of which have been discussed previously. Although steric restriction of the incoming aggregates could alone account for the apparent overperfusion in the most basal section sampled, a selective decrease of the blood flow rate in this area due to the resultant increased resistance to flow would promote further aggregation of the disproportionately large number of single spheres entering the coronary circulation. Similarly, the predominance of single spheres in the anterior wall could be explained entirely on the basis of their entry in a concentrated stream. However, the apparent decreased interaction of these spheres, as evidenced by the absence of regional distribution differences when all the microspheres per aggregate were counted, suggests a selective increase of the flow rate in this region. Since distribution differences in sections and regions were absent following left atrial injection, inadequate mixing of microspheres with left ventricular injection, and not injection of a large number of microspheres, may be responsible for this potential redistribution of flow. Whether the microsphere distribution pattern reflects the volume flow of red blood cells is tentative, although certain differences are expected on the basis of red blood cell deformability and the larger microsphere diameter (see Chien et al., 1984). Finally, the inconsistency between these results and those of other studies provides further evidence for the inadequacy of mixing of microspheres with left ventricular injection.

It has been suggested that the catheter position, rather than the injection site, is the critical factor influencing the degree of mixing of microspheres in anesthetized rats (Kobrin et al., 1984). However, the relatively large diameter catheter (PE-50, o.d. = 1 mm) used for left ventricular injection in the above study may have modified the pattern of

blood flow in the aortic root to some degree, resulting in more variable streaming of microspheres. Differences between the variability of coronary blood flow measurements determined by left ventricular and left atrial microsphere injection may, therefore, not be detected when the spheres are injected simultaneously (Flaim et al., 1984). Smaller catheters (PE-10, o.d. = 0.6 mm), as used in the present study, appear to have a negligible effect on microsphere streaming; variability in coronary blood flow measurements following left atrial injection is unaltered when a second catheter is placed in the left ventricle via the right carotid artery (Wicker, P., personal communication).

Although mixing of microspheres following left ventricular injection was shown to be inadequate for accurate measurements of regional myocardial perfusion, the significant degree of aggregation observed following left atrial injection of a large number of spheres was also more pronounced in the subendocardial layers. It may be inferred that left atrial injection also results in incomplete mixing of microspheres in the aortic root. Alternatively, the uneven distribution of microsphere aggregates could result from myocardial blood flow alterations that may occur secondary to left atrial injection of a large number of spheres in rats (Stanek et al., 1983), as suggested previously from our microsphere distribution data for left ventricular injection. Therefore, we examined the transmural distribution of aggregates following an injection protocol generally used for myocardial blood flow determinations in rats with the radioactive microsphere technique. Although the percentage of microspheres present as aggregates was significantly lower with this injection, the uneven pattern of aggregation still persisted. Thus, the transmural distribution of microsphere aggregates does not appear to be influenced by the number of microspheres injected. However, we do not

have sufficient data to reject the possibility that left atrial microsphere injection alters myocardial flow, although this seems an unlikely consequence with injection of a small number of microspheres. Similarly, without directly observing microsphere flow at the entrance to the coronary ostia, we cannot support the alternative possibility of inadequate mixing of microspheres with left atrial injection.

A more reasonable approach to explain the uneven transmural distribution of microsphere aggregates with left atrial injection is to assume that microsphere aggregates enter the coronary circulation when injected at this site, not due to inadequate mixing, but to microsphere interaction in the aortic root (and possibly, although less likely, in the left atrial or ventricular cavity). Preferential accumulation of aggregates in the subendocardial layers due to axial streaming would account for the uneven transmural pattern of aggregation observed with left atrial injection. Although microsphere interactions are more pronounced at higher microsphere concentrations, it is reasonable that such interactions would also be responsible for the slight, but significant gradient of aggregation observed with injection of a relatively small number of microspheres. The results from our study of regional microsphere distribution following left atrial injection in animals with left ventricular hypertrophy also raise the possibility that microsphere aggregation in the coronary vasculature may be function-dependent. A shift in the transmural gradient of aggregation was observed in hypertrophied hearts, with a predominance of aggregates in the subendocardial layers. It is speculated that myocardial shear forces and/or extravascular compressive forces during left ventricular contraction result in aggregation of microspheres lodged in or traversing the same vessel, especially in the subendocardium. The altered pattern of aggregation in hypertrophied hearts could

then, in part, be explained by enhancement of these forces due to hypertension. Further discussion of these results is allocated to the following section.

In conclusion, estimates of myocardial blood flow distribution which are based on measurements of tissue radioactivity following injection of radioactive microspheres may be distorted by various degrees due to the uneven distribution of microsphere aggregates. The degree of error, which indicates the adequacy of mixing of microspheres with blood, was shown to depend on the site of microsphere injection. In addition, the occurrence of aggregates within the coronary vasculature and, thus, the probability of overestimating total myocardial blood flow values, also increases with increasing numbers of microspheres entering the coronary circulation. This was shown to be a function of both the injection site and the total number of microspheres injected. If a sufficient degree of aggregation occurs in the larger coronary vessels, total myocardial blood flow may actually be decreased. Therefore, the results of the present study indicate that left ventricular injection of microspheres is unsuitable for determining myocardial blood flow and its regional distribution. For more accurate measurements, left atrial injection is recommended. Furthermore, the number of microspheres injected should be minimized, while assuring delivery of an adequate number of microspheres to each tissue sample. However, this necessitates an increased number of experiments to compensate for the larger degree of variability associated with injecting less microspheres.

Regional Distribution of Non-radioactive Microspheres in Hypertrophied Myocardium

The results of the second experimental series indicate that the transmural pattern of microsphere aggregation in the left ventricular myocardium is not exclusively a function of the injection site. Structural and functional alterations of the left ventricle in response to a chronic pressure overload also appear to have a significant affect on the distribution of microspheres in the coronary vasculature.

The significantly higher degree of aggregation across the wall of the hypertrophied left ventricle implies delivery of a greater number of microspheres to the coronary circulation. Estimates of the average number of microspheres per unit of tissue in control and hypertrophied myocardium support the idea of an increased resting coronary blood supply. Similarly, the shift in the transmural gradient of aggregation implies a concentrating effect of the increased wall thickness on streaming aggregates and/or a disproportionate increase of the coronary blood supply to the subendocardial layers. Estimates of the transmural distribution of "single" microspheres per unit of tissue (each aggregate was counted as 1 microsphere) suggest an increased subendocardial flow that is limited to the innermost two deciles of the wall thickness. A higher resting coronary blood flow implies that myocardial oxygen requirements are increased in left ventricular hypertrophy. As a result, the coronary flow reserve at any given coronary perfusion pressure is decreased, especially in the subendocardium. Unfortunately, actual coronary blood flow values could not be calculated to allow for quantitative comparison between the animal groups.

In normal hearts, in vivo measurements have demonstrated large, complex deformations within the midventricular wall as the muscle fibers shorten

during left ventricular contraction; whereas the outer 2/3 of the wall deformed very similarly, exhibiting linear displacements only, the subendocardium was dominated by large, angular deformations or shear (Fenton et al., 1978). Differential distortion of the coronary vasculature by myocardial shear forces could cause microspheres lodged in or traversing the same vessel to aggregate. If such a mechanism of aggregate formation also exists in hypertrophied hearts, the faster heart rates in animals with left ventricular hypertrophy may have contributed substantially to the shift in the transmural gradient of aggregation by increasing the rate of systolic distortion of the subendocardial vasculature. Extension of this effect over a relatively greater wall thickness is also possible due to the pronounced hypertrophy of subendocardial muscle fibers (Lund and Tomanek, 1978; Rakusan et al., 1980; Tomanek et al., 1986). If the pattern of vascular distortion is maintained with hypertrophy, the flow behavior of aggregates formed in relatively larger vessels alone would contribute to the greater variability in the number of microspheres per unit of tissue. However, histometric analysis of the muscle fiber orientation in normal and hypertrophied ventricles (Tezuka, 1975; Tezuka and Takahashi, 1976) suggests an altered pattern of vascular distortion, especially in the innermost two deciles of the wall thickness. If this structural adaptation ensures, in part, the most effective contractile force of individual fibers during systole, not only will aggregate formation be displaced, but it may also be enhanced. Thus, although anatomical (Borg and Caulfield, 1981) and physiological (Downey et al., 1974; Hess and Bache, 1979) studies suggest that myocardial shear forces have a negligible effect on the distribution of blood flow, one cannot exclude the possibility of a significant effect on the distribution of microspheres.

Systolic vascular compression due to intramyocardial tissue pressure

development and muscle fiber contraction (Downey et al., 1974) represents another possible mechanism for intracoronary aggregate formation. Unlike myocardial shear forces which, if effective, limit aggregate formation to the subendocardium, vascular compression is expected to increase aggregate formation with increasing wall depth in parallel with the transmural tissue pressure gradient and, to a smaller extent, cause uniform aggregate formation across the wall in parallel with contraction. The direct contribution of each compressive force to aggregate formation, however, is relatively independent of wall thickness (Spaan et al., 1983). The variation between normal and hypertrophied ventricles in the amount and transmural distribution of aggregates formed by vascular compression must, therefore, be related to a concentrating effect of the increased wall thickness on streaming aggregates, as previously discussed, and to altered vascular compression. Specifically, if compressive forces are stronger in hypertrophy (Tomanek et al., 1986), the resultant increase in aggregate formation across the wall would be greater than that expected with tachycardia alone.

It should be noted that this interpretation of our data is based on microsphere distribution measurements of a high spatial resolution. In contrast, low resolution measurements, i.e., the subendocardial/subepicardial ratios, indicated normalization of the flow behavior of microspheres and, thus, of red blood cells, in pressure-overloaded, hypertrophied ventricles. Since regional myocardial blood flow data which are based on measurements of tissue radioactivity are similar for comparable experimental conditions (see Table 1A in the Appendix), a definite limitation of the radioactive microsphere technique is indicated, namely the inability to detect localized changes of myocardial flow.

Advantages and Disadvantages of the Technique

To determine regional myocardial blood flow distribution with the radioactive microsphere technique, multiple tissue sampling is required. The degree of resolution will be determined by the number of pieces into which the myocardium is sectioned (Malsky et al., 1977). Although one study reported transmural myocardial blood flow profiles in deciles of the left ventricular wall thickness in porcine hearts (Hearse et al., 1986), the degree of resolution of transmural myocardial perfusion measurements obtained in larger animals is generally much lower (2-4 transmural tissue samples). To accurately measure transmural myocardial perfusion in smaller animals is technically difficult with the radioactive microsphere technique. The average left ventricular wall thickness of the control rats used in the present study approximated only 3.5 mm (assuming an average muscle fiber diameter of 16.6 μ m and a total of 211 muscle fiber layers across the left ventricular wall (Table 4)). Thus, with the exception of Vetterlein and Schmidt (1980) and Wicker et al. (in press) who measured rat myocardial flow in subendocardial and subepicardial halves of the left ventricular wall, transmural myocardial perfusion has not been determined in rats with the conventional methods used. With the morphometric technique described in this study, a continuous representation of non-radioactive microsphere distribution across the left ventricular wall may be obtained, allowing for measurement of regional myocardial perfusion in rats with a high spatial resolution.

Another advantage of the present technique is that the muscle fibers and their orientation across the left ventricular wall are visible. The regional distribution of microspheres may, therefore, be related to the myocardial morphology. The structural complexity of the left ventricular

wall has been well characterized (Streeter et al., 1969; Fenton et al., 1978; Greenbaum et al., 1981). For example, regional variability in the wall thickness and in the distribution of muscle fiber layers among the three major orientations (subendocardial, midwall, and subepicardial) is considerable. Similar observations were made in the present study (Table 4); subendocardial and midwall fibers predominated, and were present in approximately equal proportions, while subepicardial fibers were present in the smallest proportion. Thus, by direct observation of the microsphere distribution to the major muscle fiber orientations, a more sensitive index of transmural myocardial perfusion (i.e., SEN/SEP by the orientation method; see pg. 48) may be obtained, whereas calculation of the SEN/SEP blood flow ratio from radioactivity measurements in "subendocardial" and "subepicardial" layers of the left ventricular wall obtained by cutting the tissue may be misleading.

The advantage of visualizing the muscle fibers when determining transmural myocardial perfusion is also apparent when one considers the morphological changes accompanying various experimental interventions. For example, although the muscle fiber arrangement in pressure overloaded hypertrophied ventricles remains essentially the same as in normal hearts (Table 4; Korecky and Rakusan, 1983; Tezuka, 1975; Tezuka and Takahashi, 1976), subendocardial muscle fibers have been observed to undergo greater hypertrophy than those of the midwall and subepicardium (Lund and Tomanek, 1978; Rakusan et al., 1980; Tomanek et al., 1986). Thus, the "subendocardial" and "subepicardial" tissue samples from the hypertrophied ventricles may not correspond to those from the control ventricles when identical tissue sampling protocols are applied with the radioactive microsphere technique. Consequently, inaccurate comparisons may be made.

A third advantage of the present technique is that the distribution of myocardial blood flow is determined by microscopic counting of non-radioactive microspheres rather than by measurement of tissue radioactivity. The radioactive microsphere technique assumes a direct proportionality between the tissue radioactivity and the microsphere number (Heymann et al., 1977) and, therefore, of the blood flow. However, the size of the microspheres injected may vary (see Microsphere Size i. Coulter Counter-Channelyzer Analysis, pg. 56), a finding that is consistent with other reports (e.g. Flaim et al., 1984). Thus, it is more reasonable to presume that the tissue radioactivity is proportional to the microsphere volume rather than the number. By microscopic counting of non-radioactive microspheres, one can avoid the faults following radioactivity detection of microspheres (and of microsphere aggregates) varying in volume. Additional problems associated with a radioactive label (e.g. radiation hazards, radioactivity leaching into the suspending medium, and energy overlap between different labels) are also eliminated. The accuracy of measurements of myocardial blood flow distribution is, therefore, increased.

Variable myocardial regions void of microspheres were occasionally observed following left ventricular injection of a very large number of microspheres ($\geq 5 \times 10^6$). This was attributed to blockage by a large microsphere aggregate(s) of a coronary vessel(s) supplying this region. Consequently, the myocardial blood flow distribution pattern would be significantly altered. Additional microspheres entering the coronary vasculature would either become trapped behind the microsphere aggregate(s), further aggravating the problem, or be diverted to regions of altered flow. It is speculated that this occurrence is independent of the total number of microspheres injected, based on the finding that the transmural gradient of

microsphere aggregation was not significantly altered following injection of a relatively small number of microspheres (1.5×10^5) into the left atrium; i.e., the adequacy of mixing of microspheres with the blood does not appear to depend on the number of microspheres injected. The degree to which the myocardial blood flow distribution pattern would be altered with left ventricular injection of a smaller number of microspheres, however, would probably decline. If blood flow is measured in relatively large myocardial tissue samples, as is normally done with the radioactive microsphere technique, a region lacking microspheres may not be detected. Consequently, mean myocardial blood flow values for that region will be underestimated, while blood flow to other regions will be overestimated. The variability of regional flow measurements will also increase. With the non-radioactive microsphere technique described in this study, such errors can be avoided.

There also are disadvantages of applying our morphometric technique using non-radioactive microspheres to measure regional myocardial blood flow distribution. One problem concerns the accuracy of the results. According to Buckberg et al. (1971), when a sample (organ, tissue specimen, or arterial reference sample) receives only a small fraction of the cardiac output, the variability of microsphere distribution to that sample should approximate a Poisson distribution if the experiment is repeated many times under identical conditions. Therefore, to assure a measurement of flow correct to within 10% of the actual flow at the 95% confidence level, at least 400 microspheres of each type injected must be present per sample. That is, errors in calculated flow are expected in 5% of microsphere injections; if the minimal number of microspheres required for accuracy (400) is present, the error of measurement should not exceed 10%. Smaller numbers of microspheres, however, are associated

with larger errors and lower precision. Therefore, to increase the accuracy of the regional non-radioactive microsphere distribution measurements in the present study, a very large number of spheres was injected. Since most measurements were of a high spatial resolution, however, their reliability may be questioned. Tables 3A-5A in the Appendix list the average total number of microspheres counted in each myocardial region sampled per heart as estimated from the mean values shown in Table 1 and Figures 6-8, 9-10 (upper panels), Table 5 and Figures 11-14 (upper panels), and Table 8, for experimental series 1-3 respectively. Also shown is the corresponding percentage error of measurement calculated by the following equation (see Buckberg et al., 1971):

$$\text{error} = \left(\frac{1.96\sqrt{x}}{x} \right) 100$$

where x = mean number of microspheres per sample
 $\sqrt{x}$ = standard deviation

Measurements of low spatial resolution, and those that had required extensive tissue sampling, were the most accurate. The least accurate measurements were of transmural non-radioactive microsphere distribution in deciles of the left ventricular wall thickness, with errors of measurement ranging from 15.3%-26.9% in the first experimental series, and 11.2%-22.3% in the second series. Errors were most pronounced when each aggregate was counted as 1 microsphere, especially following left atrial injection in control rats. Despite these large errors, which exceed the accepted standard for accuracy described above, the mean number of non-radioactive microspheres in deciles of the left ventricular wall thickness may not necessarily over- or underestimate the flow provided that a sufficient number of experiments have been carried

out (Buckberg et al., 1971). In the present study, the standard error of measured values did not exceed 9.2%. Furthermore, empirical studies have shown that a minimum of 400 microspheres is not required per tissue sample to accurately measure cardiac output and its regional distribution (Ishise et al., 1980: 200 microspheres per tissue sample), or to demonstrate the significance of any given difference in flow (present study: < 310 microspheres per tissue sample; Meyer et al., 1982: < 100 microspheres per tissue sample). Therefore, the present results of regional microsphere distribution in the left ventricular myocardium, which are in agreement with measurements of regional myocardial perfusion generally reported by others using the radioactive microsphere technique (see Table 1A in the Appendix), are considered valid.

Increasing the total number of microspheres injected will increase the accuracy of measurements with the present technique, provided that myocardial function or flow is not altered. When 5×10^6 microspheres were injected, the smallest number of spheres delivered to a myocardial sampling region (i.e., section, region, decile, orientation) was 67 when all the microspheres per aggregate were counted (Table 3A in the Appendix). Therefore, to ensure delivery of a minimum of 400 microspheres to each myocardial sampling region while maintaining the tissue sampling protocol described in this study would require injection of approximately 3×10^7 microspheres. However, the percentage of capillaries occluded by microspheres with this injection, as estimated from the capillary density of the myocardium and the number of "single" microspheres per volume of tissue (each aggregate was counted as 1 microsphere), will approximate 92% as opposed to 15% with injection of the smaller microsphere dose, assuming a similar degree of microsphere aggregation with either injection. The actual

fraction of occluded capillaries with injection of the larger microsphere dose will obviously be lower than 92% due to the higher degree of aggregation with injection of a greater number of microspheres. Nevertheless, occlusion of the coronary vasculature following injection of the smaller microsphere dose was tolerated by the majority of experimental animals. Injection of larger numbers of microspheres, however, generally resulted in death. Thus, accuracy with the present technique may be increased only by increasing the size and/or the number of sampling sites. A greater number of experiments may also be required. This would, however, aggravate another problem associated with this technique, namely the extensive time required for data collection.

To appreciate the extent of this problem, the total time required to collect data for the first experimental series ($n=20$) is compared to that for a hypothetical series ($n=20$) in which the sampling area has been adjusted to ensure a minimum of 400 microspheres per unit of tissue (Table 9). The times reported are based on my experience with the sampling technique (~ 1 hr/sampling site), and apply to a regular work schedule.

Table 9. Approximate duration of data collection for the first experimental series, and for a hypothetical series in which the sampling area was adjusted to ensure a minimum of 400 microspheres per unit of tissue.

	First Experimental Series	Hypothetical Series
minimum number of microspheres per unit of tissue	67*	400
tissue volume (mm ³)	0.0571**	0.3410
sampling sites	12	72***
n	20	20
total time required for data collection (months)	1.5	9

* see Table 3A in the Appendix

** estimated tissue volume = (left ventricular wall thickness/10) x section thickness x width of sampling site x number of sampling sites
= 0.35 mm x 0.016 mm x 0.85 mm x 12

*** estimated number of sampling sites = $0.341 \text{ mm}^3 / (0.35 \text{ mm} \times 0.016 \text{ mm} \times 0.85 \text{ mm})$

It is evident that the present technique for determining regional myocardial perfusion in smaller animals is both time and cost inefficient. Thus, if the technique is to be used in future studies, the data collection protocol should be modified. Possible improvements are listed in the following section entitled "Technical Recommendations".

Another disadvantage of the present technique is that serial determinations cannot be made in the same animal using carbonized non-radioactive microspheres. This necessitates an increased number of experiments for comparing two (or more) experimental conditions. Recently, however, Shen et al. (1985) have made available polystyrene non-radioactive microspheres coloured with water insoluble dyes. Nine distinct colours were produced, allowing for 9 discrete determinations per experimental animal. Unfortunately several precautions must be taken when using these microspheres for multiple flow determinations. As for any microsphere type, consistency in size and shape is necessary for microsphere distribution to be proportional to red blood cell flow. Secondly, the mixing/injection/staining protocols must not interfere with the integrity of the spheres, as this will preclude data collection. Thirdly, multiple microsphere injections must not significantly alter systemic hemodynamics or regional myocardial flows. Heart rate and mean arterial pressure should, therefore, be monitored during the injection period. Constraints imposed in smaller animals on the total volume of fluid which may be introduced into the circulation during microsphere injection and saline flush, and on the total number of microspheres which may be injected (present study: 5×10^6) will limit the number of flow determinations per experimental animal. To compensate for the greater degree of variability associated with injecting less microspheres of one colour, the tissue sampling area and/or the number of experiments will have to be increased. Thus, it is

doubtful whether studies of regional myocardial perfusion using coloured, as opposed to carbonized non-radioactive microspheres will require less time for data collection. However, several considerations favouring the use of coloured spheres include the decreased between-heart variability in measured values when experimental animals are used as their own controls, the shorter time spent on surgical instrumentation, microsphere injection, and histological preparation, and the decreased cost requirements for microspheres and animals. In addition, the distribution pattern of coloured spheres may uncover information concerning their flow behavior. Recently, Reeves and Rakusan (in press) found that sequential left ventricular injection of two distinct microsphere suspensions, each suspension containing microspheres of one colour, resulted in the occurrence of two types of aggregates within the coronary vasculature: aggregates containing microspheres of one colour, and aggregates containing microspheres of both colours. This supports our view that the occurrence of aggregates within the coronary vasculature may, in part, be the consequence of random distribution of single microspheres.

Finally, because a relatively long time interval is necessary to inject a large number of microspheres suspended in a large volume of fluid, the frequency response of the present technique is relatively slow. Therefore, transient alterations in regional myocardial blood flow distribution (e.g. Sestier et al., 1978) cannot be measured.

Technical Recommendations

i. General Application Our success with the present technique in obtaining reliable estimates of regional myocardial blood flow distribution at a level of resolution previously unattained in rats justifies its use in future studies. With proper application and the necessary refinements, flow measurements with this technique should permit a detailed characterization of the coronary circulation in the various models of disease that have been developed in the rat. With appropriate tests, it is probable that this technique will also allow accurate measurement of regional blood flow distribution in other organs and small animal models.

ii. Data Collection Semi- and fully automated particle counter/image processing systems offer a readily available solution to considerably improve the accuracy and efficiency of the data sampling technique described in this study. These systems range from the relatively inexpensive digital image analyzer which permits either direct counting and mapping of non-radioactive microspheres by the operator or distribution analysis when coupled with a computer, to the extremely expensive, automated video image data processor. In all practical consideration, the system of preference will depend on the available financial resources.

By relating the data output to the corresponding histological sections, the relationship between organ flow and structure may then be defined. It should be realized, however, that neither microsphere distribution measurements obtained by counting all the microspheres nor those obtained by counting each aggregate as one microsphere accurately represent actual organ flow. Until the direct contribution to the distortion of organ flow by each of the

various factors which have been implied to affect the flow behavior of microspheres is known, a measurement of microsphere distribution midway between the range of measurements that may be obtained with this technique should provide a reliable flow estimate.

iii. Calculation of the Subendocardial/Subepicardial Blood Flow Ratio

The standard index of transmural myocardial perfusion, i.e., the subendocardial/subepicardial blood flow ratio, is generally determined from measurements of flow in the inner- and outermost "layers" of the left ventricular wall. Unless flow is uniformly distributed across the wall, however, the calculated subendocardial/subepicardial ratio may vary depending on which portions of the wall are sampled. Variable tissue sampling may, in part, explain the discrepancies in transmural myocardial blood flow determinations obtained by different laboratories. Several studies support the concept that a more sensitive measure of transmural myocardial perfusion should be adopted unless measurements are of a high resolution.

Using an autoradiographic technique for assessing the continuous distribution of regional myocardial blood flow at a high level of resolution in dogs, Malsky et al. (1977) found that the flow reduction in tissue made ischemic by coronary artery occlusion was accompanied by flow redistribution favouring the subepicardium. This is consistent with other studies in which transmural blood flow distribution in ischemic myocardium, as expressed by the subendocardial/subepicardial blood flow ratio, was measured at a relatively low level of resolution using the radioactive microsphere technique (Becker et al., 1971; Gross et al., 1984). Unlike these studies, however, Malsky et al. (1977) observed that the greatest reduction in flow occurred approximately one-third of the distance from the subendocardium to the subepicardium. Significantly

higher flows were found at the subendocardial surface. Probably to avoid masking the severity of this localized blood flow deprivation, a subendocardial/subepicardial blood flow ratio was not calculated as for normal hearts with uniform transmural perfusion.

For accurate determination of coronary blood flow in rats following left ventricular injection of microspheres, Idvall et al. (1979) recommended that the inner lining of the left ventricle be excised (5-10% of myocardial tissue) prior to measurement. The inappropriately high coronary blood flow values generally obtained in rats following left ventricular, but not left atrial injection of microspheres (Bartrum et al., 1974; Idvall et al., 1979; Kobrin et al., 1984; Wicker and Tarazi, 1982b) were believed to arise from adherence of microspheres to the endocardial surface of the left ventricle (Idvall et al., 1979). This was not observed in the present study. As previously discussed, however, we have found indirect evidence to suggest that microspheres do not accurately measure flow to the innermost subendocardial layers of the left ventricular wall, especially following left ventricular injection. Therefore, we still support the recommendation by Idvall et al. (1979), although for different reasons.

Using a Doppler system to measure blood flow velocities in an epicardial and intramural artery in open-chest, anesthetized dogs, Chilian and Marcus (1982) hypothesized the existence of a significant coronary capacitor in the epicardium. This capacitance was believed to influence measurements of phasic coronary blood flow, thereby distorting the actual flow. Some supportive data have been obtained from analysis of transmural microsphere distribution in the present study. Although a capacitive influence of epicardial arteries on the distribution of microspheres is not evident from our measurements in normal hearts, the significant variability in the number of microspheres in the

outermost decile of the left ventricular wall thickness in hypertrophied hearts suggests that the capacitance of epicardial arteries is elevated with hypertrophy to a level which would significantly influence microsphere flow. This elevated capacitance, which may be ascribed to luminal enlargement (Tomanek et al., 1986), could cause microspheres to be "stored" temporarily in the epicardial arteries, probably during systole, but how this affects microsphere flow to the epicardial capillaries is unclear. Nevertheless, this unexpected finding suggests that the outer 10% of the left ventricular wall thickness should also be excised before the subendocardial/subepicardial blood flow ratio is determined.

Finally, because significant changes in transmural myocardial blood flow appear to be localized, at least with pressure-induced left ventricular hypertrophy (present study) and experimental myocardial infarction (Malsky et al., 1977), myocardial tissue with intermediate flow should not be incorporated into the calculation of the ratio of subendocardial/subepicardial flow, as this may also decrease the sensitivity of the measurement. Our data show that this area of intermediate flow is located approximately half of the distance between the subendocardium and the subepicardium, and occupies approximately 20% of myocardial tissue.

Therefore, a sensitive measurement of transmural myocardial perfusion can be obtained, even if the measurement is of a low spatial resolution, by excising approximately 40% of myocardial tissue prior to calculation of the subendocardial/subepicardial blood flow ratio: 10% of the innermost subendocardial layers, 10% of the outermost subepicardial layers, and 20% of the midwall.

SUMMARY

Measurements of regional myocardial blood flow obtained using the radioactive microsphere technique are subject to variation, and may be misleading. There is some evidence to suggest that this variation may, at least in part, be due to a difference in the distribution of microspheres and red blood cells. Unfortunately, an appropriate method to analyze microsphere flow in the coronary vasculature is lacking due to technical limitations. Therefore, we have developed a morphometric technique that has permitted, for the first time, a detailed investigation of the distribution of individual microspheres in various regions of the left ventricular myocardium in anesthetized rats. Based on our results, we could draw reasonable inferences concerning microsphere flow.

Non-radioactive microspheres were injected, and their distribution in cross-sections of the left ventricular myocardium was determined by direct observation. Our results have shown that microsphere aggregation in the coronary vasculature is a significant problem, and may influence measurements of total myocardial blood flow and its transmural distribution when data are based on measurements of tissue radioactivity following injection of radioactive microspheres. Specifically, it was shown that left ventricular injection is unsuitable for myocardial blood flow determinations due to inadequate mixing of microspheres with the blood. Inadequate mixing resulted in delivery of a greater number of microspheres to the coronary circulation, and an increased degree of microsphere aggregation that was more pronounced in the subendocardial layers, probably due to axial streaming. For greater accuracy, left atrial injection is recommended. However, uneven transmural distribution of microsphere aggregates is also a problem following

left atrial injection, but this is less pronounced compared with left ventricular injection. Although we cannot rule out the possibility that left atrial injection also results in incomplete mixing of microspheres with the blood, it appears more likely that aggregation with left atrial injection is dependent only on random microsphere interactions proximal to the coronary ostia and within the coronary vasculature. Injection of a relatively small number of microspheres resulted in a lower degree of aggregation, indicating that these interactions are more pronounced with increasing numbers of microspheres entering the coronary circulation. Aggregates that enter the coronary circulation would then accumulate mainly in the subendocardial layers due to axial streaming. This interpretation of our data is supported by our study in which the distribution of microspheres was determined in hearts made hypertrophic by a chronic pressure overload. The higher degree of aggregation observed in these hearts may be explained by a higher resting coronary blood supply that was most pronounced in the innermost subendocardial layers and/or greater accumulation of aggregates in the subendocardium due to the extended axial streaming that is expected with left ventricular wall thickening.

In conclusion, morphometric analysis of the regional distribution of non-radioactive microspheres in sections of the left ventricular myocardium from anesthetized rats has shown that measurements of myocardial blood flow distribution obtained using the radioactive microsphere technique may be distorted by the uneven distribution of microsphere aggregates. To minimize errors in myocardial flow determinations, we recommend that left atrial injections of a relatively small number of microspheres be used.

APPENDIX

Table 1A. Summary of data from several selected studies which have used current methods to measure the regional distribution of myocardial blood flow in the left ventricular wall under various experimental conditions.

Abbreviated Terms:

LV = left ventricle, left ventricular

LA = left atrium, left atrial

LVFW = left ventricular free wall

ant, lat, post = anterior, lateral, posterior LVFW

SEN, MID, SEP = subendocardium, midwall, subepicardium

SEN/SEP = subendocardial/subepicardial blood flow ratio

LMCA, RMCA = left and right main coronary artery

LCA = left coronary artery

LAD = anterior descending branch of LCA

LC = circumflex branch of LCA

MBF = myocardial blood flow

rMBF = regional distribution of MBF

LVH = left ventricular hypertrophy

RH = reactive hyperemia

RM, NRM = radioactive and non-radioactive microspheres

hr = heart rate

sig = significant

ns, nsd = not significant, not significantly different

↑, ↓, → = increase, decrease, no change

<, > = less than, greater than

DIFFUSIBLE INDICATOR TECHNIQUE

1) Intramyocardial Isotope Injection

Technique	Results	Reference												
<u>dog (anesthetized):</u> NaI ¹³¹ clearance	MBF at least 25% lower in SEN compared to SEP	Kirk and Honig, 1964a												
<u>dog (anesthetized):</u> ¹³³ Xe clearance	MBF 30% lower in SEN compared to SEP; progressive ↑ of MBF from SEN to SEP	Andersen et al., 1969												
<u>rabbit (anesthetized):</u> -hydrogen clearance	<table><tr><td></td><td><u>SEN/SEP</u></td></tr><tr><td>control</td><td>0.88</td></tr><tr><td>8% O₂</td><td>0.81*</td></tr><tr><td>1st CO exposure</td><td>0.83</td></tr><tr><td>2nd CO exposure</td><td>0.76*</td></tr><tr><td colspan="2">* = sig from control</td></tr></table>		<u>SEN/SEP</u>	control	0.88	8% O ₂	0.81*	1st CO exposure	0.83	2nd CO exposure	0.76*	* = sig from control		Kleinert et al., 1980
	<u>SEN/SEP</u>													
control	0.88													
8% O ₂	0.81*													
1st CO exposure	0.83													
2nd CO exposure	0.76*													
* = sig from control														
-arterial O ₂ content ↓ ~30% by inspiration of CO or low O ₂ gas mixture	-under both hypoxic conditions MBF ↑, but to a lower extent in SEN													
<u>dog (paralyzed):</u> ¹³³ Xe clearance at various coronary perfusion pressures (CPP)	<table><tr><td><u>CPP (mmHg)</u></td><td><u>rMBF</u></td></tr><tr><td>74-119</td><td>-autoregulation of MBF present in SEN and SEP</td></tr><tr><td></td><td>-SEN/SEP ns from 1.00</td></tr><tr><td>60-74</td><td>-SEP MBF ↑; SEN MBF ↑ (sig)</td></tr><tr><td>45-59</td><td>-SEN and SEP MBF ↑ (sig), but ↑ more pronounced in SEN</td></tr></table>	<u>CPP (mmHg)</u>	<u>rMBF</u>	74-119	-autoregulation of MBF present in SEN and SEP		-SEN/SEP ns from 1.00	60-74	-SEP MBF ↑; SEN MBF ↑ (sig)	45-59	-SEN and SEP MBF ↑ (sig), but ↑ more pronounced in SEN	Haunso, 1981		
<u>CPP (mmHg)</u>	<u>rMBF</u>													
74-119	-autoregulation of MBF present in SEN and SEP													
	-SEN/SEP ns from 1.00													
60-74	-SEP MBF ↑; SEN MBF ↑ (sig)													
45-59	-SEN and SEP MBF ↑ (sig), but ↑ more pronounced in SEN													

ii) Intravascular Isotope Injection

<u>dog (tranquilized):</u> ⁸⁶ Rb uptake following aortic root injection, vena cava infusion, and intravenous injection/infusion	⁸⁶ Rb uptake highest near SEN surface, with a progressive ↑ towards SEP (SEN/SEP > 1.00)	Gillespie and Love, 1967
---	---	--------------------------

Technique	Results	Reference												
<u>dog (anesthetized):</u> ^{133}Xe uptake following bolus LV injection	-MBF 15% higher in SEN compared to SEP -correcting for probability that activity in LVFW partly due to ^{133}Xe uptake directly from LV cavity or to ^{133}Xe - containing blood on SEN sur- face, uniform rMBF suggested -rMBF nsd between myocardium supplied by LAD and LC, or between base and apex	Bagger, 1972												
<u>dog (anesthetized):</u> ^{133}Xe uptake following bolus injection into aortic root	^{133}Xe uptake between SEN and SEP (SEN/SEP = 0.98), between base and apex, or between LAD- and LC-perfused myocardium nsd	Bagger, 1977												
<u>^{14}C dog (conscious):</u> ^{14}C -antipyrine uptake following LA infusion -frozen tissue samples sectioned for macroauto- radiography, providing a continuous graphic representation of radioactivity from which quantitative rMBF measure- ments were obtained	-SEN/SEP = 1.12 (ns from 1.00) -minor transmural inequa- lities of MBF	Malsky et al., 1977												
<u>^{131}I dog (anesthetized):</u> ^{131}I -iodoantipyrine uptake following LMCA infusion	<table><tr><th>area</th><th>SEN/SEP</th></tr><tr><td>base</td><td>1.00</td></tr><tr><td>apex</td><td>1.00</td></tr><tr><td>ant</td><td>1.10</td></tr><tr><td>lat</td><td>1.07</td></tr><tr><td>post</td><td>1.12</td></tr></table>	area	SEN/SEP	base	1.00	apex	1.00	ant	1.10	lat	1.07	post	1.12	Griggs and Nakamura, 1968
area	SEN/SEP													
base	1.00													
apex	1.00													
ant	1.10													
lat	1.07													
post	1.12													
<u>dog (anesthetized):</u> uptake of ^{86}Rb injected as a bolus into the coronary circulation when MBF limited to systole	MBF highest in SEP, with a progressive $\downarrow$ towards SEN (SEN/SEP = 0.52)	Downey and Kirk, 1974												

Technique	Results	Reference								
<u>dog (anesthetized):</u> uptake of ^{86}Rb or ^{42}K injected as a bolus into the coronary circulation when MBF limited to systole in A) control hearts B) hearts ejecting into a severed aorta (LV pressure absent) C) isovolumetrically contracting hearts (LV pressure present)	-with perfusion over the entire cardiac cycle, MBF highest in SEN, with a progressive $\downarrow$ towards SEP (SEN/SEP = 1.40) -when MBF limited to systole, <table><tr><th></th><th>SEN/SEP</th></tr><tr><td>A</td><td>0.37</td></tr><tr><td>B</td><td>1.00</td></tr><tr><td>C</td><td>0.62</td></tr></table>		SEN/SEP	A	0.37	B	1.00	C	0.62	Downey et al., 1974
	SEN/SEP									
A	0.37									
B	1.00									
C	0.62									

<u>^{86}Rb dog (anesthetized):</u> ^{86}Rb uptake was compared at different mean arterial (MAP) or intraventricular (IVP) pressure levels in normal, ischemic (LC ligation), and fibrillating myocardium	-in normal myocardium, IVP changes did not affect the uniform rMBF (SEN/SEP ns from 1.00) -in ischemic myocardium, <table><tr><th>MAP (mmHg)</th><th>SEN/SEP</th></tr><tr><td>40</td><td>1.25*</td></tr><tr><td>100</td><td>0.83*</td></tr><tr><td>152</td><td>0.57*</td></tr></table> * = sig from each other -in fibrillating myocardium, <table><tr><th>IVP (mmHg)</th><th>SEN/SEP</th></tr><tr><td>5</td><td>1.00*</td></tr><tr><td>16</td><td>0.91</td></tr><tr><td>26</td><td>0.83*</td></tr></table> * = sig from each other	MAP (mmHg)	SEN/SEP	40	1.25*	100	0.83*	152	0.57*	IVP (mmHg)	SEN/SEP	5	1.00*	16	0.91	26	0.83*	Cutarelli and Levy, 1963
MAP (mmHg)	SEN/SEP																	
40	1.25*																	
100	0.83*																	
152	0.57*																	
IVP (mmHg)	SEN/SEP																	
5	1.00*																	
16	0.91																	
26	0.83*																	

Technique	Results	Reference
<u>dog (anesthetized):</u>		
uptake of ^{86}Rb infused into LMCA in	SEN/SEP A i) 0.94 ii) 1.07** iii) 0.28* **	Moir and DeBra, 1967
A i) control [normal coronary perfusion pressure (CPP), normal left intraventricular pressure (LIVP)]	B N H i) 1.15** 1.19* ** ii) 1.04 1.12* ** iii) 1.07* ** 1.09* **	
ii) LV hypertension [normal CPP, + LIVP]	C i) 1.23* ** 1.20* **	
iii) ischemia [+ CPP, normal LIVP]	* = sig from 1.00 ** = sig from control N = normotensive H = hypertensive	
B with injection of coronary vasodilators		
i) dipyridamole		
ii) noradrenaline		
iii) vasopressin		
C with injection of β -blockers		
i) propranolol		
<hr/>		
<u>dog (anesthetized):</u>	-when LAD not occluded, ^{133}Xe activity between SEN and SEP in LC-perfused myocardium nsd	Bagger, 1977a
uptake of ^{133}Xe injected as a bolus into aortic root during optimal stenosis (LC progressively constricted, producing vasodilation of area it supplies, until diastolic RH to 10 sec occlusion disappeared)	-when LAD occluded, ^{133}Xe activity in SEP ~ 2X higher than in SEN in LC-perfused myocardium	
<hr/>		
iii) Inert Gas Clearance		
<hr/>		
<u>human (anesthetized):</u>	-small inhomogeneities of rMBF in representative patient without coronary artery disease	Cannon et al., 1975
uptake of ^{133}Xe injected as a bolus into LMCA or RMCA	-analysis could not distinguish between SEN and SEP MBF, but provided index of average MBF across LVFW	

INTRAVASCULAR ISOTOPE INJECTION VS RADIOACTIVE MICROSPHERE TECHNIQUE

Technique	Results	Reference												
<u>isolated, Langendorff, perfused, nonworking dog heart preparation:</u>														
RM of different sizes, 125 _I -antipyrine (I-Ap), 42 _K , or 86 _{Rb} injected into aortic inflow	-all tracers showed relatively higher MBF in SEN compared to SEP (SEN/SEP > 1.00) - 42 _K , I-Ap, and small diameter RM showed similar results; large diameter RM were deposited preferentially in areas of high MBF, exaggerating the apparent heterogeneity of rMBF	Yipintsoi et al., 1973												
<u>dog, sheep (anesthetized):</u> -LA injection of RM (25, 15, 9, 1-10 (filtered) μ m diameter; 0.5-3.0 x 10 ⁶ / injection) -radioactive-antipyrine sometimes simultaneously infused over 15-60 sec	-total MBF values were independent of RM size when % of untrapped RM known -with any pair of RM injected, larger RM were in excess in SEN, except for 25 μ m diameter RM, which were in excess of 15 μ m diameter RM in areas of higher MBF, whether it was in SEN or SEP -rMBF ~ uniform with 9 μ m diameter RM and antipyrine (SEN/SEP ns from 1.00)	Utley et al., 1974												
<u>dog (anesthetized):</u> bolus injection of ¹³³ Xe and RM (9 μ m diameter; 6-9 x 10 ⁶ /injection) into aortic root following A) progressive hemodilution until diastolic RH response to 10 sec LC occlusion disappeared (optimal hemodilution), and LAD occlusion B) 10 sec LCA occlusion to produce maximal coronary vasodilation (rMBF deter- mined at peak diastolic RH)	-in controls, both tracers showed uniform rMBF (SEN/SEP = 1.14; RM) <table> <tr> <td></td><td>SEN/SEP</td><td>¹³³Xe</td></tr> <tr> <td>RM</td><td></td><td></td></tr> <tr> <td>A</td><td>0.58*</td><td>0.55*</td></tr> <tr> <td>B</td><td>1.14</td><td>1.03</td></tr> </table> * = sig from 1.00		SEN/SEP	¹³³ Xe	RM			A	0.58*	0.55*	B	1.14	1.03	Bagger, 1978
	SEN/SEP	¹³³ Xe												
RM														
A	0.58*	0.55*												
B	1.14	1.03												

RADIOACTIVE MICROSPHERE TECHNIQUE

Technique	Results	Reference
dog (conscious; anesthetized):	RM diameter (μm)	SEN/SEP
LA injection of RM (14-61 μm diameter; $0.07-1.60 \times 10^5$ / injection, depending on radioactivity; twice, 8×10^6 RM with low activity were injected)	51-61 20-23 14 9	2.5 1.4 1.3 ~1.0
dog, sheep, lamb (anesthetized):		
LV or LA RM injection (8-50 μm diameter)	-equal concentration/g of 8 μm diameter RM in SEN and SEP	Buckberg et al., 1971
	-with 14-50 μm diameter RM, the larger the RM size, the greater the distribution to SEN	
dog (anesthetized):	RM diameter (μm)	SEN/SEP
LA injection of 15 and 50 μm diameter RM ($< 4 \times 10^6$ /injection)	15 50	1.12* 1.45* **
	* = sig from 1.00 ** = sig compared to 15 μm diameter RM	
dog (conscious; anesthetized):	RM (25 μm) /g	Eng et al., 1984
RM and NRM (25 and 50 μm diameter) injected into LV to determine rMBF (RM) and focal myocardial necrosis (NRM)	SEN 259 $\pm$ 120 MID 193 $\pm$ 87 SEP 167 $\pm$ 68	
	-SEN/SEP = 1.55 -highest variability in SEN -necrotic lesions larger and more frequent in MID and SEP	

Technique	Results		Reference
dog (anesthetized): 1.15-21.6 x 10 ⁶ RM (7-10 μ m diameter) injected simultaneously (sim) or sequentially (seq) into LA	AREA	normalized MBF (%) sim seq	Falsetti et al., 1975
	base 1	105.2 103.4*	
	2	97.9 96.8*	
	3	97.6 97.9	
	apex 4	99.1 102.0	
	* = sig from each other		
	ant	103.0 97.7*	
	lat	103.0 102.6*	
	post	96.1 100.8	
	* = sig from each other		
	SEN	104.4* 104.9**	
	MID	100.0* 100.0**	
	SEP	95.5* 95.2**	
	*,** = sig from each other		
	-SEN/SEP (avg) = 1.15		
	-variability of MBF to SEN, MID, and SEP was 28.7 $\pm$ 10.2%, 30 $\pm$ 11.3%, and 34.5 $\pm$ 12.4% ($\pm$ SD) respectively		
dog (anesthetized): 3.3 x 10 ⁶ labelled human albumin micro- aggregates (15 μ m diameter) injected into LV	-MBF rates and intravas- cular blood volume + from SEP to SEN (sig), and from base to apex in both SEN and SEP (sig)		Eliassen et al., 1982
	-SEN/SEP = 1.24 in basal, middle, and apical myocardium		
dog (anesthetized): RM (15 μ m diameter) injected into LV (3 x 10 ⁶ / injection) before and after CO poisoning	-in control, SEN/SEP (avg) = 1.37; heterogeneity in SEN/SEP across different LVFW regions (sig) (range: 1.17-1.57)		Einzig et al., 1980
	-with CO poisoning, SEN/SEP < 1.00 (sig)		

Technique	Results	Reference
<u>dog (conscious):</u> RM (7-10 μ m diameter) injected into LA (3 x 10 ⁶ /injection) A) over entire cardiac cycle B) when MBF limited to systole C) during subsequent RH (B > 20 sec) D) when MBF limited to equivalent period in diastole	<u>SEN/SEP</u> A 1.29**; progressive $\uparrow$ MBF from SEN to SEP B 0.38* **; SEP MBF $\uparrow$; progressive $\uparrow$ MBF from SEP to SEN C 6.4* **; SEP MBF $\uparrow$; SEN MBF $\uparrow$ (sig); [for equivalent peak RH following coronary occlusion, SEN/SEP = 1.73 over entire cardiac cycle] D ns from A * = sig from A ** = sig from 1:00	Hess and Bache, 1976

<u>dog (conscious):</u> RM (7-10 μ m diameter) injected into LA (3 x 10 ⁶ /injection) A) over entire cardiac cycle B) when MBF limited to systole C) when MBF limited to diastole D) during total coronary artery occlusion	<u>SEN/SEP</u> A 1.13 B 0.34* ** C 1.15 D 0.28* ** * = sig from A ** = sig from 1:00	Hess and Bache, 1979
---	--	-------------------------

<u>dog (conscious):</u> RM (15 μ m diameter) injected into LA (0.8-2.0 x 10 ⁶ /injection) at 3 of the following times: control (preocclu- sion), and 0.1, 2, 6, 24, or 96 hours after LAD or LC occlusion	<u>SEN/SEP</u> A B C 1.28 1.04* D 1.30 1.12* * = sig from A A = conscious (control) B = anesthetized, open-chest C = LAD-perfused myocardium D = LC-perfused myocardium	Bishop et al., 1976
--	---	------------------------

-after sudden LAD or LC
occlusion, MBF $\uparrow$ (sig) in
ischemic tissue; $\uparrow$ most severe
in SEN, less so in SEP, and
least in marginal region of
the infarct; MBF $\uparrow$ (sig) to
nonischemic tissue

-by 24 hours postocclusion,
MBF $\uparrow$ (moderate) in all areas
except central SEN core, with
further $\uparrow$ 96 hours postocclusion

Technique	Results						Reference
		<u>SEN/SEP</u>					
	<u>con</u>	<u>0.1</u>	<u>2</u>	<u>6</u>	<u>24</u>	<u>96</u>	
A	1.28	1.35	1.17	1.21	1.26	1.24	
B	1.28	1.08	0.81	0.92	1.02	1.02	
C	1.29	0.82	0.36	0.53	0.78	0.82	

A = nonischemic tissue
 B = marginal region
 C = ischemic tissue
 con = control
 0.1, 2, 6, 24, 96 = time post-occlusion (hours)

dog (anesthetized):
 3 x 10⁷ RM (15 μ m diameter)
 injected into left carotid
 artery or LC when
 A) autoregulation intact
 B) autoregulation absent
 C) autoregulation absent;
 MBF and coronary per-
 fusion pressure restored
 to normal

spatial heterogeneity of
rMBF:

Sestier et al.,
 1978

	<u>SEN/SEP</u>
A	1.20
B	0.63
C	1.20

-not a component of
 autoregulation

temporal heterogeneity
of rMBF:

-periodicity from high to
 low MBF of ~35-90 sec in
 small myocardial regions;
 phenomenon is random; MBF
 cycles in SEN and SEP have
 different periodicities

-component of autoregulation;
 when autoregulation abolished,
 temporal heterogeneity $\downarrow$

-because spatial heteroge-
 neity exists, temporal hete-
 rogeneity must occur to
 assure adequate rMBF

Technique	Results	Reference	
<u>dog (conscious; anesthetized):</u>			
LA injection of RM (7-10 μ m diameter)	A) circumferential SEN and SEP MBF ~ homogeneous;	Cobb et al., 1974	
A) in controls (conscious)	transmural gradients in most regions; SEN/SEP = 1.06-1.16		
B) with anesthesia, thoracotomy, and pericardiotomy	B) SEN and SEP MBF $\uparrow$; transmural gradients only in papillary muscle regions; SEN/SEP = 0.95-1.13		
C) during adenosine infusion in dosages that produced maximum MBF $\uparrow$ in A (i) and B (ii)	C) -SEN and SEP MBF $\uparrow$; SEN MBF < SEP MBF -circumferential SEP MBF homogeneous; SEP MBF $\uparrow$ in Ci and Cii nsd -circumferential SEN MBF not homogeneous; SEN MBF $\uparrow$ higher in Ci than in Cii [SEN/SEP (Ci) = 0.81-0.99; SEN/SEP (Cii) = 0.63-0.78]		
<hr/>			
<u>isolated, unloaded, diastolic-arrested, dog heart preparation:</u>			
RM injection (15 and 8 μ m diameter; 2 x 10 ⁵ /injection) during maximal vasodilation (adenosine)	-SEN/SEP = 1.62 (range: 1.11-1.85) -measurements in 8 layers showed successive $\uparrow$ in maximal MBF from SEP towards deeper layers; with a slight $\uparrow$ in deepest SEN layers (layers 7-8); greatest variability in SEN	Wüsten et al., 1977	
<hr/>			
<u>dog (anesthetized):</u>			
LA injection of RM (8-10 μ m diameter; 3 x 10 ⁷ /injection) during maximal vasodilation (carbocromen) at	-in LC-perfused myocardium,	Ellis and Klocke, 1979	
A) normal preload	SEN/SEP		
B) $\uparrow$ preload	A 0.76 B 0.55*		
	* = sig from A		
-LC cannulated and perfused (diastole only) through an external circuit of rigid tubing originating in left subclavian artery	-control SEN/SEP (A) probably underestimated; untrapped RM in large arteries may lodge in SEP during systole; to test this, external perfusion circuit was vented to the atmosphere during systole to prevent untrapped RM from being redistributed to SEP during systole [SEN/SEP (A) = 0.99; SEN/SEP (B) = 0.80*]		

Technique	Results	Reference
<u>dog (anesthetized):</u> RM (14 μ m diameter) injected into LA (1.2×10^6 / injection) in normal (N) and ischemic (I) myocardium at A) normal LV end-diastolic pressure (normal preload) B) $\uparrow$ LV end-diastolic pressure ($\uparrow$ preload)	SEN/SEP A B N 1.06 1.14 I 0.71 0.45	Kjekshus, 1973
<u>dog (conscious):</u> LA injection of RM (7-10 μ m diameter; 3×10^6 /injection) during ventricular pacing under A) control conditions B) maximal coronary vaso- dilation (adenosine)	A) rMBF remained essentially uniform with hr $\uparrow$ B) rMBF uniform at 100 bpm; with hr $\uparrow$ (150-250 bpm), SEP MBF $\rightarrow$, while SEN MBF (and SEN/SEP) $\uparrow$ in propor- tion to hr $\uparrow$; SEN MBF $\uparrow$ most marked in deepest layer	Bache and Cobb, 1977
	SEN/SEP 100 150 200 250 A 1.10 1.18* 1.13 1.04 B 1.00 0.81* 0.56* 0.40*	
	100, 150, 200, 250 = hr (bpm) * = sig from values at 100 bpm	
<u>dog (tranquilized):</u> LV injection of RM (15 μ m diameter; 2×10^5 (range: $0.05-5 \times 10^5$)/ injection) during A) control conditions B) tachycardia (151, 193 bpm)	A) SEN/SEP = 1.26 (sig > 1.00) B) total MBF $\uparrow$; SEN/SEP $\uparrow$ in proportion to hr $\uparrow$ (sig from control only at 193 bpm); SEN/SEP $\geq$ 1.00 -rMBF nsd between basal, middle, and apical myocardium	Neill et al., 1973
<u>dog (conscious):</u> LA injection of RM (7-10 μ m diameter) at rest and during 3 levels of treadmill exercise	-at rest, SEN MBF > SEP MBF in all regions of LVFW (range: 1.12-1.33) -with exercise, MBF $\uparrow$ to all regions in proportion to hr $\uparrow$; transmural MBF gradient was abolished (SEN/SEP ns from 1.00)	Ball et al., 1975

Technique	Results	Reference
<u>dog (anesthetized):</u> LAD injection of RM (9 μ m diameter; 8×10^5 /injection) during maximal vasodilation (adenosine) in controls (A), and following LAD injection of lidocaine (B) and isoproterenol (C)	- with lidocaine, + local contractility and coronary extravascular resistance - with isoproterenol, + systolic wall thickening <div style="text-align: center;"> <u>SEN/SEP</u> A 1.02 B 1.28* A 1.08 C 0.75* * = sig from A </div>	Marzilli et al., 1979
<u>dog (anesthetized):</u> RM (8-10 μ m diameter) injected into LA ($6-8 \times 10^5$ / injection) during A) distal aortic constric- tion (systolic and diastolic hypertension) B) proximal aortic constric- tion (+ LA pressure) C) ventricular pacing (shortened diastole)	<div style="text-align: center;"> <u>SEN/SEP</u> control 1.01 A 1.11* B 0.37* C 0.98 * = sig from 1.00 </div> - when SEN/SEP sig from 1.00, MBF to MID intermediate to MBF to SEN and SEP	Buckberg et al., 1972
<u>dog (anesthetized):</u> - mild LVH induced by banding of ascending aorta - after 6 weeks, LA injection of RM (9 μ m diameter)	<div style="text-align: center;"> <u>SEN/SEP</u> control 1.11 LVH 1.13 </div>	O'Keefe et al., 1978
<u>dog:</u> - chronic aortic stenosis (AS) produced with a Jacobson cuff, resulting in moderate/ severe LVH (LV weight 67% higher than in control) LA injection of RM (15 μ m diameter)	with AS, SEN/SEP + (sig) compared to control	Bishop et al., 1974

Technique	Results		Reference
dog (conscious):	<u>SEN/SEP</u>		Zile et al., 1985
-LVH induced by aortic banding at 7 weeks of age	A	1.21	
	Bi	1.10	
	Bi1	0.89*	
-rMBF studied 1 year later in			
A) controls	* = sig from A		
B) LVH i) compensated [fractional shortening and peak systolic pressure ns from A]			
ii) cardiac failure [fractional shortening + (sig) and peak systolic pressure + (sig) compared to A]			
dog (conscious):	<u>SEN/SEP</u>		Marcus et al., 1976
-moderate LVH (LV weight 50% higher compared to controls) induced by 1 kidney-Goldblatt hypertension	A	B	
	control	1.30	0.80
	LVH	1.20	0.90
-rMBF determined in the absence (A) or presence (B) of maximal coronary vasodilation (adenosine)	B) 5-6X LV MBF + in both control and LVH		
dog (anesthetized):	total SEN and SEP MBF (ml/min/g LV) + in LVH in both normal and vasodilated states		O'Keefe and Hoffman, 1976
-LVH induced by supra-valvular aortic constriction			
-after 6 weeks, RM injected into LA under normal conditions, and in presence of vasodilation (adenosine, carbochromen)	<u>Normal</u>	<u>total MBF</u>	<u>SEN/SEP</u>
	control	1.33	1.05
	LVH	1.00	1.13
	<u>Vasodilation</u>		
	control	5.23	0.55
	LVH	3.02	0.54

Technique	Results	Reference																				
<u>dog (anesthetized):</u> -moderate LVH (LV weight 50% higher than in control) induced by single kidney renal vascular hypertension -LA injection of RM (7-10 and 15 μ m diameter; 3.5-5.5 x 10 ⁶ /injection) A) at rest B) during LV pacing (200 bpm) C) during maximal vasodilation (adenosine)	<table><tr><td></td><th colspan="3">SEN/SEP</th></tr><tr><td></td><th>A</th><th>B</th><th>C</th></tr><tr><td>control</td><td>1.32</td><td>1.29</td><td>0.95</td></tr><tr><td>LVH + HT</td><td>1.40</td><td>1.17*</td><td>0.94</td></tr><tr><td>LVH</td><td>1.38</td><td></td><td>1.01</td></tr></table> <p>HT = hypertension * = sig in paired comparison between control and pacing</p> <p>-MBF between sections (base to apex), between regions (ant, lat, post, septal) or between layers (SEN, MID, SEP) nsd between control and LVH; SEN perfused homogeneously</p> <p>-progressive + MBF from SEN to SEP</p>		SEN/SEP				A	B	C	control	1.32	1.29	0.95	LVH + HT	1.40	1.17*	0.94	LVH	1.38		1.01	Mueller et al., 1978
	SEN/SEP																					
	A	B	C																			
control	1.32	1.29	0.95																			
LVH + HT	1.40	1.17*	0.94																			
LVH	1.38		1.01																			
<u>dog (conscious):</u> -severe LVH (heart weight 87% higher than in control) induced by banding of ascending aorta at 6 weeks of age -rMBF determined (9 μ m diameter RM) 1 year later at rest (A) and during moderate exercise (B)	<table><tr><td></td><th colspan="2">SEN/SEP</th></tr><tr><td></td><th>A</th><th>B</th></tr><tr><td>control</td><td>1.32</td><td>1.21</td></tr><tr><td>LVH</td><td>1.26</td><td>1.05*</td></tr></table> <p>* = sig from control</p>		SEN/SEP			A	B	control	1.32	1.21	LVH	1.26	1.05*	Holtz et al., 1977								
	SEN/SEP																					
	A	B																				
control	1.32	1.21																				
LVH	1.26	1.05*																				
<u>dog (conscious):</u> RM (15 μ m diameter) injection at various levels of treadmill exercise in controls and in animals with LVH	<p>-at rest,</p> <table><tr><td></td><th>SEN/SEP</th></tr><tr><td>control</td><td>1.34</td></tr><tr><td>LVH</td><td>1.27</td></tr></table> <p>-with exercise, SEN/SEP + in proportion to hr + ; in both control and LVH, SEN/SEP > 1.00, even during heavy exercise</p>		SEN/SEP	control	1.34	LVH	1.27	Mueller et al., 1979														
	SEN/SEP																					
control	1.34																					
LVH	1.27																					
<u>dog (conscious):</u> -LVH induced by perinephritic hypertension -rMBF determined at rest (A; hr = 98 bpm) and at 2 levels of treadmill * exercise (B; hr = 201 bpm) (C; hr = 248 bpm)	<table><tr><td></td><th colspan="3">SEN/SEP</th></tr><tr><td></td><th>A</th><th>B</th><th>C</th></tr><tr><td>control</td><td>1.24</td><td>1.30</td><td>1.21</td></tr><tr><td>LVH</td><td>1.19</td><td>1.18*</td><td>1.07*</td></tr></table> <p>* = sig from control</p>		SEN/SEP				A	B	C	control	1.24	1.30	1.21	LVH	1.19	1.18*	1.07*	Bache et al., 1985				
	SEN/SEP																					
	A	B	C																			
control	1.24	1.30	1.21																			
LVH	1.19	1.18*	1.07*																			

POSITION EMISSION COMPUTERIZED TOMOGRAPHY

Technique	Results	Reference
<u>dog (anesthetized):</u> $H_2^{15}O$ clearance following LMCA injection	uniform rMBF	Parker et al., 1978
<u>dog (anesthetized):</u> -following intravenous bolus injection of $H_2^{15}O$, data from 7 simultaneous transaxial planes were collected -data corrected for vascular pool radioactivity with use of tomographic data obtained after inhala- tion of $C^{15}O$	-uniform rMBF -tomographic distribution of $H_2^{15}O$ correlated closely with tomographic distribution of macroaggregated albumin RM (10-35 μm diameter; 4×10^6 injected into LV) ($r = 0.93$), and with rMBF determined by postmortem analysis with carbonized RM (15 μm diameter; 4×10^6 injected into LV) ($r = 0.95$)	Bergmann et al., 1984
<u>dog (anesthetized):</u> -LA injection of ^{14}C -human albumin RM (10-20 μm diameter; $4-5 \times 10^6$ / injection) -myocardial tissue samples matched to tomographic slices were well-counted for calculation of relative rMBF	-rMBF uniform 2, 20 and 40 min after injection -with LAD occlusion, MBF to ant 51% of normal flow -rMBF correlated closely with results obtained with carbonized RM (9 μm diameter; $4-5 \times 10^6$ injected into LA)	Wilson et al., 1984

Table 2A. Fixation and staining procedure for frozen tissue sections.

-Periodic Acid Schiff (PAS)

Solution		Staining Time (min)
1. Fixative		1
acetic acid	5.0 ml	
absolute alcohol	95.0 ml	
distilled water	100.0 ml	
2. Running tap water		5
3. 1% Periodic acid		10
periodic acid	1.0 g	
distilled water	100.0 ml	
4. Running tap water		5
5. Schiff reagent		25
distilled water	450.0 ml	
basic fuchsin	2.5 g	
hydrochloric acid, 1.0 N	50.0 ml	
potassium metabisulfite	5.0 g	
activated charcoal	1.88 g	
		-boil. Slowly add:
		-swirl 1 min. Filter through 2 filter papers into dark flask. Cool to 50°C. Slowly, while stirring, add:
		-swirl 2 min. Stopper and refrigerate 24 hr. Remove from refrigerator, warm to room temperature, and add:
		-shake vigorously 1 min. Filter immediately through 2 filter papers. Reagent should be clear and colourless; if not, repeat charcoal step. Keep away from light. Store in refrigerator.

Continued...

6.	0.4% Sodium metabisulfite (repeat X 2)	2
	sodium metabisulfite 0.4 g	
	distilled water 100.0 ml	
	-use fresh solution	
7.	Running tap water	5
8.	Saturated alcoholic picric acid	2
	picric acid 7.0 g	
	absolute alcohol 142.5 ml	
	distilled water 7.5 ml	
	-stir. Filter through 2 filter papers.	
9.	Running tap water	5
10.	Mordant	1
	phosphomolybdic acid 10.0 g	
	phosphotungstic acid 10.0 g	
	distilled water 400.0 ml	
	-prepare 24 hr ahead. Filter through 2 filter papers.	
11.	Fast green	15-18
	fast green 2.5 g	
	acetic acid 2.5 ml	
	distilled water 197.5 ml	
	-stir. Heat in oven (55°C) 1 hr. Filter.	
12.	1% Acetic acid	0.5
	acetic acid 1.0 ml	
	distilled water 99.0 ml	
13.	50% Alcohol	1
	absolute alcohol 50.0 ml	
	distilled water 50.0 ml	
14.	70% Alcohol (repeat)	2
	absolute alcohol 70.0 ml	
	distilled water 30.0 ml	
15.	95% Alcohol	2
	absolute alcohol 95.0 ml	
	distilled water 5.0 ml	
16.	Absolute alcohol (repeat)	2
17.	absolute alcohol 50.0 ml	2
	xylene 50.0 ml	
18.	Xylene (repeat)	2-3

Table 3A. The average total number of microspheres counted in each myocardial region sampled per heart as estimated* from the mean values shown in Table 1 and Figures 6-8, 9-10 (upper panels) for animals receiving left ventricular (n=10) and left atrial (n=10, except for overall values (n=17)) microsphere injection. The corresponding percentage error of measurement is shown to the right.

	LV (-)		LV (+)		LA (-)		LA (+)	
<u>Overall</u>	972	6.3%	1452	5.1%	732	7.2%	960	6.3%
<u>Sections</u>								
1 base	256	12.3%	395	9.9%	154	15.8%	198	13.9%
2	243	12.6%	365	10.3%	150	16.0%	189	14.3%
3	247	12.5%	355	10.4%	143	16.4%	182	14.5%
4 apex	228	13.0%	338	10.7%	149	16.1%	187	14.3%
<u>Regions</u>								
anterior	367	10.2%	536	8.5%	201	13.8%	249	12.4%
lateral	309	11.2%	451	9.2%	181	14.6%	224	13.1%
posterior	299	11.3%	466	9.1%	215	13.4%	283	11.7%
<u>Deciles</u>								
1 SEN	95	20.1%	153	15.8%	53	26.9%	67	23.9%
2	105	19.1%	164	15.3%	55	26.4%	72	23.1%
3	104	19.2%	160	15.5%	59	25.5%	79	22.1%
4	105	19.1%	159	15.5%	57	26.0%	73	22.9%
5	99	19.7%	146	16.2%	63	24.7%	84	21.4%
6	92	20.4%	140	16.6%	65	24.3%	81	21.8%
7	89	20.8%	133	17.0%	60	25.3%	76	22.5%
8	89	20.8%	130	17.2%	58	25.7%	73	22.9%
9	93	20.3%	127	17.4%	60	25.3%	74	22.8%
10 SEP	102	19.4%	142	16.4%	66	24.1%	78	22.2%
<u>Orientations</u>								
SEN	422	9.5%	673	7.6%	229	13.0%	296	11.4%
MID	473	9.0%	722	7.3%	258	12.2%	336	10.7%
SEP	157	15.6%	224	13.1%	95	20.1%	116	18.2%

LV = left ventricular injection; LA = left atrial injection;
 (-) = each aggregate was counted as 1 microsphere; (+) = all
 microspheres per aggregate were counted; SEN = subendocardium;
 MID = midwall; SEP = subepicardium

Continued...

- * For mean values expressed as "MS/decile" (overall, sections, regions, deciles), where MS = microspheres,

total number of MS counted = (MS/decile) x number of sampling sites

where number of sampling sites:

(overall) = 120 (4 sections x 3 regions x 10 deciles)
(sections) = 30 (3 regions x 10 deciles)
(regions) = 40 (4 sections x 10 deciles)
(deciles) = 12 (4 sections x 3 regions)

For mean values expressed as "MS/layer" (orientations),

total number of MS counted = (MS/layer) x number of layers (Table 4)
x number of sampling sites

where number of sampling sites:

(orientations) = 12 (4 sections x 3 regions)

Table 4A. The average total number of microspheres counted in each myocardial region sampled per heart as estimated* from the mean values shown in Table 5 and Figures 11-14 (upper panels) for animals with left ventricular hypertrophy (n=7) and their sham-operated controls (n=7). The corresponding percentage error or measurement is shown to the right.

	SHAM (-)		SHAM (+)		LVH (-)		LVH (+)	
<u>Overall</u>	936	6.4%	1248	5.5%	1488	5.1%	2148	4.2%
<u>Sections</u>								
1 base	238	12.7%	317	11.0%	375	10.1%	529	8.5%
2	227	13.0%	298	11.4%	370	10.2%	532	8.5%
3	230	12.9%	310	11.1%	378	10.1%	561	8.3%
4 apex	236	12.8%	318	11.0%	367	10.2%	530	8.5%
<u>Regions</u>								
anterior	294	11.4%	394	9.9%	458	9.2%	662	7.6%
lateral	302	11.3%	401	9.8%	508	8.7%	727	7.3%
posterior	335	10.7%	448	9.3%	525	8.6%	763	7.1%
<u>Deciles</u>								
1 SEN	99	19.7%	138	16.7%	191	14.2%	294	11.4%
2	119	18.0%	164	15.3%	198	13.9%	309	11.2%
3	104	19.2%	137	16.7%	168	15.1%	252	12.3%
4	92	20.4%	130	17.2%	147	16.2%	214	13.4%
5	92	20.4%	125	17.5%	127	17.4%	176	14.8%
6	87	21.0%	118	18.0%	121	17.8%	172	14.9%
7	77	22.3%	99	19.7%	120	17.9%	168	15.1%
8	79	22.1%	102	19.4%	135	16.9%	183	14.5%
9	82	21.6%	104	19.2%	124	17.6%	166	15.2%
10 SEP	100	19.6%	125	17.5%	160	15.5%	219	13.2%
<u>Orientations</u>								
SEN	416	9.6%	568	8.2%	679	7.5%	1001	6.2%
MID	370	10.2%	491	8.8%	560	8.3%	785	7.0%
SEP	142	16.4%	176	14.8%	263	12.1%	354	10.4%

SHAM = sham-operated controls; LVH = left ventricular hypertrophy; (-) = each aggregate was counted as 1 microsphere; (+) = all microspheres per aggregate were counted; SEN = subendocardium; MID = mid-wall; SEP = subepicardium

* refer to Table 3A for details

Table 5A. The average total number of microspheres counted in each myocardial region sampled per heart as estimated* from the mean values shown in Table 8 for animals receiving left atrial microsphere injection (n=6). The corresponding percentage error of measurement is shown to the right.

	Single		Aggregated		Total	
<u>Orientations</u>						
SEN	1058	6.0%	81	21.8%	1139	5.8%
MID	1179	5.7%	86	21.1%	1265	5.5%
SEP	792	7.0%	59	25.5%	851	6.7%

SEN = subendocardium; MID = midwall; SEP = subepicardium

* total number of MS counted = (MS/orientation) x number of sampling sites

where number of sampling sites = 45 (45 sections)
MS = microspheres

REFERENCES

Andersen, H., Bagger, H. and Gotzsche, H. Non-uniform blood flow in the left ventricular wall of dogs measured by the Xe-133 wash-out technique. *Acta Physiol. Scand.* 76: 376-382, 1969.

Archie, J.P., Jr. Minimum left ventricular coronary vascular resistance in dogs. *J. Surg. Res.* 25: 21-25, 1978.

Archie, J.P., Jr., Fixler, D.E., Ulliyot, D.J., Hoffman, J.I.E., Utley, J.R. and Carlson, E.L. Measurement of cardiac output with and organ trapping of radioactive microspheres. *J. Appl. Physiol.* 35: 148-154, 1973.

Armour, J.A. and Randall, W.C. Canine left ventricular intramyocardial pressures. *Am. J. Physiol.* 220: 1833-1839, 1971.

Bache, R.J. and Cobb, F.R. Effect of maximal coronary vasodilation on transmural myocardial perfusion during tachycardia in the awake dog. *Circ. Res.* 41: 648-653, 1977.

Bache, R.J., Vrobel, T.R., Ring, W.S., Emery, R.W. and Andersen, R.W. Regional myocardial blood flow during exercise in dogs with chronic left ventricular hypertrophy. *Circ. Res.* 48: 76-87, 1981.

Bache, R.J., Alyono, D., Vrobel, T.R., Robb, J.F., Homans, D.C. and Dai, X.-Z. Myocardial blood flow during exercise in left ventricular hypertrophy produced by aortic banding and arterial hypertension. *Circ.* 72(III): 74 (abstr.), 1985.

Baer, R.W., Payne, B.D., Verrier, E.D., Vlahakes, G.J., Molodowitch, D., Uhlig, P.N. and Hoffman, J.I.E. Increased number of myocardial blood flow measurements with radionuclide-labeled microspheres. *Am. J. Physiol.* 246: H418-H434, 1984.

Bagger, H. The distribution of Xe-133 in left and right ventricular walls of dog hearts after left ventricular bolus injection. *Acta Physiol. Scand.* 84: 275-283, 1972.

Bagger, H. Distribution of coronary blood flow in the left ventricular wall of dogs evaluated by the uptake of Xe-133. *Acta Physiol. Scand.* 99: 421-431, 1977.

Bagger, H. Regional blood flow in the left ventricular wall of dogs with a graded coronary artery stenosis. *Acta Physiol. Scand.* 100: 154-164, 1977a.

Bagger, H. Distribution of maximum coronary blood flow in the left ventricular wall of anesthetized dogs. *Acta Physiol. Scand.* 104: 48-60, 1978.

Baird, R.J., Manktelow, R.T., Shah, P.A. and Ameli, F.M. Intramyocardial pressure. A study of its regional variations and its relationship to intraventricular pressure. *J. Thorac. Cardiovasc. Surg.* 59: 810-823, 1970.

Ball, R.M., Bache, R.J., Cobb, F.R. and Greenfield, J.C., Jr. Regional myocardial blood flow during graded treadmill exercise in the dog. *J. Clin. Invest.* 55: 43-49, 1975.

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

Becker, L.C., Fortuin, N.J. and Pitt, B. Effect of ischemia and antianginal drugs on the distribution of radioactive microspheres in the canine left ventricle. *Circ. Res.* 28: 263-269, 1971.

Bergmann, S.R., Fox, K.A.A., Rand, A.L., McElvany, K.D., Welch, M.J., Markham, J. and Sobel, B.E. Quantification of regional myocardial blood flow in vivo with $H_2^{15}O$. *Circ.* 70: 724-733, 1984.

Bishop, S., Franklin, D., Ross, J., Jr. and Bloor, C. Ultrastructural alterations of hypertrophied canine left ventricular subendocardium. *Circ.* 50(III): 13 (abstr.), 1974.

Bishop, S.P., White, F.C. and Bloor, C.M. Regional myocardial blood flow during acute myocardial infarction in the conscious dog. *Circ. Res.* 38: 429-438, 1976.

BMDP Statistical Software Manual. Dixon, W.J., ed. Department of Biomathematics, University of California, Los Angeles, 1985.

Borg, T.K. and Caulfield, J.B. The collagen matrix of the heart. *Fed. Proc.* 40: 2037-2041, 1981.

Brandi, G. and McGregor, M. Intramural pressure in the left ventricle of the dog. *Cardiovasc. Res.* 3: 472-475, 1969.

Buckberg, G.D., Luck, J.C., Payne, D.B., Hoffman, J.I.E., Archie, J.P. and Fixler, D.E. Some sources of error in measuring regional blood flow with radioactive microspheres. *J. Appl. Physiol.* 31: 598-604, 1971.

Buckberg, G.D., Fixler, D.E., Archie, J.P. and Hoffman, J.I.E. Experimental subendocardial ischemia in dogs with normal coronary arteries. *Circ. Res.* 30: 67-81, 1972.

Cannon, P.J., Sciacca, R.R., Fowler, D.L., Weiss, M.B., Schmidt, D.H. and Casarella, W.J. Measurement of regional myocardial blood flow in man: description and critique of the method using xenon-133 and a scintillation camera. *Am. J. Cardiol.* 36: 783-792, 1975.

Chien, S., Usami, S. and Skalak, R. Blood flow in small tubes. In: *Handbook of Physiology. Sec. 2, The Cardiovascular System, Vol. IV, Microcirculation, Part 1.* Renkin, E.M. and Michel, C.C., eds. American Physiological Society, Bethesda, Md., 1984.

Chilian, W.M. and Marcus, M.L. Phasic coronary blood flow velocity in intramural and epicardial coronary arteries. *Circ. Res.* 50: 775-781, 1982.

Clausen, G., Tyssebotn, I., Kirkebo, A., Ofjord, E.S. and-Aukland, K. Distribution of blood flow in the dog kidney. III. Local uptake of 10 μ m and 15 μ m microspheres during renal vasodilation and constriction. *Acta Physiol. Scand.* 113: 471-479, 1981.

Cobb, F.R., Bache, R.J. and Greenfield, J.C., Jr. Regional myocardial blood flow in awake dogs. *J. Clin. Invest.* 53: 1618-1625, 1974.

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

Creasy, R.K., Kahanpaa, K.V. and de Swiet, M. Trapping of radioactive microspheres in the pregnant and non-pregnant rabbit. *Acta Physiol. Scand.* 90: 252-259, 1974.

Cutarelli, R. and Levy, M.N. Intraventricular pressure and the distribution of coronary blood flow. *Circ. Res.* 12: 322-327, 1963.

Dole, W.P. and Bishop, V.S. Regulation of coronary blood flow during individual diastoles in the dog. *Circ. Res.* 50: 377-385, 1982.

Domenech, R.J. Regional diastolic coronary blood flow during diastolic ventricular hypertension. *Cardiovasc. Res.* 12: 639-645, 1978.

Domenech, R.J., Hoffman, J.I.E., Noble, M.I.M., Saunders, K.B., Henson, J.R. and Subijanto, S. Total and regional coronary blood flow measured by radioactive microspheres in conscious and anesthetized dogs. *Circ. Res.* 25: 581-596, 1969.

Downey, H.F., Bashour, F.A., Boatwright, R.B., Parker, P.E. and Kechejian, S.J. Uniformity of transmural perfusion in anesthetized dogs with maximally dilated coronary circulations. *Circ. Res.* 37: 111-117, 1975.

Downey, H.F., Crystal, G.J. and Bashour, F.A. Asynchronous transmural perfusion during coronary reactive hyperaemia. *Cardiovasc. Res.* 17: 200-206, 1983.

Downey, J.M., Downey, H.F. and Kirk, E.S. Effects of myocardial strains on coronary blood flow. *Circ. Res.* 34: 286-292, 1974.

Downey, J.M. and Kirk, E.S. Distribution of the coronary blood flow across the canine heart wall during systole. *Circ. Res.* 34: 251-257, 1974.

Einzig, S., Nicoloff, D.M. and Lucas, R.V., Jr. Myocardial perfusion abnormalities in carbon monoxide poisoned dogs. *Can. J. Physiol. Pharmacol.* 58: 396-405, 1980.

Eliassen, P., Amtorp, O., Tondevoid, E. and Haunso, S. Regional blood flow, microvascular blood content and tissue haematocrit in canine myocardium. *Cardiovasc. Res.* 16: 593-598, 1982.

- Ellis, A.K. and Klocke, F.J. Effects of preload on the transmural distribution of perfusion and pressure-flow relationships in the canine coronary vascular bed. *Circ. Res.* 46: 68-77, 1979.
- Eng, C., Cho, S., Factor, S.M., Sonnenblick, E.H. and Kirk, E.S. Myocardial micronecrosis produced by microsphere embolization. Role of an α -adrenergic tonic influence on the coronary microcirculation. *Circ. Res.* 54: 74-82, 1984.
- Falsetti, H.L., Carroll, R.J. and Marcus, M.L. Temporal heterogeneity of myocardial blood flow in anesthetized dogs. *Circ.* 52: 848-853, 1975.
- Fenton, T.R., Cherry, J.M. and Klassen, G.A. Transmural myocardial deformation in the canine left ventricular wall. *Am. J. Physiol.* 235: H523-H530, 1978.
- Ferrans, V.J. Cardiac hypertrophy: morphological aspects. In: *Growth of the Heart in Health and Disease*. Zak, R., ed. Raven Press, New York, 1984.
- Flaim, S.F., Morris, Z.Q. and Kennedy, T.J. Dextran as a radioactive microsphere suspending agent: severe hypotensive effect in rat. *Am. J. Physiol.* 235: H587-H591, 1978.
- Flaim, S.F., Nellis, S.H., Toggart, E.J., Drexler, H., Kanda, K. and Newman, E.D. Multiple simultaneous determinations of hemodynamics and flow distribution in conscious rat. *J. Pharmacol. Methods* 11: 1-39, 1984.
- Forbes, M.S., Rennels, M.L. and Nelson, E. Innervation of myocardial microcirculation; terminal autonomic axons associated with capillaries and postcapillary venules in mouse heart. *Am. J. Anat.* 149: 71-92, 1977.
- Fortuin, N.J., Kaihara, S., Becker, L.C. and Pitt, B. Regional myocardial blood flow in the dog studied with radioactive microspheres. *Cardiovasc. Res.* 5: 331-336, 1971.
- Foster, D.O. and Frydman, M.L. Comparison of microspheres and $^{86}\text{Rb}^+$ as tracers of the distribution of cardiac output in rats indicates invalidity of $^{86}\text{Rb}^+$ -based measurements. *Can. J. Physiol. Pharmacol.* 56: 97-109, 1978.
- Gillespie, W.J. and Love, W.D. Gradients in the regional rates of myocardial rubidium-86 clearance in tranquilized dogs. *Circ. Res.* 20: 606-615, 1967.
- Greenbaum, R.A., Ho, S.Y., Gibson, D.G., Becker, A.E. and Anderson, R.H. Left ventricular fibre architecture in man. *Br. Heart J.* 45: 248-263, 1981.
- Griggs, D.M., Jr. and Nakamura, Y. Effect of coronary constriction on myocardial distribution of iodoantipyrine- ^{131}I . *Am. J. Physiol.* 215: 1082-1088, 1968.
- Griggs, D.M., Jr., Tchokoev, V.V. and Chen, C.C. Transmural differences in ventricular tissue substrate levels due to coronary constriction. *Am. J. Physiol.* 222: 705-709, 1972.

Gross, G.J., Lamping, K.G., Warltier, D.C. and Hardman, H.F. Effects of three bradycardiac drugs on regional myocardial blood flow and function in areas distal to a total or partial coronary occlusion in dogs. *Circ.* 69: 391-399, 1984.

Hales, J.R.S. and Cliff, W.J. Direct observations of the behaviour of microspheres in microvasculature. *Bibl. Anat.* 15: 87-91, 1977.

Harell, G.S., Dickhoner, W.A. and Breiman, R.S. The simultaneous visualization of microspheres and blood flow in the microvascular bed of the hamster cheek pouch. *Microvasc. Res.* 13: 203-210, 1977.

Haunso, S. Lower limits of blood flow autoregulation in different myocardial layers of the left ventricular free wall of dogs. *Acta Physiol. Scand.* 112: 349-350, 1981.

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

Henry, P.D., Shuchleib, R., Borda, L.J., Roberts, R., Williamson, J.R. and Sobel, B.E. Effects of nifedipine on myocardial perfusion and ischemic injury in dogs. *Circ. Res.* 43: 372-380, 1978.

Hess, D.S. and Bache, R.J. Transmural distribution of myocardial blood flow during systole in the awake dog. *Circ. Res.* 38: 5-15, 1976.

Hess, D.S. and Bache, R.J. Transmural right ventricular myocardial blood flow during systole in the awake dog. *Circ. Res.* 45: 88-94, 1979.

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

Hoffman, J.I.E. and Buckberg, G.D. Transmural variations in myocardial perfusion. In: *Progress in Cardiology.* Yu, P.N. and Goodwin, J.F., eds. Lea & Febiger, Philadelphia, 1976.

Holtz, J., Restorff, W.v., Bard, P. and Bassenge, E. Transmural distribution of myocardial blood flow and of coronary reserve in canine left ventricular hypertrophy. *Basic Res. Cardiol.* 72: 286-292, 1977.

Horn, H., Field, L.E., Dack, S. and Master, A.M. Acute coronary insufficiency: pathological and physiological aspects. An analysis of twenty-five cases of subendocardial necrosis. *Am. Heart J.* 40: 63-80, 1950.

Idvall, J., Aronsen, K.F., Nilsson, L. and Nosslin, B. Evaluation of the microsphere method for determination of cardiac output and flow distribution in the rat. *Eur. Surg. Res.* 11: 423-433, 1979.

Ishise, S., Pegram, B.L., Yamamoto, J., Kitamura, Y. and Frohlich, E.D. Reference sample microsphere method: cardiac output and blood flows in conscious rat. *Am. J. Physiol.* 239: H443-H449, 1980.

Jennings, R.B., Sommers, H.M., Smyth, G.A., Flack, H.A. and Linn, H. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Arch. Pathol.* 70: 68-78, 1960.

Johnson, J.R. and Di Palma, J.R. Intramyocardial pressure and its relation to aortic blood pressure. *Am. J. Physiol.* 125: 234-243, 1939.

Johnson, L.L., Sciacca, R.R., Ellis, K., Weiss, M.B. and Cannon, P.J. Reduced left ventricular myocardial blood flow per unit mass in aortic stenosis. *Circ.* 57: 582-590, 1978.

Kaihara, S., van Heerden, P.D., Migita, T. and Wagner, H.N., Jr. Measurement of distribution of cardiac output. *J. Appl. Physiol.* 25: 696-700, 1968.

Kirk, E.S. and Honig, C.R. An experimental and theoretical analysis of myocardial tissue pressure. *Am. J. Physiol.* 207: 361-367, 1964.

Kirk, E.S. and Honig, C.R. Nonuniform distribution of blood flow and gradients of oxygen tension within the heart. *Am. J. Physiol.* 207: 661-668, 1964a.

Kirk, R.E. In: Experimental Design: Procedures for the Behavioral Sciences. 2nd ed. Brooks/Cole Pub. Co., Monterey, Calif., 1982.

Kjekshus, J.K. Mechanism for flow distribution in normal and ischemic myocardium during increased ventricular preload in the dog. *Circ. Res.* 33: 489-499, 1973.

Kleinert, H.D., Scales, J.L. and Weiss, H.R. Effects of carbon monoxide or low oxygen gas mixture inhalation on regional oxygenation, blood flow, and small vessel blood content of the rabbit heart. *Pflugers Arch.* 383: 105-111, 1980.

Kobrin, I., Kardon, M.B., Oigman, W., Pegram, B.L. and Frohlich, E.D. Role of site of microsphere injection and catheter position on systemic and regional hemodynamics in rat. *Am. J. Physiol.* 247: H35-H39, 1984.

Korecky, B. and Rakusan, K. Effects of hemodynamic load on myocardial fibre orientation. In: Cardiac Adaptation to Hemodynamic Overload, Training and Stress. pp. 297-303, 1983.

Laughlin, M.H., Armstrong, R.B., White, J. and Rouk, K. A method for using microspheres to measure muscle blood flow in exercising rats. *J. Appl. Physiol.* 52: 1629-1635, 1982.

Ljungqvist, A. and Unge, G. The proliferative activity of the myocardial tissue in various forms of experimental cardiac hypertrophy. *Acta Pathol. Microbiol. Scand. Sec. A.* 81: 233-240, 1973.

Ludwig, L. Capillary pattern of the myocardium. In: Methods Achiev. Exp. Pathol., vol. 5, Functional Pathology of the Heart. Bajusz, E. and Jasmin, G., eds. Basel, Karger, 1971.

Lund, D.D. and Tomanek, R.J. Myocardial morphology in spontaneously hypertensive and aortic-constricted rats. *Am. J. Anat.* 152: 141-152, 1978.

Luyet, B.J. A method for increasing the cooling rate in refrigeration by immersion in liquid N₂ or other boiling baths. *Biodynamica* 8: 331, 1961.

Makowski, E.L., Meschia, G., Droegemueller, W. and Battaglia, F.C. Measurement of umbilical arterial blood flow to the sheep placenta and fetus in utero. Distribution to cotyledons and the intercotyledonary chorion. *Circ. Res.* 23: 623-631, 1968.

Malik, A.B., Kaplan, J.E. and Saba, T.M. Reference sample method for cardiac output and regional blood flow determinations in the rat. *J. Appl. Physiol.* 40: 472-475, 1976.

Malik, A.B. and Gēha, A.S. Cardiac function, coronary flow and MVO₂ in hypertrophy induced by pressure and volume overloading. *Cardiovasc. Res.* 11: 310-316, 1977.

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

Malsky, P.M., Vokonas, P.S., Paul, S.J., Robbins, S.L. and Hood, W.B., Jr. Autoradiographic measurement of regional blood flow in normal and ischemic myocardium. *Am. J. Physiol.* 232: H576-H583, 1977.

Marchetti, G.V., Merlo, L., Nosedà, V. and Visioli, O. Myocardial blood flow in experimental cardiac hypertrophy in dogs. *Cardiovasc. Res.* 7: 519-527, 1973.

Marcus, M.L. In: The Coronary Circulation in Health and Disease. McGraw-Hill, New York, 1983.

Marcus, M.L., Mueller, T.M., Kerber, R.E. and Abboud, F.M. Regional left ventricular perfusion in hypertrophied ventricles during maximal vasodilation. *Circ.* 54(II): 43 (abstr.), 1976.

Marzilli, M., Goldstein, S., Sabbah, H.N., Lee, T. and Stein, P.D. Modulating effect of regional myocardial performance on local myocardial perfusion in the dog. *Circ. Res.* 45: 634-641, 1979.

Mazur, P., Leibo, S.P. and Chu, E.H.Y. A two-factor hypothesis of freezing injury. Evidence from chinese hamster tissue-culture cells. *Exp. Cell Res.* 71: 345-355, 1972.

McDevitt, D.G. and Nies, A.S. Simultaneous measurement of cardiac output and its distribution with microspheres in the rat. *Cardiovasc. Res.* 10: 494-498, 1976.

Mendell, P.L. and Hollenberg, N.K. Cardiac output distribution in the rat: comparison of rubidium and microsphere methods. *Am. J. Physiol.* 221: 1617-1620, 1971.

- Meyer, H.H., Vetterlein, F., Schmidt, G. and Hasselblatt, A. Measurement of blood flow in pancreatic islets of the rat: effect of isoproterenol and norepinephrine. *Am. J. Physiol.* 242: E298-E304, 1982.
- Millard, R.W., Baig, H. and Vatner, S.F. Cardiovascular effects of radioactive microsphere suspensions and Tween 80 solutions. *Am. J. Physiol.* 232: H331-H334, 1977.
- Mittmann, U., Bruckner, U.B., Keller, H.E., Kohler, U., Vetter, H. and Waag, K.-L. Myocardial flow reserve in experimental cardiac hypertrophy. *Basic Res. Cardiol.* 75: 199-206, 1980.
- Moir, T.W. Subendocardial distribution of coronary blood flow and the effect of antianginal drugs. *Circ. Res.* 30: 621-627, 1972.
- Moir, T.W. and DeBra, D.W. Effect of left ventricular hypertension, ischemia and vasoactive drugs on the myocardial distribution of coronary flow. *Circ. Res.* 21: 65-74, 1967.
- Moline, S.W. and Glenner, G.G. Ultrarapid tissue freezing in liquid nitrogen. *J. Histochem. Cytochem.* 12: 777-783, 1964.
- Monroe, R.G., LaFarge, C.G., Gamble, W.J., Kumar, A.E. and Manasek, F.J. Left ventricular performance and coronary flow after coronary embolization with plastic microspheres. *J. Clin. Invest.* 50: 1656-1665, 1971.
- Mueller, T.M., Marcus, M.L., Kerber, R.E., Young, J.A., Barnes, R.W. and Abboud, F.M. Effect of renal hypertension and left ventricular hypertrophy on the coronary circulation in dogs. *Circ. Res.* 42: 543-549, 1978.
- Mueller, T.M., Marcus, M.L., Eastham, C.L. and Tipton, C.M. Effects of treadmill exercise in dogs with pressure-induced left ventricular hypertrophy. *Fed. Proc.* 38: 955 (abstr.), 1979.
- Neill, W.A., Phelps, N.C., Oxendine, J.M., Mahler, D.J. and Sim, D.N. Effect of heart rate on coronary blood flow distribution in dogs. *Am. J. Cardiol.* 32: 306-312, 1973.
- Nishiyama, K., Nishiyama, A. and Frohlich, E.D. Regional blood flow in normotensive and spontaneously hypertensive rats. *Am. J. Physiol.* 230: 691-698, 1976.
- O'Keefe, D.D. and Hoffman, J.I.E. Regional coronary blood flow and vascular resistance in experimental left ventricular hypertrophy (LVH) in dogs. *Circ.* 54(II): 43 (abstr.), 1976.
- O'Keefe, D.D., Hoffman, J.I.E., Cheitlin, R., O'Neill, M.J., Allard, J.R. and Shapkin, E. Coronary blood flow in experimental canine left ventricular hypertrophy. *Circ. Res.* 43: 43-51, 1978.
- Parker, J.A., Beller, G.A., Hoop, B., Holman, B.L. and Smith, T.W. Assessment of regional myocardial blood flow and regional fractional oxygen extraction in dogs, using ^{15}O -water and ^{15}O -hemoglobin. *Circ. Res.* 42: 511-518, 1978.

Permutt, S. and Riley, R.L. Hemodynamics of collapsible vessels with tone: the vascular waterfall. *J. Appl. Physiol.* 18: 924-932, 1963.

Phibbs, R.H. and Dong, L. Nonuniform distribution of microspheres in blood flowing through a medium-size artery. *Can. J. Physiol. Pharmacol.* 48: 415-421, 1970.

Poujeol, P., Chabardes, D., Bonvalet, J.P. and de Rouffignac, C. Glomerular filtration rate and microsphere distributions in single nephron of rat kidney. *Pflugers Arch.* 357: 291-301, 1975.

Rakusan, K. In: *Methods and Achievements in Experimental Pathology, Vol. 5, Functional Morphology of the Heart.* Bajusz, E. and Jasmin, G., eds. S. Karger, Basel, 1971.

Rakusan, K. and Poupa, O. Differences in capillary supply of hypertrophic and hyperplastic hearts. *Cardiologia* 49: 293-298, 1966.

Rakusan, K. and Rajhathy, J. Distribution of cardiac output in unanesthetized rats after hemorrhage and in hemorrhagic shock. *Can. J. Surg.* 16: 360-365, 1973.

Rakusan, K. and Blahitka, J. Cardiac output distribution in rats measured by injection of radioactive microspheres via cardiac puncture. *Can. J. Physiol. Pharmacol.* 52: 230-235, 1974.

Rakusan, K., Moravec, J. and Hatt, P.-Y. Regional capillary supply in the normal and hypertrophied rat heart. *Microvasc. Res.* 20: 319-326, 1980.

Rakusan, K. and Korecky, B. Regression of cardiomegaly induced in newborn rats. *Can. J. Cardiol.* 1: 217-222, 1985.

Reeves, W.J. and Rakusan, K. Capillary blood in the heart is concurrent as indicated by the method of coloured microspheres. *Can. J. Physiol. Pharmacol.*, in press.

Rouleau, J., Boerboom, L.E., Surjadhana, A. and Hoffman, J.I.E. The role of autoregulation and tissue diastolic pressures in the transmural distribution of left ventricular blood flow in anesthetized dogs. *Circ. Res.* 45: 804-815, 1979.

Rudolph, A.M. and Heymann, M.A. The circulation of the fetus in utero. Methods for studying distribution of blood flow, cardiac output and organ blood flow. *Circ. Res.* 21: 163-184, 1967.

Sapirstein, L.A. Fractionation of the cardiac output of rats with isotopic potassium. *Circ. Res.* 4: 689-692, 1956.

Sasaki, Y. and Wagner, H.N., Jr. Measurement of the distribution of cardiac output in anesthetized rats. *J. Appl. Physiol.* 30: 879-884, 1971.

Sasayama, S., Ross, J., Jr., Franklin, D., Bloor, C.M., Bishop, S. and Dilley, R.B. Adaptations of the left ventricle to chronic pressure overload. *Circ. Res.* 38: 172-178, 1976.

Schaper, W. Comparative arteriography of the collateral circulation. In: The Collateral Circulation of the Heart. North-Holland, Amsterdam, 1971.

Schaper, W. In: The Pathophysiology of Myocardial Perfusion. Sec. 1, Methods. Elsevier/North-Holland, New York, Amsterdam, 1979.

Sestier, F.J., Mildenerberger, R.R. and Klassen, G.A. Role of autoregulation in spatial and temporal perfusion heterogeneity of canine myocardium. *Am. J. Physiol.* 235: H64-H71, 1978.

Shell, W., Kligerman, M., Chang, B.-L., See, J., Meerbaum, S. and Corday, E. Measurement of myocardial blood flow with non-radioactive microspheres. *Circ.* 72(III): 191 (abstr.), 1985.

Spaan, J.A.E., Bruinsma, P. and Laird, J.D. Coronary flow mechanics of the hypertrophied heart. In: Cardiac Left Ventricular Hypertrophy. ter Keurs, H.E.D.J. and Schipperheyn, J.J., eds. Martinus Nijhoff, Boston, 1983.

Stanek, K.A., Smith, T.L., Murphy, W.R. and Coleman, T.G. Hemodynamic disturbances in the rat as a function of the number of microspheres injected. *Am. J. Physiol.* 245: H920-H923, 1983.

Streeter, D.D., Jr., Spotnitz, H.M., Patel, D.P., Ross, J., Jr. and Sonnenblick, E.H. Fiber orientation in the canine left ventricle during diastole and systole. *Circ. Res.* 24: 339-347, 1969.

Tezuka, F. Muscle fiber orientation in normal and hypertrophied hearts. *Tohoku J. Exp. Med.* 117: 289-297, 1975.

Tezuka, F. Morphometrical analysis of cardiac hypertrophy: left ventricular shape and number of muscle-fiber layers across left ventricular wall. *Tohoku J. Exp. Med.* 138: 1-6, 1982.

Tezuka, F. and Takahashi, T. Pathology of cardiac hypertrophy in pressure overload. *Jap. Circ. J.* 40: 1111-1118, 1976.

Tomanek, R.J., Palmer, P.J., Peiffer, G.L., Schreiber, K.L., Eastham, C.L. and Marcus, M.L. Morphometry of canine coronary arteries, arterioles, and capillaries during hypertension and left ventricular hypertrophy. *Circ. Res.* 58: 38-46, 1986.

Tsuchiya, M., Walsh, G.M. and Frohlich, E.D. Systemic hemodynamic effects of microspheres in conscious rats. *Am. J. Physiol.* 233: H617-H621, 1977.

Tuma, R.F., Vasthare, U.S., Irion, G.L. and Wiedeman, M.P. Considerations in use of microspheres for flow measurements in anesthetized rat. *Am. J. Physiol.* 250: H137-H143, 1986.

Utley, J., Carlson, E.L., Hoffman, J.I.E., Martinez, H.M. and Buckberg, G.D. Total and regional myocardial blood flow measurements with 25 μ , 15 μ , 9 μ and filtered 1-10 μ diameter microspheres and antipyrine in dogs and sheep. *Circ. Res.* 34: 391-405, 1974.

Vetterlein, F. Investigation of local blood flow by non-radioactive microsphere mapping in cleared tissue slides. Basic Res. Cardiol. 70: 401-405, 1975.

Vetterlein, F. and Schmidt, G. Effects of isoprenaline on functional capillary density in the subendocardial and subepicardial layer of the rat myocardium. Basic Res. Cardiol. 75: 526-536, 1980.

Voorhees, A.B., Baker, H.J. and Pulaski, E.J. Reactions of albino rats to injections of dextran. P. Soc. Exp. Biol. Med. 76: 254-256, 1951.

Warren, D.J. and Ledingham, J.G.G. Measurement of cardiac output distribution using microspheres. Some practical and theoretical considerations. Cardiovasc. Res. 8: 570-581, 1974.

Weber, K.T., Scott, G., Janicki, J.S. and Shroff, S. The normal regulation of coronary blood flow. In: Coronary Artery Spasm and Thrombosis. Goldberg, S., ed., 1983.

Wicker, P. and Tarazi, R.C. Coronary blood flow in left ventricular hypertrophy: a review of experimental data. Eur. Heart J. 3(A): 111-118, 1982.

Wicker, P. and Tarazi, R.C. Importance of injection site for coronary blood flow determinations by microspheres in rats. Am. J. Physiol. 242: H94-H97, 1982a.

Wicker, P. and Tarazi, R.C. Coronary blood flow measurements with left atrial injection of microspheres in conscious rats. Cardiovasc. Res. 16: 580-586, 1982b.

Wicker, P., Abdul-Samad, M., Rakusan, K., Tarazi, R.C. and Healy, B. Effects of chronic exercise on the coronary circulation in conscious rats with renovascular hypertension. Hypertension, in press.

Wiedeman, M.P. and Tuma, R.F. Cine-photomicrography of distribution of microspheres in the peripheral vasculature. Bibl. Anat. 18: 207-209, 1979.

Wilson, R.A., Shea, M.J., De Landsheere, C.M., Turton, D., Brady, F., Deanfield, J.E. and Selwyn, A.P. Validation of quantitation of regional myocardial blood flow in vivo with ¹¹C-labeled human albumin microspheres and positron emission tomography. Circ. 70: 717-723, 1984.

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

Wisnes, A. and Kirkebo, A. Regional distribution of blood flow in calf muscles of rat during passive stretch and sustained contraction. Acta Physiol. Scand. 96: 256-266, 1976.

Wusten, B., Buss, D.D., Deist, H. and Schaper, W. Dilatory capacity of the coronary circulation and its correlation to the arterial vasculature in the canine left ventricle. Basic Res. Cardiol. 72: 636-650, 1977.

Yipintsoi, T., Dobbs, W.A., Jr., Scanlon, P.D., Knopp, T.J. and Bassingthwaite, J.B. Regional distribution of diffusible tracers and carbonized microspheres in the left ventricle of isolated dog hearts. *Circ. Res.* 33: 573-587, 1973.

Yoran, C., Covell, J.W. and Ross, J., Jr. Rapid fixation of the left ventricle: continuous angiographic and dynamic recordings. *J. Appl. Physiol.* 35: 155-157, 1973.

Zile, M.R., Hoshino, P.K., Oxendine, J. and Gaasch, W.H. Myocardial blood flow with left ventricular failure in pressure overload hypertrophy. *Circ.* 72(III): 4 (abstr.), 1985.