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STORAGE AND TRANSIT OF RED BLOOD CELLS IN SKELETAL MUSCLE OF THE CAT

by

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Department of Biophysics

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
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ABSTRACT

When isolated cat gastrocnemius muscles are perfused with cell-free solutions, the red cells are washed out in a manner that suggests that there are at least three compartments to the red cell store within the vascular bed. These are distinguished by great differences in the perfusion flow per unit volume through each (Groom, Song and Campling 1973). The most poorly perfused of these was shown to be made up of some of the reticulocytes within the muscle. The mechanism which causes the slow release of these cells was believed to be a special adhesiveness of the surface of the cells themselves. The origin of the other compartments was not determined but it was suggested that they were similar to the poorly perfused areas that others have found in resting muscles using tracer clearance methods.

To examine the latter hypothesis, red cell washout from isolated cat gastrocnemius muscles was studied by perfusion of these muscles with isotonic cell-free solutions. Cell concentrations in the venous outflow were determined with a Celloscope counter. Three procedures were used to modify the perfusion of the vascular beds of different sets of muscles. 1.) The muscles were stimulated to twitch at $4.sec^{-1}$. 2.) The muscles were resting and care was taken to avoid reactive hyperemia at the beginning of perfusion. 3.) The vascular bed of each muscle was dilated with sodium nitroprusside. In all cases the red cell washout pattern was very similar to that reported by Groom *et al*. (1973) for resting muscles.

In addition, in some of the vasodilated muscles, the washout of ^{125}I-labelled albumin as a label of the plasma was studied. The pattern of plasma washout was similar to that of the cells, including those of the most poorly perfused compartment.

To determine whether a property of the reticulocytes themselves would be solely responsible for their slow clearance from the muscle vascular bed, the following was done in other experiments. Heat-treated red blood cells from the cat, human red blood cells, glutaraldehyde-fixed cat red blood cells, neuraminidase-treated cat red blood cells and red cells obtained from the red pulp of the cat spleen were injected in suspensions of about 10^{10} cells-ml^{-1} intra-arterially into muscles which had been perfused to clear them of most of the original cells. The recovery of cells from the venous outflow and the resistance to perfusion through the muscle were measured in order to detect trapping of these abnormal cells in the vascular bed. Only in the case of the fixed cells was the recovery different from normal cells and only the fixed and heat-treated cells affected the resistance to perfusion to a greater degree than normal cells.

It is concluded that 1.) The inhomogeneity of perfusion of the red cell store is not caused by the action of physiological control mechanisms regulating the perfusion of various parts of the vascular bed. 2.) The trapping of cells in the slow compartment is accompanied by a similar trapping of plasma; therefore it must be in a part of the bed where the red cells occlude the vessel lumen.

3. The trapping of reticulocytes in the vascular bed is confined to some small part of the bed and results from some interaction between that part and some property of these cells.

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I wish to thank my parents, Mr. and Mrs. William Bowden, for their spiritual and material support during the time I worked on this thesis, and my sister, Mrs. Margaret McDonald for patiently deciphering and typing my manuscript.

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CHAPTER I.

BACKGROUND.

1. Introduction: an overview of the problem

The supply of blood to the skeletal musculature of animals and man has been an important area of anatomical and physiological investigation for more than a century. The survival of an animal (and perhaps a human) depends in a large part on the ability of the muscles to respond to a variety of unpredictable demands for locomotion as the organism confronts a changing and possibly threatening environment.

The energy use of skeletal muscle is thus extremely variable, being as much as fifty times the resting value during maximal exertions. (Astrand and Rodahl 1970, p.3)

Since, in the long term, the supply of oxygen to the fissues by the blood limits the energy available for muscular work, the demand for blood flow is also quite variable. It would be wasteful of energy to maintain a continuous blood supply to all of the muscles sufficient for their maximum exertions; these events are quite seldom in the life of an animal, and somewhat more so in man. Thus it is not surprising that the actual blood flow through muscle is extremely variable, it can be less than one-tenth its maximum value in some muscles at rest (Folkow and Halicka 1968; Hudlicka, 1973,p. 14). It is subject to control by a variety of systems whose full extent is still not understood. The reserve capacity to perfuse the skeletal muscles with blood is an important part of the ability of an organism to adapt to many situations and conserve energy in the cardiovascular system; described as "cardiovascular fitness" it is a concern of many people today.

The function of the blood supply to skeletal muscle is to deliver oxygen and nutrients to the working muscle fibres and to remove the products of metabolism. The effectiveness of this service is not only a function of blood flow. The distribution of the flow through the muscle mass and the barriers to the diffusion of oxygen and nutrients from the blood to the tissue and of metabolites back to the blood also affect the service, and may limit the performance of the muscle.

All of these factors have been studied extensively both in animals and man. It has been found that both the behaviour of blood as as fluid and its distribution through the vascular beds of organs are very complex subjects. The relation between the two has been studied; but not extensively.

The red blood cell is important both in its function as the principal carrier of oxygen in the blood and also in its properties which determine the fluid nature of blood. Its passage through the vascular bed of skeletal muscle determines the pattern of the delivery of oxygen, which is required for muscular work. However many of the investigators who have studied the distribution of blood flow through muscle have used methods or theories that give little attention to the red blood cell. In their analysis (e.g. Renkin 1959a) the blood is assumed to be a uniform source or sink for a tracer which is exchanged with the tissue and the distribution of blood flow is deduced from the nature of this exchange.

The majority of the investigators who have studied the fluid dynamics of blood have recognized the role of the red blood cell in determining the behaviour of blood as a whole. They

have restricted themselves in their detailed studies to the flow of blood through artificial systems, single vessels or simple networks in living organisms. A detailed discussion of the behaviour of blood in the whole vascular bed of an organ is forbiddingly complex. It would have to consider how the diameters and lengths of blood vessels and the properties of the red blood cell work together to determine the paths that the cells take through the vascular system.

Some investigators have considered the distribution of the passage of red cells through the vascular bed of different organs, including skeletal muscle. A recent investigation of this sort by Groom, Song and Campling (1973) gave results which suggested that:

a) the pattern of blood flow as traced by the cells is quite inhomogenous,

b) that this inhomogeneity must be, in part, the result of differences in physical character among the red cells themselves.

This thesis reports the investigation of these results and further conclusions about them, and hence about how the characteristics of the red blood cells affect their passage through the vascular bed of skeletal muscle.

2. The structure of this thesis

The remainder of this chapter is a review of some of the literature on skeletal muscle blood flow, with particular emphasis on the distribution of blood flow. It describes how it may be non-uniform, how it is controlled and how investigators deduce aspects of its pattern from their findings. It also reviews some of our present knowledge about blood as a fluid, with reference to its behaviour in living blood vessels and, in particular, how the physical properties of the red cell affect its distribution through the perfused vascular bed.

It then discusses those studies of the kinetics of the passage of the red blood cells through muscles which precede those reported here. The paper by Groom _et al._ (1973) served as the starting point for these investigations and their results, mentioned before, are discussed in more detail.

Chapter II describes a number of investigations using techniques similar to those of Groom _et al._ (1973) to determine whether the inhomogeneities of the red cell store in muscle were related to the inhomogeneities of blood perfusion of the tissue whose existence other authors deduced by other means in resting muscle. The methods are somewhat similar in each case, so the chapter begins with a section devoted to methods and the remaining sections deal with the purposes, results and interpretation of each of the four series of experiments. Since each of these was done in part to resolve questions

concerning the previous series, I thought it best to present and discuss the results of each before discussing the purpose of the next.

Chapter III reports on the exploration of the possible relationship between the character of the red blood cell and its passage through the vascular bed of skeletal muscle. Since the methods differ from those of Chapter II, these are described at the beginning of the chapter and the results and discussion follow.

Chapter IV is a summary of the conclusions of the two previous chapters and in it their implications for the study of blood flow in skeletal muscle are discussed in order to develop questions for further study.

3. The pattern of the vascular bed in skeletal muscle.

a Methods of study

Most of the vascular beds of vertebrate skeletal muscles are hidden from view by the large muscle masses. Microscopic studies have been done *in vivo* on the surface vessels of large muscles (Krogh 1929; Honig,Frierson and Patterson 1970) and in those muscles which are thin enough for transillumination, such as the rat cremaster (Smaje, Zweifach and Intaglietta 1970) the cat tenuissimus (Eriksson and Myrhage 1972) and the rat extensor hallucis proprius (Myrhage and Hudlicka 1976). Studies of the vascular bed have been done on sections of large muscles taken after death or as biopsy (e.g. Casley-Smith, Green, Harris and Wadey 1975). These observations have yielded ideas as well as information about the pattern of the vascular bed, which have been applied to the understanding of the living circulation through larger muscles. They give us some of the details; the overall pattern is still hidden.

b Details of the pattern

i The arrangement of muscle tissue.

The basic cellular unit of muscle tissue is the fibre. It is roughly cylindrical in shape, about 20 μm to 50 μm in width in mammalian muscles (Eriksson and Myrhage 1972; Plyley and Groom 1975), and extends without interruption from one tendon to its opposite. The array of fibres restricts the pattern of the blood

vessels to the spaces between the individual fibres.

Muscle fibres are heterogeneous in their metabolic demand for oxygen and have been divided into three classes; red or slow fibres, white or fast fibres and intermediate fibres. Muscles in which red fibres predominate have a higher resting blood flow and oxygen consumption per unit weight than those in which the fibres are of mixed types (Reis, Wooten and Hollenberg 1967; Folkow and Halicka 1968; Hilton, Jeffries and Vrbova 1970; Reis and Wooten 1970) and have a greater density of capillaries (Reis and Wooten 1970). In muscles with a mixture of fibre types, it has been shown that the number of capillaries around each red fibre is greater than the number around each fibre of the other types of fibres (Romanul 1965). This has been considered in explaining the observed heterogeneity in blood flow in resting muscle (Hudlicka 1973, p.43). Eriksson and Myrhage (1972) report that the average numbers of capillaries around the fibres of different types is similar, but the red fibres are narrower, leading to a locally greater density of capillaries in their area.

ii The capillaries

The capillaries are arranged among the muscle fibres in a dense network. It is here that the exchange of oxygen, carbon dioxide, nutrients and metabolites between the blood and the tissue primarily occurs, because of their large surface area and thin, permeable walls (Landis and Pappenheimer 1963).

The average diameter of capillaries in mammalian skeletal muscles is near 5 or 6 μm (5.3μm in the cat tenuissimus according

to Eriksson and Myrhage (1972)). The diameters vary somewhat among capillaries in a single muscle, and even along the length of a single capillary.

Most of the capillaries run parallel to the muscle fibres for a variable distance whose average value may be 500 μm to 1000 μm (Eriksson and Myrhage 1972; Myrhage and Hudlicka 1976; Honig, Feldstein and Frierson 1977) in mammalian muscles. The standard deviation of capillary lengths is about one-half the mean value in rat (Honig et al. 1977) and frog (Plyley, Sutherland and Groom 1976) muscles, indicating that the lengths are extremely variable. Capillaries are joined to each other by connecting capillaries (capillary anastomoses) which run diagonally or perpendicularly around the fibres. These join the capillaries every 200 μm to 250 μm of their length, on the average (Eriksson and Myrhage 1972; Myrhage and Hudlicka 1975). The pattern of these branches and rejoinings appears to be quite random (Plyley et al. 1976).

Muscle sections perpendicular to the fibres are also perpendicular to the majority of capillaries. The density of capillaries per mm^2 of cross-sectional area of the tissue has been an important concept in muscle circulation physiology since the observations of Krogh (1919a and b) and more will be said on this subject in the section of the control of the distribution of blood flow. The anatomical density in a number of muscles has been reviewed by Plyley and Groom (1975). They find that the density in many vertebrate muscles has an average value about 1.5 times the density of fibres. Their values for the average capillary density in

muscles range between 341 per mm^2 and 1440 per mm^2. The distribution of capillaries is not uniform; the number around one fibre may be between zero and eight. This may be related to the observations of Romanul (1965) cited above.

iii The arteries and arterioles

These vessels deliver the blood to the capillaries from the major arteries of the muscle. They are surrounded by layers of smooth muscle in the medial layers of their walls. The structure and function of the walls of arteries is a complex topic; I will discuss only the effect of the contraction of the smooth muscle on the distribution of blood flow.

A detailed study of the distribution of lengths and diameters among branches of the arterial tree has been published (Gross and Intaglietta 1973) for the cat tenuissimus muscle and these have been used to estimate volumes and resistances in the various parts of the arterial tree (Eriksson and Lisander 1972a). These authors regarded the arterioles as a set of parallel branching networks without interconnection among them, although other authors report anastomoses between arterioles (Hudlicka, 1973, p.3). In muscles with more than one feeding artery there are connections between the arteries at various levels, so that there may be a "collateral" flow from one artery to the vascular bed supplied by the other. (Manthey, Gaehtgens, Hirche, Hombach and Steinhagen 1974).

The terminal arterioles have been of principal interest to those who study the distribution of blood flow, because they may

have a principal role in determining the distribution of flow to the capillaries beyond them. Their vascular smooth muscle is innervated (Rhodin 1967) and is also responsive to local stimuli such as oxygen (Duling and Berne 1971; Burton and Johnson 1972; Prewitt and Johnson 1976; Klabunde and Johnson 1977) hyperosmolality (Gray 1971) and local pressure (Klabunde and johnson 1977; Henrich and Johnson 1978). This local responsiveness may be principally directed towards regulating the perfusion of the individual capillaries, although it will also affect the total resistance to blood flow through the muscle. The responses of the larger arterioles and arteries to neural or hormonal stimuli from the central nervous system and glands may principally serve to alter the overall resistance(Mellander 1970).

iv The veins and venules

The veins and venules form a system very much like that of the arterial tree; indeed the vessels often occur in pairs, one of each kind,in muscle tissue (Eriksson and Myrhage 1972). They have muscular walls and are responsive to hormonal and neural stimuli (Hudlicka 1973, p. 40). The diameters of venules and veins are everywhere larger than those of the corresponding arterioles and arteries, therefore their resistance to flow is lower and their content of blood is larger. The data given by Gross and Intaglietta (1973) show that the volume of a venular tree in the cat tenuissimus is 2.7 times that of the corresponding arterial tree. The contractions of the veins and venules in muscle in response to nervous stimuli,if they exist, do not have a great effect on the total blood volume

(Lesh and Rothe 1969). The passive collapse of the veins in response to the pressure induced by fibre contraction has an important role in augmenting muscle perfusion (Folkow, Gaskell and Waaler 1970). Contraction of the veins and venules probably has no effect on the distribution of blood flow through the capillaries.

v Special Circulations

Arteriovenous anastomoses are vessels larger than capillaries (about 10μm in diameter) which run directly from an arteriole to a venule by-passing the capillaries. They have muscular walls and these are innervated. Zweifach (1939) described their presence and role in the rat mesentery. Eriksson and Myrhage (1972) found very few of these in the muscle tissue itself of the cat tenuissimus but found them to be relatively common, "about one or two per muscle", in the fascia between the muscles. These vessels have also been observed in tha rat biceps femoris tendon by Grant and Payling Wright (1970) and near the free margins of the rat spinotrapezius by Zweifach and Metz (1955). Their presence has been inferred by Barlow,Haigh and Walder (1961) from the poor clearance of $^{24}Na^{+}$ ions from the tendons and fascia of the cat gastrocnemius muscle.

vi Summary

The muscle vascular bed can be considered to be an array of parallel channels (the capillaries) with a common supply and drainage system. There are numerous interconnections between these channels. They vary greatly in length and somewhat in diameter. Except for the arteriovenous anastomoses, which are scarce in muscle,

the channels are variable but homogeneously distributed in their resistance to perfusion and their density in the tissue. There are mechanisms which can greatly alter the perfusion of individual capillaries or groups of capillaries in response to local or neural stimuli.

4. The red blood cell in the microcirculation

a The composition of blood and the red cell

Blood is a suspension of cells in a solution of proteins and electrolytes called plasma. Plasma is normally a Newtonian fluid with a viscosity 1.7 times that of water (Charm and Karland 1972). From 30 to 50 % of the total volume of blood is occupied by red cells in mammals; about 1% by the other formed elements, the white cells and platelets.

When the red cells of mammals are at rest in a dilute suspension they have the shape of discs, with concave sides. Their diameters in man are 7.65 $\pm$ 0.67 (SD) μm; their volumes 98 $\pm$ 16 (SD) μm^3 (Fung 1977a). Cat red cells are smaller, their average diameter being 5.8 μm and average volume 57 μm^3(Altman and Dittmer pp. 110 and 119). The variation in size of human red blood cells is described by Canham and Burton (1968) and Fung (1977a). That of other species is probably similar.

The red blood cell is a flexible membrane surrounding a fluid of viscosity about 6 cP (Fung 1977a) called the cytoplasm. The cell is very easily deformed by the shear forces within the circulation (Fung 1977a) and almost never is seen in its disc-like shape in flowing blood (Goldsmith 1971; Miyamoto and Moll 1971; Burton 1972 p. 41). The deformation has a limit in that the membrane area and volume cannot change (Rand and Burton 1964; Canham and Burton 1968). A consequence of this is that there is a minimum diameter for cylindrical tubes through which the cell can pass without damage; for human cells this is near 2.5 μm (Jay 1973). Normal red

cells pass through larger tubes, although they may be smaller than the normal diameter of the cell, with very little resistance (Rand and Burton 1964; Braasch and Jenett 1968; Jay,Rowlands and Skibo 1972). The deformability of normal red cells reduces the apparent viscosity of whole blood to about 4 cP which is much less than that of suspensions of the same concentration of cells made rigid by fixation (Chien, Usami Dellenback and Gregersen 1967; Ham Dunn, Sayre and Murphy 1968).

Normal red blood cells suspended in plasma show a slight tendency to adhere to one another in stacks called rouleaux. In parts of the microcirculation these form spontaneously when flow is arrested, but break up instantly when it is restored. (Branemark 1968; Eriksson and Lisander 1972b). The adhesion depends upon the concentration of fibrinogen in the plasma (Chien, Usami, Taylor, Lundberg and Gregersen 1966; Chien, Usami, Dellenback Gregersen, Nanninga and Guest 1967). It is very easily increased by artificial means, such as the addition of high-molecular-weight Dextrans (Fajers and Gelin 1959; Eliasson and Samelius-Broberg 1965).

b The red blood cell in the microcirculation

The red blood cell, by its deformability and to a lesser extent by its adhesiveness, influences the bulk fluid properties of blood. Blood is a non-Newtonian fluid with a yield stress and an apparent viscosity that decreases with increasing rates of shear (Charm and Kurland 1972). The apparent viscosity given before is found at shear rates greater than 100 sec^{-1} where it is relatively constant (Chien, Usami, Dellenback and Gregersen 1967; Djojosugito,

Folkow, Oberg and White 1970). The shear rate dependence of blood viscosity may not be important in the microcirculation (Rosenblum 1977).

The red cell deformability causes other anomalies of blood flow, which not only affect the resistance to flow in the microcirculation but also the distribution of the red cells themselves through the vascular beds of various organs, including skeletal muscle.

Human blood flowing in tubes or blood vessels whose diameters are between 8 μm and 1000 μm shows a definite marginal layer which is deficient in red cells (Krogh 1929 pp. 5,6; Bloch 1962; Charm and Kurland 1972; Burton 1972, p.41). The cause of this is deformability and shape of the red cells which causes them to move across velocity gradients in the blood to regions of higher flow velocity (Mason and Goldsmith 1969). The fraction of the volume occupied by the cells in such vessels (the tube haematocrit) is less than that in the blood in larger vessels which supply them (Fahraeus 1929; Barbee and Cokelet 1971). The red blood cells move more quickly on the average than the plasma so that these vessels carry cells and plasma at rates proportional to the large vessel haematocrits. The difference in average velocity between the cells and plasma has been observed in glass tubes and the living mesenteric microcirculation (Gaehtgens, Benner, Schickendantz and Albrecht 1976) and can be up to 15% of the plasma velocity.

Plasma skimming is a phenomenon which may be a consequence of this (Krogh 1929, p.7; Johnson 1971). Where blood flows from a vessel into two branches, the branch with greater flow receives a greater concentration of cells, if the ratio of flows is greater than 1.5 (Johnson 1971).

The two effects, the Fahraeus effect and plasma skimming, both tend to cause red blood cells to travel through a vascular bed more quickly than plasma, on the average; the former by causing them to travel more rapidly in a vessel and the latter by directing them to vessels of faster flow. Johnson (1971) considers the Fahraeus effect to be more important. The difference in circulation times has been observed; this is described in Section 6.

The Fahraeus-Lindqvist effect is an apparent reduction in the resistance of blood flow through narrower tubes, when compared to that of a Newtonian fluid of the viscosity shown by blood in bulk flow (Fahraeus and Lindqvist 1931). This reduction depends upon the diameter of the tube (Haynes 1960) and may be a consequence of the Fahraeus effect (Barbee and Cokelet 1971) as it occurs in tubes or vessels of the same diameter. The resistance to blood flow in the living vascular beds of muscles is also lower than that predicted by the apparent viscosity of blood (Whittaker and Winton 1933; Djojosugito et al.1970).

The phenomena described above all cause the distribution of the red cells through the vascular bed of muscles to be different from that of a Newtonian fluid in the same bed and also from a solute of the plasma. Thus neither the patterns of the blood vessels themselves nor the distribution of a tracer in the plasma will predict in a simple way the distribution of the red cells.

c Abnormalities of the red blood cell

Red blood cells may differ in their physical properties from the normal cell in a variety of ways. Many differences can be produced artificially; some of these are described, with their consequences for the circulation in muscle in Chapter III. Here I shall consider only those variations which may occur in a normal mammal, which may lead to differences in the way cells pass through the blood vessels. Immature red cells, called reticulocytes are of special interest to this study; they are described in the introduction to Chapter III.

The normal life span of red cells in the circulation is about 120 days in man (Burton 1972, p. 32) and as the cells age they lose membrane (Smith, La Celle and La Celle 1977) and the reduced deformability that results may lead to their being trapped in the microscopic pores of the spleen sinuses (Canham and Burton 1968; Chen and Weiss 1973). Whether this affects the passage of the aged cells through the microcirculation is not known.

In mammals which are diseased or injured the adhesion of red cells to each other increases greatly and red cells may form large cemented masses in slowly-flowing blood which sink to the lower sides of blood vessels (blood sludge) (Knisely 1965). Plasma skimming increases (Gelin 1961) and there is evidence that the distribution of blood flow through skeletal muscle becomes non-uniform (Appelgren 1972).

Thus the adhesiveness of red cells may affect its passage through the vascular bed. This may be of no consequence to a healthy mammal, but the observer of the microcirculation may affect what

he sees by his method of preparation.

5. The perfusion of skeletal muscle by blood

a Theories on the control of the distribution of blood flow

i Introduction

The pattern of the blood flow through the vascular bed of skeletal muscle is modified by the contraction of arterial smooth muscle, which alters the diameters of the arteries and arterioles thus changing their resistance to blood flow. The control of the smooth muscle by nerves, hormones and local chemicals is a complex topic reviewed by a number of authors (Haddy and Scott 1968; Mellander 1970; Ross 1971; Hudlicka 1973). This thesis is concerned with the pattern of blood flow itself so that the effects rather than the causes of arteriolar vasoconstriction are of more signficance here.

ii The pre-capillary sphincter

Although they have not been observed in skeletal muscles (Eriksson and Myrhage, 1972) the idea of the pre-capillary sphincter (Zweifach 1939) as the means whereby flow through individual capillaries can be altered or even arrested has had great currency in skeletal muscle physiology (Folkow and Mellander 1960; Mellander 1970; Honig, Freirson and Patterson 1970; Wiedeman, Tuma and Mayrowitz 1976). The variation in the numbers of perfused capillaries in muscles has been observed repeatedly (Krogh 1919a and b, Honig et at. 1970; Eriksson and Lisander 1972a; Eriksson and Myrhage 1972; Honig, Feldstein and Frierson 1970). The change in the numbers of perfused capillaries overall may not be as large as the increase in the average rate of

flow through individual capillaries (Burton and Johnson 1972) and this may be controlled by the terminal arterioles. The temporary arrests in flow noted in individual capillaries may be the result of temporary plugging by the adhesion of red cells (Branemark 1971) or white cells (Eriksson and Lisander 1972a and b) within each capillary.

iii Functional capillary density and surface area

The idea that the arrest of flow in capillaries may serve to regulate the oxygen delivery to the tissue by regulating the density of perfused capillaries originated with Krogh (1919a). It has had great influence on the study of muscle blood flow (e.g. Stainsby and Otis 1964; Granger and Shepherd 1973). If it is true it follows that the capillary surface area between the flowing blood and tissue must also be regulated, and change with changes in the tissue metabolism.

The surface area of capillaries is an important part of the exchange of many tracers between the blood and tissue. These tracers such as ^{42}K (Renkin 1959a) ^{86}Rb (Renkin and Rosell 1962) and ^{24}Na (Lassen 1967) have a somewhat limited permeability through the capillary wall, therefore the clearance of these tracers from the tissue (Lassen 1967) or their extraction from the blood (Renkin 1959a; Yudilevich, Renkin, Alvarez and Bravo 1968) depends upon the area available for exchange through the capillary wall as well as the rate of blood flow. The exchange of tracers has been used to support the idea that in resting muscles there are poorly-perfused areas, where the area available for exchange is less per unit volume of tissue than in well-perfused areas. These become well-perfused in working muscle so that the area per unit volume of tissue increases (Renkin 1959a and

b; Renkin and Rosell 1962; Renkin Hudlicka and Sheehan 1966).

Another measure of the capillary surface area is the total conductance of the capillary walls to water, as described by Mellander (1960) and Kjellmer (1964). This also increases in working muscle (Kjellmer 1964; Kjellmer and Odelram 1965; Lundvall Mellander and Sparks 1967; Gosselin and Audino 1971; Beer and Yonce 1972). The disadvantages of this method are that it demands assumptions about the capillary pressure and the conductance per unit area of the capillary wall which are hard to verify. It also shows nothing about the distribution of flow among those capillaries where the exchange of fluid takes place.

The methods which employ diffusible tracers may also be complicated by factors other than the blood perfusion of the capillaries. If the diffusion of the solute within the tissue itself is limited the concentration differences between the blood and tissue may differ from what they are presumed to be when one interprets the clearance or equilibration studies in terms of changes in capillary surface area per unit volume of tissue. This would make the estimates of changes in this area and hence the capillary density unreliable.

<u>iv</u> Nutritional and shunt flow

The vascular bed of skeletal muscle has at rest a great heterogeneity of capillary lengths, flow velocities and hence transit times (Honig *et al.*1977). The changes in relative perfusion of the vascular bed in reactive and exercise hyperemia are superimposed in some manner upon the continuous variations in flow velocity in

individual capillaries.

Such changes may come about in several distinct ways. The average fraction of a time interval in which an individual capillary is without flow may decrease so that at any given moment the number of perfused capillaries is greater. Alternatively, there may be special capillaries in which the flow only occurs during vasodilatation.

This idea is supported by the results of Hyman and Lenthall (1962) who show that, when a tracer with limited diffusibility ($^{125}I^-$) is injected intra-arterially into a vasodilated muscle, it is distributed in a pattern different from that when it is injected intra-arterially into a resting muscle. The injections made when the muscle is vasodilated are cleared by blood perfusion at constant flow at a rate which increases if the muscle is vasodilated during the clearance. The injections made when the muscle is at rest, however, are not cleared at greater rates by constant blood flow if the muscle is vasodilated. The tracer must be distributed in the tissue by pathways in the vasodilated muscle which are not open in the resting muscle. These are considered to be nutritional pathways; those open in resting muscle are largely non-nutritional.

An alternative view of the distinction between nutritional and non-nutritional or "shunt" pathways is that of Chien (1971) who postulated that the blood may flow through channels which are incapable of exchange with the tissue as well as those which can exchange. Applying this theory to the results of Yudilevich _et al_. (1968) gave inconsistent results for the portions of blood flow to each type of pathway and the exchange capacity for the nutritional flow; thus it is

unlikely that the distinction is this sharp.

The observations by Barlow and Walder (1964) which lead to their suggestion that there are two parallel circuits in the vascular bed of skeletal muscle (in addition to that through the fascia and tendons) are based on studies of the changes in resistance to blood flow throughout the muscle in response to different stimuli. The lack of anatomical uniqueness or differences in the exchange character of the two circuits they describe makes it difficult to compare their results with those of others.

What I have described so far are essentially models of skeletal muscle blood flow. These models are used to explain observations about transcapillary exchange. What they have in common is the idea that the perfusion of the vascular bed by blood in resting muscle is not uniform, and it may become more uniform on vasodilatation in the muscle, particularly in the case of reactive or metabolically-induced hyperemia.

b Direct evidence for the non-uniformity of blood flow in muscle

Many authors, in interpreting the results of tracer studies have used a variation of the "single- capillary" model (Renkin 1959a; Renkin 1967; Crone 1970; Middleman 1972). This model assumes that blood flows through a single tube at a constant rate and diffusion of the tracer occurs radially across the tube wall between the blood (in which it is at a uniform concentration varying only down the length of the tube) and the tissue (in which it has a constant concentration). The failure of such a model to predict the results of equilibration (Renkin 1959a) or indicator dilution studies (Yudilevich *et al.* 1968) can be taken as evidence of

non-uniform perfusion of the tissue. It gives no idea what the actual pattern of flow may be unless a more complex model can fit the results more precisely. Renkin (1959a) showed that a model with parallel ciruits with different values for the area available for tracer exchange per unit volume of tissue perfused would fit the results of his equilibration studies. Yudilevich et al.(1968) suggest that these circuits may differ in transit-time as well. More recently Rose and Goresky (1976) have applied a model based on different capillary transit-times to the indicator-dilution study of sucrose extraction in the dog heart. It produces a better result in the normal heart than one of uniform capillary transit times.

Another sort of evidence of inhomogeneity of blood perfusion of the tissue comes from the clearance of tracers deposited in muscle. A number of studies on the human forearm with non-diffusible tracers (Fries, Schnaper and Lilienfeld 1957) and diffusible but'permeability-limited' tracers (Dobson and Warner 1957; Linde and Wahren 1964) have shown a multiexponential course for the washout of the labels. Chapter II shows how such results can be interpreted to show different rates of blood flow per unit volume in the vascular bed or the tissue itself. Other studies of isolated muscles have shown a similar pattern of clearance of diffusible tracers (Prentice, Stahl, Dial and Pontiero 1955; Renkin 1971).

Another class of studies on blood flow distribution are those in which ^{133}Xe is used as a tracer. Xenon is soluble in lipids, and the capillary wall provides no barrier to its passage from the tissue to the blood and vice versa. The tracer reaches equilibrium in its distribution between the blood and the tissue in a time which

is short compared with that of the passage of blood through the same tissue. When this is the case it is as if the blood washes the whole tissue free of the tracer, and its rate of disappearance is directly proportional to the blood flow per unit volume of the tissue. Sejrsen and Tonnesen (1968) describe the theory; it is similar to that given in Chapter II of this thesis for the interpretation of the red cell washout pattern. As shown in that chapter, it is sometimes possible to show the presence of parallel compartments in which the flow per unit volume is different. This is true when the washout curves themselves have a multiexponential form. This is the case in the ^{133}Xe washout studies describer by Kjellmer, Lindbjerg, Prerovsky and Tonnesen (1967) and Kjellmer and Prerovsky (1968). They find more than one compartment in resting and exercising muscle. This, along with their observation that the blood flow measured by the ^{133}Xe clearance technique is not equal to that measured directly, has been cited as evidence of parallel pathways in the skeletal muscle vascular bed (Groom _et al_. 1973; Hudlicka 1973, p. 42.). Sejrsen and Tonnesen(1968) suggest that the observed clearance pattern could be due to shunting between the artery and the vein of the tracer by diffusion during the washout, and that the blood flow per unit volume of the muscle is more uniform than the clearance studies suggest. Again the understanding of blood flow patterns is complicated by the problem of extra-vascular diffusion.

In summary there is good evidence of an inhomogeneous distribution of blood flow through skeletal muscle. The nature of

the inhomogeneity is not clear. Is it due to variation in the anatomical density of capillaries, or to variations in the perfusion of the available capillaries? Does the continual change in blood flow velocity in the individual capillaries explain the inhomogeneity of flow in the vascular bed, or does it hide subtle distinctions between circuits through the bed that play special roles in tissue nutrition?

6. The red cell as a tracer of blood flow distribution

a The advantages of the red cell as a tracer

The red blood cells are contained within the vascular bed of an organ so they will only be influenced by the pattern of the perfusion of the bed itself. Also the red cells are naturally present, and if methods are available for detecting and counting them they are an easily obtained tracer.

The microscopic studies of blood flow have depended upon the red cell as a tracer. All the microscopists cited above, who have given us data on local variations of blood flow, have used the velocity of the red cell as a measure of blood flow rate. This is largely because of the simplicity of the techique and the fact that the cells fill the whole luminal diameter of the capillaries, so that their velocity in these vessels must be very close to the average velocity of the plasma around them.

b The disadvantages of the red cell as a tracer

Because of plasma skimming and the Fahraeus effect, the red cell is known to be inadequate as an indicator of blood flow distribution and the vascular volume in many tissues. The ratio of red cells to plasma in many organs is less than that in venous blood (Gibson, Seligman, Peacock, Aub, Fine and Evans 1946; Allen and Reeve 1953; Everett, Simmons and Lasher 1956; Pappenheimer and Kinter 1956; Friedman 1959; Polosa and Hamilton 1963). The "extra plasma" is believed to be within parts of the vascular bed where the above-mentioned phenomena reduce the red cell concentration. In muscles,

the intravascular hæmatocrit is 0.82 times that of venous blood (Polosa and Hamilton 1963). The circulation time for the red cells through isolated muscles is about 0.80 that for plasma (Baker and O'Brien 1964; Groom 1966).

c The red cell and the perfusion of the vascular bed

A number of investigations by Baker (Baker and O'Brien 1964; Baker 1965; Moore and Baker 1971) have been done on red blood cell and albumin distribution volumes within the dog forelimb and gracilis muscle. In the forelimb it was shown that the ratio of the red cell distribution volume to that of the albumin increased on vasodilatation and that these volumes, as measured by the transit times increased more than the actual volume. The results on the forelimb show an inhomogeneous perfusion in the unvasodilated state, but this could be due to the variety of tissue (skin and muscle) perfused. In the gracilis muscle it was shown that the volume available to red cells increases in reactive hyperemia more than that of the plasma. All these observations are consistent with the idea that the number of well-perfused vessels increases upon vasodilatation. They also show that the red cell distribution in the perfused vascular bed is a function of the physiological state of the bed itself.

d The red cell washout experiments

The paper by Groom et al.(1973) which defines the starting point of this work describes the use of the red blood cell in a new way as a tracer of blood flow distribution. A muscle is isolated and perfused with a cell-free solution via the artery and the outflow

from the vein is collected in small samples. The cell concentration in the outflow can be determined with a particle counter and is plotted as a function of volume perfused through the muscle. What distingishes this from the short washouts of Polosa and Hamilton(1963) is the extent that the cell counting technique allows the concentration to be followed during the perfusion. Concentrations of only one part in one hundred thousand of that of the venous blood are easily determined. The extensive perfusion reveals poorly perfused parts of the cell store otherwise undetectable.

Similar experiments had been performed on the spleen (Song and Groom 1971a and b, Groom and Song 1971). They revealed that the large red cell store of the spleen was washed out as if it were three parallel "compartments" each receiving a different fraction of the total inflow and each containing its own store of cells. The theory which produces such a model is described in Chapter II.

What was significant about this model in the spleen was that each compartment was found to have an anatomical or functional counterpart in the spleen. The best perfused corresponded to the vascular pathways (Song and Groom 1971b), the intermediate to free cells in the red pulp (Song and Groom 1971b, Groom and Song 1971) and the most poorly perfused to bound immature and abnormal cells in the pulp (Song and Groom 1971a and b; 1972; 1974; Groom, Song, Lim and Campling 1971). The correspondence between the mathematically defined compartments and visible parts of the red cell store made the extent of the latter measurable for the first time.

The muscle washout experiments, analyzed the same way showed four compartments in the red cell store. The similarity in half-

lives between the most poorly perfused compartments of the organs suggested that abnormal cells made up the slow compartment of muscle; this was found to be the case.

Of the remaining compartments, the two less well perfused had half-lives in a ratio of less than one to five. This suggested according to criteria developed by van Liew (1962) that they may be the result of a continuum of similar wasbout processes, having half-volumes distributed according to a single-peaked density function over a range of values. The final description of the red cell store had only three parts: the best perfused, an intermediate compartment and the most poorly perfused, which contained about half of reticulocytes. The best perfused had 98% of the flow but only 82% of the cells, the most poorly perfused, with 1% of the cells,washed out as if it received only 0.002% of the flow. What was the significance of the compartments?

The presence of the slow compartment in the red cell store of the vascular bed in muscle was ascribed to a unique adhesiveness of these cells, which may cause their retention in any part of the circulation. Was it strictly a property of the reticulocytes that retained them in the muscle?

CHAPTER II.

KINETICS OF RED CELL WASHOUT FROM SKELETAL MUSCLE (ELECTRICALLY-STIMULATED, RESTING AND VASODILATED, RESPECTIVELY) AND THE KINETICS OF PLASMA WASHOUT FROM VASODILATED SKELETAL MUSCLE.

1. General introduction

The results reported by Groom, Song and Campling (1973) raised several questions. Their discovery of an apparent inhomogeneity in the circulation of red blood cells in the cat gastrocnemius muscle, as shown by the several "compartments" in their model was explained only in part. They had shown that the most slowly washed-out compartment in their model was largely due to some characteristic of the reticulocytes, or immature cells, but they had not explained the existence of two or more compartments in addition to this in the resting muscle model.

The studies of the equilibration of tracers between blood and tissue discussed before have in many cases led to the postulate that there are poorly-perfused areas in resting muscle which become well-perfused through the action of specific control mechanisms in exercising muscles. This change is the hypothetical explanation for the generally observed increase in the rates of clearance or equilibration of tracers that occurs in exercising muscle, either independently of blood flow rate or out of proportion to it. The experiments described here are an attempt to determine whether these changes in the distribution of blood flow in contracting muscle would be reflected in the red cell washout kinetics.

The first series of experiments was thus done on muscles that were stimulated electrically to twitch repeatedly, but otherwise were prepared in the same manner as reported by Groom _et al_.(1973). The results of these experiments and a consideration of the method

suggested that the washout kinetics of resting muscle should be re-investigated with better procedures. This was the purpose of the second series of experiments. The third series of experiments studied the washout kinetics of muscles treated with a vaso-dilator drug so that all the active smooth muscle tone of the vascular bed was abolished. This series was done to see whether such activity had any effect on the red cell washout kinetics at all. In this series, once the pattern of the red cell washout had been established, an additional question was considered. The red cell washout pattern may be unique to the cells or a function of the vascular bed which could affect any part of its contents. To decide which was the case, the washout of a label of the plasma was also studied.

2. Methods

a General introduction

In general, the method for determining the red cell washout kinetics is as follows. The organ is isolated from the animal, and access is gained to the vascular bed through the artery and vein. A perfusant solution is forced into the artery under either constant pressure or constant flow conditions, and the outflow from the vein is collected. The red cell concentration in samples of the outflow is measured and plotted on a graph as a function of the total volume of perfusant passed through the organ when the sample was taken. The resulting graph is called the washout curve and is analysed in terms of a model which describes the processes which determine its shape.

This basic scheme describes the experiments reported here as well as those reported by Groom _et al_.(1973). The details of the method used here are different. Some differences are related to the special nature of the various experiments,e.g. washout from contracting muscle was studied using muscles which were induced to twitch during the washout. Others are improvements in the general technique which were made for both physical and physiological reasons.

Because of the general similarity of the methods used in the three series of experiments described here, a general description of the methods is given below. Certain details apply to some of the series of experiments and not to others; they will be referred to in the particular descriptions that follow.

_b_ Perfusion solutions and their preparation

Three different recipes were used for the perfusant solutions used in these experiments. Their compositions are listed in Table I (solution IV of the Table is described in the next chapter). Solution I is the same as that used by Groom _et al_.(1973). Solution II is modified from the recipe given by McEwen (1956) by the addition of glucose, and Solution III is the bicarbonate-buffered Ringer's solution described by Renkin (1962) containing 5% (w/v) Dextran T-40 (Pharmacia Ltd.). Each solution recipe was adopted because the previous one proved inadequate for the purposes of the experiments.

Solution I, used in the experiments on exercising muscle, as well as those reported by Groom _et al_.(1973), was found to be

inadequately buffered. In equilibrium with the 95% O_2-5% CO_2 gas mixture used to oxygenate the solution, it showed a pH between 6.2 and 6.6, on analysis with a Corning Model 165 pH-blood gas analyser. This was unphysiologically low. Solution II was well-buffered, but during aeration in a heated bath it formed a precipitate in some experiments. It was also evident that some colloid was required in the perfusate to counteract the tendency of the perfused muscle to accumulate fluid and become edematous. Solution III was found to be stable and well-buffered to pH 7.4 in equilibrium with the gas mixture. Dextran T-40 was used rather than the more commonly used Dextran T-70 because of the latter's tendency to cause abnormal aggregation of cat red blood cells (Eliasson and Samelius-Broberg 1963) Solutions I and II were prepared with distilled water less than 24 hours before use. They were filtered three times through 0.22 µm Millipore filters, and stored in a refrigerator. Solution III was prepared in two components, one (Component I) with Dextran, bicarbonate and glucose, the other (Component II) with the remaining solutes. In the experiments in which it was used, sodium nitroprusside was added to the perfusant solution. Since the nitroprusside is unstable in solution, it was dissolved in Component I on the day of use, the components were then mixed and made up to volume with distilled water. The solution was then filtered just before use. The purpose of using the separate components was to prevent the calcium, magnesium, and bicarbonate ions from forming insoluble precipitates during the agitation required to dissolve the Dextran and nitroprusside.

Table I

Compositions of Perfusion and Cell Suspension Solutions

Solute	Concentration in milligrams per litre			
	I	II	III	IV
NaCl	9000	8100	7305	8000
$CaCl_2$	240	240	277	100
KCl	420	420	321	200
$NaHCO_3$	200	1900	2100	
$MgCl_2 \cdot 6H_2O$			203	100
Na_2HPO_4				1150
$NaH_2PO_4 \div H_2O$		164		
KH_2PO_4				200
Glucose	1000	1000	1000	
Dextran T-40			50,000	

Before the perfusion experiments the solutions were transferred to 1 l or 2 l flasks and placed in a temperature-controlled bath at 38°C. They were equilibrated with a 95% O_2-5% CO_2 gas mixture by bubble aeration before and during the perfusion.

c Surgical procedure

Each experiment usually required one cat, although in some of the experiments with contracting muscle it was possible to use the two legs of one animal. Each cat was anesthetized with 40 mg/kg sodium pentobarbital (Nembutal, Abbott Laboratories Ltd. Montreal). The skin and outer muscle layers of one leg were cut and parted by blunt dissection and thermocautery to expose the gastrocnemius-plantaris muscle group. This muscle group was then isolated, except for the branches of the popliteal artery and vein which entered it. In doing this, the fat and layers of fascia were parted by blunt dissection. All other blood vessels entering the muscle were sectioned between ligatures. Nerves, except for the sciatic nerve proximal to the muscle, were tied in two places and cut between the ties. The sciatic nerve was also tied and cut, but in the case of the experiments in contracting muscle, this was postponed until the muscle was otherwise isolated. The tendons were ligated by ties of crochet cotton and severed by thermocautery.

Branches of the popliteal artery and vein which did not enter the gastrocnemius-plantaris muscle group were tied and cut in order to isolate these vessels for at least 8 mm of their length proximal to the muscle for cannulation.

In preparation for the cannulation 500 U/kg of heparin

were injected intravenously into the cat. Then both the popliteal artery and vein were cannulated in the direction towards the muscle and the muscle was removed to the experimental apparatus. Cannulae were made of Intramedic polyethylene tubing (Clay-Adams Division of Becton-Dickinson and Co., U. S. A.) of the following sizes: PE 190(1.19 mm i. d.), PE 90 (0.86 mm i. d.) PE 50 (0.58 mm i. d.). The greater part of each cannula was of PE 190 tubing, the arterial cannula being 10 cm long, the venous, 18 cm. Tips were made of PE 90 tubing, 1 cm long,to fit the artery. Both cannulae were filled with heparinized saline before use by attaching them to a saline-filled syringe with a blunted hypodermic needle.

The above technique, identical to that used by Groom et al. (1973), was used on the experiments on contracting muscles. It required that the blood flow through each muscle be interrupted from the beginning of the cannulation to the beginning of the perfusion of the muscle in the apparatus. This time ranged from 5 to 20 min depending on one's skill and good fortune with the cannulation. The interruption of flow could have an effect upon the vascular pattern (reactive hyperemia) as well as the properties of the red cells in the muscle (Knisely 1965; Braasch 1971). For this reason, another method was used for the experiments on resting and vasodilated muscles.

In these experiments the isolation of the muscle was the same as before and included the sectioning between ligatures of all nerves and tendons, and of all blood vessels except the branches of the popliteal artery and vein which enter the muscle. The technique of cannulation of these vessels was modified to avoid the interruption of blood flow. Both of these vessels extend beyond their insertions into

the gastrocnemius-plantaris muscle group to form the tibial artery and vein. These extensions,rather than the popliteal vessels themselves, were cannulated without interrupting the flow to the muscle itself. The artery required a tip of Intramedic PE 50 tubing on the cannula, and the vein, one of PE 90 tubing. Because the muscle could not then be moved from its place near the tibia of the cat without interrupting its blood supply, the experimental arrangement was modified to allow the perfusion of the muscle where it lay on the operating table. This is referred to as the *in situ* preparation, although the muscle was almost completely isolated from the cat.

d Experimental apparatus

The muscle, when it was removed from the cat, was attached at its resting length to a framework made of Lucite by the strings tied to its tendons. This served to maintain the muscle at its resting length throughout the experiment and also to position the cannulae so as to prevent the twisting of tha artery and vein. The muscle, on its frame, was placed in a mineral oil bath heated to 37°C *via* a heat exchanger by a Haake recirculating pump with a heater and thermostat. This system is shown in Figure I.

When the muscle was left *in situ*, the tibia of the cat was placed near the edge of the operating table and the muscle was covered with saline-moistened gauze and wrapped loosely with Saran Wrap. The muscle was heated by an infrared lamp which was connected *via* a relay to a Yellow Springs thermistor thermometer placed near the muscle. When the thermometer indicated a temperature above 37°C, the lamp was turned off automatically, otherwise it was on. This arrangement is shown in Figure II.

e Perfusion of the muscle

The perfusion apparatus delivers the solution from the reservoir of perfusate previously described to the arterial cannula. Two systems were used, one providing controlled flow and the other controlled pressure.

The controlled-flow system, shown with the isolated muscle perfusion in Figure I, uses a roller pump(Sigmamotor Model AE-40) to draw the solution from the reservoir through Tygon tubing and deliver it to the cannula. The flow rate varies periodically as the roller moves through one revolution, but it is independent of the resistance to flow presented to the delivery outlet, as long as the pressure there is within reasonable limits. A 10 ml air-filled syringe with a locked plunger was attached to a side branch of the delivery tube. During the perfusion the trapped air served to reduce the cyclic variation in pressure caused by the variation in pump flow. Pressure at the cannula was measured by a Statham P23 DC pressure transducer and recorded by a Dynograph chart recorder. The gauge was calibrated before each experiment by measuring the indicated pressure when it was at the base of a 1 m column of water, and when it was open to air.

The controlled-flow system allows the total volume of fluid perfused through the muscle to be calculated simply by multiplying the pre-set flow rate of the pump by the elapsed time since the beginning of perfusion. Any deficiency of outflow volume with respect to inflow volume would be indicative of leakage or accumulation of fluid within the tissue. The Statham transducer gives a continuous record of perfusion pressure which, if the flow rate

be held constant, is proportional to the vascular resistance of the muscle. This record can therefore be used as one index of the viability of the preparation during the experiment. The disadvantage of this system is that the shear stresses in the vascular bed, which might well determine the rates of release of red blood cells, are uncontrolled, as they are a function of the perfusion pressure. From this point of view, a system which controls the perfusion pressure would be better. Also the tubing of the pump and the air-chamber are elements of the system which are difficult to clean and may harbour contaminants of the perfusate solution which are undesirable.

For these reasons the experiments with resting and vasodilated muscles were performed with a constant pressure apparatus as shown in Fig. II. The reservoir holding the perfusate solution was sealed so that the escape of the gas mixture aerating the solution could be controlled. This, and the control of the pressure of the inflowing gas, allowed the pressure in the reservoir and delivery tube to be set at any desired pressure between 10 and 130 cm of water. This pressure was monitored by the transducer used before. It remained independent of the perfusate flow as long as this flow was much less than the flow of gas through the reservoir.

The procedure for the perfusion was different in the case of the isolated muscle and in situ perfusions. The perfusion of the isolated contracting muscle was started simply by ensuring that the pump and delivery tube were filled with perfusate and connecting the arterial cannula to the outlet. The pump was turned on and the samples were collected starting with the first drop of outflow. The concentration in the outflow at 0 ml perfused was presumed

Figure I. The apparatus used for perfusion of an electrically-stimulated, isolated muscle at constant flow.

The following apparatus are identified by number:

1. Temperature-controlled bath
2. Perfusate reservior
3. Sigmamotor pump
4. Air-chamber
5. Pressure transducer
6. Mineral-oil bath with muscle
7. Haake thermoregulated bath with pump
8. Fraction collector
9. Stimulator

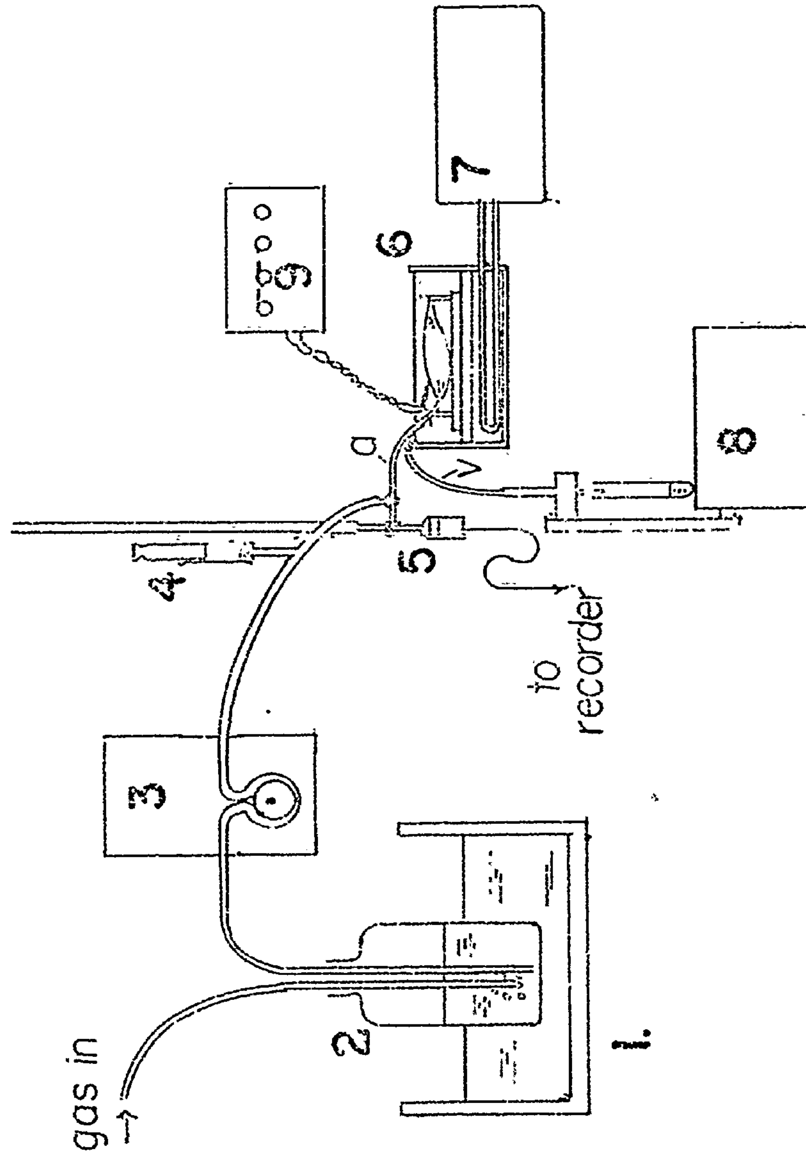
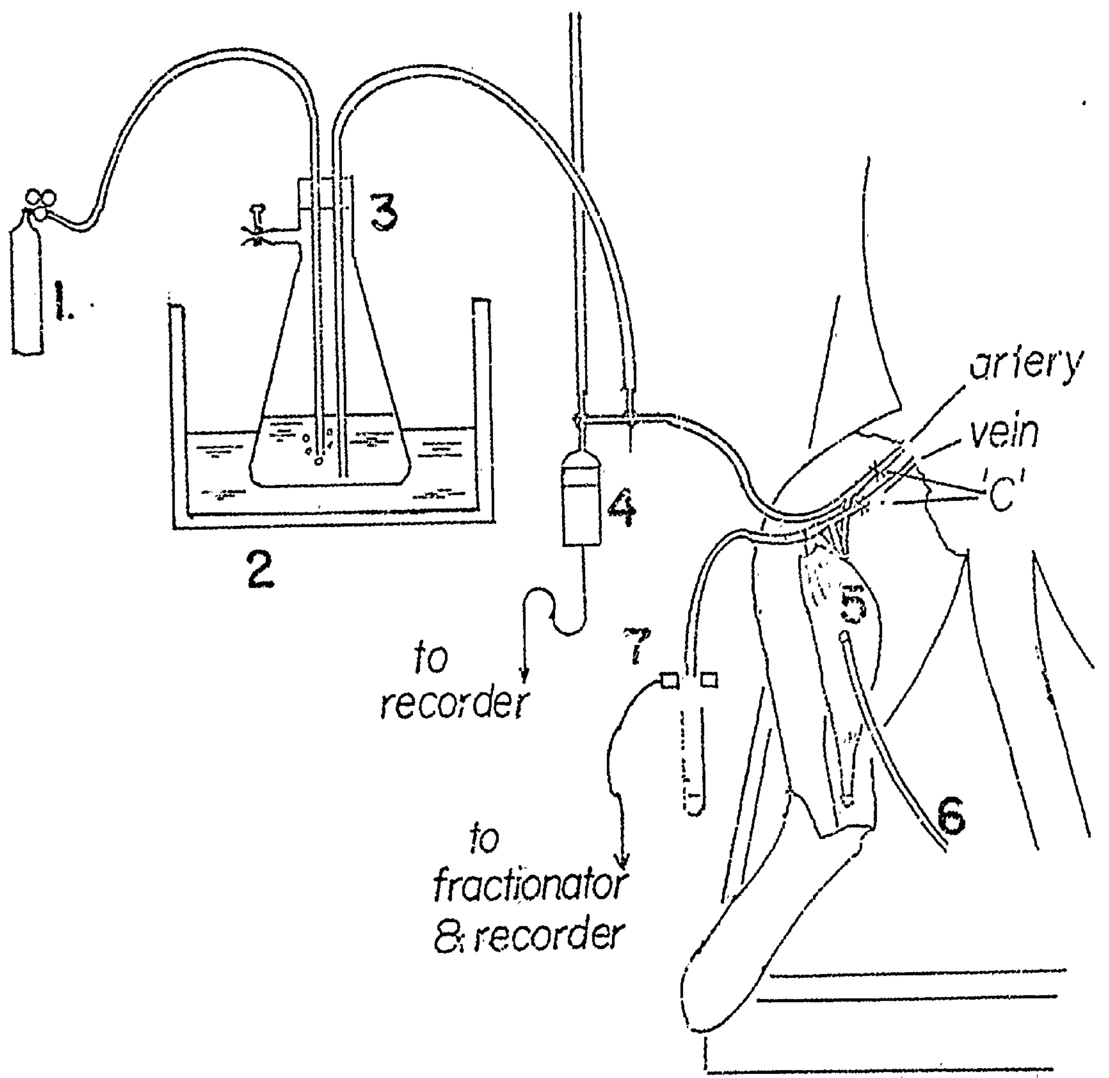

gas in
1
2
3
4
5
6
7
8
9
a
v
to recorder

Figure II. The perfusion at constant pressure of a muscle *in situ*.

The following apparatus are identified by number:

1. Gas cylinder with pressure regulator.
2. Thermostat-regulated heated bath.
3. Sealed reservoir of perfusate with hosecock to control the escape of gas.
4. Pressure gauge.
5. Muscle *in situ*. (The covering is not shown.)
6. Temperature transducer for the regulator.
7. Drop counter.

The artery and vein are clamped at the points 'C' before perfusion.



1.
2
3
4
to
recorder
5
6
7
to
fractionator
& recorder
artery
vein
'C'

to be equal to that of peripheral venous blood sampled elsewhere (e.g. at the contralateral limb).

The experiments in which the muscle was perfused *in situ* were started by a different procedure. The arterial cannula was attached to the perfusion apparatus and the stopcock positioned to connect the pressure gauge to the artery so that the arterial pressure delivered to the muscle by the cat could be measured. The venous cannula was positioned in the fraction collector and the blood flowing from the muscle diverted to this cannula by clamping the popliteal vein with a haemostat. Thus the blood flow rate through the muscle could be measured and the collected samples used for determining the concentration of red blood cells corresponding to 0 ml of perfusion. The muscle was perfused by the cat in this way for a minute or so. Then the popliteal artery was clamped and the stopcock of the perfusion apparatus turned to admit the perfusate from the reservoir to the cannula. The interruption of flow to the muscle was less than 5 seconds long. The beginning of the perfusion was set at the time the stopcock was turned.

f The collection of samples

The venous cannula was held with its free end below the level of the muscle and was as short as possible in order to keep the venous pressure at a minimum value. The strategy for collecting the outflow from this cannula was changed between the experimental groups in an effort to improve the accuracy of the results.

Groom *et al.* (1973) collected samples at pre-determined

times after the start of perfusion, and did this manually. The first sample was not collected until after 0.5 min of perfusion, the second at 1.0 min. It was found, and this will be obvious when the results are presented, that more samples were necessary at the beginning, because of the rapid change in the red cell concentration in the outflow. It was also found, during preliminary experiments, that the expression of the red cell concentration as a function of volume perfused through the muscle produced a more consistent result than the expression of the concentration as a function of the time elapsed after the start of the perfusion. For these reasons a strategy for collecting samples at pre-determined volumes perfused, rather than at pre-determined times was followed and automatic sample collection was tried.

In the experiments with contracting muscles perfused at a constant flow rate a fraction collector controlled by a timing mechanism (Gilson Mini-Escargot Fractionator, Gilson Medical Electronics Inc. U.S.A.) was used to collect the first 25 ml of outflow. Since the flow rate was constant and known, the volume in each tube could be determined by the setting of the timer. The experiments with restin. muscle perfused at constant pressure used a different fraction collector (Gilson Model FC-80E Micro-Fractionator) which was controlled by a drop-counter. This could be used to collect pre-set volumes even when the flow rate was unknown. However the mechanism of the fractionator required that the venous cannula be somewhat long, about 20 cm, and thus added a certain volume to the system being washed out.

The most successful system used a tray which accepted a row of calibrated disposable plastic cups of the sort used in automatic biochemical analyzers, and which was held by hand and moved along as each cup was filled to a pre-determined calibration mark. This allowed the use of a very short cannula.

After the first 25 ml of perfusion the venous outflow was directed into a graduated cylinder and the samples were collected in test tubes as the meniscus rose past pre-determined points. The contents of the test tubes were replaced by adding water to the contents of the cylinder. After 225 ml of perfusion, the outflow was collected in 50 ml beakers. The time of collection of each sample was noted in all experiments performed with constant pressure perfusion.

g Cell counting

A Celloscope counter (Particle Data Inc. U.S.A.) was used to measure the cell concentrations in the samples. Dilution of most samples was required, as the counter was capable of accurate counts of only a small range of concentrations. The diluent used was Diluton (BDH Chemicals Ltd. Canada). Samples of the diluent and the perfusate solution were also counted to determine the background due to particles in these solutions. In undiluted samples the perfusate background was subtracted from the sample count. In diluted samples, the dilution was over ten times, thus the perfusate background was usually negliglble. The diluent background was then subtacted from the sample count. The concentration of cells in the sample was calculated from two counts

of the diluted sample

$$C = \frac{V_D + V_s}{V_s} \cdot \frac{1}{V_c} \left(\frac{N_1 + N_2}{2} - N_B \right)$$

where

C is the cell concentration per ml.

V_D the volume of diluent used,

V_s the volume of the sample used,

V_c the volume of the diluted sample counted by the Celloscope (always equalled 0.5 ml),

N_1 & N_2 the two counts obtained from the Celloscope,

N_B the appropriate background count.

An electronic discriminator in the Celloscope prevented the counting of particles significantly smaller than the red blood cells of the cat. These counts were used in the analysis described below.

h Analysis of the results

The results of a washout experiment plotted as a graph of the concentration of a tracer versus time of the volume perfused since the beginning of the washout, may, if analyzed, yield information about the processes by which the tracer is released from the system under study, and thus about the system itself.

Let us consider the ideal washout of a simple system, a tank of volume V filled with a fluid containing a tracer such as a dye at a concentration C. Fluid is pumped into the tank at a constant rate F, and drains from the tank at the same rate. To prevent

complication of the theory, we assume that the concentration is everywhere uniform in the tank and that the fluid draining from it shares this concentration. The tank then contains a quantity C.V of the tracer and loses it at the rate C.F. Therefore

$$\frac{d}{dt}(C \cdot V) = -C \cdot F$$

or

$$\frac{1}{C}\frac{dC}{dt} = -\frac{F}{V} \qquad (1)$$

Equation (1) has the solution

$$C(t) = C_o e^{-(F/V)t} \qquad (3)$$

of alternatively

$$\log_e C(t) = \log C_o - \frac{F}{V}t \qquad (3a)$$

where C_o is an arbitrary constant equal to the concentration at an arbitrary time t=0.

Equation (3a) shows that if $\log_e C(t)$ is plotted as a function of t the graph will be a straight line of slope -(F/V) and intercept at t=0 of $\log C_o$. It suggests that real washout curves may be most easily understood if the concentration is plotted as a function of time on a semi-logarithmic graph. This is what has been done with the data presented here as well as in many other reports.

Do the real data fit a straight line? In many cases yes - beyond a certain point. At volumes of perfusion less than this point the line of the data curves upward, suggesting that the

actual washout is more complex than the simple model proposed so far.

Consider, therefore, a more complex model, consisting of n tanks of different volumes v_i, $(i = 1...n)$, each receiving a different fraction f_i of the total flow F delivered by a common channel, and each contributing its own fraction f_i to the total flow F leaving by a common drain. Figure III illustrates this. Each tank receives a flow f_iF and thus, by analogy with equation 3, the concentration c_i in the tank i is described by

$$c_i(t) = (C_0)_i \; e^{-(f_iF/v_i)t} \qquad (4)$$

The concentration in the outflow will be the result of mixing streams of the concentrations c_i in the ratios f_i and will equal

$$C\,(t) = \sum_{i=1}^{n} c_i(t)\cdot f_i \qquad (5)$$

To limit the number of variables involved, we assume that, at time t=0, the concentrations in all tanks are equal so that the values $(C_0)_i$ are equal to one value C_o. Equation 5 becomes, with the appropriate substitution from equation 4:

$$C(t) = \sum_{i=1}^{n} C_of_ie^{-(f_iF/v_i)t} \qquad (6)$$

Equation 6 can be transformed into a form in which the outflow concentration is a function of the total volume of solution perfused by substituting $V=F\cdot t$ where V is the volume perfused through the whole system.

$$C(V) = C_o \sum_{i=1}^{n} f_i e^{-(f_i/v_i)\,V} \qquad (7)$$

The quantity $-(f_i/v_i)$ is called the decay constant of the ith tank.

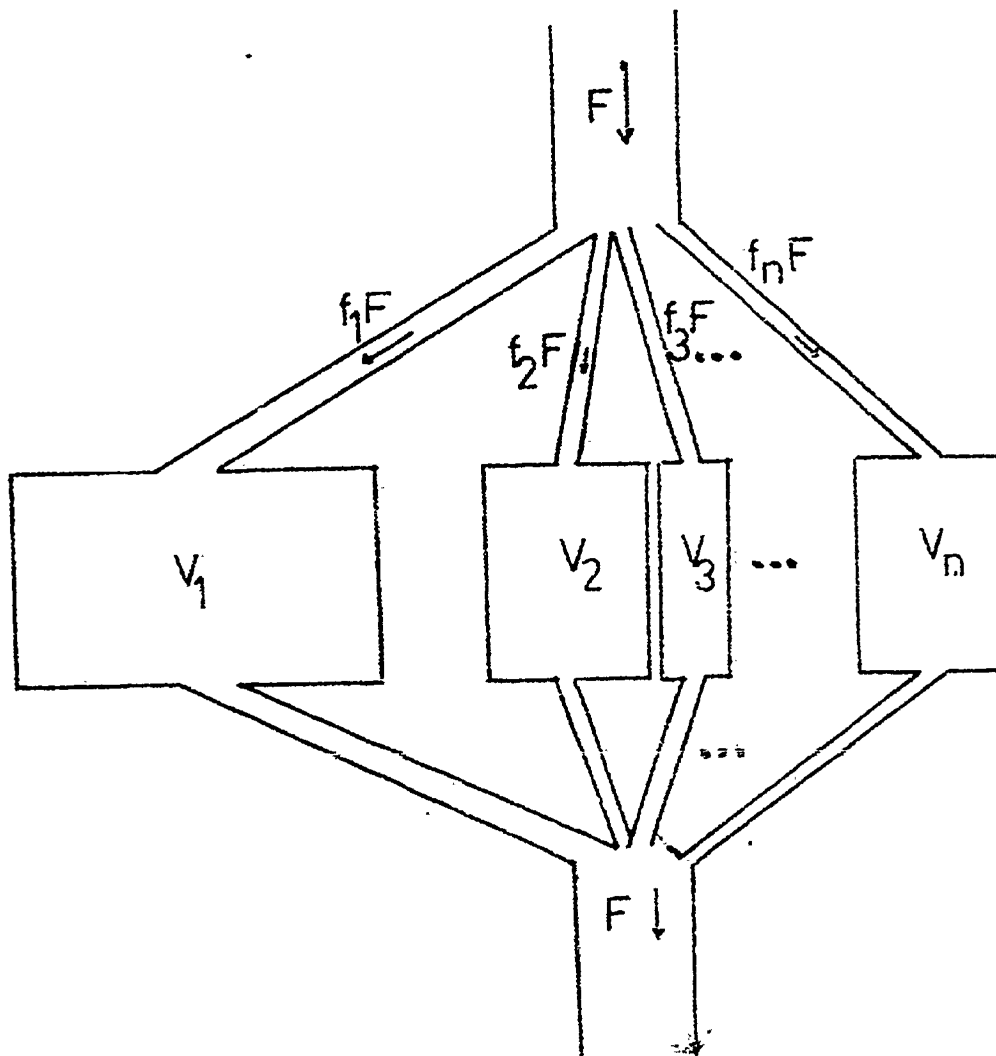
The application of this model is best shown by an arbitrarily chosen example, in which there are two tanks, the first (i=1) having a value of f_1 ten times the value f_2 for the second, but each having the same volume v_i so that the decay constant of the first is ten times that of the second. Figure IV shows the values predicted for each of the terms of equation 7, as well as the sum on both linear and logarithmic scales, as a function of volume perfused. One can see by the graphs that tank 1 does not contribute a significant amount of tracer to the outflow beyond a certain volume perfused. The logarithm of the total concentration thereafter follows a straight line with slope $-(f_2/v_2)$ and intercept $\log_e(C_o f_2)$.

If these lines were composed of data points, a point could be chosen, to the right of which the points could be fitted to a straight line by regression formulae. This would yield experimental values of f_2 and v_2 from the original graph. In actual experiments the data may contain some random error, and the selection of this cutoff point is somewhat arbitrary and can be difficult.

If the concentrations shown by the straight line are subtracted from the original graph, the remainder is the concentrations due to tank 1. The logarithms of these concentrations can be fitted to another straight line of slope $-(f_1/v_1)$ and intercept $\log_e(C_0 f_1)$. This

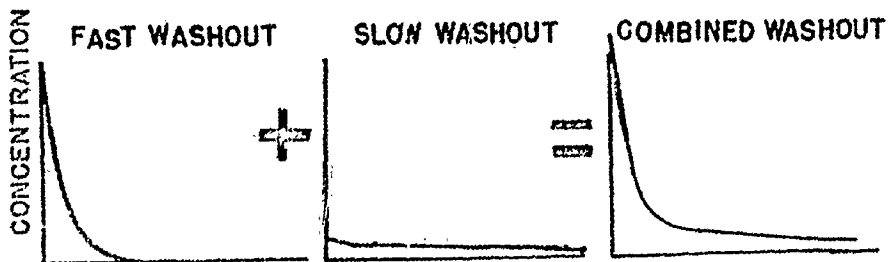
Figure III.

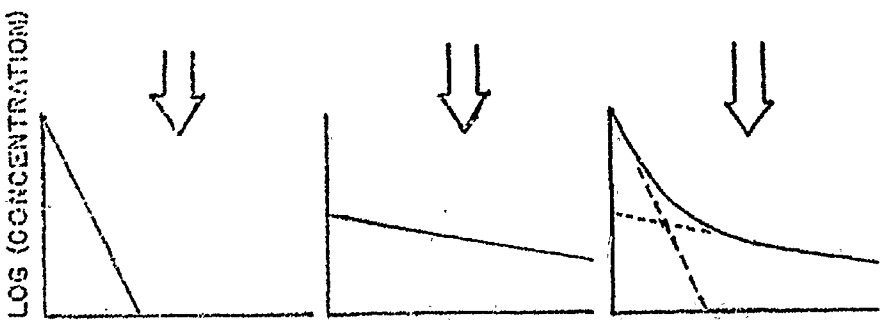
The washout of n compartments (tanks) in parallel.



F
f_1F
f_2F
f_3F...
f_nF
V_1
V_2
V_3 ...
V_n
...
F

Figure IV. The washout kinetics of a tracer from a two compartment system. The upper graphs show the concentration on a linear scale as a function of the volume perfused. The lower graphs show the same concentrations on a logarithmic scale. The concentration of the right hand graphs is the sum of the concentrations shown in the two graphs to the left of each.





WASHOUT OF A TWO-COMPONENT SYSTEM

(theory of analysis)

technique of analysing a graph by successive fittings to a straight line and subtractions is called curve-peeling and has many applications in physics and biology.

A similar process may be applied to real experimental results to obtain a model in terms of the tanks I have described. However, there are qualifications.

The results must be similar to the arbitrary example shown in that there are a relatively small number of compartments with clearly-separated values of the decay constants. Van Liew (1962) has shown from models that, if the decay constants are not in the ratio of 1 to 5 or greater, it is not possible to assume that the curve is the result of the washout of two distinct compartments rather than a continuum of compartments in which the decay constants have a single-peaked distribution.

A curve-peeling of a graph of washout data yields a model, according to our assumptions, with a number of tanks or compartments depending upon the number of times the fitting and subtraction has to be done in order to account for all the points in the last fitting. Other facts can be deduced from the values of the intercepts and slopes of the lines. At zero volume perfused, the concentration of the tracer is given as

$$C(0) = C_0 \sum_{i=1}^{n} f_i$$

so that the intercepts of the straight lines, corresponding to the values $\log_e(C_0 f_i)$, yield the fractions f_i of the total flow in each compartment.

The total store of tracer in each compartment is given by C_0v_i and can be calculated by dividing the intercept of the component on the linear scale (C_0f_i) by its slope on the logarithmic scale (f_i/v_i). This is identical to computing the area under the exponential curve by integrating from zero volume perfused to infinity. Each value of C_0v_i divided by the sum of these values, which is the total area under the washout curve, gives the fraction of the total store of tracer that is originally held by each compartment.

It has become conventional to describe exponentially decaying processes in time by a 'half-life' rather than a decay constant, as it seems easier to understand the processes by saying that half of whatever is left of a substance will disappear in a given time. By analogy, I will define the 'half-volume', which is that volume of solution which must be perfused through the whole system before a given compartment has lost half of its tracer. Algebraically,it is

$$(V_{\frac{1}{2}})_i = (v_i/f_i).\ \log_e 2$$

and it is proportional to the reciprocal of the decay constant.

Thus a model may be constructed for certain types of washout behaviour that are more complex than the simple exponential decay of concentration with volume perfused. The model involves some arbitrary assumptions and also arbitrary choices of cutoff points in its application. Under certain circumstances it can yield a useful interpretation of the results, as in the work of Song and Groom (1971a; see also Groom and Song 1971; Song and Groom 1971b and 1972; Groom, Song, Lim and Campling 1971). At the very

least, in those situations where the process of curve-peeling can be applied, it yields a description of the results which uses a small number of parameters, the values of $(V_{\frac{1}{2}})_i$, f_i and v_i which can be used to compare the results with others from similar systems.

In the experiments described here there was considerable variation from point to point in the results from some individual washouts. This is similar to the case described by Groom et al. (1973). This variation, as I have mentioned, makes curve peeling difficult by obscuring the cutoff points of the successive regressions. Groom and his co-workers solved this problem by computing the averages of the logarithms of the concentrations obtained at each volume perfused in several experiments. The resulting curve of averages was smooth enough to be peeled easily. It also showed those characteristics which were common to all the indiviual washout experiments, as opposed to those peculiar to each. However, it gave no idea of the variation which might occur if other sets of experiments were similarly done and analysed. For this last reason I have attempted to apply the curve-peeling technique to the results of individual washouts, using the peeling of the average curve of the set as a guide, in order to obtain samples of the distribution of possible values of the parameters of the various compartments that were apparent in the average curve. From these samples, the mean and standard error of the mean of these values could be calculated, and comparisons between the results of experiments done under different conditions could be performed using standard statistical tests.

For the sake of comparison, the values of volume of solution

perfused were divided by the weight of the muscle (see below) to produce a normalized value for each point.

The computer program QCPEL, written in BASIC, and listed in Appendix I, was used to perform the curve-peelings of the individual washout graphs of a set and compile the results for the various parameters together. It required that the cutoff points be determined beforehand from inspection of the graph or manual curve-peeling.

i Other measurements

In many of the experiments the muscle gained weight and became visibly edematous during the experiment. The degree to which this occurred was measured by weighing the muscle immediately after the perfusion. Weighing the muscle before perfusion was not usually done because of the constraints of the method. However this weight could be estimated by taking 1% of the weight of the cat, which was always measured. This estimate was shown in other experiments to be a good one. The calculated gain in weight was divided by the original weight of the muscle to produce a normalized weight gain. In muscles perfused at constant flow, the vascular resistance was estimated as the quotient of perfusion pressure and the pre-set flow rate. In muscles perfused at constant pressure, the timing of the collection of the samples provided an estimate of the flow rate but this was available only for certain volumes perfused. The resistance could be calculated by dividing the measured pressure by the estimated flow rate.

It is important to remember that the resistance calculated this way

is not an intrinsic property of the muscle, but depends greatly upon the perfusion pressure and other variables. However most of the experiments described here were done at similar pressures of perfusion and flows. Thus the calculated resistance may serve for comparison between the various types of preparations studied. I have expressed resistance in a form often used by physiologists, the PRU (Peripheral Resistance Unit). A muscle is said to have 1 PRU if for every millilitre of flow per minute per 100 gm tissue, there is 1 mm Hg perfusion pressure. This form takes into account the variations in muscle weight and perfusion pressure that occured within a set of experiments.

3. The red cell washout kinetics of contracting muscle

a Introduction

The response of the vascular bed to physical activity in skeletal muscle has been modelled in isolated muscle preparations by stimulating the muscles to twitch maximally at frequencies between two and eight times a second (Kjellmer 1964; Renkin, Hudlicka and Sheehan 1966; Folkow and Halicka 1968; Moore and Baker 1971). At these frequencies the blood flow is increased to nearly its maximum value and indices of the distribution of blood flow show the most pronounced differences between the contracting muscles and a resting muscle. At higher frequencies of stimulation the twitches of the muscle reduce the blood flow by their action in compressing the blood vessels (Folkow and Halicka 1968), although higher flow rates may occur after the stimulation is stopped. A cycle of stimulation and rest would not be suitable for a washout experiment because the theory has the assumption that the system under study remains constant in its pattern during the washout process. For these reasons, then the red cell washout of a contracting muscle was studied by perfusing muscles which were stimulated to twitch four times a second. If the non-uniformities in the distribution of flow that gave rise to the separate compartments in the model that Groom *et al*.(1973) proposed for the red cell store of resting muscle were due to the same causes as those which led to the non-uniform perfusion of resting muscles inferred by other authors, the red cell washout kinetics should be

very much different in contracting muscle.

b Details of the method

Twelve gastrocnemius muscles were isolated from nine cats, weighing 1.9 to 2.7 kg, in the manner described in the previous section. They were attached to the Lucite frame and placed in the mineral oil bath at 37°C. The sciatic nerve of each was sectioned between ligatures just before the isolation. Two platinum wire electrodes, which served to stimulate the muscle to twitch, were attached to the cut stump of the nerve attached to the muscle. The muscle was induced to twitch four times per second by means of pulses applied to the electrodes from a Grass Model S-88 stimulator. The pulses were of 1 ms duration and between 0.5 and 1.5 V amplitude, the actual amplitude being the minimum which elicited the maximum possible twitch of the muscle, as judged by visual observation. Kjellmer(1964) demonstrated that thisstimulation did not stimulate the action of vasoconstrictor nerve fibres in the sciatic nerve. Solution I was perfused through the muscle at a constant flow of 5.0 $ml \cdot min^{-1}$. The apparatus was exactly as shown in Figure I. The collection of samples and the cell concentration determinations were done as described before.

In seven of these experiments the muscle was stimulated to twitch for ten minutes before it was completely isolated, i. e. when the nerve, the popliteal artery and the popliteal vein were still intact. The pulses were of the same voltage,duration and frequency as used subsequently during the perfusion and

were applied directly to the uncut nerve. This had no effect on the blood pressure of the cat, indicating that no central circulatory reflexes were excited by the stimulation of the uncut nerve.

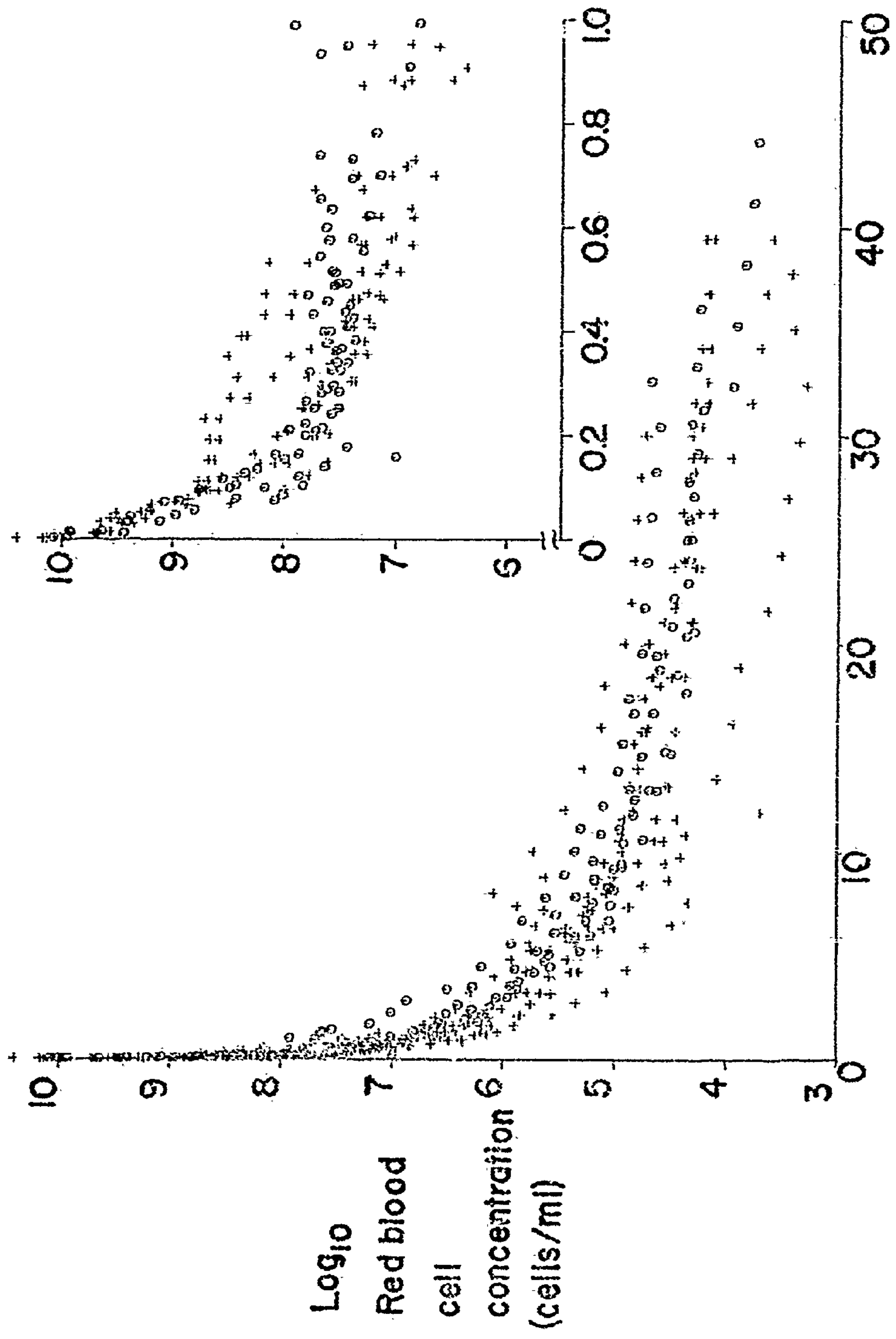
c Results

Figure V shows the results of the individual experiments in the form of the logarithms of the cell concentrations plotted against the volume of solution perfused per gram of tissue. The experiments in which the muscles were stimulated for ten minutes before perfusion are shown as distinct from the others, but the graph shows that their washout kinetics are not distinct from those of the others. For this reason all the results were considered as a single group in the subsequent analysis.

Since in each experiment the samples were taken at different values for the volume of solution perfused per gram of tissue, it is not possible to produce a graph of the average concentration for each volume perfused as Groom et al. (1973) did previously. The concentrations in each experiment, corresponding to certain arbitrarily selected volumes per gram were obtained by geometric interpolation between the measured concentrations on either side of the selected volumes perfused and the averages with the standard errors of the mean of these values are shown in Figure VI.

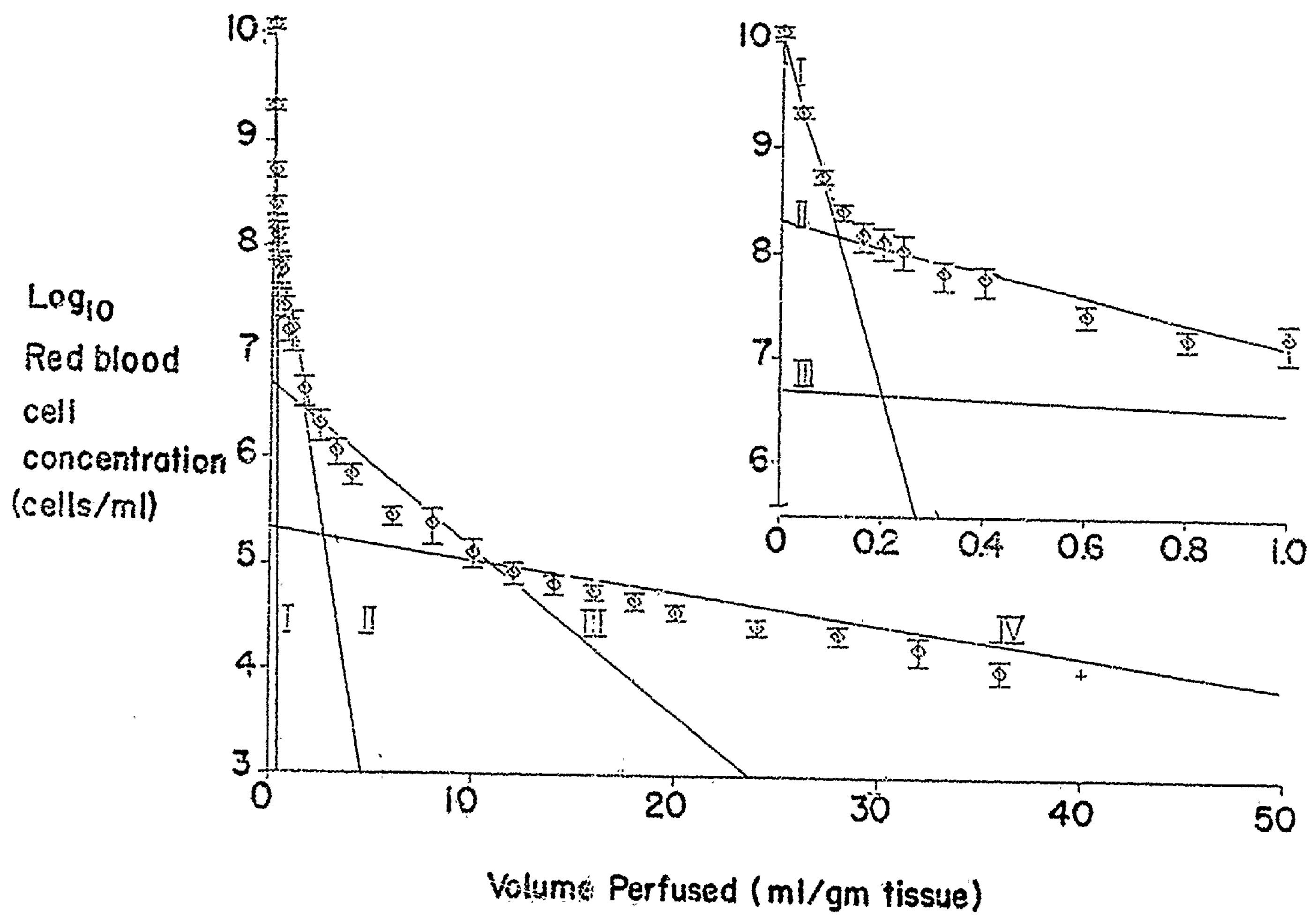
An analysis of the results based on the previously explained procedure of curve-peeling showed four components. The averages of the parameters of these components, with standard errors of the mean, are shown in Table II, and the lines corres-

Figure V. The red cell washouts of twelve cat gastrocnemius muscles. The results from muscles that were stimulated before perfusion are shown thus: (+) the others are shown thus: (o). The inset shows the results from the first ml / gm of perfusion on an expanded scale.



Log10
Red blood
cell
concentration
(cells/ml)
Volume Perfused (ml/gm tissue)

Figure VI. The logarithms of the averages (± SEM) of the concentrations obtained by interpolation from the results shown in Figure V. The lines have the average slopes and intercepts given for the compartments in Table II. Because the lines are given slopes and intercepts which are the averages of those of the curve-peelings of the individual washouts, rather than being derived from the curve-peeling of the average curve shown here, they are not necessarily coincident with the points.



Log10
Red blood
cell
concentration
(cells/ml)
10
9
8
7
6
5
4
3
0
10
20
30
40
50
Volume Perfused (ml/gm tissue)
I
II
III
IV
0.2
0.4
0.6
0.8
1.0

Table II Parameters of the compartment model derived from the curve-peeling of the red cell washout data obtained from contracting muscles.

Parameter	Comp. I	Comp. II	Comp. III	Comp. IV
Half-volume	0.0182	0.266	1.41	10.7
± S.E.M.	± 0.0018	± 0.045	± 0.091	± 1.15
(ml/gm)				
Intercept	1.11×10^{10}	2.03×10^{8}	5.10×10^{6}	2.02×10^{5}
± S.E.M.	0.25×10^{10}	0.81×10^{8}	1.25×10^{6}	0.66×10^{5}
(cells/ml)				
Percent of				
total flow	97.68	2.26	0.059	0.0022
± S.E.M.	0.90	0.90	0.014	0.0007
Percent of				
total Cells	78.18	17.68	3.30	0.85
± S.E.M.	4.55	4.36	0.80	0.16

Total of Intercepts: $1.128\times10^{10} \pm 0.248\times10^{10}$ cells/ml.

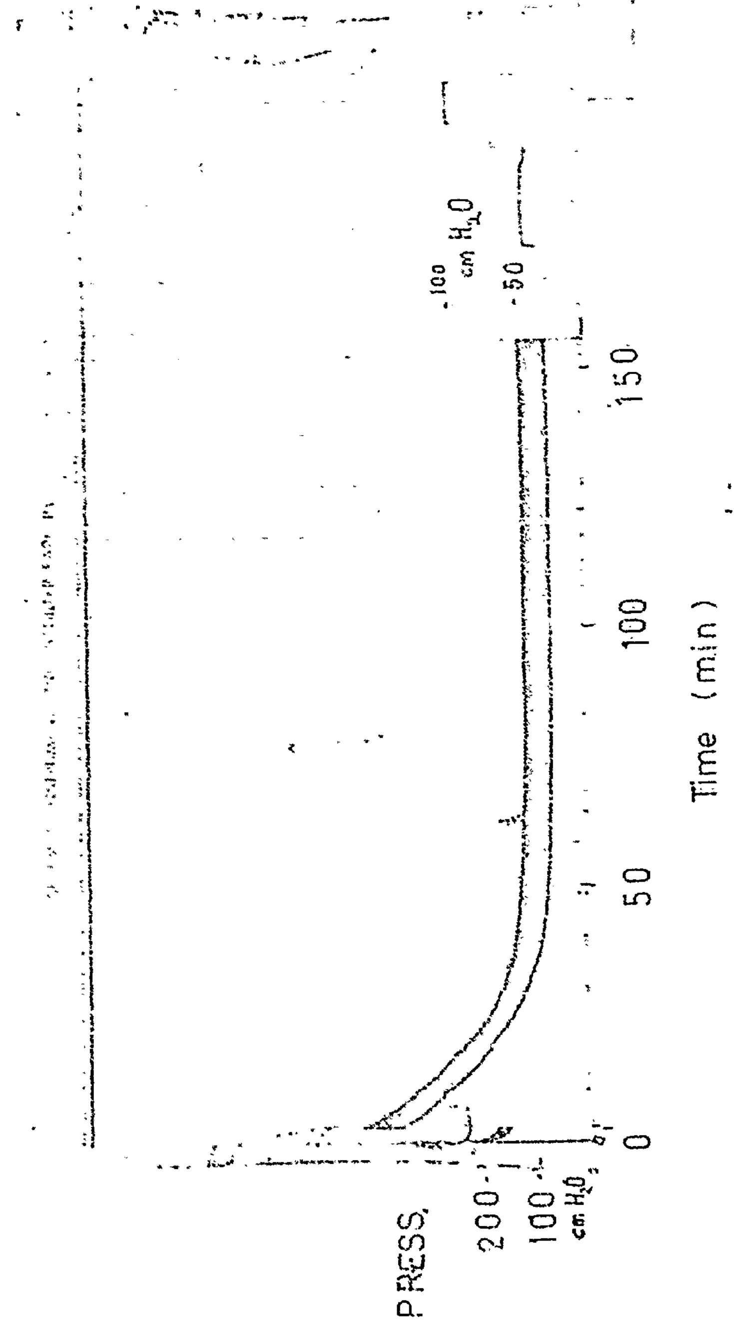
Total of cells stored: $3.206\times10^{8} \pm 0.292\times10^{8}$ cells/gm.

ponding to the average intercept and the average slope are drawn in on Figure VI over the averages of the interpolated concentrations. Since these lines have slopes and intercepts which are the averages of these parameters for the several curve-peelings, they do not necessarily lie on the lines of the average points on Figure VI. This is most evident in Compartment IV where the variation in the logarithms of the concentrations is the greatest, and the curve-peeling was done on some results which did not extend to the volumes of solution perfused that the average graph does. This lack of coincidence between the lines of the "average" compartments and the average curves is also evident in Figures X, XII and XIV.

The pressure recorded at the arterial cannula showed a distinct pattern in each of the two groups of experiments. In those experiments in which stimulation did not begin until the perfusion began, the pressure rose to very high values, greater than 200 cm H_2O, before the flow began. The fluid delivered by the pump compressed the air in the syringe rather than flowing through the muscle. When the flow started the pressure decreased, rapidly at first, and then more slowly (Figure VII). After 15 to 20 min of perfusion, the pressure reached a value which it held for most of the perfusion. These values had an average of 57±15(SD) cm H_2O. In the other experiments, the initial rise in pressure did not occur (Figure VIII). The steady pressures in these experiments had an average of 73±45 (SD) cm H_2O. The steady pressures obtained in the two groups of experiments were not significantly different.

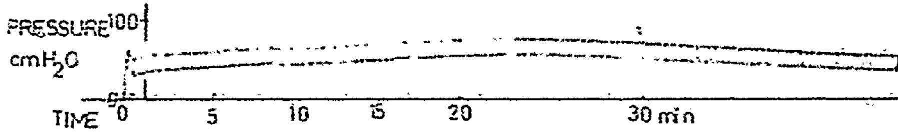
From the estimated weights of the muscles, the pre-set

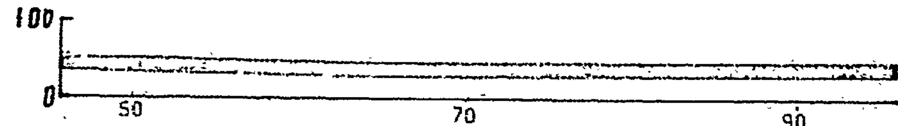
Figure VII. Pressure record of the perfusion of a muscle that was not stimulated before isolation. The break in the curve at 5 min is the result of an imposed change in the recorder's sensitivity from that indicated by the scale on the left to that of the scale on the right.

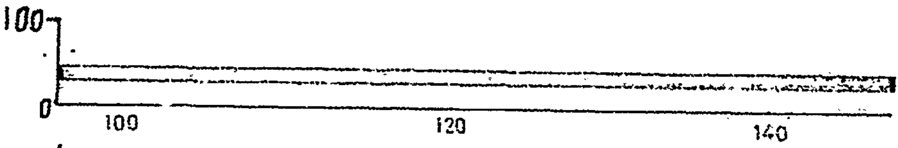


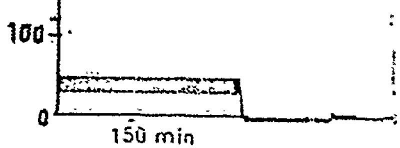
PRESS.
200
100
cm H_2O
0
50
100
150
Time (min)
100
cm H_2O
50

Figure VIII. The record of perfusion pressure in a muscle which was stimulated for ten minutes before isolation.


PRESSURE
100
cmH2O
TIME
0
5
10
15
20
30 min


100
0
50
70
90


100
0
100
120
140


100
0
150 min

flow rate, and the measured pressures, the normalized resistance to flow was calculated for the period in each experiment where the level pressure was obtained. These resistances had an average value of 1.87 PRU ± 0.26 (SEM).

Almost all of the muscles gained weight during the perfusion due to the accumulation of perfusant fluid. This weight gain was most rapid at the end of the perfusion. In nine of the experiments the weight gain was measured and ranged from zero to 100% of the muscle's original estimated weight. The average gain was 47%.

In all the experiments, the response of the muscles to stimulation, as observed in visible twitches, decreased rapidly at the start of perfusion and usually persisted only as a slightly visible "tic" in the fibres after twenty-five millilitres of perfusion.

In a subsequent experiment, in which the Achilles tendon of the isolated muscle was connected to a strain gauge force transducer, it was found that the force of contraction decreased to less than five percent of the original force after five minutes of Ringer's perfusion. It could be restored temporarily by increasing the stimulating voltage and also by increasing the perfusate flow rate, even after 500 ml of perfusion.

d Discussion of the results

The purpose of the preparation was to have the vascular bed in a state corresponding to that in a maximally-exercising muscle. The various observations, other than the washout kinetics, should be compared to what is expected in such a state.

The normalized flow resistance in the muscles, apart from

the initially high values, had an average of 1.87 PRU. When this is compared to the lowest value determined from the results of Folkow and Halicka (1968) in the cat gastrocnemius, about 2.2 PRU, it is obviously lower, but the comparison is, of course, not valid. Blood in the vascular bed shows more resistance to flow than saline. The measurements of Djojosugito, Folkow, Oberg and White (1970) suggest that blood has an apparent absolute viscosity in the cat hindlimb, at high perfusion pressures, of 2.3 cP. This is about three times the viscosity of saline at 37 $^{\circ}$C, which is 0.7 cP (Weast, 1974). Thus the resistance to flow is somewhat higher than what might be expected. However, it is still much lower than that which could be found in resting muscle, where the resistance to blood flow is about 11 PRU. (Folkow and Halicka 1968). Thus there is a definite vasodilatation.

The high pressures at the beginning of the perfusions, in those muscles which were not stimulated before perfusion, may have been due to a vasoconstriction caused by hormones such as noradrenaline or angiotensin released by the cat before the isolation of the muscle. The muscles which were stimulated before isolation showed low initial resistances to flow. Cobbold, Folkow, Kjellmer and Mellander (1963) have shown that metabolic vasodilatation can overcome the effects of vasoconstrictor stimuli, which may be the reason for the difference in the two sets of experiments.

The failure of the muscles to contract maximally during the perfusion is likely due to a shortage of oxygen in the perfusate.

The solubility coefficient (α) of oxygen in water at 35 C is 0.024 ml O_2 (STPD) atmosphere ml H_2O (Lange, 1952). Thus the oxygen content of the solution has a maximum value of 0.95 α in equilibrium with 95% O_2 at atmospheric pressure. The delivery of oxygen by the perfusate is the arteriovenous concentration difference times the flow. With the average flow per unit weight used in these perfusions, the delivery must be less than 0.57 ml·(min-100 gm tissue)$^{-1}$, using the given value of α, if the venous concentration is greater than zero. This is just equal to the value given by Folkow and Halicka (1968) for the oxygen consumption of resting cat gastrocnemius muscles, and far less than that required by exercising muscles. Thus, a perfusion of this sort will maintain a stimulated muscle in an extreme state of hypoxia because the oxygen supply is insufficient even for a resting muscle.

This may not necessarily be a fault in the preparation, if it is the vascular state that is of interest. Concerning the cause of the vasodilatation in exercise, most authors postulate that it is the absence of oxygen or the excess of some product of the metabolism that causes the change from the resting state (Ross, 1971; Stainsby 1973). A similar vasodilatation, reactive hyperemia, is produced by a few minutes of occlusion of the blood flow. Many authors have argued that the two cases have the same cause and similar effects on the distribution of blood flow, although Hilton (1968) has suggested that there are more factors involved in exercise vasodilatation.

The gain in weight of almost all the muscles during perfusion

is most likely the result of fluid being forced through the capillary endothelium by the hydrostatic pressure in the capillaries. Since this pressure is not balanced by any protein oncotic pressure in the perfusate, as it is to a great extent in blood-perfused capillaries (Landis and Pappenheimer 1963), it would be expected to cause abnormal swelling. This swelling was somewhat more evident here than in the resting muscle perfusions done by Groom et al. (1973) and this might be taken as evidence that the capillary bed is more extensively perfused in this preparation. The variability of the weight gain, and the possibility that there may be other causes, such as a higher venous pressure, make any hypothesis difficult to defend.

At present the only results that bear comparison with the red cell washout curves are those of Groom et al. (1973). I have re-expressed these results in the same form as Table II by multiplying their values of $T_{\frac{1}{2}}$ by their average flow rate (6.3 ml per minute), and dividing by their average muscle weight (25 gm) to obtain the half volumes $V_{\frac{1}{2}}$. I have also renumbered their compartments in increasing order of $V_{\frac{1}{2}}$ as in Table II. The other entries in Table III, which shows the results of this calculation are the same as in their Table I, except for the total cell store, which is also divided by the muscle weight.

What is most immediately obvious about the results of the present experiments is their similarity to the results obtained on resting muscle. In each case there are four components to the washout curve, and the half-volumes are similar. The other parameters are also closely similar. Compartment II in the

Table III The compartment model of Groom, Song, and Campling (1973) for the red cell washout of resting muscles. The compartments have been re-numbered in order of increasing half-volumes for comparison with Table II.

Parameter	Comp I	Comp II	Comp III	Comp IV
Half-volume ml/gm	0.0228	0.19	0.99	11.87
Intercept (cells/ml)	8.73×10^{9}	1.63×10^{8}	9.40×10^{6}	1.98×10^{5}
% Total Flow	98.1	1.83	0.11	0.002
% Total Store	82.1	13.1	3.8	0.98

Total cells 3.5×10^{8} cells/gm

Total of intercepts 8.9×10^{9} cells/ml

present results has a slightly larger fraction of the total cell store and also of the total flow, but the difference can hardly be called significant. There are no major changes in the washout kinetics similar to the one that Groom & Song (1971) obtained by administering noradrenaline to the cat spleen.

Thus we have a paradox; the contracting muscle, in which others have observed notable changes in the distribution of blood flow, does not have a different washout pattern from that observed by Groom *et al.* (1973) in resting muscle.

To conclude that the red cell washout does not show the changes in distribution of blood flow that occur when a muscle is contracting, it must be verified that both the present results and those of Groom *et al.*(1973) are obtained from muscles which exhibit the patterns of flow regulation typical of each case. I have mentioned a few reasons why the present experiments should show the pattern typical of exercising muscle such as the relatively low resistance to perfusion and the evidence for an oxygen shortage. However, the resting muscle experiments with which they are to be compared must also be examined in the same light.

I have mentioned that reactive hyperemia and the vasodilatation of exercise, called functional hyperemia, are often attributed to a common cause and have been considered similar in their effects on the distribution of blood flow. Reactive hyperemia will follow an occlusion of blood flow as short as 1 min (Barcroft, 1963). Thus in any experiment in which the blood flow is interrupted, it is usually necessary to restore blood perfusion before the muscle

is considered to have returned to the normal resting state. Groom et al. (1973) washed out muscles immediately after a long interruption of flow, so that their muscles must have been in a state of reactive hyperemia at the start of the washout. The short supply of oxygen may have allowed this state to persist throughout the washout experiment, as it is known that a deficiency of oxygen slows the recovery from reactive hyperemia (Haddy and Scott 1968). It should also be recalled that the solution used by those authors was identical to the solution I used here, which was somewhat acidic (pH 6.8) when in equilibrium with the gas used to aerate it. Perfusion by acidic blood causes a vasodilatation in skeletal muscle (Deal and Green 1954) and acidity has been considered among the possible mechanisms by which the metabolism of exercising muscle causes the associated vasodilatation (Haddy and Scott 1968). Thus the perfusion solution may well cause a hyperemia in the muscle, whether or not it is exercising.

For these reasons, the similarity between the present results and those of Groom et al. (1973) should not be surprising. It takes considerable care to maintain a muscle in the normal resting state, whereas the vasodilatation associated with exercise might be surprisingly easy to obtain.

4. The red cell washout kinetics of resting muscle

_a_ Methods

The discussion of the last section described two possible reasons for the similarity of the results of the washout of contracting and resting muscles. They were the possibility of reactive hyperemia in the isolated muscles and the poorly controlled pH of the perfusate used. Both these errors are of more serious consequence in the resting muscle, as they tend to cause changes similar to those induced in working muscle. It was considered necessary to re-examine the red cell washout kinetics of resting muscle in a way that would overcome these faults.

This involved two changes in method, both of which are described in detail in Section I of this chapter. They are the use of a well buffered perfusate solution (Solution II of Table I), and a method by which the muscle is prepared for washout perfusion without interrupting the normal blood flow. This was the procedure described before as _in situ_ perfusion. At this time also, it was decided to use constant pressure perfusion for the reasons mentioned before.

Five cats weighing 1.5 to 4.0 kg were used in this series of experiments. The preparation and perfusion apparatus were as shown in Figure II. Perfusion was at a constant pressure of 150 cm water in four of the experiments and 130 cm of water in the fifth. The drop-counting fractionator used to collect the samples required a longer cannula as mentioned before. This added a 'dead space' of 0.3 ml to the volume washed out, but because the open end

was lower than the vein to counteract the pressure difference from flow in the cannula, it did not affect the venous pressure.

b Results

The perfusion started in all cases with a fairly high flow rate, which did not change during the first minute of perfusion. There was no initial high resistance to flow, as had been found in some of the exercising muscle washouts and in some of the experiments described by Groom et al. (1973). The resistance to flow at the beginning of the washout had an average value of 5.8 ± 1.6 (SD) PRU. In three experiments, the resistance to blood flow before the perfusion began was also measured: this had an average value of 14.7 ± 4.8 (SD) PRU. From these determinations the ratio of the resistance to blood and saline perfusion at the beginning of the washout could be calculated; this had an average value 2.9 ± 1.11 (SD). After 25 ml of perfusion, the resistance to flow decreased, so that at 30 ml of perfusion it had an average value of 3.8 ± 0.7 (SD) PRU. After this point, the resistance in some muscles remained low or decreased even further; in others, which became noticeably edematous, the resistance rose again to values equal to, or greater than, the initial resistance.

The muscles became edematous and gained weight during the perfusions, much in the same way as noted before in the exercising muscles. The weight gain ranged from a negligible amount to 70% of the original weight of each muscle and had an average value of 41% of the original weight.

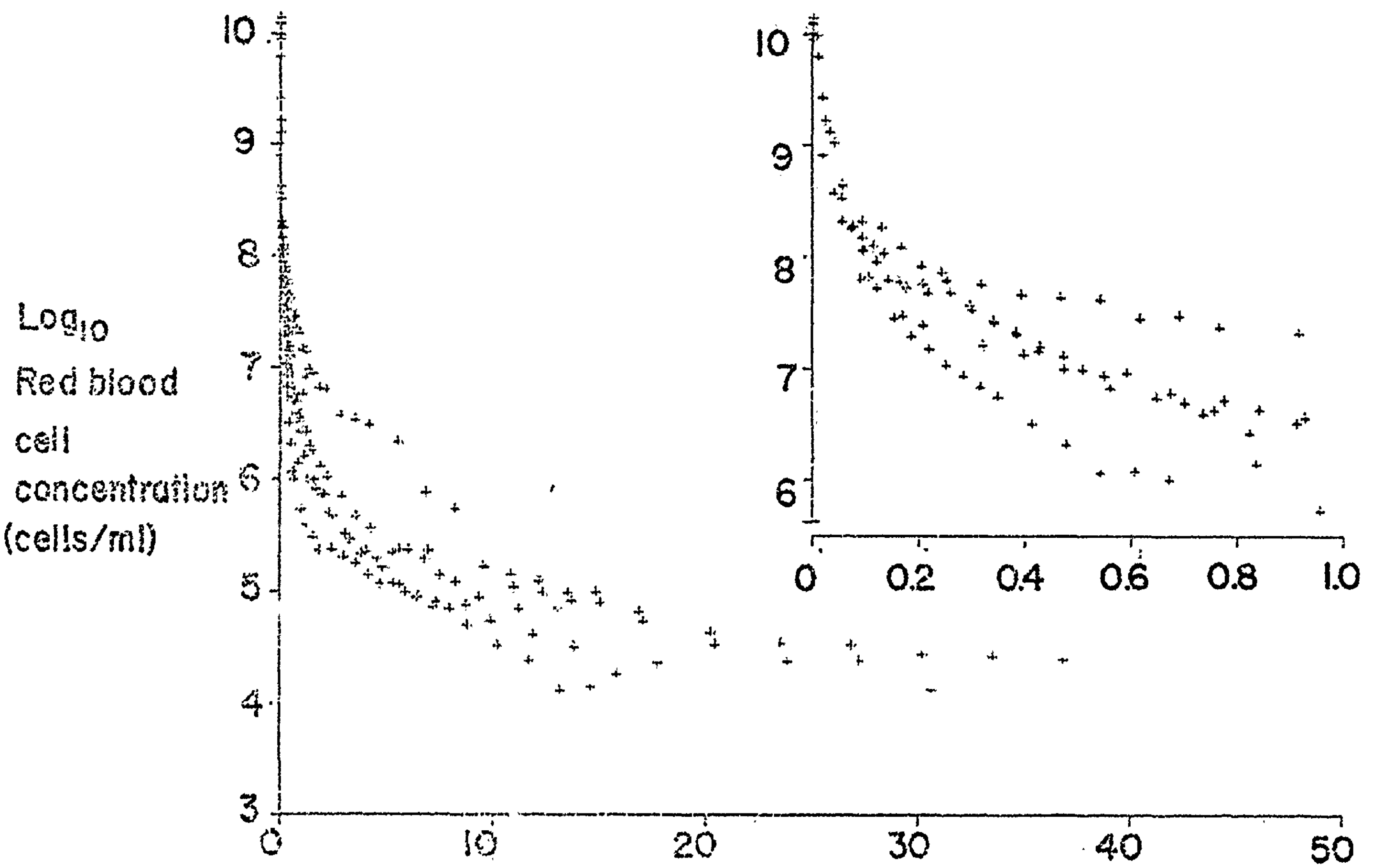
The red cell washout data for the five experiments are shown

in Figure IX and the averages of interpolated values for certain selected volumes perfused are shown in Figure X. A curve peeling, applied to these results yielded four components. The averages and standard errors of the mean for the parameters of these components are shown in Table IV. The lines with the average slope and intercept for each component are shown in Figure X.

c Discussion

Do these muscles represent resting muscles during the washout? The principal evidence available is their resistance to flow. Their resistance to blood flow before isolation is somewhat higher than the average value reported by Folkow & Halička (1968) for resting cat gastrocnemius muscles. This suggests that normal vascular smooth muscle activity is present, although it might be modified by the action of hormones such as noradrenaline. The resistance to perfusion measured after 5 ml of perfusate has passed through the muscle is essentially determined by resistance of the muscle to perfusion by Ringer's solution, as the washout graphs show that the blood cells are very quickly replaced by Ringer's at the start of perfusion. The fist millilitre of perfusion, about 0.05 ml. per gram, reduces the cell concentration to about one eighth of the valuesin venous blood and it continues to fall from that value. The change in resistance noted at this point was a fall to one part in 2.6 of the resistance to blood flow. Assuming the value of 2.3 cP that Djojosugito et al.(1970) obtained for the effective viscosity of blood in the vascular bed at high perfusion pressures and a viscosity of 0.7 cP for the saline solution,

Figure IX. Red Cell Washouts of five resting muscles. The inset shows the results from the first ml/gm of perfusion on an expanded horizontal scale.



Log10
Red blood
cell
concentration
(cells/ml)
10
9
8
7
6
5
4
3
0
10
20
30
40
50
0.2
0.4
0.6
0.8
1.0
Volume Perfused (ml/gm tissue)

Figure X. The averages with standard error of the mean of the concentrations obtained by interpolation from the results shown in Figure IX.

The lines have the average slopes and intercepts given for the compartments in Table IV. Because the lines are given slopes and intercepts which are the averages of those of the curve-peelings of the individual washouts, they are not necessarily coincident with the avearage points shown here.

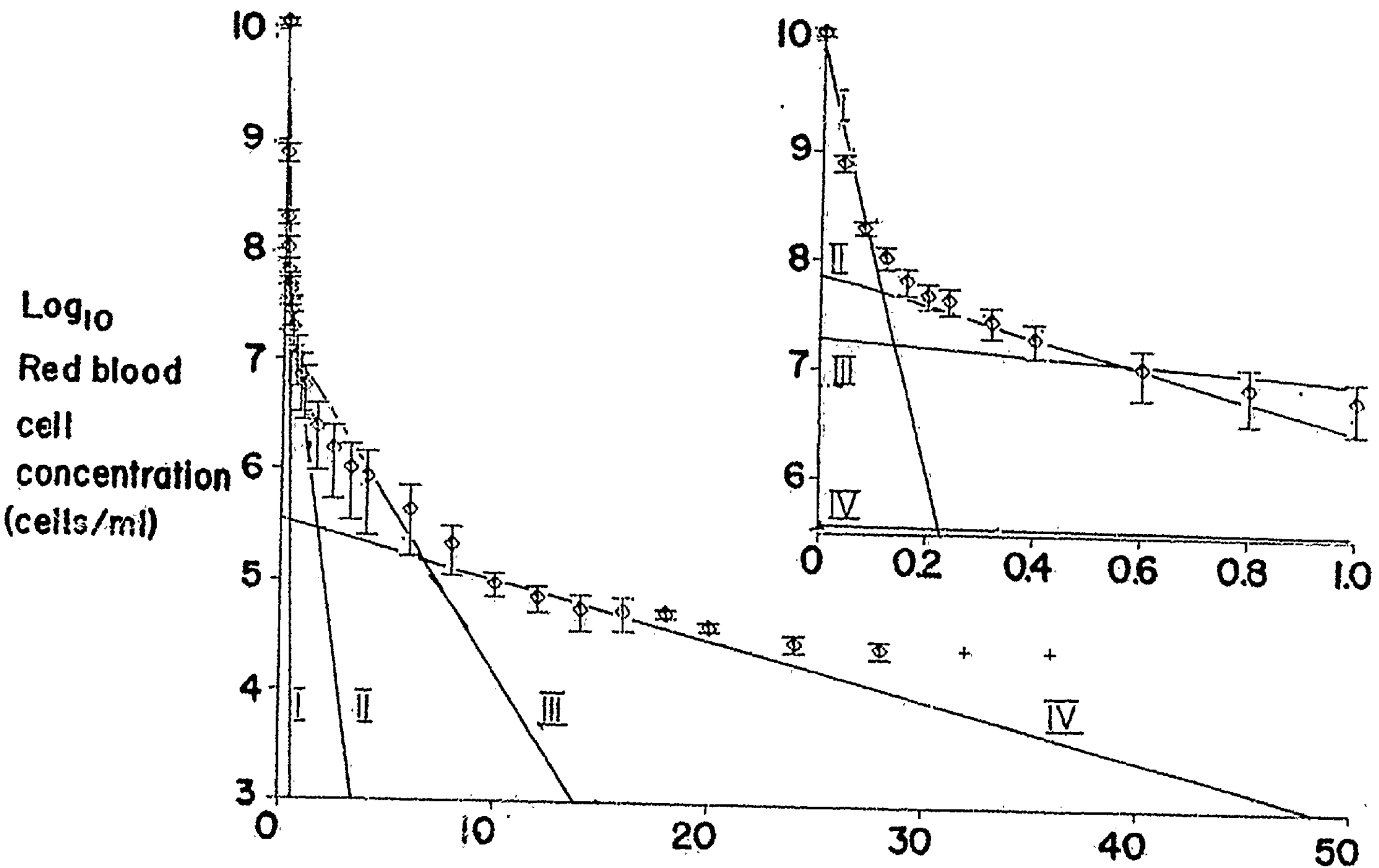

Log10
Red blood
cell
concentration
(cells/ml)
10
9
8
7
6
5
4
3
0
10
20
30
40
50
I
II
III
IV
0.2
0.4
0.6
0.8
1.0
Volume Perfused (ml/gm tissue)

TABLE IV Parameters of the compartment model derived from the curve-peeling of the red cell washout data obtained from resting muscles.

Parameter	Comp. I	Comp. II	Comp. III	Comp. IV
Half-volume	0.0158	0.213	0.972	5.67
± S.E.M.	± 0.003	± 0.045	± 0.247	± 1.26
(ml/gm)				
Intercept	8.11×10^9	7.70×10^7	1.90×10^7	3.69×10^5
± S.E.M.	2.19×10^9	3.16×10^7	1.19×10^7	0.71×10^5
(cells/ml)				
Percent of				
total flow	98.63	1.12	0.24	0.0057
± S.E.M.	± 0.55	± 0.46	±0.14	± 0.0015
Percent of				
total cells	81.77	9.39	7.30	1.54
± S.E.M.	± 5.56	± 2.11	± 4.30	± 0.33

Total of Intercepts: $8.208\times10^9 \pm 2.205\times10^9$ (S.E.M.)

(cells/ml)

Total of cells stored: $1.889\times10^8 \pm 0.246\times10^8$ (S.E.M.)

(cells/gm muscle)

the ratio of viscosities is 3.3, which is somewhat larger than the ratio of blood flow resistances measured, although the difference is hardly to be considered significant. Thus the change in resistance might well be explained by the change in viscosity alone, without postulating that there is any change in vascular tone. The occurrence of such changes is not excluded, however.

Later in the perfusion there is a drop in flow resistance which must be due to a change in vascular dimensions, since the concentration of cells is extremely low at the time. The lowest resistance found in these muscles is higher than that found in the contracting muscles before, but the change does indicate that the resting vascular tone is lost.

The decline of vascular smooth muscle tone, on exposure to Ringer's solutions or other plasma substitutes is an observation which has been known for some time and has much supporting evidence in the literature, but has not been directly or quantitatively studied.

Wurzel (1963) reviewed evidence from previous authors that exposure to plasma increased the contractions of vascular smooth muscle. The earliest such evidence was published by Ludwig in 1868. There is the possibility that these early observers were noting the effects of hormones in the plasma that were not identified until later. However Wurzel, in his own experiments, showed that many of the hormones known at that time could not be responsible for the effect. Later, Bohr and Johannson (1966) studied the effect of plasma on the contractility of isolated smooth

muscle strips, and found that there was a factor in plasma that caused contractions of the muscle, and in lower doses, increased the contractions caused by other stimuli. Bohr, Verrier and Sobieski (1971) presented evidence that the factor was not a protein and had a very small molecular weight.

The experimenters who have perfused the vascular bed of skeletal muscles with blood substitutes and have also studied the resistance to flow, have used either a maximally dilated bed (Whittaker and Winton 1933; Djojosugito et al. 1970) or a vascular bed in which constriction is induced by the use of barium chloride (Folkow and Lofving 1956; Baekstrom, Folkow, Ken[illegible]ick, Lofving and Oberg 1971). The reasons given for the choice are often the experimenters' inability to maintain the same vascular tone during switches from blood perfusion to saline perfusion. The constrictor effects of barium chloride are of long duration, so that, in this case it is possible to make such changes without immediate changes in vascular tone.

Of this problem, Bohr and Johannson (1966) wrote:

> This is in accord with Furchgott's observation and our own that most isolated vascular smooth muscle in a PSS (Physiological Saline Solution) bath remains completely relaxed unless it is acted upon by a stimulating agent. The observation that vascular smooth muscle loses its state of partial contraction when removed from its in situ environment to the isolated bath of PSS and that partial contraction may be restored by the addition of plasma, constitutes indirect evidence that the plasma constrictor may be responsible for non-neurogenic vascular tone in situ."

Stainsby (1973) wrote:

> "Although the past literature contains a few reports that autoregulation does not occur normally when artificial non-plasma containing perfusates are used, most of the knowledge in this area has only been passed around in the halls at meetings; such negative data too often end up in a drawer and not in print . . . The necessity for a substance or substances in plasma to be present in the perfusate before autoregulation will occur ought to have been clarified long ago. . . "

The present report may help to remedy the deficiency that Stainsby sees in the scientific literature.

The rise in the resistance to perfusion, which was noted in some muscles at the end of the perfusion, is probably related to the gain in muscle weight by fluid accumulation. The extravascular fluid is compressed in the muscle capsule, and the pressure bears on the veins, causing their resistance to flow to rise. This hypothesis is supported by the observation that the gain in weight is often seen to accelerate with time, as the high venous resistance raises the capillary pressure, thus increasing the rate at which fluid passes into the tissue.

Despite the changes in perfusion resistance the red cell washout pattern obtained from these muscles resembles that of the stimulated muscles a great deal.

The two curves for the washouts were compared using the sets of interpolated values at the pre-selected volumes-perfused used in the preparation of the average curves in Figures VIII and X. The values at each point obtained from each of the twelve washouts of stimulated muscle and the five of resting muscles were compared as two groups of data. Student's t-test, as described by Ractliffe

(1962), was used to test the hypothesis that the means of the groups were equal. This test failed ($p < .05$) at only two points, 0.02 ml/gm and 0.04 ml/gm perfusion. Here the means of the interpolated concentrations were significantly lower in the resting muscle. Beyond 1 ml/gm of perfusion, the results gave a probability greater than 0.5 for the hypothesis of equal means.

The compartment analysis may reflect, as well as significant differences between the curves, other factors which might bias the analysis. In particular, the smaller half-volumes of Compartments III & IV of the resting muscle probably reflect the fact that these muscles were not perfused to the same extent as the others on a per-unit-weight basis as can be seen from a comparison of the original washout graphs Figs. V and IX. This has led to a poorer definition of the slope of Compartment IV in the resting muscles.

A more significant difference would be related to the difference between the curves at the points mentioned before. This difference is not obvious in the model parameters listed in Tables II and IV, because both the intercepts and slopes of the Compartments I and II of the resting muscle are not significantly different from those of the corresponding compartments of the contracting muscles. However, the total cell store of Compartment I in the resting muscle is smaller ($p < .1$) and this is reflected in the fact that the total cell store per gram of the resting muscles is about three-fifths of that of the contracting muscle.

We can conclude that there is a real difference between the washout kinetics of resting and contracting muscles. The

fastest decaying, and hence best perfused compartment in the model has a smaller cell store than the corresponding one in contracting muscle. This is well in accord with the theories concerning the changes in blood perfusion of muscles from the resting state to exercise vasodilatation.

However, this series of experiments has shown that the central question, that of the causes of the inhomogeneities of perfusion which cause our distinct compartments, remains unanswered. The separate compartments are almost the same in character, and exactly the same in number, in contracting and resting muscles. I have also shown that the washout of the muscle beyond 30 ml from which the data for compartments III and IV are obtained causes a vasodilatation in the muscle which renders the method insensitive to changes in the vascular state. Clearly, the pattern that Groom _et al._ (1973) found so intriguing has little to do with the physiological control of the distribution of blood flow in muscle.

5. The red cell washout kinetics of vasodilated skeletal muscle.

a Introduction

The experiments on contracting and resting muscles have shown that the red cell washout method is a poor one for studying the effects of physiological control mechanisms on the distribution of blood flow in skeletal muscle. As a reference, it was considered useful to study the washout kinetics of the red cells from a muscle in which all the normal physiological control mechanisms had been abolished by the use of a vasodilator drug, so that the pattern of blood flow was determined by the pattern of the passive, dilated vascular bed.

To do this required a vasodilator whose effect was independent of the nervous control of the vascular smooth muscle and could overcome it and also one whose effect could be maintained from the start of the washout perfusion to its finish.

Among the drugs that have a vasodilator mechanism, those of interest included ones which had been used to dilate the muscle vascular bed in attempts to obtain complete capillary filling, such as the nitrites (Krogh 1919a; Martin, Wooley and Miller 1932) or papaverine (Plyley and Groom 1975). The nitrites are either insoluble in water (amyl nitrite) or somewhat unstable in solution (sodium nitrite) and their effects are short-lasting, so that they could not be given in the form of a pre-injection before the perfusion, nor could they be dissolved in the perfusate for continuous infusion. Papaverine presents a similar problem, for

it is soluble only in very acidic solutions, and quite insoluble in buffered solutions at pH 7.4.

The drug finally selected for the vasodilator was sodium nitroprusside. It has a pronounced vasodilator effect which has been compared to that of the nitrites, however on a weight for weight basis it is about 50 to 1000 times more potent in intact animals (Johnson 1929). In artificially perfused organs it overcomes the constrictor effect of adrenaline; in this respect it is about three times as potent as sodium nitrite. It is soluble in water or saline, and this is the usual mode by which it is administered as a vasodilator (Tuzel 1974). Such solutions are unstable,but last long enough for the few hours required for the perfusions, keeping 62% of their original concentration after eight hours in a heated bath at 37°C in a well illuminated room (Frank, Johnson & Rubin 1976).

The effect of sodium nitroprusside on the distribution of blood flow has not been well studied in isolated limbs or organs. However, it has been suggested that the clinically observed effects are due to an extreme dilatation of the large vessels, especially the veins, with a moderate dilatation of the resistance vessels (Bhatia and Frohlich 1973). In this it resembles the effects of sodium nitrite, for Ablad and Mellander (1963) showed that the effect of the latter substance on cat skeletal muscle was to dilate the capacitance vessels extremely, with only a moderate effect on the resistance to blood flow. Mellander and Johansson (1968) state that the effects of nitrite and nitroglycerin

are similar, and that they do not affect the precapillary sphincters in skeletal muscles very much. This is somewhat paradoxical if it is assumed that the nitrites are general smooth muscle relaxants, but the mechanism of their action is not perfectly understood. Despite this, the suitability of sodium nitroprusside for infusion as a solute in the perfusate solution, as well as its extreme potency, made it an attractive choice for a vasodilator drug.

b Methods

Experiments were carried out on nine muscles from nine cats weighing between 2.9 and 6.0 kg. The cats were chosen for their size because this made cannulation of the small branches of the popliteal artery and vein easier. The muscles were prepared for perfusion *in situ* as were the resting muscles. Solution III of Table I, to which was added 0.01% (w/v) sodium nitroprusside, was used as the perfusate. In these experiments a study of the plasma washout pattern was also performed, which required that after the preparation of the muscle a period of one hour be allowed for complete equilibration of the injected ^{125}I-albumin in the circulation. After this time the vein draining the muscle was clamped proximal to the cannulated side branch, and the venous outflow was diverted through the cannula. Ten to 20 mg of sodium nitroprusside in 1 to 2 ml of saline were injected intra-arterially to the muscle to cause a complete vasodilatation before the washout began. A few minutes were allowed for blood perfusion from the artery to displace the

saline from the muscle. During this time it was possible in some experiments to estimate the change in flow resistance after the injection. The sample which was used as the venous blood concentration reference for the washout was collected at this time. The artery supplying the muscle with blood was then clamped, and the perfusate from the reservoir was allowed to flow _via_ the arterial side branch through the muscle. The samples were collected manually from the open end of a venous cannula 10 cm in length. The pressure used in the perfusion was between 60 and 130 cm of water.

_c_ Results

In three muscles of the nine both the initial resistance to blood flow before treatment with nitroprusside and the resistance after treatment was calculated from available measurements. The average fall in resistance was to 0.31 $\pm$ 0.02 (SD) of the original value. The flow of Ringer's solution encountered a resistance slightly greater than that of blood flow in the same muscle. The resistance to Ringer's perfusion remained generally constant near its original value. As in the resting muscle experiments, there was no initial high resistance to flow. The average value of the resistance at 5 ml of perfusion was 4.71 $\pm$ 1.22 (SD) PRU, and at 150 ml of perfusion it was 4.99 $\pm$ 1.90 (SD) PRU.

Only three of the muscles showed visible weight gains during the perfusion. These gains had an average value of 38%. The other muscles gained less than 10% of their original weight despite

the fact that they were perfused by up to 825 ml of Ringer's.

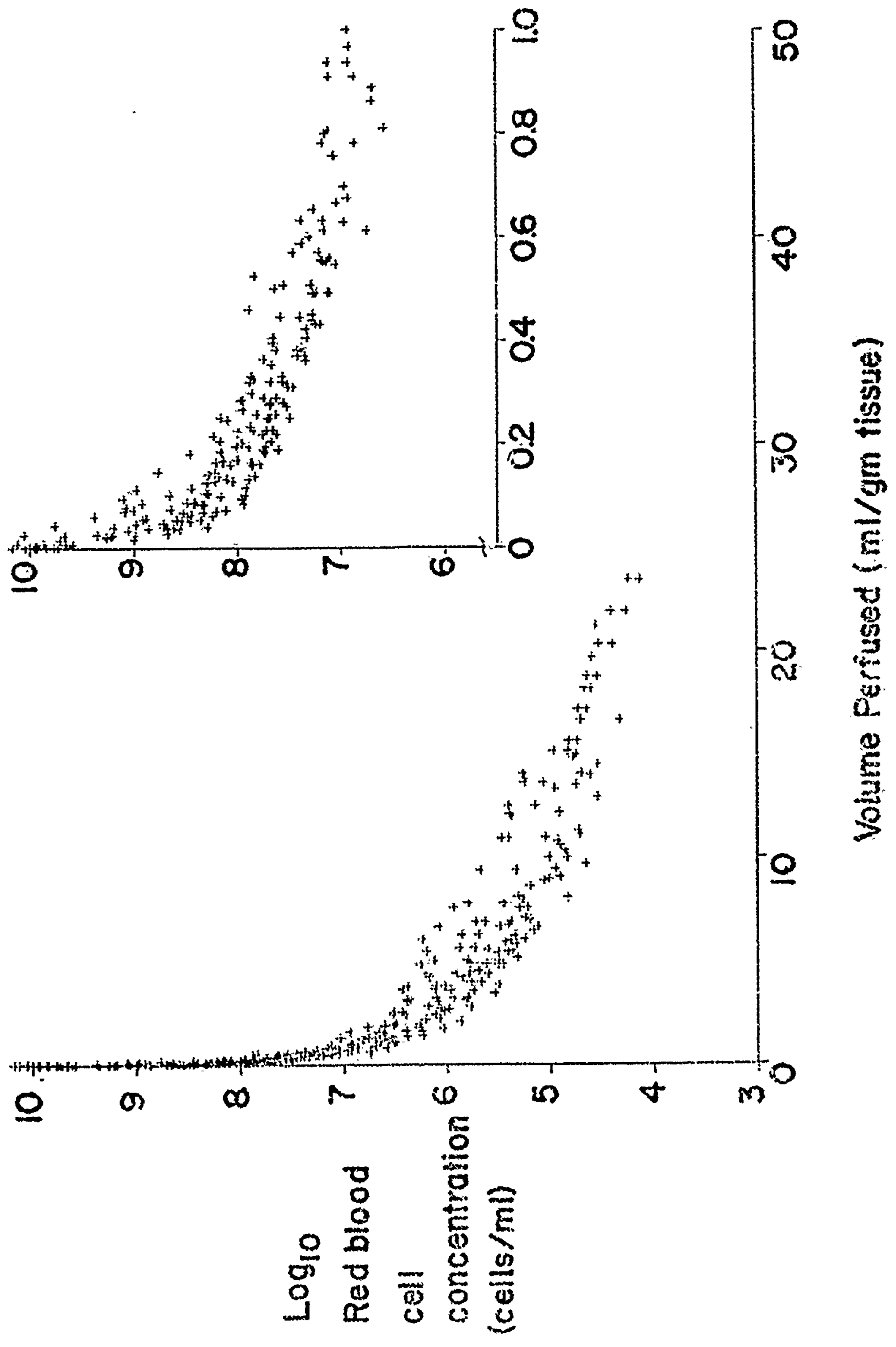
Figure XI shows the red cell concentrations plotted as a function of the normalized volume perfused for all of the muscles. Figure XII shows the averages with standard errors of the mean for interpolated values as determined by the method used for the other experiments. Because of the greater size of the muscles (36 ± 7(SD) gm), and the practice of perfusing all muscles by the same volume, these results do not extend to as great volumes-perfused-per-gram as do the others.

The curve-peeling process applied to these results in the same way as to the others yielded a four-component model whose parameters are given in Table V. The lines with the average slope and intercept are shown in Figure XII.

d. Discussion of the results

The resistance to flow in the muscles is not as low as that encountered in the contracting muscle. This is not surprising in view of the fact that the Dextran-containing Ringer's used in these experiments is 2.5 times as viscous as the saline used in the other experiments. (This value for the ratio, determined with as Ostwald viscometer at 37°C, is in accord with the results given by Baekstrom et al.(1971)). The resistance to saline flow measured in the contracting muscles should be multiplied by the ratio of viscosities to obtain the corresponding resistance to the flow of the Dextran-containing perfusate used here. When this is done, the resulting resistance in contracting muscle is almost equal to the resistances measured in this series of experiments. It

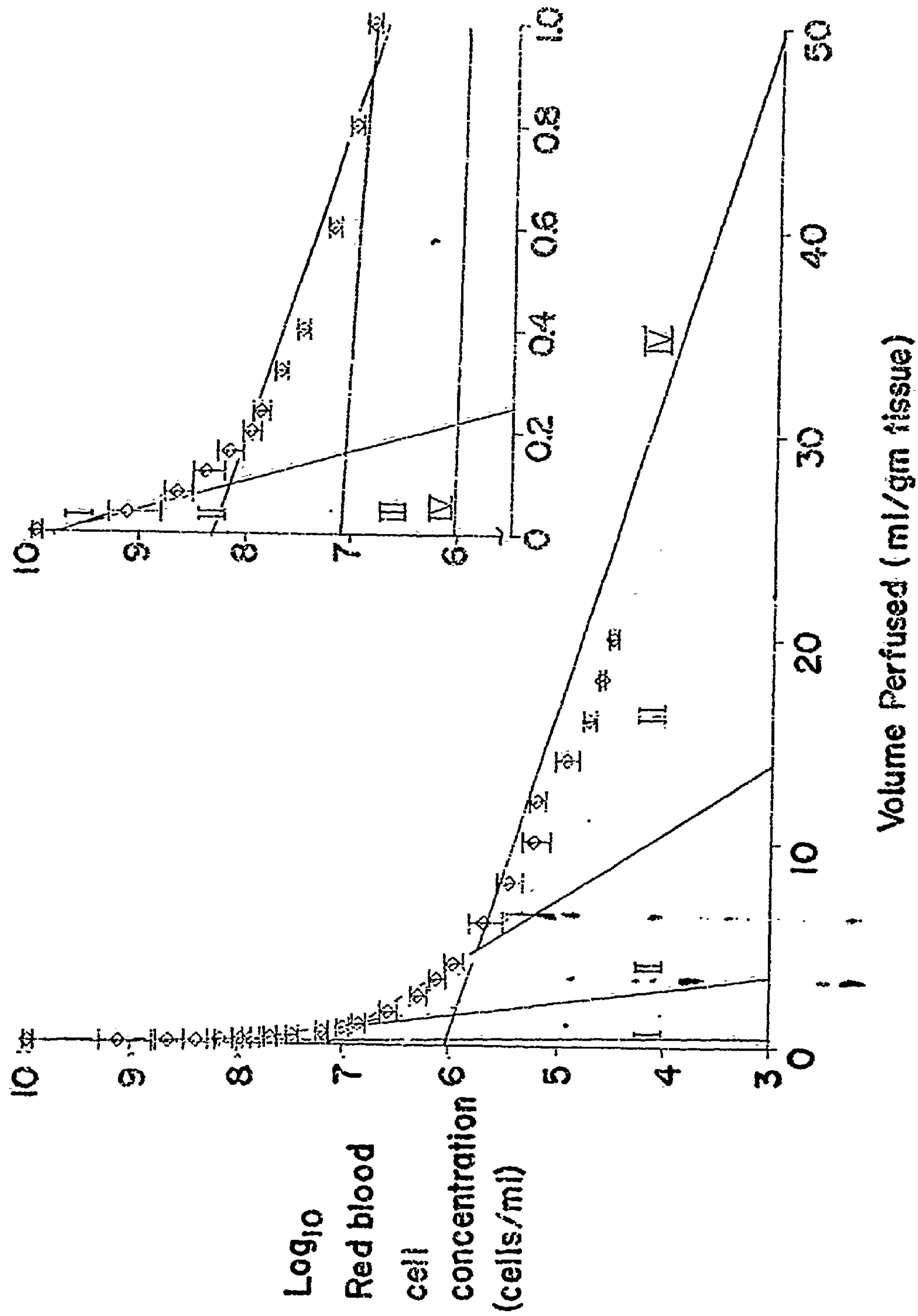
Figure XI. Red cell washouts of nine vasodilated muscles. The inset shows the first ml/gm of perfusion on an expanded horizontal scale.



Log10
Red blood
cell
concentration
(cells/ml)
10
9
8
7
6
5
4
3
0
10
20
30
40
50
0
0.2
0.4
0.6
0.8
1.0
Volume Perfused (ml/gm tissue)

Figure XIII. The logarithms of the averages with standard error of the mean of the concentrations obtained by interpolation from the results shown in Figure XI.

The lines have the average slopes and intercepts given for the compartments in Table V. Because they are derived from the curve-peelings of the individual curves, they are not necessarily coincident with the average concentrations shown on the same graph.



Log$_{10}$ Red blood cell concentration (cells/ml)
Volume Perfused (ml/gm tissue)
10
9
8
7
6
5
4
3
0
10
20
30
40
50
0.2
0.4
0.6
0.8
1.0
I
II
III
IV

TABLE V Parameters of the compartment model derived from the curve-peeling of the red cell washout data obtained from vasodilated muscles.

Parameter	Comp. I	Comp. II	Comp. III	Comp. IV
Half-volume	0.0174	0.187	1.01	5.01
$\pm$ S.E.M.	$\pm$ 0.0016	$\pm$ 0.035	$\pm$ 0.17	$\pm$ 0.83
(ml/gm)				
Intercepts	6.36×10^9	2.14×10^8	1.30×10^7	1.02×10^6
$\pm$ S.E.M.	1.72×10^9	0.61×10^8	0.52×10^7	0.41×10^6
Percent of total flow	95.84	3.85	0.28	0.027
$\pm$ S.E.M.	$\pm$ 1.36	$\pm$ 1.34	$\pm$ 0.13	$\pm$ 0.014
Percent of total cells	67.59	21.45	7.18	3.73
$\pm$ S.E.M.	$\pm$ 4.21	$\pm$ 3.27	$\pm$ 2.11	$\pm$ 1.61

Total of Intercepts: $6.59 \times 10^9 \pm 1.75 \times 10^9$ (S.E.M.)
(cells/ml)

Total of cells stored: $2.18 \times 10^8 \pm 0.66 \times 10^8$ (S.E.M.)
(cells/gm muscle)

was found in later experiments that it was not possible to further dilate the vascular bed of the muscle with large dosed of nitroprusside at the end of the perfusion. It is reasonable to conclude that the muscle is maximally vasodilated during the perfusion.

The relatively low weight gain in these experiments, as compared to the others, could have three possible causes. One is the presence of the Dextran, which establishes 2.5 mm Hg colloid osmotic pressure in the perfused vascular bed to counteract the effect of the hydrostatic pressure forcing fluid into the tissue . Another is the possibility that the capillary hydrostatic pressure may be lowered as a result of the effect of the nitroprusside on the venous resistance. A third explanation is that the muscles were not perfused to the same extent per unit weight as the others. Each of these may have contributed in part to the difference.

Because of the obvious differences in vascular state between these muscles and the resting muscles, the red cell washout kinetics would be expected to be most similar to those of the contracting muscles.

These results were compared using Student's t-test with those from the contracting muscles, in the manner described previously when comparing the results from resting and contracting muscles. At only two points is there a significant difference, one at 0 ml perfused, the other at 12 ml/gm perfused. A possible explanation of the first point, at which the concentration in the vasodilated muscles is lower, is that the

saline used to inject the sodium nitroprusside was not replaced completely by the arterial blood before the washout. The other point seems to represent a vagary in the actual results rather than any trend in the difference between the curves. It is not surrounded by other points with similar differences. For this reason I consider the difference to be without significance.

The compartment models derived from the two curve-peelings differ in the last two compartments, but as the points to which the lines correspond are not themselves different, no real significance can be attached to the differences between the compartments. The total cell stores and the totals of the intercepts of the two models are not significantly different.

These results also can be compared with those obtained from the resting muscles. Surprisingly, the same application of Student's t-test shows no significant differences at the 5% level, but the same tendency for the concentrations obtained from resting muscles to be lower, in the region between 0.2 and 1.0 ml/gm perfusion, is evident here as in the comparison between the resting and contracting muscles.

Thus the vasodilated muscles are more similar to the contracting muscles, but the difference between the two groups is only barely significant. These results suggest that the vasodilatation produced in contracting muscle and that produced by sodium nitroprusside are indistinguishable by the red cell washout technique. As we have mentioned before, this technique is insensitive in the most part to changes in the physiological states of the muscle so that such a conclusion is not unexpected.

The results of these experiments confirm the idea that the inhomogeneities represented by the compartments in our models of the muscle circulation are quite unrelated to the inhomogeneities observed by Barlow, Haigh, and Walder (1961), or Renkin (1959a) or the other observers who have found their inhomogeneities to be sensitive to physiological control mechanisms. The red cell washout inhomogeneities are a consequence of some other factor, such as the pattern of the dilated vascular bed, or the properties of the red cells themselves.

6. The washout of plasma from a vasodilated skeletal muscle

a Introduction

The previous sections of this chapter have discussed the red cell washout kinetics of the cat gastrocnemius muscle without reference to the other major component of the blood, the plasma. The experiments described in this section examined how the plasma is washed from the vasodilated vascular bed of the muscle using the same perfusion technique as used for the cells.

There are several mechanisms which may cause the overall kinetics of the plasma washout to be different from that of the red blood cells. They include the Fahraeus effect, plasma skimming, and the adhesion of red cells within the vascular bed.

The degree of separation of the cells and plasma caused by the Fahraeus effect and plasma skimming is determined by the pattern of blood vessel diameters and flow velocities in the vascular bed, and thus the magnitude of these effects as observed under the microscope cannot predict the overall difference in transit times in the organ. The adhesion of the cells in a vascular bed has been very clearly demonstrated by Song and Groom (1971b and 1972) in the spleen. Here there is definite binding of immature and abnormal cells to the endothelial cells and macrophages lining the sinusoids of the red pulp. Such a phenomenon would not be expected in muscle, except for the observation by Groom _et al_. (1973) that reticulocytes form a large part of the slow compartment of the red cell store in skeletal muscle vasculature. Such binding of the red cells to the vascular endothelium may occur in any part of the circulation.

Comparative studies have been done on both the transit time (Baker and O'Brien 1964; Groom 1966) and the distribution volume (Allen and Reeve 1953; Polosa and Hamilton 1963; Moore and Baker 1971)of red cells and plasma in muscle tissue, but none have approached the problem using the extensive perfusion technique used here. Without exception, the studies done have shown that the volume available for plasma in resting muscles is larger than that available for cells, by a factor of about 1.1 to 2.2. The difference is reduced in vasodilated muscles (Moore and Baker 1971).

The present experiments were intended to show whether the washout by perfusion with Ringer's solution would show differences between the red cell and plasma stores in the vascular bed of skeletal muscle. Of particular interest was the question whether the slowest washed-out compartment (number IV of the models described here) of the red cell store would have a parallel compartment in the plasma store. The absence of such a compartment has been noted in the cat spleen (Levesque and Groom 1976a). Also of interest was whether the general kinetics of the plasma washout would show the effects of plasma skimming and the Fahraeus effect on the distribution of red cells and plasma in the vascular bed.

_b_. Methods

The preparation of the experimental apparatus and the cat gastrocnemius-plantaris muscle group was carried out as described previously for the red cell washout of vasodilated muscle. This preparation was the most stable and successful of those tried,

and thus was used as the basis for the comparison of plasma and red cell washouts. Results described here are from the last four experiments whose red cell washouts were discussed in Section 5. One ml of radioiodinated (^{125}I) human serum albumin solution (20 μCi per ml, Frosst Pharmaceuticals) was injected intravenously into each cat after the isolation of the muscle, except for the artery and vein, was complete. One hour was allowed for intravascular equilibration of the radioiodinated albumin to occur with the cat's blood.

The beginning of the perfusion was as described previously for the red cell washout experiments. The outflow from the venous cannula was collected as before except that between 25 and 225 ml of perfusion alternate samples were taken to provide the red cell and plasma determinations.

c Determination of the concentration of the radioactivity in the outflowing perfusate.

The counting apparatus was a Nuclear-Chicago Model DS-202 integral assembly, consisting of a well-type Thallium activated sodium iodide (NaI-(Tl)) scintillation crystal, a photomultiplier tube and a lead shield, with a Nuclear-Chicago Model 8741 Pulse-height analyzer with timer and scaler.

The well of the scintillation crystal could accommodate samples up to 5 ml in volume for counting with maximum efficiency. Samples collected during the first 25 ml of perfusion in 1.5 ml plastic vials were counted directly in the vial. The red cell concentration was determined from a diluted aliquot from the same sample after

the radioactivity count was finished.

Samples taken after this were in larger volumes. To increase the count rate, and thus reduce the time required for a count of given accuracy, the samples were concentrated into a volume of 5 ml or less for counting. This was done by adding about 1 ml of a concentrated solution of ordinary serum albumin as a carrier and then adding a saturated solution of trichloroacetic acid drop by drop to the sample until no more white precipitate was produced. The sample was then centrifuged until the precipitate was compacted within the bottom of the centrifuge tube. If the tube could fit within the well of the crystal, the counting was done on the sample as it was, otherwise the supernatant was poured off and the precipitate was transferred to a smaller tube, using 4 M sodium hydroxide to resuspend it if this was necessary. This treatment meant that the red cell count had to be done on alternate samples, or if the sample was large enough, on an aliquot of the sample drawn before the treatment. The count of the contents of a 50 ml beaker in the same volume as a 0.5 ml vial allowed us to measure the concentration of the label as it decreased over four orders of magnitude during the perfusion.

The pulse-height analyser was calibrated with a Cesium-137 source. The spectrum of output pulses from the amplifier did not have a clearly-defined peak when an iodine-125 sample was counted, so the setting of the discriminators was generally that which provided the best discrimination between sample counts and background.

Radioactivity in a sample was measured by timing the interval required to obtain 10,000 counts with the sample in place, or

1000 counts, if the activity was exceptionally low. The count rate of background events with no sample in place was measured regularly. A sample of the venous outflow before perfusion began served as the standard for each experiment.

The concentration of the plasma label was calculated as a fraction of that in venous blood by the following formula:

$$\frac{C}{C_o} = \frac{(t_b/t_s - 1)}{(t_b/t_o - 1)} \div \frac{V_o}{V_s}$$

where

C is the concentration of the label in a given sample,

C_o is the concentration in the venous blood sample,

t_b is the time required for 10,000 counts of background events,

t_s is the time required for 10,000 counts with the sample in place

t_o is the time required for 10,000 counts with the sample of venous blood in place,

V_o is the volume of the sample of venous blood

V_s is the sample volume.

C and V_s apply to the sample before it was concentrated by precipitation. Since it is reasonable to expect that C will be directly proportional to C_o which in turn will depend upon the ratio of the amount of label injected to the blood volume of the cat, the values of C/C_o were calculated and used for comparisons between experiments.

d Results

The muscles whose plasma washouts are described here were not exceptional among those which were described before in the section on red cell washouts, as far as their red cell washouts kinetics or their resistance to perfusion was concerned. They were among the largest muscles used; their weights ranged from 33 to 50 gms.

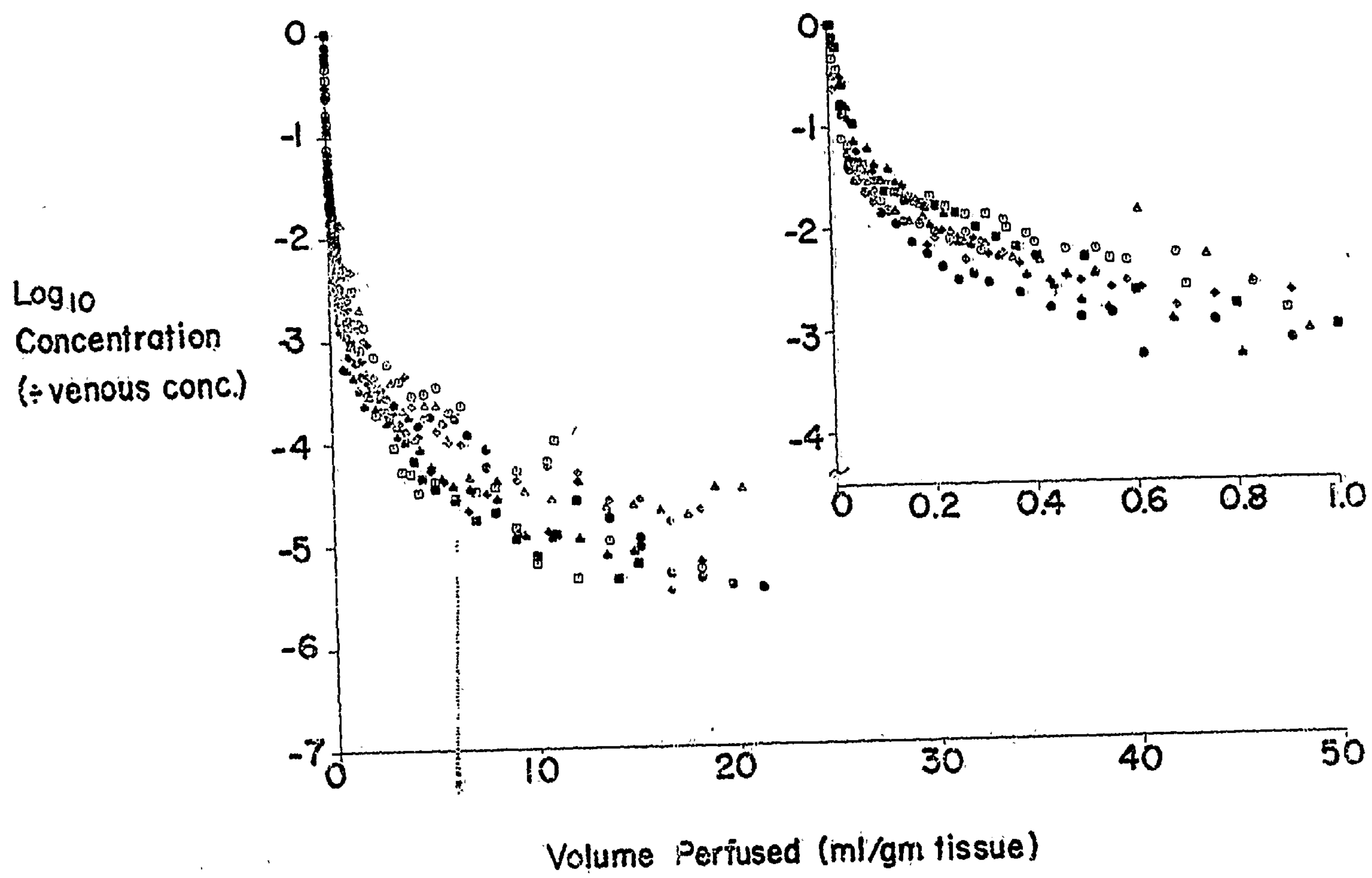
The values of C/C_0 from the radioactivity determinations are plotted in Figure XIII, which also shows the red cell washout results from the same experiments. The red cell concentrations have been divided by that which was obtained in venous blood in the same experiment, so that they can be compared on this basis with the plasma determinations. Figure XIV shows the averages of interpolated values at certain volumes perfused per unit weight, as was shown before for contracting muscles. The method of curve-peeling was applied to these results as described before, and yielded a four-compartment model, whose parameters are listed in Table VI. The lines with the average slopes and intercepts are shown in Figure XIV.

e Discussion

The most remarkable feature of the plasma washout kinetics as presented in the Figures XIII and XIV is their similarity to the red cell washout kinetics observed before. This can be tested by plotting the normalized concentrations of the plasma label against the normalized concentrations of the red cells obtained in the same samples or interpolated from the concentrations in neighbouring samples. Figure XV is such a plot, and although it shows that the concentrations fall nearly in step with each other, it also emphasizes the differences from absolute identity between the two

FIGURE XIII. Red cell and albumin washout results from four muscles. The results from each muscle are separately identified for comparison.

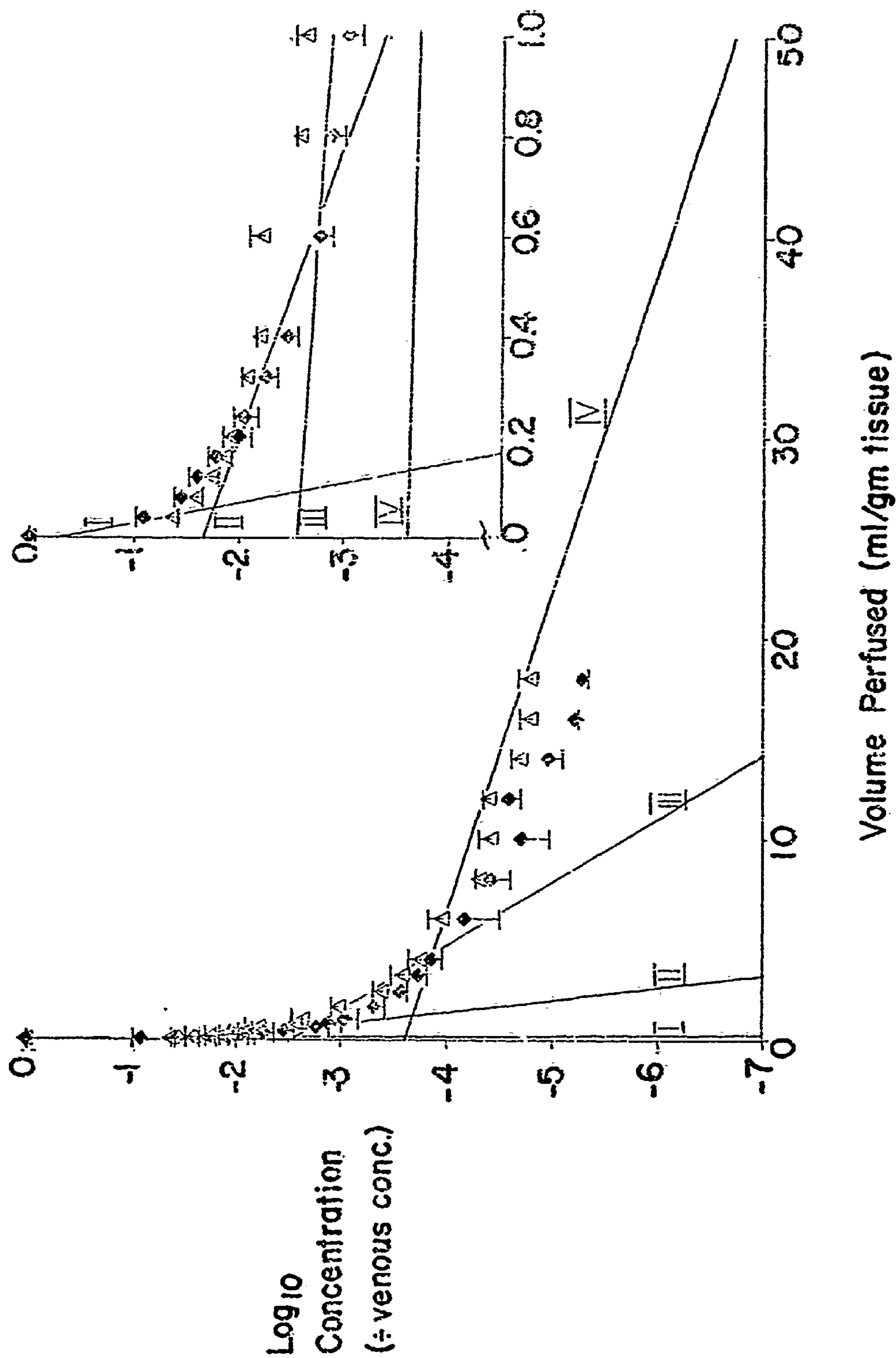
Muscle	Albumin	Red cells
1.	○	●
2.	□	■
3.	△	▲
4.	◇	◆



Log10
Concentration
(÷ venous conc.)
0
-1
-2
-3
-4
-5
-6
-7
0
10
20
30
40
50
0
-1
-2
-3
-4
0
0.2
0.4
0.6
0.8
1.0
Volume Perfused (ml/gm tissue)

Figure XIV. The averages with standard errors of the mean for the concentrations of red cells and albumin at selected volumes perfused obtained by interpolation from the results shown in Figure XIII.
The lines correspond to the compartments listed in Table VI for the albumin washout.

△ albumin
◆ red cells



Log10
Concentration
(÷ venous conc.)
Volume Perfused (ml/gm tissue)
0
-1
-2
-3
-4
-5
-6
-7
0
10
20
30
40
50
0.2
0.4
0.6
0.8
1.0
I
II
III
IV

TABLE VI Parameters of the compartment model derived from the washout of labelled plasma from vasodilated skeletal muscle.

Parameter	Comp. I	Comp. II	Comp. III	Comp. IV
Half-volume	0.0130	0.191	0.829	4.86
± S.E.M.	± 0.0007	± 0.050	± 0.237	± 0.93
(ml/gm)				
Intercepts	0.442	0.0143	7.48×10^{-3}	2.47×10^{-4}
± S.E.M.	± 0.031	± 0.0016	$\pm 4.83 \times 10^{-3}$	$\pm 1.17 \times 10^{-4}$
(÷ Venous blood conc.)				
Percent of total flow	95.42	3.10	1.42	0.058
± S.E.M.	± 0.87	± 0.25	± 0.82	± 0.030
Percent of total store	44.80	21.09	26.46	7.65
± S.E.M.	± 2.17	± 5.47	± 7.42	± 1.99

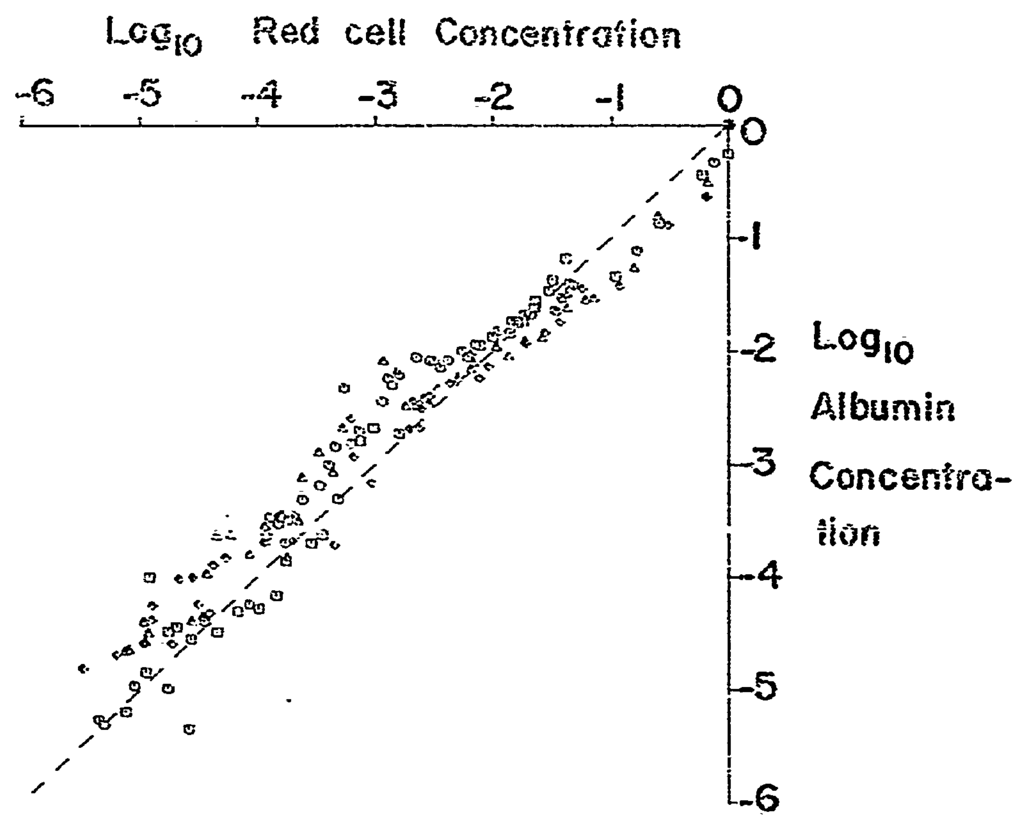
Total of Intercepts/ venous blood concentration:

0.464 ± 0.037 (S.E.M.)

Total store of plasma/ venous blood concentration:

$1.836 \times 10^{-2} \pm 0.214 \times 10^{-2}$ ml/gm

Figure XV. Red cell concentration plotted against the albumin concentration in the same sample, or interpolated from neighbouring samples in the same experiment. Both are normalized by dividing by the venous blood concentration in the same experiment. The symbols are the same as used for the albumin washout graph in Figure XIII.



Log10 Red cell Concentration
-6
-5
-4
-3
-2
-1
0
0
-1
-2
-3
-4
-5
-6
Log10 Albumin Concentration

washout processes.

Of particular interest in the first 0.1 ml per gm of perfusion where the albumin concentration falls significantly below that of the red cells in the same samples. This affects the definition of the fastest decaying compartment in the model of the plasma store, so that relative to the total, it has a smaller volume than that of the corresponding compartment of the cell store. The intercept of this compartment is somewhat less than expected; the totals of the intercepts should be 1.0. The remainder of the intercept of the curve, which is not fitted by the exponential components, may by due to a fifth "compartment" but, since only one point is available to fit to this, that at 0 millilitres of perfusion in most cases, it would be difficult to define. This points out a weakness in the method; the samples at finite intervals are not sensitive to rapid changes in the outflow concentration. Because of this, the washout kinetic studies done this way may show large errors in estimating the total flow and store of the best-perfused parts of the circulation, although the relative distribution of flow and capacity of the more poorly-perfused parts are well-defined.

Compartments II and III of the model are more similar in half-volume than the corresponding compartments of the red cell store in the vasodilated muscles. By the criterion of Van Liew (1962) they may well be the result of a single-peaked continous distribution of washout processes, and therefore should not be considered as separate entities. This is also true of the red cell washouts, as will be discussed later. The difference between the

plasma and the red cell compartment models reflects a real difference in the washout curves, as is shown by the fact that the points on the graph in Figure XV tend to lie above the line of identity in the region of $C/C_0 = 10^{-3}$.

The existence of Compartment IV in the plasma washout, and the related fact that the plasma concentrations follow the red cell concentrations down to five orders of magnitude below the concentration in venous blood was quite unexpected. It had been shown that there is no slow compartment of the plasma washout of the spleen, corresponding to the slow compartment of the red cell store of that organ (Levesque and Groom 1976a). That the muscle washout shows the similarity between the two washouts observed, suggests that whatever mechanism traps the cells of the slow compartment in the muscle also traps a corresponding volume of the plasma, or at least the plasma label.

Such an idea is not inconsistent with the notion that the adhesiveness of the cells to the vascular endothelium is responsible for their retention. It is well-known that the red cells of most mammalian species are deformed to some degree as they pass through the capillaries in many tissues. The usual pattern of the capillary blood flow has the cells squeezing through in single file, separated by boluses of plasma (Branemark 1971). One model postulates that the resistance to the motion of the red cell is governed by the presence of a lubricating layer of plasma between the cell membrane and the adjacent capillary endothelium, which exists in a balance between the elastic compressive force of the cell and the hydrodynamic forces caused by the pressure differences driving the cell

along the capillary (Fitz-Gerald, 1972). It seems plausible that, if a red cell were to adhere within a capillary, it would block the passage of plasma around it, and thus prevent the clearance of all the plasma back to the previous branch in the capillary network. Thus the slow release of the red cells with perfusion would be accompanied by the parallel slow release of plasma from the capillary bed.

It is possible that a slow washout of albumin from a muscle may be due to the extravascular trapping of plasma, perhaps due to abnormal filtration from the vascular bed. However, in such a case the decay of the concentration of the label would not necessarily be parallel to that of the cells, as is observed here.

The principal conclusions that can be drawn from these experiments are that the plasma store in muscle is washed out in a manner very similar to that of the cells. The best-perfused part of the plasma store is smaller in percent of the total volume than the corresponding part of the red cell store. The poorly-perfused parts are similar to the corresponding parts of the red cell store even to the slowest-cleared compartment. Whatever mechanisms are responsible for the inhomogeneities of perfusion of the red cell store also seem to impose the same inhomogeneities upon the plasma store. This suggests that the causes may be in the geometry of the vascular bed, rather than in any peculiar properties of the cells themselves, but it is not possible to dismiss the idea of a cellular cause entirely, because of the nature of flow in capillaries.

CHAPTER III.

THE TRAPPING OF ABNORMAL RED CELLS DURING A SINGLE TRANSIT THROUGH SKELETAL MUSCLE.

1. Introduction

a The trapping of reticulocytes in the circulation

The work described in the previous chapter had failed to produce any explanation for the inhomogeneities in the pattern of red blood cell washout in terms of the functional control of the distribution of blood flow through the vascular bed of skeletal muscle. Indeed the inhomogeneities persisted in the absence of any physiological control of blood flow distribution and were discovered under conditions in which this control was abolished. Clearly the inhomogeneities had other causes.

The results of the plasma label washout did not necessarily exclude the idea that the behaviour of red cells themselves was responsible for the washout pattern. As noted before, the plasma in the capillaries of skeletal muscle might well be retained by cells that block the entire capillary lumen. This is quite different from the spleen, where the sinuses and open spaces of the pulp allow plasma to be cleared more quickly than the cells (Levesque and Groom, 1976a).

Groom et al. (1973) suggested that the slow clearance of cells in the "slow compartment" of the muscle was due to the same process as the similar clearance of cells in the spleen. They observed that cells collected after 30 min of perfusion showed a high percentage of reticulocytes. These cells, they had shown earlier, made up the slow compartment of the red cell store of the spleen of the cat (Groom, Song, Lim and Campling 1971). The cells were also shown to adhere to the endothelial cells and macrophages of

the splenic pulp when spleens were perfused and examined under the microscope (Song and Groom 1972; Song and Groom 1974). Because of this the cells in the slow compartment of muscle were believed to be slowly cleared because of their adhesion to the walls of the blood vessels in that organ. This suggested a general phenomenon in which reticulocytes adhered within any vascular bed, because of an inherent "stickiness" of their surface. The large number of reticulocytes stored in the spleen would then be due to the large surface area that that organ presents to the blood. In muscle, the slow component was about 1% of the store of cells in the vascular bed; this is similar to the fraction of normal blood that is composed of reticulocytes (Wintrobe 1961). Thus the slow component of the muscle washout pattern was the consequence of a general trapping of cells in vascular beds due to some special property of reticulocytes and similar cells that caused them to adhere to vascular walls.

What properties of reticulcytes that would cause their retention in the spleen and also in the vascular bed of skeletal muscle? This is a complex and confusing question that involves the physical and biochemical properties of the red cell, both immature and mature, the spleen and the behaviour of cells in the peripheral circulation.

Song and Groom (1971a and b ; 1972;1974) and others (Jandl 1960; Card and Valberg 1967; Wade 1973) provided evidence that the spleen sequesters reticulocytes or immature red cells and releases them slowly except, in some animals, under stress (Geiger, Song and Groom 1973; Wade 1973). The mechanism whereby these cells are

selectively trapped is not completely understood, although Song and Groom (1974) have demonstrated that the cells adhere to the surfaces of macrophages and endothelial cells, rather than being trapped in the narrow fenestrae in the endothelium surrounding the sinuses that exist in some species such as the dog. This latter mechanism of trapping was postulated by Canham and Burton (1968) as a mechanism for trapping abnormal red cells and has been demonstrated by Leblond (1973) and Chen and Weiss (1973) as the mechanism whereby cells with inclusion bodies are trapped in the spleen.

The stickiness of the surface of reticulocytes has been known for some time (Key 1922) but its cause is not understood. Jandl (1960) showed that reticulocytes produced by bleeding or phenylhydrazine poisoning in rabbits tended to clump spontaneously and to agglutinate when exposed to Coomb's serum or polyvinylpyrrolidone. These cells may be different from the reticulocytes produced normally, as they are unusually large and short-lived in the circulation (Sorbie and Valberg 1970). The cause of the adhesiveness may be a protein on the surface which binds to plasma proteins which bind cells together.

Reticulocytes are among the least dense of the red cells of a mammal (Leif and Vinograd 1964; Danon and Marikovsky 1964) and are somewhat larger than mature cells (Key 1921). As mentioned before, abnormal bulk physical properties of red cells may be important in their being trapped by the spleen, but these properties do not seem to be the cause of the trapping of reticulocytes.

b The models used in these experiments

i General

To test the hypothesis that the mechanisms that cause the trapping of reticulocytes in muscle are the same as those that lead to their being trapped in the spleen, the next series of experiments was performed. In each experiment cells with a specific abnormality were injected intra-arterially into a muscle that had been perfused with cell-free solution to clear it of its original store of cells. Evidence of the trapping of the injected cells was sought from the cells leaving *via* the vein after the injection, and also from changes in the resistance to flow of perfusate in the muscle during and after the injection. A similar series of experiments has been performed on the feline spleen (Levesque and Groom 1977; Levesque 1977) and has shown trapping of a variety of types of abnormal cells in that organ on a single transit.

The choice of the types of abnormal cells used in both series of experiments was somewhat broad, but was restricted to types of abnormal cells that had been studies by other investigators, both *in vitro*, to determine their physical properties and *in vivo*, with respect to their survival in the circulation. Most of the types also have been widely used, either in clinical or laboratory studies so that their preparation is reasonably simple and reliable.

Within this constraint, a choice was made that would explore changes in both the surface properties (adhesiveness) and bulk properties (deformability, shape and size). Although the studies mentioned before indicate that the sequestration in the spleen of

immature cells was due to a surface property, it was of interest to explore the relative sensitivity of the vascular bed of skeletal muscle to abnormal bulk properties of the cells as well as abnormal surface adhesiveness, to see whether the former may cause sequestration in muscle.

The five types of abnormal red cells tested are described below.

ii Cells obtained from the splenic pulp

Levesque and Groom (1976 a and b) described a method whereby cells could be obtained from the red pulp of the cat spleen by stimulating it to contract with noradrenaline. The cells obtained from the last fraction expelled thus come from blood with a very high hematocrit (75-80%) which is strong evidence that they come from the red pulp of the spleen. Song and Groom (1971a) and Groom et al.(1971) showed that these cells have a high fraction of reticulocytes corresponding to the 'slow' compartment in their model of the spleen and Geiger, Song and Groom (1974) showed that the bound cells are in fact released when the spleen is stimulated to contract. Thus it is to be expected that cells obtained by stimulating the spleen to contract would contain a high proportion of immature cells, although it would be difficult to estimate beforehand what this fraction is. The remainder would be free cells from within the pulp,and they would have been "conditioned" by the acidic, oxygen-poor environment of the pulp. Levesque (1977) showed that 69% of these cells are retained in the spleen after a single transit, as opposed to 15% of normal cells.

Clearly, these cells exhibit properties that lead to their retention in and slow clearance from the spleen and are most likely to be the immature cells which are retained in both spleen and the vascular bed of muscle.

iii Neuraminidase-treated cells

Treatment of red blood cells of several mammalian species with the enzyme neuraminidase removes the sialic acid portions from the glycoproteins of the membrane surface. With the sialic acid the electric charge of the cell surface is also removed, as shown in studies of the electrophoretic mobility of the cells (Cook,Heard and Seaman 1961; Eylar, Madoff, Brody and Oncley 1962). The surface charge has been shown by this method to affect the aggregation of cells by Dextrans (Jan and Chien 1971) and polylysine (Marikovsky, Danon and Katchalsky 1966). It is suggested by these authors and others (Brooks 1975) that the normal surface charge of red cells, which is negative in all cases, acts to repel cells from each other at close range, thus inhibiting the process whereby cells are drawn together to form rouleaux. Thus, in this case, the adhesion of cells to each other is increased by the removal of the surface charge.

It should be noted that immature or young red blood cells in man have a normal complement of surface charge and it is the older cells that have the least surface charge (Marikovsky, Danon and Katchalsky 1966). It is also true that neuraminidase treatment of red cells does not enhance their adhesion to protein-coated glass plates (Ponder 1965) even when their surface charge is

reduced to zero. Thus, neuraminidase treatment produces an enhanced adhesiveness which is quite unlike that of immature cells.

Nevertheless, the treatment of red blood cells by neuraminidase is an effective method for ensuring their sequestration by the spleen. Up to 70% of treated cells are retained on a single passage through the cat spleen (Levesque 1977). Neuraminidase-treated cells injected into the peripheral circulation of rabbits are retained by the spleen and liver (Durocher, Payne and Conrad 1975; Gattegno, Bladier, and Cornillot 1975). The loss of sialic acid which occurs during the ageing of a cell may mark it specifically for trapping and destruction by the cells of the reticulo-endothelial system, particularly in the spleen (Bocci 1976). Thus, although neuraminidase-treated cells share with immature cells the property of being trapped in the spleen, they may have this property for entirely different reasons. Thus this model is useful in determining whether it is the increased adhesiveness of immature cells (as shown in their tendency to form rouleaux) that is related to their being trapped in the vascular bed of muscle.

<u>iv</u> Human Red Blood Cells

The use of mammalian cells of a different species was considered, not because of any antigen-antibody mechanism proposed for the retention of cells in the vascular bed, but because of the differences in size among red blood cells from different mammals. Cat red blood cells are 5.7 microns in diameter (Altman and Dittmer 1971, p.119) and have a mean corposcular volume of 57 cubic microns. Human cells are larger with a mean measured diameter of 7.8 microns and

a mean volume of 96 μm^3 (Fung 1977a). Jay (1973) has shown that human cells can be drawn into glass capillary tubes of less than 2.5 μm diameter without permanent damage, this diameter being far smaller than that of the capillaries in cat muscle (5.3 μm in the cat tenuissimus muscle, according to Eriksson and Myrhage (1972)). However the human cells may require more energy to deform them into the shape required to pass through the capillaries, and if they were trapped in the vascular bed, it would indicate that this energy is an important factor in the passage of red cells through the muscle vascular bed.

v Heat -treated cells

It has been known for some time that mammalian red blood cells exposed to heat are damaged in a specific way which causes them to be trapped by the spleen or liver without prior cell destruction. This is true only of certain procedures of heat-treatment; excessive heat or prolonged exposure will destroy the cells *in vitro* (Ham, Sayre, Dunn and Murphy 1968). Treatment of a washed suspension of cells for 15 to 20 min at 49°C to 50°C has been shown to produce cells which do not spontaneously agglutinate or hemolyse but are trapped almost exclusively in the spleen on injection into an intact animal or human (Harris, McAlister and Prankerd 1957; Wagner, Razzak, Gaertner, Caine and Feagin 1962; Crome and Mollison 1964: Kimber and Lander 1964; Marsh, Lewis and Szur 1966). Ham *et al.* (1968) showed that there are changes in the shape and deformability of human and dog red blood cells treated this way which increase their resistance to filtration through pores of

diameter less than that of the normal cells, and also increase the bulk viscosity of suspensions of the cells. These changes are explained by their observations that the heated cells lose part of their membrane by "budding" off small pieces. This should reduce their surface-area-to-volume ratio. They suggest that the budding is the result of contracture of the proteins of the membrane, which also might cause the membrane to be less deformable. Either change would reduce the deformability of the cell, and hence both the bulk viscosity of cell suspensions and the filterability of cells. According to the model proposed by Canham and Burton (1968, also Canham, 1969), and confirmed with respect to cells containing inclusion bodies by the electron microscope observations of Chen and Weiss (1973) and Leblond (1973), this reduction in deformability would cause the trapping of cells by the fenestrae in the sinus walls of the spleen. Levesque and Groom (1977) have shown that about 35% of an injection of heat-treated cells are trapped in the spleen on a single transit. They show that this trapping is in the faster-flowing circuits through that organ and immobilises a large part of its cell store. This suggests that the trapping of heat-treated cells injected into the spleen is in the blood vessels of the organ rather than in the red pulp.

The fact that heat-treated cells are concentrated in the spleen when injected into an intact animal or man argues that trapping of these cells in the vascular bed is not a major phenomenon elsewhere in the body. However in a vascular bed in which all the perfusing blood is passed through the capillary bed, such as that of skeletal muscle, such trapping may be impossible without blocking

the vascular pathway. This would, of course, bring the pressure difference existing between the arterial and venous ends of the pathway on the trapped cell, and may cause its release, if the force required were not too great. This might make possible a situation in which cells of reduced deformability do not remain in the vascular bed in great numbers, but might be trapped in those parts of the circulation which are easily bypassed by the blood flow.

vi Fixed cells

The treatment of red blood cells with chemical fixatives such as formaldehyde or glutaraldehyde causes them to become rigid without changing their shape (Morel, Baker and Wayland 1971; Jay and Canham, 1972). The resistance to deformation of fixed cells is extremely great; they do not pass through filters of pore diameter less than the cell diameter and do not deform in packing by centrifugation at 15,000 G (Chien, Usami, Dellenback and Gregersen 1967). The bulk viscosity of suspensions of these cells becomes infinite, i.e. they behave as a solid, at hematocrits of about 60%.

The injection of cells fixed with 1 to 2% gluteraldehyde solutions, which produce the above characteristics, into a living animal or man has not been reported, but cells fixed in weaker solutions (0.125%) are trapped selectively in the spleen in man, rabbit and rat (Deloach, Peters, Pinkard,Glen and Ihler 1977). Higher concentrations of glutaraldehyde in the fixation solution result in the cells being trapped in the liver. This is parallel to the case with heat-treated cells in which treatments beyond the

temperatures described before result in trapping of the treated cells in the liver rather than the spleen (Crome and Mollison 1964; Kimber and Lander 1964).

Levesque (1977) has shown that, when cat cells treated in 1% glutaraldehyde solutions are injected into the isolated cat spleen, none of the cells leave the organ if the perfusion pressure is not raised to unphysiologically high levels. The fixed cells represent an extreme case of a cell abnormality resulting in trapping in the spleen. This model would then determine whether it was possible that any decreased cell deformability would result in the trapping of cells in the vascular bed of muscle.

The five types of cells listed above represent a selection from the possible types of abnormal cells which are trapped in the spleen and may also be trapped in the vascular bed of skeletal muscle.

c The constraints on the method

The experimental study of the trapping of these abnormal cells is subject to a few constraints. Ideally the experiment that would bear comparison with the washout studies described in the last chapter would start with the muscle filled to equilibrium with abnormal cells and perfused in the manner described before. However, this is not practical, both for the obvious difficulty in removing all of the original cells and leaving the muscle in a good state of health, and also in the impossibility of refilling it to equilibrium with abnormal cells. Compromises had to be made with these problems.

The first compromise is that the muscle vascular bed would not be completely cleared of the "slow compartment" cells, but would be perfused only to remove the majority of the original cells in it. The injections of abnormal cells would then have to be large enough so that their subsequent departure from the muscle via the vein could be detected above the clearance of the remaining original cells in the muscle at the same time. Implicit in this method is the hypothesis that the number of trapping sites for abnormal cells is very large compared to both the number of cells remaining in the muscle and the number injected.

Although the above hypothesis is adopted through necessity, it is not unreasonable. The number of slow compartment cells is somewhat less than the total number of reticulocytes stored in the muscle (Groom et al. 1973), but is of the same order of magnitude. This suggests that the number of slowly released cells is determined by the number of reticulocytes present. Also, the contrary hypothesis, that a certain finite part of the vascular bed is the exclusive site of cell trapping, does not seem reasonable in the absence of any evidence, despite many microscopic studies, of special parts of the vascular bed of muscle that might have this function.

Another number which is important in determining the effect of an injection of abnormal cells is the number of possible pathways that would take an injected cell from the artery to the vein. If the number of injected cells is much larger than this and every cell is trapped in the vascular bed, the resultant blockage of

pathways would impede the passage of not only the following cells but the suspending medium as well. The highly deformed state of red blood cells travelling through peripheral capillaries makes it possible that the cell could adhere to all of the wall surface of a capillary cross-section at once and thus block the flow of the suspending medium in that passage. The resistance to perfusion during the passage of a large number of injected cells through each pathway would be a sensitive indicator of a trapping mechanism even in the absence of trapping itself. Recalling the discussion of the fact that heat-treated cells are not normally sequestered in the peripheral circulation of an intact animal, it is probable that cells which do adhere in the vascular bed are forced out by a transient increase in the perfusion pressure which may be the only evidence that large-scale trapping of the cells is possible.

The number of pathways through a cat gastrocnemius muscle from the artery to the vein may be estimated from the following formula:

$$N_t = \frac{100 \cdot D_c \cdot V}{L}$$

where N_t is the total number of pathways, D_c is the capillary density in mm^{-2} in cross-sections through the tissue, V is the volume of the tissue, and L is the mean path length in cm between branches in the capillary network. For D_c I shall take a value, 405 mm^{-2} obtained by Willner (1977) using PAS staining of cat gastrocnemius muscle sections. L is more difficult to determine as longitudinal studies of the vascular bed of the cat gastrocnemius

similar to those of Plyley, Sutherland and Groom (1970) on the frog sartorius have not been done. Eriksson and Myrhage (1972) give a value, 200 microns, for this length in the cat tenuissimus muscle and I shall borrow this for the gastrocnemius. Assuming the density of the muscle to be 1 gm per cm^3, V can be estimated by the weight of the muscle in grams. Thus for a 25 gm muscle, typical of those studied, N_t is 5.0×10^7 pathways.

I have chosen injections of about twenty times this number of cells. They correspond to about 0.1 ml of blood at normal haematocrit. Larger injections would not approximate instantaneous injections into the 0.6 ml vascular volume of the muscle, and would confuse the determination of the transit characteristics.

The transit characteristics, explained in more detail in the section on methods of this chapter, correspond to the washout characteristics of the cells stored in the muscle described before. They are an indication, in a quantitative way, of what volume is available to the injected cells, and how this volume is perfused by the injection.

Because of the presence of the original red cells of the muscle during the experiment it is not possible to determine directly whether a small portion of the injected cells passes very slowly through the vascular bed. What can be determined is the time or perfusion volume, that is required for the "average" of the bulk of the cells to pass through the muscle. The permanent or long-term trapping of a small fraction of the cells must be determined by other means.

One possible method is to compare the number of cells recovered

in a short interval, corrected for the release of the cells originally in the muscle, with the number injected. This method, for reasons explained later, has limited accuracy. A method of possibly greater sensitivity is to compare the resistance to perfusion flow in the muscle after the passage of the majority of the injected cells with that before the injection, to see whether the passage of the perfusate is impeded by the remaining abnormal cells.

2 Methods

a General

In these experiments normal and abnormal cells were injected intra-arterially into muscles which had been perfused in order to wash out the original cells to a very low outflow concentration. The outflow from the vein was collected in serial samples and the red cell concentration was measured as a function of the volume perfused by direct counting of the cells. At the same time, or in similar experiments, the resistance to the flow of the cell suspension during the transit was measured.

From these experiments four indices of the possible effects of trapping of the cells were calculated. The effects considered were: transit characteristics that differed from those of normal cells in the same preparation, the loss of cells during the transit in the muscle, a temporary change of the flow resistance during the passage of the cells, and a permanent change in the resistance to flow in the muscle once the majority of the cells had passed.

b Perfusion and cell suspension solutions

The muscles were perfused with solution III as listed in Table I. Sodium nitroprusside (0.1 $gm \cdot l^{-1}$) was dissolved in the solution in order to maintain vasodilatation of the muscle. The red cells were washed and suspended (see below) in phosphate buffered saline ((PBS), solution IV of Table I (Dulbecco and Vogt 1954).

c Preparation of the muscles

The preparation of the muscles for perfusion was exactly as described before for the red cell washout experiments *in situ*. Each muscle was perfused *via* side branches of the popliteal artery and vein. Aheating pad placed below the muscle served in place of the heat lamp to keep the muscle at 37°C. The pad was controlled by the same system that was used before to control the heat lamp.

d Preparation of the cells for injection

Red blood cells from the same cat were used in all experiments except for those with fixed cells, where, in some experiments, cell were saved from a previous preparation. About 10 ml of blood were withdrawn through a polyethylene cannula from the jugular vein into polyethylene centrifuge tubes into which 0.1 ml of heparin solution (500 U per ml) had been placed. The blood was centrifuged at 2600 g for 15 min and the plasma and buffy coat were removed from the packed cells. The cells were then resuspended in five to eight volumes of PBS with gentle agitation and separated again by centrifugation at 2600 g for ten minutes. This washing was repeated three times, each time with new PBS.

Those blood cells that were used for the normal cell injections (controls) were resuspended after the third washing in an equal volume of PBS. The resuspended cells had a haematocrit of about 40%, and a cell concentration of about 10^{10} cells per ml, a concentration slightly larger than that of the cells in normal blood.

The five types of abnormal cells described before were prepared for these experiments. They were: 1.) Human cells, 2.) Cells from the

splenic red pulp, 3.) Heat-treated cells, 4.) Neuraminidase-treated cells and 5.) Glutaraldehyde-fixed cells.

For the human cells, blood was obtained by veni-puncture from a normal donor and placed in a heparinized Vacutainer (Becton-Dickinson, U.S.A.). The cells were separated from the plasma and washed three times in PBS as described above for cat cells.

Red cells from the splenic pulp were obtained by the method of Levesque and Groom (1976a). The spleen of the same cat used in the experiment was isolated and the common artery and vein were cannulated up to the point of branching. Blood was allowed to drain freely from the venous cannula and then 0.5 mg of noradrenaline in 0.1 ml of saline was injected intra-arterially. About 10 ml of blood were expelled by the contracting spleen, of which all but the last two or three millilitres was discarded. This blood has been shown to come from the red pulp of the spleen (Levesque and Groom, 1976a and b). The cells were separated from the plasma and washed and resuspended in PBS as described for normal cat cells.

Heat-treated cells were prepared from the suspension of washed, normal cells, obtained as described above, by immersing the test-tube of the suspension for 20 minutes with gentle agitation in a water bath maintained at 50°C. This follows the method of Marsh, Lewis and Szur (1966) except that, in their method, packed cells were treated. The cell suspensions were used without rewashing after treatment.

Neuraminidase-treated cells were prepared from normal cat cells, washed as described before, except that the packed cells were not resuspended after the final centrifugation. To a 1 ml sample

of the packed cells was added 0.1 ml of <u>Vibrio</u> <u>Cholerae</u> Neuraminidase solution (300 U per ml, BDH Chemicals, England). The suspension was incubated for 1 hr at 37 C in a water bath with gentle agitation. After treatment the cells were washed again three times in PBS. This method is adapted from that of Gattegno, Bladier and Cornillot (1975), who show that it removes all the sialic acid residues from rabbit red blood cells.

Washed normal cells were fixed by suspension in five volumes of 1% isotonic phosphate-buffered glutaraldehyde solution. These cells were separated from the fixative by centrifugation and washed three times again in PBS. Violent agitation was usually required to re-suspend the packed fixed cells after each centrifugation. The cells were resuspended in five or ten volumes of PBS before injection.

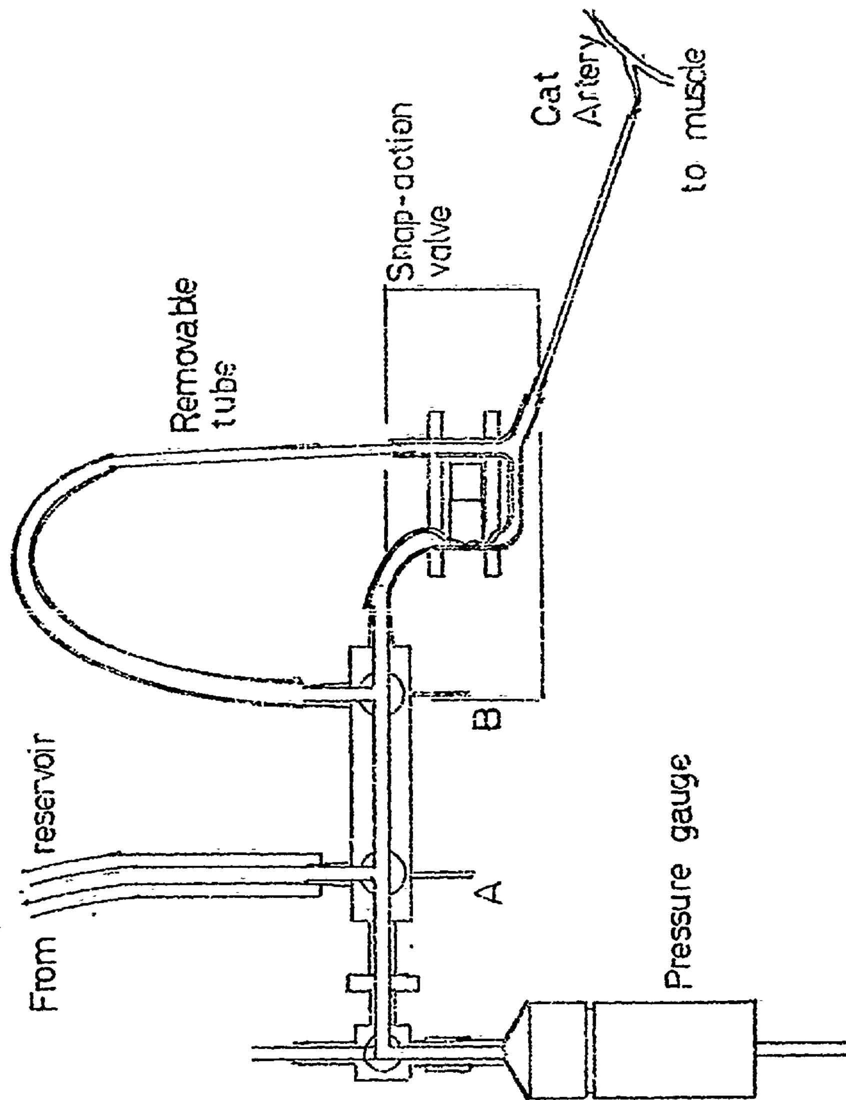
<u>e</u> Apparatus

The perfusion apparatus was, in most parts, the same as that used in the muscle washout studies. Both constant-flow and constant-pressure perfusions were tried but for the majority of the experiments described here, and all those on abnormal cells, constant pressure perfusion was used.

The principal modification of the perfusion apparatus was the addition of the assembly shown in Figure XVI. This assembly permitted the introduction of a "slug" of cell suspension into the arterial inflow without affecting the inflow pressure or flow rate.

Referring to Figure XVI and Figure II, the reader can note that the stopcock manifold containing stopcocks "A" and "B" replaces

FIGURE XVI. Apparatus for the perfusion of muscles modified to permit the injection of cell suspensions during perfusion. The snap-action valve is in the position that clamps shut the lower passage from stopcock 'B'.


From reservoir
A
Pressure gauge
B
Removable tube
Snap-action valve
Cat Artery
to muscle

the single stopcock of Figure II with stopcock "A", and provides the additional stopcock "B" which can direct the inflow from the reservoir to either or both of the passages leading from it.

The lower of these passages, which are made of thin walled latex rubber tubing 3.2 mm in diameter, passes through one arm of the "snap-action valve", forms a T-junction with the upper passage and is connected to the arterial cannula. The cannula is 5 cm of the PE 190 tubing described before, with a 1 cm tip of PE 50 tubing.

The upper passage, passing through the other arm of the "snap-action valve" is like the lower, apart from its greater length and the "removable tube", a 5 cm length of PE 240 tubing which serves to hold the "slug" of cell suspension before it is injected. It has an interior volume of 0.11 ml.

The "snap-action valve" is the toggle and mechanism of an electric switch. Two projections of the plastic case of the switch were drilled to hold the latex tubing of the passages in such a way that the toggle of the switch clamped one or other of them firmly shut under the pressure of the springs of its mechanism. Thus the toggle provided a ready means of instantly switching the arterial inflow from one passage to the other.

During the normal perfusion of the muscle, both stopcock "B" and the snap-action valve were set to allow flow through the lower passage. Before an injection, the polyethylene tube was removed from the upper passage, drained of the Ringer's in it and attached by a tube to a 1 ml syringe. The tube was filled with the cell suspension to be injected by immersing its free end in the suspension and withdrawing the plunger of the syringe

until the suspension rose above the tube.

The tube, still attached to the syringe, was then replaced in the system, its free end fitted in the tubing nearest the snap-action valve first. The syringe was then removed and the upper end of the tube fitted into the latex tube coming from the stopcock "B". The latex tubes were compressed slightly to expel any air that was visible in the tubing near the connections before they were made. Stopcock "B" was then turned to admit the inflow to both passages. At the moment the collection began, the snap-action valve was switched to admit the flow from the upper passage rather then the lower. This moment was taken as the origin in the graphs and computations of time or volume perfused during the injection.

The purpose of this apparatus was to inject a measurable quantity of the red cell suspension into the arterial inflow in a pattern that, although it did not approximate the ideal of an instantaneous injection too well, allowedan estimate of the transit time or volume of the vascular bed studied. The volume of cell suspension used, 0.11 ml, is quite large when compared with the vascular volume of the muscle (about 0.6 ml); and injection of 0.11 ml by means of a syringe would distort the pattern of pressure and flow in the vascular bed, if it were done quickly enough to be short compared to the transit time. The present method takes advantage of the inflowing stream to flush the removable tube clear of cells.

The method has faults. The open ended plastic tube does not control the volume of injected cells as well as some other systems

might, such as the closed glass cylinder used by Groom, Morris and Rowlands (1957). The number of cells injected may depend on the position and shape of the menisici at the ends of the tube after it is filled and the connection with the syringe is broken. For this reason, the total number of cells injected in a given experiment was not estimated by measuring the concentration of cell in the suspension used as "stock" for the injection, and estimating the volume of the tube. Rather, the tube was loaded with the cell suspension and installed in the apparatus just as was done in an actual injection. Its contents were then flusned through the arterial cannula into a graduated tube with 10 ml of Ringer's solution. The contents of the tube were then counted and the volume was measured to estimate the number of cells injected from that stock in the actual experiments. This was done three times in order to estimate the variation in the number of cells injected as well as the mean value.

The other major fault of the apparatus is that the cells are not all injected immediately into the arterial cannula. The flow in the tube during the perfusion is laminar and the cells nearest the tube wall are cleared from the tube much more slowly than the rest. The result of this is that the concentration of injected cells plotted against the volume perfused has a skewed profile which reflects this clearance as well as the time required for the cells to pass to the end of the arterial cannula. This profile, as measured in a sham experiment in which the arterial cannula was drained into the collection apparatus is shown on a

linear scale in Figure XVII, and on a semilogarithmic scale in Figure XVIII.

f Sample collection

In preliminary experiments the venous outflow was collected in test-tubes in a hand-held rack. The collection was on a drop-by-drop pattern and test-tube received varying multiples of the drop volume according to a pre-determined plan. The results of these experiments showed that, after an injection of normal cells, the concentration of cells in the outflow as a function of volume perfused had the form shown on a linear scale in Figure XVII, and on a semilogarithmic scale in Figure XVIII. The major characteristic of this graph is the single peak of concentration at less than a millilitre of perfusion followed by the relatively slow fall to background levels. Two results influenced the design of the collection technique for subsequent measurements. They are the roughly exponential decline of the concentration to background levels after 3 ml of perfusion (most apparent on the semilogarithmic graph, Figure XVIII), and the fact that after 3 ml of perfusion well over 95% of the collected cells have been recovered (most apparent on the linear graph, Figure XVII). The linear graph also shows that there are relatively large errors in the concentration measurements at the peak of the curves. These errors make estimates of the total number of cell collected and the mean transit time (or mean transit volume) somewhat unreliable; they indicate the need for an improved method of collecting samples.

FIGURE XVII. Linear graph of cell concentration as a function of volume perfused after the beginning of the injection.

- ⊥○⊤ - from the arterial cannula
- ⊥●⊤ - from the vein of a perfused muscle.

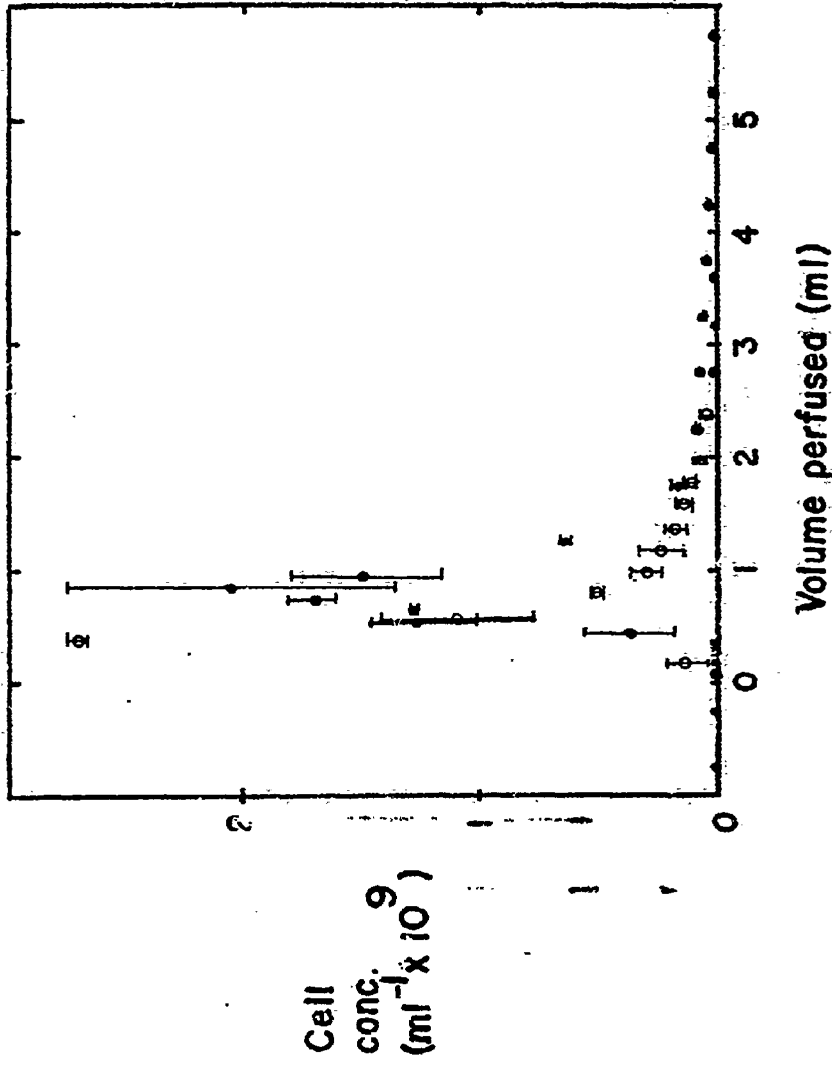
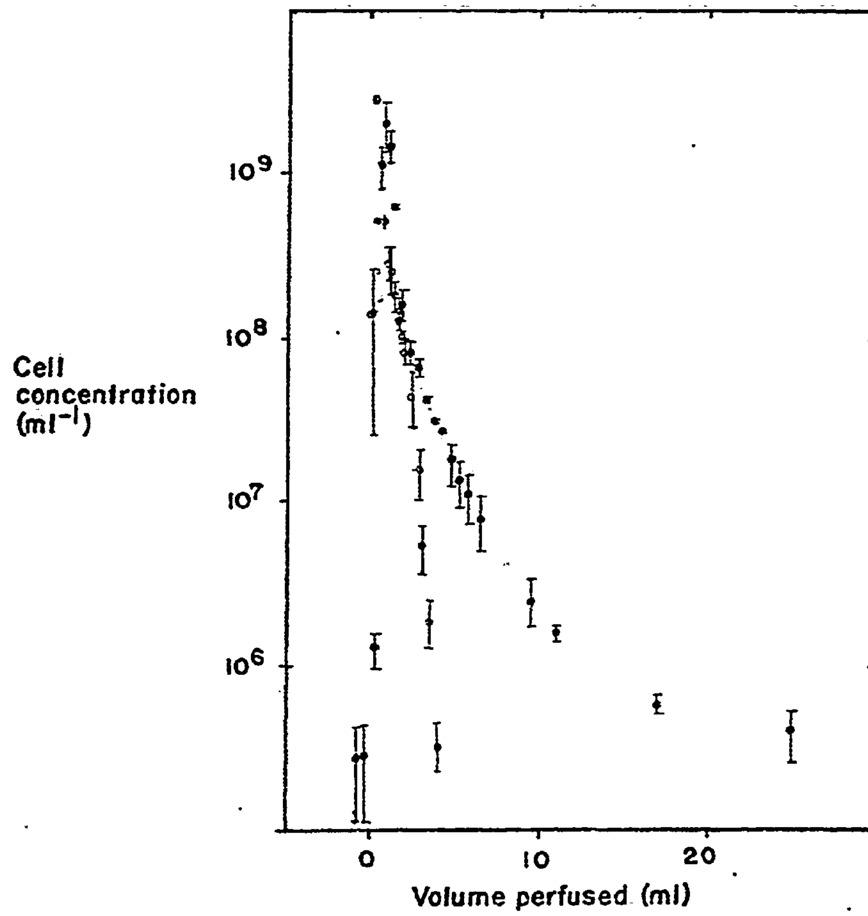

Cell
conc.
(ml^{-1} x 10^{9})
Volume perfused (ml)
0
1
2
3
4
5
0
1
2

Figure XVIII. Semi-logarithmic graph of cell concentrat-
tion.as a function of volume perfused
after the beginning of the injection.

- from the arterial cannula
- from the vein of a perfused muscle.



Cell
concentration
(ml⁻¹)
10^9
10^8
10^7
10^6
0
10
20
Volume perfused (ml)

Two improvements were made in the procedure. One was the use of a rail mounted rack for the sample collection tubes which made accurate timing of the collection of samples more simple. The other was the pre-filling of the collection tubes with 0.5 or 1.0 ml of Diluton diluent before they were used to collect the samples. Each sample, whose volume was otherwise 0.05 to 0.1 ml, was instantly diluted on collection by a known volume of diluent and the evaporation of the sample in the collection tube, which had been a major source of error, was greatly reduced. About 30 tubes, each accepting 0.1 ml of the outflow could be accommodated on the rack. The first 3 ml of outflow was collected this way. After the tubes were filled a beaker was placed under the venous cannula to collect a further 10 to 15 ml of the outflow.

In the majority of the experiments, which were performed with constant-pressure perfusion, it was considered more practical to measure the concentration of cells as a function of volume perfused through the muscle rather than as a function of time. For this reason the rack of collection tubes was moved by hand rather than by an electric motor, so that variations in the flow rate would not affect the position of each sample on a "volume-perfused" scale. An electronic drop counter was installed under the free end of the venous cannula, and it provided a signal to change test-tubes at every fourth drop. The volume of the drops was assumed to be constant, and it was measured by collecting a predetermined large number of drops from the same cannula in a graduated cylinder.

The concentration of cells obtained in each sample was determined using a Celloscope counter as described before for the washout

experiments. Correction was made for the volume of diluent placed in each collection tube before the sample was collected.

g) Determination of the total number of cells collected and the mean transit volume

The total number of cells collected after an injection and the mean transit volume were calculated from the concentrations and the volumes of the collected samples.

The muscles were perfused with approximately 100 ml of Ringer's solution to remove most of the red cells. At this point the remaining red cells left the muscle at a concentration of 10^5 to 10^6 cells per millilitre. It was considered necessary to subtract some estimate of this background of cleared cells from the concentrations in the samples in order to estimate the concentrations of cells that came from the injected cells. This background was estimated by taking samples before the injection and after the beakers had been filled, more than 10 ml of perfusion later. The first samples collected after the instant of injection were also considered for this purpose, since they were taken before the first of the injected cells passing through the muscle had reached the venous cannula.

Whichever of the concentrations in these samples was the least was used as an estimate of the background, and it was assumed to be constant concentration over the period that the collections were made. An exception was made when the concentration measured before the injection was higher than the others. In this case the concentration in samples taken immediately after the injection was usually much lower than this, but not as low as that measured after

the cell injection had passed. It was assumed that this background was artificially raised by the clearance of a small amount of cell suspension into the muscle from the injection apparatus which occurred as the result of changing between the two passages during the loading of the removable tube. This operation was done immediately before the background sample which preceded the injection was taken. Thus the concentration of cells cleared from the muscle was assumed to fall exponentially towards the constant value with a rapid decay rate. The same rate constant characterized the fall in concentration from the peak value after an injection, as in Figure XVII. The decay constant was assumed to be one decade per millilitre, or that which fitted the concentrations after the start of the injection, whichever was greater. The background concentrations were never much greater than 10^6 cells per millilitre whereas peak concentrations of the cells in the venous outflow were close to 10^{10} cells per millilitre. Thus the background was not a significant part of the total of cells collected, although its omission could have a significant effect upon the estimate of the transit characteristics, in particular the mean transit volume.

After the subtraction of the background concentration from the measured concentrations, the remaining cells were presumed to come from the injection itself. The total number of cells could be estimated from the volumes of the samples and the concentrations in each.

$$N_R = \sum_{i=1}^{30} c_i v_o + c_b v_b$$

where N_R is the number of recovered cells, c_i the concentration of cells in the ith small sample, v_o the small sample volume, c_b the concentration of cells in the beaker and v_b the volume of outflow in the beaker.

To describe the distribution of concentration with volume perfused I defined the "mean transit volume" in analogy with the "mean transit time" used by theorists such as Zierler (1965) to describe indicator studies where the flow character in the system studied is not directly measureable. In this theory, the density of transit times of the indicator is assumed to be a function h(t) of time t. The quantity

$$\int_{t_1}^{t_2} h(t)dt$$

can be interpreted as the fraction of the total of the indicator that requires time intervals between t_1 and t_2 in duration to pass through the system, or alternatively as the fraction of the total of a bolus of indicator injected at time t=o that is collected during the times between t_1 and t_2 after this. The first concept suggests the notion of the mean transit time, that is the average of all the transit times. This would be determined by an integration, over all times, of the density function h(t) multiplied by the time t.

$$\bar{t} = \int_0^\infty t \cdot h(t)dt \qquad (1)$$

The second relates h(t) to the measured concentrations of the indicator in the outflow after an injection

$$h(t) = \frac{F \cdot c(t)}{q} \qquad (2)$$

where F is the flow rate, c(t) is the concentration of the tracer in the outflow at time t after the injection, and q is the total quantity of tracer injected.

In the experiments described here the flow rate was not necessarily constant, as is required for equation (2) to apply. Concentration of cells was measured as a function of volume perfused through the muscle rather than of time. In analogy with the above equation I defined

$$f(v) = \frac{c(v)}{q} \qquad (3)$$

where c(v) is the concentration of the indicator, in this case red blood cells, at volume v perfused after the injection. f(v) can be thought of as a density function of transit volumes and a "mean transit volume" is defined by

$$\bar{v} = \int_0^{\infty} v \cdot f(v)\, dv \qquad (4)$$

Knowledge of the flow rate is not necessary in the calculation or interpretation of $\bar{v}$ as it is in the case of the mean transit time. This is not to say that the mean transit volume is necessarily independent of the flow rate; rather it is expected that the variation of the mean transit volume with flow rate is less than that of the mean transit time. The reason for this is that the models by which

the transit time is interpreted assume that the transit time is related to the flow rate by a constant which is a property of the system studied, i.e.

$$\bar{t} = \frac{V}{F} \qquad (5)$$

where V is a distribution volume for the indicator. The mean transit volume derived above would be simply equated with this distribution volume by analogy. For this reason I have used mean transit volumes measured at slightly different rates of flow as estimates of the same volume which I presume to depend only upon the vascular bed of the muscle and the cell type injected.

The estimation of $\bar{v}$ is accomplished by first estimating f(v), at values of v corresponding to the mid-points of the periods in which each of the samples was taken, as the concentration in the sample divided by the total number of cells recovered.

$$f\left\{v = (j+\tfrac{1}{2})v_0\right\} = \frac{c_j}{\Sigma\, c_i v_i + c_b v_b} \qquad (6)$$

In the integration, this is equivalent to the assumption that the concentration varies linearly during the time that one sample is taken, which is accurate enough for the small samples which are closely spaced. However, the large sample in the beaker in each collection has a contribution to the mean transit volume which is not negligible; this contribution must be calculated using some more appropriate assumption for the distribution of concentration with volume perfused.

Noting the results of the preliminary experiments, I decided

that a single exponential function of volume perfused over the interval in which the beaker was filled would be this model. The function was chosen so that (i) the concentration would begin at that of the last small sample and (ii) the total number of cells that the function predicted for the contents of the beaker would be equal to the number of cells actually collected minus the background. Thus the beaker contribution to the mean transit volume is (apart from division by the total number of cells):

$$\int_{v_{30}}^{v_{30}+v_b} v \cdot C_o e^{-kv} dv = \frac{c(v_{30})}{k} (v_{30} + 1/k) \qquad (7)$$

where $c(v_{30})$ is the concentration of the 30th or last sample at v_{30} equal to 30 times v_o, and k is chosen so that

$$\frac{c(v_{30})}{k} (1 - e^{-kv_b}) = c_b v_b \qquad (8)$$

Equation (8) is solved by iterated guesses which converge rapidly as kv_b is usually fairly large. The computer program "GEORGE" which is described in full in Appendix II performs this calculation. The contents of the beaker are referred to, appropriately, as the "tail" of the collection in the program description.

In summary, the integral of equation (4) is estimated by:

$$\bar{v} \simeq \frac{\sum_{j=1}^{30} (j+\frac{1}{2})\, v_o^{2} \cdot c_j + \frac{c(v_{30})}{k}\,(30v_o + 1/k)}{\sum_{j=1}^{30} c_j v_o + c_b v_b}$$

The contribution of each of the small samples to the mean transit volume is estimated by the fractional concentration, its average "volume perfused" and the sample interval v_o.

The computer program "GEORGE" written in BASIC uses the raw data of the Celloscope counts, dilutions and estimates of the sample volumes to perform the calculations described above. It is listed in Appendix II.

h Determination of the effects on flow resistance of the passage of cells.

Since the perfusion of the muscles was at constant pressure, the flow rate was the variable which followed changes in the flow resistance in the muscle. In some of those experiments in which samples were taken for the determination of the mean transit volume, the times at which the samples were taken were recorded on a chart recorder (Beckman Dynograph) and provided a record of the flow rate. In other experiments the outflow from the venous cannula was allowed to fall into a beaker attached to a force transducer (Grass Model ft103 c) connected to the Dynograph so that its weight was continuously recorded as it was filled. From this record, which was calibrated by pipetting 1.0 ml samples of the perfusate into the beaker while the gauge recorded its weight, the flow rate was measured. The contents of the beaker also served to determine the number of

cells recovered during the injection.

To assess the effect of the passage of cells on the flow rate through the muscle two indices of changes in the flow rate were calculated from measurements of the flow rate at three points in the record. These points were

1. immediately before the injection of cells,
2. over the interval between 0.3 and 0.8 ml of perfusion after the injection,
3. between 7 and 15 ml of perfusion after the injection.

The measured flow rates at these points are called F_1, F_2, and F_3 in later equations. The first of the two indices is the index of temporary change in flow rate F_t

$$F_t = \frac{2F_2}{F_1 + F_3}$$

which will be less than 1 if the flow rate is reduced temporarily by more than one-half of any permanent change that might occur. The second is the index of permanent change in flow rate F_p

$$F_p = \frac{F_3}{F_1}$$

which will be 1 if the flow rate is unchanged and less than 1 if the flow rate is decreased after the passage of the cells. These indices were used to compare the results of injections through different muscles at different flow rates in order to determine whether any cell type has a consistent effect upon the resistance to perfusate flow in the muscle.

i General procedure

An outstanding feature of the results greatly influenced the design and interpretation of the experiments. It was discovered during preliminary experiments that none of the abnormal cell types tested, with the exception of the fixed cells, was retained to any great extent in the muscle, nor did their injection have any obvious permanent effect on the flow resistance in the muscle. These results, which will be described in detail later, invited the economy of using the same muscle for several injections of abnormal cells, alternating with control injections of normal cells as a standard for comparison. The fixed cells always had a permanent effect on the flow resistance and were never completely recovered. They could only be tested once on a muscle; it was then never used for further injections. The injections of fixed cells were done on four muscles and were the last injections made on each.

The arterial pressure used for these experiments was between 15 and 20 cm H_2O. The low pressure was used to allow any property of the cells that would lead to their retention or adhesion to have the maximum effect. Blood flow obtained at these pressures was about 0.05 ml per gram tissue per minute. After perfusion of the muscle with about 100 ml of Ringer's to displace the original cells, a series of injections was performed.

Table VII shows the order in which normal and abnormal cells were tested in the ten muscle preparations whose results are described here. The general design was to accomplish the following aims:

1. The transit volumes of abnormal cells were to be compared with those of normal cells injected into the same muscle. To do this, injections of normal and abnormal cells, in which the transit volumes were measured, were alternated.

2. Tests were to be made of each type of abnormal cell in at least three muscles. To accomplish this with a small number of preparations, more than one type of abnormal cell was tested in each of the last six experiments. An attempt was made to permute the order of abnormal cell injections so that those tested first in one muscle would be tested later in another.

3. Extra data on the flow resistance effects and recovery of normal and abnormal cell injecticns was to be obtained from each preparation after the eight racks of test-tubes used for the transit-volume determinations had been filled.

3 Results

a Indices

The individual results of the ten experimental preparations are given in Tables VIII to XV. The types of cells were considered separately and the results from all experiments on the same type of cell were taken together.

Because each experiment was done on a different muscle with a different lot of cells for each type, the direct comparison of mean

TABLE VII

The order of injections used in the study of abnormal red cell transit characteristics.

Expt. #	1	2	3	4	5	6	7	8	9	10
Inj. #1	C	C	C	C	C	C*	C	C	C	C
2	M	S	N	T	N	N*	S	M	T	T
3	C	C	C	C	C	C*	C	C	C	(C)
4	M	S	N	(T)	N	N*	S	M	T	T
5	C	C	C	C	C	C*	C	C	C*	C
6	M	S	N	T	S	S*	M	T	M*	N
7	C	C	C	C	C	C*	C		C*	C
8	M	S	N	T	S	S*	M		M*	N
9				N*	C*	N*	C*		C*	C*
10				N*	S*	S*	S*		T*	T*
11				N*	N*	F*	M*		C*	N*
12						F*	C*		T*	C*
13						F*	S*		F*	T*
14							M*			N*
15							F*			C*
16										T*
17										N*
18										F*

Key: C- Normal cat red blood cells.
F- Gluteraldehyde-fixed cat red blood cells
M- Normal Human red blood cells
N- Neuraminidase-treated cat red blood cells
S- Red blood cells obtained by draining the cat spleen
T- Heat-treated cat red blood cells.

Notes:
1. Injections marked in brackets were not included in results because of collection errors.
2. Runs marked with asterisks were used to measure flow effects rather than mean transit volume.

TABLE VIII Results of the transit characteristic experiments

Expt. #1. Muscle weight 23.2 gm

Run	1	2	3	4	5	6	7	8
Cell Type	C	M	C	M	C	M	C	M
Cells Recovered ($\times 10^{-9}$)	0.68	0.53	0.71	0.45	0.66	0.48	0.74	0.53
M.T.V. (ml)	0.81	0.91	1.07	0.89	0.83	0.88	0.91	1.09

Cells injected; C, o.741 $\pm$ 0.020 (SD) (n=3)

($\times 10^{-9}$) M, 0.421 $\pm$ 0.064 (SD) (n=3)

Expt. #2. Muscle weight 30.2 gm

Run	1	2	3	4	5	6	7	8
Cell Type	C	S	C	S	C	S	C	S
Cells Recovered ($\times 10^{-9}$)	-	0.72	1.00	0.68	1.00	0.70	0.93	0.71
M.T.V. (ml)	1.12	0.95	1.00	0.96	1.10	0.94	0.97	1.00

Cells Injected: C, 0.886 $\pm$ 0.007 (SD) (n=3)

($\times 10^{-9}$) S, 0.739 $\pm$ 0.006 (SD) (n=3)

TABLE IX Results of the transit characteristic experiments (continued)

Expt. #3 Muscle weight 38 gm

Run	1	2	3	4	5	6	7	8
Cell Type	C	N	C	N	C	N	C	N
Cells Recovered ($\times 10^{-9}$)	0.82	0.80	0.84	0.73	0.90	0.77	0.90	0.69
M.T.V. (ml)	0.96	0.87	0.95	0.85	0.96	0.87	1.10	0.95
F_t								
F_p								

Cells Injected: C, 0.889 ± 0.087 (SD) (n=3)
($\times 10^{-9}$) N, not determined.

Expt. #4 Muscle weight 32.7 gm

Run	1	2	3	4	5	6	7	8
Cell Type	C	T	C	(T)	C	T	C	T
Cells Recovered ($\times 10^{-9}$)	0.88	0.77	0.91	-	0.86	0.80	0.99	0.76
M.T.V. (ml)	1.14	1.26	1.11	-	1.06	1.22	1.17	1.27
F_t	1.14	0.85	1.01	-	0.96	0.94	1.04	0.98
F_p	1.00	0.99	0.99	-	0.69	0.77	0.92	0.65

TABLE X Results of the transit characteristic experiments (contined)

Expt. #4(continued) Muscle weight 32.7 gm

Run	9	10	11
Cell Type	N	N	N
Cells Recovered ($\times 10^{-9}$)	0.69	0.63	0.69
M.T.V. (ml)			
F_t			
F_p			

Cells Injected: ($\times 10^{-9}$) C 0.852 $\pm$ 0.108 (SD) (n=3)
T, 0.768 $\pm$ 0.070 (SD) (n=3)
N, 0.759 $\pm$ 0.017 (SD) (n=3)

Expt. #8 Muscle weight 28.5 gm

Run	1	2	3	4	5	6
Cell Type	C	M	C	M	C	T
Cells Recovered ($\times 10^{-9}$)	0.87	0.57	0.77	0.55	0.96	0.89
M.T.V. (ml)	0.91	0.96	0.96	0.98	0.89	1.07
F_t	-	0.89	-	1.08	1.01	0.90
F_p	-	1.17	-	1.05	1.05	0.78

Cells Injected: ($\times 10^{-9}$) C, 1.087 $\pm$ 0.167 (SD) (n=3)
M, 0.542 $\pm$ 0.011 (SD) 9n=3)
T, 0.884 $\pm$ 0.025 (SD) (n=3)

TABLE XI Results of the tranist characteristic experiments (contined)

Expt. # 5 Muscle weight 35.3 gm

Run	1	2	3	4	5	6	7	8
Cell Type	C	N	C	N	C	S	C	S
Cells Recovered ($\times 10^{-9}$)	0.93	0.86	0.81	0.56	0.77	0.63	0.70	0.65
M.T.V. (ml)	0.91	1.06	0.99	0.99	0.99	1.20	0.96	0.96
F_t	1.01	-	-	-	-	-	0.77	1.04
F_p	0.98	-	-	-	-	-	0.84	1.16

Run	9	10	11
Cell Type	C	S	N
Cells Recovered ($\times 10^{-9}$)	0.90	0.75	0.65
M.T.V. (ml)	-	-	-
F_t	1.03	0.71	0.78
F_p	0.94	0.85	0.64

Cells Injected: ($\times 10^{-9}$)

C 0.960 $\pm$ 0.040 (SD) (n=3)

S 0.703 $\pm$ 0.035 (SD) (n=3)

N 0.657 $\pm$ 0.028 (SD) (n=3)

TABLE XII Results of the transit characteristic experiments (continued)

Expt. #6 Muscle weight 18.1 gm

Run	1	2	3	4	5	6	7	8
Cell Type	C	N	C	N	C	S	C	S
Cells Recovered ($\times 10^{-9}$)	0.94	0.73	0.93	0.84	0.91	0.65	0.88	0.69
M.T.V. (ml)	-	-	-	-	-	-	-	-
F_t	0.83	0.97	0.93	0.81	0.75	0.82	0.97	1.03
F_p	0.86	1.27	1.04	0.91	0.90	0.93	1.03	1.26

Run	9	10	11	12	13
Cell Type	N	S	F	F	F
Cells Recovered ($\times 10^{-9}$)	0.83	0.70	0.028	0.029	0.037
M.T.V. (ml)	-	-	-	-	-
F_t	1.02	1.00	0.94	0.67	0.62
F_p	1.01	1.03	0.88	0.88	0.92

Cells Injected: ($\times 10^{-9}$)

C 0.908 $\pm$ 0.066 (SD) (n=3)

N 0.758 $\pm$ 0.072 (SD) (n=3)

S 0.695 $\pm$ 0.156 (SD) (n=3)

F 0.063 $\pm$ 0.036 (SD) (n=3)

TABLE XIII Results of the transit characteristic experiments (continued)

Expt. #7 Muscle weight 35 gm

Run	1	2	3	4	5	6	7	8
Cell Type	C	S	C	S	C	M	C	M
Cells Recovered (× 10^{-9})	0.77	0.90	0.70	0.98	0.75	0.48	0.72	0.50
M.T.V. (ml)	0.95	0.94	0.90	1.00	1.00	1.06	0.89	0.96
F_t	-	-	1.06	0.89	1.01	0.78	0.90	0.88
F_p	-	-	0.90	0.90	0.99	0.92	0.89	1.00

Run	9	10	11	12	13	14	15
Cell Type	C	S	M	C	S	M	F
Cells Recovered (× 10^{-9})	0.81	0.98	0.51	0.71	0.97	0.51	0.030
M.T.V. (ml)	-	-	-	-	-	-	-
F_t	0.96	0.95	0.81	0.95	0.92	0.94	-
F_p	1.00	1.05	0.94	1.10	0.93	1.02	3.05

Cells Injected (× 10^{-9}):

C, 0.897 $\pm$ 0.146 (SD) (n=3)

S, 0.969 $\pm$ 0.026 (SD) (n=3)

M, 0.544 $\pm$ 0.050 (SD) (n=3)

F, 0.154 $\pm$ 0.024 (SD) (n=3)

TABLE XIV Results of the transit characteristic experiments (continued)

Expt. #9 Muscle weight 24 gm

Run	1	2	3	4	5	6	7	8
Cell Type	C	T	C	T	C	M	C	M
Cells Recovered ($\times 10^{-9}$)	0.81	0.94	0.78	1.04	0.77	0.49	0.75	0.55
M.T.V. (ml)	1.00	1.02	0.98	1.00	-	-	-	-
F_t	0.82	1.19	-	0.77	0.88	0.89	0.92	0.96
F_p	0.94	0.78	-	1.11	1.01	0.92	0.96	1.19

Run	9	10	11	12	13
Cell Type	C	T	C	T	F
Cells Recovered ($\times 10^{-9}$)	0.76	1.36	0.91	1.13	0.036
M.T.V. (ml)	-	-	-	-	-
F_t	0.99	0.95	0.89	0.89	0.13
F_p	1.03	1.02	1.04	0.94	0.35

Cells Injected: ($\times 10^{-9}$)

C, 0.901 $\pm$ 0.083 (SD) (n=3)

M, 0.554 $\pm$ 0.026 (SD) (n=3)

T, 1.123 $\pm$ 0.067 (SD) (n=3)

F, 0.154 $\pm$ 0.024 (SD) (n=3)

TABLE XV Results of the transit characteristic experiments (continued)

Expt. #10 Muscle weight 42 gm

Run	1	2	3	4	5	6	7	8
Cell Type	C	T	C	T	C	N	C	N
Cells Recovered (× 10^{-9})	0.93	0.82	-	0.80	1.08	0.84	0.95	0.89
M.T.V. (ml)	1.14	1.30	-	1.11	1.13	0.96	0.97	1.12
F_t	0.80	0.68	0.81	0.77	-	0.77	-	-
F_p	0.85	0.85	0.92	1.02	-	1.18	-	-

Run	9	10	11	12	13	14	15	16
Cell Type	C	T	N	C	T	N	C	T
Cells Recovered (× 10^{-9})	0.95	0.71	0.95	0.98	0.90	1.00	1.05	0.64
M.T.V. (ml)	-	-	-	-	-	-	-	-
F_t	0.95	0.57	0.98	1.05	0.85	0.83	0.63	0.46
F_p	1.03	0.93	0.81	1.19	0.91	1.03	1.07	0.76

Cells Injected: (× 10^{-9})

C, 1.099 $\pm$ 0.077 (SD) (n=3)

T, 0.992 $\pm$ 0.073 (SD) (n=3)

N, 1.024 $\pm$ 0.045 (SD) (n=3)

F, 0.055 $\pm$ 0.027 (SD) (n=3)

TABLE XVI Results of the transit characteristic experiments (continued)

Expt. #10		
Run	17	18
Cell Type	N	F
Cells Recovered ($\times 10^{-9}$)	1.01	0.039
M.T.V. (ml)	-	-
F_t	0.88	-
F_p	1.04	0.04

TABLE XVII. Summary of the values (Mean ± S.E.M.) of the indices R, T_1, T_2, F_t, F_p for four types of abnormal cells in muscle. F_t* and F_p* are these indices for normal cell injections.

Index	Heat-treated cells	Human Cells	Neuraminidase treated cells	Spleen Cells
R (%)	93.8 ± 4.4	102.8 ± 3.8	96.8 ± 4.0	97.0 ± 1.3
no. of experiments (muscles)	10 (4)	12 (4)	14 (4)	14 (4)
P (μ_R = 100%)	<0.1	>0.5	>0.3	>0.3
T_1	1.064 ± 0.025	1.006 ± 0.031	0.947 ± 0.039	1.004 ± 0.053
no. of experiments (muscles)	4(3)	6(3)	6(3)	6(3)
P (μ_{T_1}=1)	<0.1	>0.5	>0.5	>0.5
T_2	1.103 ± 0.026	1.053 ± 0.038	0.967 ± 0.044	1.002 ± 0.043
no. of experiments (muscles)	7(4)	8(3)	8(3)	8(3)
P (μ_{T_2}=1)	<0.01	>0.2	>0.3	>0.5
F_t	0.79 ± 0.05	0.90 ± 0.04	0.88 ± 0.04	0.90 ± 0.04
no. of experiments (muscles)	10(4)	8(3)	9(3)	9(3)
P (μ_{F_t}=$\mu_{F_t^*}$)	<0.01	>0.5	0.2	>0.5
F_p	0.88 ± 0.12	1.03 ± 0.04	1.03 ± 0.09	0.94 ± 0.06
no. of experiments (muscles)	10(4)	8(3)	6(3)	9(3)
P (μ_{F_p}=$\mu_{F_p^*}$)	<0.05	>0.1	>0.3	>0.3

transit volumes and numbers of cells recovered was not possible.

In the case of the recovery of cells the number of recovered cells was compared with the estimated number injected and a recovery index, R was calculated

$$R = N_R/\bar{N}_i \times 100\%$$

N_R is the total number of cells recovered in a particular injection, as calculated from the contents of the samples as described before, or as determined from the concentration of cells and the volume of fluid in a beaker which collected all the outflow after the injection. $\bar{N}_i$ is the average of the estimates of the number of cells injected from the calibration procedure. R can be calculated for each injection and should be independent of the small variations in $\bar{N}_i$ so that experiments using different stocks of cells can be compared. However R does not include any information about the variation in the number of injected cells, and thus a simple statistical comparison of R to 100% may lead to a false conclusion. I have therefore normalized the individual values of both N_R and N_i obtained in a given experiment from a given cell stock suspension, to the average of the values of N_i. These normalized values were then pooled for al the experiments on the same cell type and the hypothesis that the means of the normalized values of N_R and those of N_i were different was tested using Student's t-test. This takes into account the variation in N_i and at the same time pools the results of several experiments to obtain a result that may have general validity.

The mean transit volume will depend to a certain extent upon

the muscle used for study, as well as other factors, such as its preparation and the patency of its vascular bed. In many cases an injection of abnormal cells was preceded and followed by injections of normal cells. In some others there was only a preceding injection of normal cells. These injections of normal cells were used as the standard of comparison for the mean transit volume of the abnormal cells. Two indices of the ratio of mean transit volumes of abnormal and normal cells were calculated. They are:

$$T_1 = \frac{2\ \bar{v}^*}{\bar{v}_b + \bar{v}_a}$$

which can be calculated for the injections which were both preceded and followed by normal cell injections, and

$$T_2 = \frac{\bar{v}^*}{\bar{v}_b}$$

which can be calculated for almost all the abnormal cell injections. $\bar{v}^*$ is the mean transit volume of the abnormal cell injection, and $\bar{v}_b$ and $\bar{v}_a$ are the mean transit volumes of the previous and following normal cell injections, respectively. T_1 is a more selective index, in that it takes into account factors which might sequentially change the mean transit volume of normal cells from injection to injection, but it can only be applied in a few cases. These indices were compared to 1.0 which is their expected value if the mean transit volumes of the abnormal and normal cells tended to be the same in

a given muscle.

Thus, in summary, the results provide some means of assessing the possible effects of abnormal cells being injected into the muscle vascular bed. They are the loss of cells, expressed by R, the abnormality of transit characteristics, expressed by T_1 and T_2, and changes in flow resistance, expressed by comparison of the flow rate indices with those of the normal cell injections. The average values for these indices, and the significance of the relevant statistical comparisons, are given in Table XVII. With R, T_1 and T_2 are also given the average values for F_t and F_p. The relevant statistical test compares the means, using Student's t-test with those obtained with normal cells.

b Normal cat cells

In addition to serving as a standard of reference for describing the behaviour of abnormal cells, these injections were considered on their own. The distribution of transit volumes measured in each experiment was reproduced approximately in part of the output of the program "GEORGE", and always resembled the general form of Figure XVII and XVIII. In 45 injections in the ten experiments, R had a mean value of 93.2 $\pm$ 1.5 (SEM) %. The difference between R and 100% was significant overall, when the variation in the number of injected cells was taken into account as described before.

The mean transit volume was determined for 32 injections of normal cells in ten muscles. The average value is 0.988 $\pm$ 0.018 (SEM) ml. The ten muscles weighed 32.8 $\pm$ 5.3 (SD) gm. The mean transit volume is positively correlated with the weight of the muscle,

but poorly (R^2 = 0.127). The intercept of the line of best fit at zero muscle weight is 0.7 ± 3.7 (SD) ml; it is not significantly different from the volumes of the injection and collection cannulae, which total 0.35 ml. The large standard deviation in the intercept, which is related to the poor correlation, makes it quite meaningless. The mean transit volume of normal cells did not appear to be related to the position of the injection in the sequence of injections in any systematic way.

In 26 measurements, F_t for normal cells had an average value of 0.93 ± 0.02 (SEM) indicating that increased resistance to flow, relative to that with dextran-Ringer's accompanies the passage of normal cells through muscle. The difference between F_t and 1.0 is statistically significant ($p < .05$). In the same measurements F_p had an average value of 0.98 ± 0.02 (SEM) which was not significantly different from 1.0. As expected, the passage of normal cells does not have a detectable permanent effect on the resistance to flow through the muscle vascular bed.

c Fixed cells

The injections of fixed cells were done only once on each muscle where they were tried, except in one case. Thus the recovery of cells was not as precisely estimated, as was the case for other types of abnormal red cells. The mean transit volume of the recovered cells was not measured in these experiments. Visual observation of the passage of the cells from the venous cannula showed that, of the cells recovered at constant pressure perfusion, the majority were recovered in the first 3 ml of perfusate after the injection,

as with the other cell types.

In general the counts of both calibration sham injections and those of recovered cells were less reproducible than those of any other cell type tested. This is probably related to the fact that the packed fixed cells usually required violent agitation to resuspend them in saline, and it is possible that aggregates of cells were not completely broken up.

In two of the experiments, #7 and #9, the estimated number of cells injected was (1.54 $\pm$ 0.24 (SD)) $\times 10^8$. Of these, 21.4 $\pm$ 2.8 (SD) % were recovered by perfusion at the same pressure. The resistance to blood flow increased to 3.08 $\pm$ 0.28 times the original value after the injection. In one of the experiments, there was a temporary increase in resistance to 15 times the original value which accompanied the passage of the cells through the muscle and disappeared as the majority of the collected cells appeared. In the other there was no temporary change that was noticeably greater than the permanent change.

In each experiment an attempt was made to force out the trapped cells by increasing the perfusion pressure. This was done manually, as the system does not allow changing the pressure rapidly to a pre-determined value. In experiment #7 perfusion at 200 cm H_2O for 20 sec released 30% of the injected cells in 6 ml of outflow; in the other experiment perfusion at 120 cm H_2O for 60 sec released 6% of the cells in 12 ml of perfusate.

In the other experiments, #6 and #10, the stock of fixed cells was further diluted with PBS to about half the concentration used in the experiments described above. In experiment #6 the

estimated number of injected cells was $(6.27 \pm 3.64 \text{ (SD)}) \times 10^7$. This number of cells was injected three times in succession with no attempt to purge the cells between injections. The first injection was followed by the collection of 44% of the estimated number of injected cells in 18 ml of perfusate. The second yielded 46% of the injected number in 19.5 ml, the third 59% in 17.8 ml. The first two injections caused the resistance to Ringer's perfusion to rise to 1.13 and 1.14 times the previous value, the third caused a further rise to 1.08 times the previous value.

In experiment #10, the estimated number of cells injected was $(8.58 \pm 0.21) \times 10^7$. 46% of these were recovered. Purging at 60 cm H_2O recovered a further 42% of the cells in 13 ml of perfusion. Before the purging the resistance to flow increased to 17 times the original value. Purging, followed by perfusion at the higher pressure, encountered a resistance to flow less than the original value: however the determination, made at a higher pressure, does not necessarily indicate that the vascular bed is as free from blocked pathways as it was before the injection.

In summary, the few experiments done with fixed cells indicate that there is always an increase in the resistance to perfusion following an injection of these cells, but it is extremely variable. The recovery of cells, as a fraction of the number injected, increases with decreasing concentration of the injected cells, from about 20 % to about 50% over the range of concentrations used. It is possible to recover more cells by perfusion at higher pressure, but even at unphysiologically high pressures not all the cells are recovered.

4. Discussion

a The recovery of cells

The most surprising result of these experiments is the consistent failure to recover as many of the normal cells as the estimated number injected. The average of the fractions of normal cells recovered is the lowest of any of the cell types studied, except for the fixed cells.

There is some reason to question the significance of this result. The variation in the estimates of the numbers of cells injected and recovered is of the same order of magnitude as the differences between the averages of these numbers. The number of normal cells recovered was in only a few experiments significantly different from the number injected; this was shown by applying Student's t-test to the estimates of each number obtained. The statistical significance of the difference comes from the large number of tests and the consistency of the difference between the estimates of the numbers of cells injected and those recovered.

It is unlikely that the sham injection method of estimating the number injected would give results with a consistent bias. The estimate of the number recovered may be affected by the calculation of the "background" of cells leaving the muscle during the passage of the bolus of injected cells. The background was assumed to be a very small fraction of the total number of cells recovered, about 1%. This calculation presupposed that the manner in which the cells within the muscle at the beginning of an injection would leave the muscle was unaffected by the injection.

This supposition may not be true. Zweifach (1940) described a phenomenon which is relevant to this study. He was observing, under the microscope, the capillaries of frogs, which he perfused with cell suspensions as well as cell-free solutions. He noted that there were certain vessels that were always perfused and others that were not perfused when cell-free or particle-free solutions were used. He was able to observe stationary red blood cells in these vessels which were immediately flushed out when the perfusion by cell or particle suspensions was resumed. Assuming that this represents a property of blood flow which is also true in mammals, it may mean that the presence of cells in the injection bolus makes a larger part of the vascular bed available for perfusion by the bolus, but, when it is followed by the cell-free perfusate, this extra volume is isolated from perfusion and thus releases its cells very slowly.

The effect of this extra volume on the number of cells recovered is difficult to predict, for it would both release cells from its store to be collected with those from the injection when it enters, and also claim cells from the injection to replace these cells when the inflow is cleared of cells. The net loss from the injection would be greatest when the number of injected cells was largest and the store of cells in the muscle was least. It would be difficult to predict the store in the muscle from the number released per unit time; the washout kinetics are not that precisely known. It is worth noting, however, that the greatest recovery of cells was in the case of the human cells where the number injected was the smallest because of the larger size of these cells.

Whether or not this sort of exchange occurs, the results of these experiments indicate that R itself is a poor indicator of whether cells would be trapped during the passage of blood through the vascular bed of skeletal muscle in an intact mammal. Only in the case of the fixed cells, where R is clearly less than 100%, can we say that trapping is bound to occur. In the other cases, as with the normal cells, the recovery index cannot be used to decide whether cells are trapped in the muscle.

The measurements of the recovery of the fixed cells are limited in their own way. A few general observations can be made from the scanty and extremely variable results from these experiments.

First, there is a partial recovery of the fixed cells. In view of the observations of Levesque and Groom (1977) on the spleen, and those of Chien _et al_. (1967) on the filtration of fixed cell suspensions through pores of diameter similar to that of the cells, it is surprising that the fixed cells should pass through the muscle at all. It suggests that there is sufficient elasticity in the capillary wall to allow a cell of external diameter about 12% greater than the internal diameter of the vessel to pass through. It would be of interest to try this experiment on another species where the ratio of the red blood cell diameter to the capillary diameter is different.

The fact that the recovery of fixed cells as a fraction of those injected increases as the concentration decreases, suggests that there is a co-operative effect between cells that contributes to the trapping, a sort of 'log-jam' that might occur in the pre-capillary arterioles where more than one cell may appear in a cross-section at one time.

It might be proposed that fixed cells do pass through capillaries in single file, but their behaviour in bulk causes the effective viscosity of their suspensions to rise to infinity in larger vessels. The tendency of fixed cells to adhere to each other may contribute to this.

b The mean transit volumes

The transit volume-distribution shown in Figures XVII and XVIII is similar in form to those of Baker and O'Brien (1964) and Groom (1966) for labelled red cells in normal blood. Precise comparisons are difficult because of the differences in preparations used, but this indicates that the use of a cell-free perfusate does not have a significant effect on the form of the transit-volume distribution. The mean transit volume for normal cells, about 0.65 ml on the average, if the effective volume of the cannulae is subtracted from the measured result, is very close to the half-volume of Compartment I of the model derived from the washout studies. This is to be expected, as this compartment receives all but about 2% of the perfusion flow.

The mean transit volumes for the abnormal cells, other than the fixed cells, are not significantly different from that of the normal cells,except when the heat-treated cells are compared to the normal cells of the previous injection. This latter difference, and the difference in the flow during the passage of the cells, suggests that heat-treated cells are delayed in their passage through the vascular bed of skeletal muscle more than any of the other types of abnormal cells

tested, except for the fixed cells.

In experiment #4, where heat-treated cells were compared with normal cells in the same muscle, the distributions of transit volumes, as produced by the computer, were compared. The volumes perfused for the recovery of 10%, 20%, 40%, 60%, 80% and 90% of all those cells recovered were greater, in every case for the heat-treated cells. This supports the idea that the trapping of heat-treated cells is a uniform phenomenon, and not limited to some small part of the vascular bed.

c The effect on the flow rate

The changes in resistance to flow of the perfusate solution during and after the passage of the slug injections of red cells were considered to be a sensitive indicator of cell trapping. Certainly, in case of the fixed cells, there was a profound change in the resistance to flow which accompanied the trapping of relatively small quantities of red cells; about 4×10^7 cells retained within a muscle raised the flow resistance to more than three times its original value. This quantity is about 5% of the numbers injected in the tests of the other cell types and I have shown that it is difficult to detect losses of this magnitude by counting the recovered cells.

All of the cell types tested, including normal cells, produced a significant temporary reduction in the flow rate during their passage. In the case of normal cells, this change is expected as the effective viscosity of blood in the muscle vascular bed, as measured by Baekstrom *et al.*(1971), 2.3 cP, is larger than that of the

Dextran-Ringer's (about 1.8 cP). The fact that there is no permanent change in resistance suggests that the apparent loss of cells, as measured by the recovery index, is not the result of trapping.

Of the other abnormal cell types, only the heat-treated cells affected the flow resistance in a way that was significantly different from that of normal cells. The temporary increase in flow resistance, shown by an average value of F_t significantly greater than that of the normal cells, shows that there was more resistance to the passage of the heat-treated cells. The permanent change after the passage of the majority of the heat-treated cells, as shown by the significantly lower value of F_p, indicates that a small fraction of these cells may block pathways through the muscle, thus increasing its resistance to the flow of the perfusion solution.

Of the remaining cell types, human cells, spleen cells, and neuraminidase-treated cells, the most remarkable characteristic shown here is that their passage through the vascular bed of cat skeletal muscle is indistinguishable from that of the normal cat cells. In this the muscle is very different from the spleen, where injections of all of these cell types lead to the retention of some cells and heat-treated cells are not exceptional in the fraction of them which is retained.

The muscle appears to be more sensitive to abnormalities in deformability than to those in red cell size or surface character.

5 Conclusions

The purpose of this series of experiments was to determine whether the factors that cause cell trapping in skeletal muscle are the same as those which cause cell trapping in the spleen, with particular reference to the reticulocytes that are released slowly from both organs on perfusion. They have shown that cells with abnormal deformability are delayed in their passage through the muscle, and trapped to some extent, especially if the cells are rigid. Cells with abnormal surface characteristics, which lead to their trapping in the spleen, are not affected in any way in their passage through muscle. Since it was a surface characteristic that was believed by Song and Groom (1974) to be the cause of the trapping of reticulocytes in the spleen, the mechanism for trapping cells in the muscle does not seem to be the same as this. However, it seems unlikely that the bulk character of the reticulocytes is responsible for their being trapped, because the differences between reticulocytes and mature cells in these respects must be far smaller than those between mature normal cells and heat-treated cells, which are only slightly trapped. Thus it does not seem that a cell characteristic alone is responsible for the slow clearance of reticulocytes from the vascular bed of skeletal muscle. The true cause must be more complex, perhaps a combination of red cell and vascular bed properties, or a characteristic of the washout process itself.

CHAPTER IV.

GENERAL CONCLUSIONS

1 The hypotheses tested by these experiments

The washout studies of Groom et al.(1973) showed that the perfusion of the muscle vascular bed by Ringer's solution revealed a great heterogeneity in the red blood cell store in the muscle. In the first chapter I have reviewed some possible causes for this heterogeneity. They are: heterogeneity in the pattern of the vascular bed, heterogeneity in the physical properties of red cells, and heterogeneity of flow distribution through the vascular bed imposed by the action of vascular smooth muscle as a mechanism for controlling the distribution of blood flow.

The second and third chapters describe tests in which the second and third of the possible causes described above were altered or investigated in order to assess their contribution to the heterogeneity of the red cell store.

2 Physiological control mechanisms

To investigate the effect of the physiological control mechanisms that regulate the distribution of blood flow on the red cell washout kinetics, these mechanisms were modified, as in the comparison between resting and contracting muscles, or abolished, as in the vasodilated muscles.

The number and gross character of the compartments observed by Groom et al. (1973) are not affected by these mechanisms. The only change observed is a slight decrease in the volume of the best perfused compartment in the resting muscle when it is compared with

the others. This evidence does support accepted views about differences in the flow patterns between resting and working muscles (e.g. Kjellmer 1964; Renkin, Hudlicka and Sheehan 1966; Moore and Baker 1971), but it does not begin to explain the inhomogeneity of the washout of the red cell store.

Indeed, a consideration of the experimental methods of Groom et al. (1973) suggests that their washouts were performed on passively-dilated vascular beds in which smooth muscle was incapable of establishing the flow patterns of the resting muscle vascular bed. The interruption of flow, the acidity and absence of plasma in the Ringer's solution they used each would relax the vascular smooth muscle. Thus these results in themselves show that the perfusion of a dilated vascular bed is inhomogeneous as far as the red cell store traces it. This is in contrast to the conclusions of many other observers that the vasodilatation induced by the interruption of blood flow or physical work of the muscle tends to make the distribution of blood flow more homogenous.

3 Heterogeneity among the red cells

After physiological control mechanisms were dismissed as a cause for the pattern of red cell washout, inhomogeneites in the nature of the red cells were next considered. The reticulocyte differs from a mature cell slightly in size and in its adhesion in the spleen. Could these differences be responsible themselves for its sequestration in the muscle vascular bed? Chapter III describes the test of this question. Abnormal red cells were injected into the vascular bed. Except for the fixed cells the recovery and transit-time of each

of the types studied was very much like that of the normal cat cells. This shows that the muscle is not very sensitive to abnormalities in cell shape, size or deformability, except in the extreme case of the rigid fixed cells, and possibly in the case of the heat-treated cells. The muscle is insensitive to the properties of the neuraminidase-treated cells and the splenic red pulp cells that cause them to adhere within the spleen.

The difference between the muscle and the spleen in the capacity of their vascular beds to trap abnormal red cells suggests that the spleen must have a special function as a filter for the cellular part of the blood. The spleen traps red blood cells both by surface adhesion (Song and Groom 1972 and 1974) and by mechanically testing the shape and deformability of the cells (LeBlond 1973: Chen and Weiss 1973). The presence of two or more mechanisms for recognizing abnormal cells suggests a special adaptation for this task.

From a teleological point of view, it would not be satisfactory if the muscle vascular bed were as effective a trap for abnormal red blood cells as the spleen. This would reduce the effectiveness of the splenic function in the intact circulation and adversely affect the flow of blood through the muscle, since I have shown that the slow release of cells is accompanied by a slow release of plasma, indicating that the trapped cells block the flow of blood. The present results are thus not surprising.

What is surprising is that the muscle retains reticulocytes during perfusion by cell-free solutions, as does the spleen, but does not trap any abnormal cells including the

reticulocyte-rich cells from the splenic red pulp, on intra-arterial injection.

The two situations are different and certain assumption were made about the trapping mechanism when the injection studies were interpreted. The conclusion is that the trapping of the reticulocytes in the vascular bed of muscle is a limited phenomenon. The reticulocytes do not attach themselves indiscriminately to the walls of the vascular bed of skeletal muscle. Rather they must be retained in the perfused muscle by special parts of the vascular bed which, because of anatomical or flow peculiarities, are more able to hold them. In the model proposed to explain the red cell washout kinetics the slow compartment, which Groom et al.(1973) ascribe to the trapping of reticulocytes, contains less than 10^8 cells. In the perfusion before each injection experiment this compartment was not cleared of even half of its cells, so that its capacity to accept new cells would be much less than 10^8 cells. Since the injections were of 10^9 cells each, the small difference caused by trapping in the slow compartment, if it is a limited trap for reticulocytes, would not have been detected. The location or nature of the trap for reticulocytes is a mystery, for the muscle vascular bed does not appear to have any anatomical part that would favour this sort of trapping.

4 What remains to be explained

The experiments described here have dismissed, in a sense, two easily tested hypotheses about the cause of the inhomogeneity of the red cell store in skeleltal muscle. Before considering other

possible causes as hypotheses worthy of testing, it is useful to review what has to be explained by these hypotheses.

The four compartments of our model might be best described by the results from contracting muscle in Table II. I have shown that the vasodilated muscles are not significantly different from these in the actual washout kinetics, except at the beginning of the perfusion, where an experimental artifact may have altered the original cell concentration in the muscle. The vasodilated muscles also were not perfused to the same extent per unit weight, so the parameters for Compartment IV (and hence Compartment III to a lesser degree) are not as certain. The pattern for the contracting muscles is very similar to that discovered by Groom *et al.*(1973) for their "resting" muscles.

These authors assigned only three distinct compartments to their model, despite the four components of their fitting to the washout curve. The criterion of Van Liew (1962) that they used in arguing that their Compartments II and III could be the result of a continuum of similar processes rather than two separate processes is that such pairs of components have half-lives in ratios of less than one to five. In the case of the plasma washout this is certainly true of Compartments II and III; for the red cell washout the ratio of half-volumes is 5.3, so that the compartments may be the result of distinct processes. However the evidence is not conclusive, and I shall consider Compartments II and III together rather than attempt to draw distinctions between them. Thus we have three distinct parts of the red cell store: a very well perfused compartment, an array of

relatively poorly perfused ones, and a very poorly perfused one that has some relationship with the reticulocytes.

The distribution of flow among the compartments is most inhomogenous. The well perfused compartment has 98% of the total flow. It is unlikely that the remaining flow could be detected by other methods, such as those which use tracer clearance. The usual distinctions between poorly-perfused and well-perfused circulations in muscle, where they have been quantitative, leave about 80-90% of the flow to the well-perfused part (Kjellmer, Lindbjerg, Prerovsky and Tønnesen 1967; Kjellmer and Prerovsky 1968). Nakamura, Suzuki, Tsuiki and Tominaga (1972) assign 40% of the total flow in muscle to the shunt circulation.

It is misleading, however, to compare the results of tracer clearance studies with those from the red cell washout experiments. For example, a tissue rich in arteriovenous anastomoses such as the fascia and tendons of skeletal muscle (Grant and Payling Wright 1970; Eriksson and Myrhage 1972) would appear poorly perfused in a tracer clearance study (e.g. Walder 1968) because these vessels have a limited capacity for exchange. The red cell store of such a tissue may be well perfused, because these vessels have a high flow rate.

On the other hand, a tissue with a large number of "closed" capillaries would appear poorly perfused in a tracer clearance study as well. The red cell washout of such a tissue may show a different picture. If the "closed" capillaries are deficient in red blood cells due to plasma skimming, as has been suggested by some observations (Moore and Baker 1971; Johnson, Blaschke, Burton and Dial 1971), they would contribute relatively less to the red cell store

and that store would look more uniformly perfused. Alternatively, if local variations in flow such as those caused by blockage of capillaries by white cells caused temporary closings of some capillaries, tracer studies would show poor perfusion of the bed. The red cells may be slowly or rapidly washed out, depending upon the length of time that these interruptions last. Eriksson and Lisander (1972a) state that these interruptions may last from a few seconds to a minute, and Honig, Feldstein and Frierson (1977) report that the observed range of capillary transit times in the rat gracilis muscle extends from 90 msec to 43 sec. The half-volumes of Compartments I and II of the present model of the red cell washout kinetics correspond to times of 4.3 sec and 64 sec at the rather high flow rates used in these perfusions, so that blood cells held in the capillaries for intervals in these ranges may contribute to both compartments. The observation that only 11% of the capillary transit times exceeded 8 sec in the resting rat gracilis (Honig <u>et al</u>. 1977) and the fact that our perfusion flow rates were higher than those of resting muscle suggests that most of the cells held within the capillaries would appear in Compartment I despite the interruptions of flow. The clear distinction between Compartment I and the others in half-volume also indicates that a single homogenous process could not contribute to both compartments together. It seems that the compartments other than the best perfused represent parts of the vascular bed which are uniquely poorly perfused.

The distribution of volumes among the compartments of the red cell store is much more uniform. Here about twenty percent of the cells are in poorly perfused areas of the vascular bed. The presence

of such a large fraction of the red cells in such an unusual part of the vascular bed should have drawn the attention of those who have observed the microcirculation visually. The absence of such observations suggests that the red cell washout is showing something quite different from the normal circulation. What part of the dilated vascular bed could hold up to twenty percent of the red cells within a skeletal muscle, and be perfused by less than three percent of the flow so that it requires perfusion of the muscle by more than ten times its vascular volume to clear half of these cells? This is the principal question posed by the present work.

5 Poorly perfused areas in the vascular bed of skeletal muscle

I have already described some observations on the heterogeneity of capillary transit times that would make some parts of the normal vascular bed appear less well perfused than others. This heterogeneity, although physiologically significant, is much less than is required to explain the pattern of red cell washout. Abnormalities or special regions in the vascular bed of the isolated muscle may be the cause of this pattern.

The isolation of the muscle may be indirectly the cause of the poorly perfused part of the red cell store. The injury in cutting skin, fascia and tendons may promote leucocytosis and increase the blockage of capillaries by white cells. The injury may also promote aggregation of red cells, as do many other types of injury (Knisely 1965). It is expected that similar problems would affect the work of those who study the living microcirculation under the microscope. However,

whereas these authors can choose areas of the muscle which have better flow, the red cell washout studies must include all of the vascular bed, including the vessels near tendons and the surface.

Another cause for poorly-perfused areas of the vascular bed would be the ligation of a number of small blood vessels which connect the muscle to other parts of the leg. The gastrocnemius-plantaris muscle group is well-suited to perfusion studies because it is principally served by one major artery and one major vein. The other vessels may serve small parts of the vascular bed which have connections, as does that of the sural artery (Folkow, Sonnenschein and Wright 1971), with the main vascular bed at the pre-capillary level. The ligation of thses vessels would leave the corresponding vascular beds connected with the main bed but poorly-perfused. The filtration of fluid from the tissue, which is often edematous in these experiments, into the capillary beds of these areas may slowly expel the red cells. These areas cannot account for twenty percent of the red cells in the muscle, but they may contribute to the less well perfused components that correspond to Compartment III of the original four-compartment model.

6 The red cell washout process

To this point I have attempted to interpret the results of the red cell washout studies in terms of the normal passage of blood through muscle. A question remains: Does the replacement of the inflowing blood with a cell-free solution affect the pattern of perfusion of the vascular bed? Is the pattern of the washout of the red cell store by cell-free solutions a true picture of the kinetics of red cells in the normal circulation?

A factor very likely to affect the kinetics of the red cells during washout is the difference between the viscosity of the cell-free perfusate and the apparent viscosity of blood in the vascular bed. The most viscous perfusate used here, the Dextran-containing Ringer's solution, has a viscosity of about 1.8 cP at 37°C. This is much less than the apparent viscosity of blood in bulk flow. However, in the microcirculation, the resistance to blood flow is less than would be expected, because of the Fahraeus-Lindquist effect. Thus the changes in the pattern of flow and pressure distribution in the finer parts of the vascular bed when it is perfused by Ringer's solution are less than that a comparison of viscosities would predict. In the capillaries, where the greatest inhomogeneities in resistance to perfusion may occur, the ratio of the resistance to blood flow to that of a cell-free solution is only 1.5, instead of about 4 or 5, as it is in bulk flow (Jay, Rowlands, and Skibo 1972) so that the washout of cells from the capillaries ought not to be greatly different from their passage during perfusion of the bed by normal blood.

However there is evidence that this may not be the case. I have mentioned the observations of Zweifach (1940) on the perfusion of the vessels of the frog mesentery and tongue by cell-free solutions in Chapter III. The cell-free solutions tend to bypass the majority of the capillaries _via_ preferential channels, leaving them filled with red cells. The presence of cells or other particles in the perfusate is required for uniform perfusion of the capillaries.

The vascular bed of muscle is relatively free of arterio-venous anastomoses through which the capillaries could be bypassed. However the variation in lengths and resistances among capillaries

may result in some capillaries being cleared of cells more quickly than others, and these might serve as the preferential channels, allowing the perfusate to bypass the other capillaries.

This may explain the great inhomogeneity in the washout of the red blood cells by cell-free solutions, and would be a serious objection to the use of the washout method in studying the normal distribution of blood flow.

The value of the washout method may depend on the answer to a simple question. Suppose that two capillaries of different lengths carry blood in parallel between a common arteriole and a common venule. Normally blood cells would move with only slightly different velocities through each. If the vascular bed, of which these form a part, is perfused with a cell-free solution, it is likely that the shorter one will be cleared of red cells first. What will happen to the red cells in the other?

The results of Jay _et al._ (1972) suggest that the pressure difference across the capillaries, although it may be reduced by the fall in resistance in the cleared capillary, will still cause the red cells to move at a speed only slightly less than that which they would have during blood perfusion. The observations of Zweifach (1940) suggest that the red cells may remain stationary. Objections to the relevance of either observation can be made; that of Jay _et al._ (1972) was made on blood flowing in glass tubes, and that of Zweifach (1940) in a vascular bed which has many arteriovenous anastomoses. The question may only be resolved by experiment, as I suggest in the second of the proposals for further study.

7 Summary of the conclusions

The inhomogeneity of the red cell washout pattern leading to the separate components of the cell store proposed by Groom et al. (1973) is not due to the same mechanism which causes inhomogeneity in the blood flow through resting muscle and is modified in contracting or vasodilated muscle.

The poorest-perfused compartment is not due to the abnormal adhesiveness of the reticulocytes acting indiscriminately in the vascular bed as is the case in the spleen, but must be due to the trapping of these cells in som iunidentified part of the vascular bed which is more capable of holding them.

The vascular bed of skeletal muscle is generally insensitive to a variety of red cell abnormalities which lead to significant trapping of red cells in the spleen. Only red cells rendered rigid by chemical fixation are retained to any great extent on injection into the muscle circulation, and these are not completely retained.

8 Proposals for futher studies

1. Studies in which isolated gastrocnemius muscle of cats are perfused with about 0.05 ml per gram of tissue in order to clear the red blood cells of Compartment I would reveal the physical location of the remaining cells if the muscles were subsequently fixed and sectioned to allow the identification of the red cells within the tissue. These studies present difficulties because fixing the muscles by perfusion is not possible and sections thin enough to allow visual examination may be too thin to retain the red blood cells in the capillaries. If possible the distribution of the remaining red cells through the muscle tissue should be examined to determine whether the cells are uniformly distributed through the muscle mass or concentrated in areas such as the tendons, fascia, the muscle surface or tissue near ligated small vessels.

2. A study by means of intravital microscopy of the perfusion of a muscle by cell-free solutions would reveal whether such perfusion distorts the normal pattern of blood flow. This may be performed on the frog sartorius, and later on mammalian muscles such as the cat tenuissimus or rat cremaster. The object would be to estimate the numbers of cells remaining in the vascular bed after perfusion of the muscle with known volumes to see whether this correlates with the results obtained by counting cells in the venous outflow. The poorly perfused part of the cell store corresponding to Compartments II,III and IV should be quite distinct from the well perfused part.

3. Studies of the red cell washout of other muscles than the cat gastrocnemius may reveal whether the red cell washout pattern observed here is unique to the cat gastrocnemius or generally true of muscle tissue. The varying patterns of collateral circulation and anastomoses between small vessels in different muscles (Hudlicka 1973, p.3) may be reflected in different patterns of red cell washout in the poorly-perfused parts of the circulation, if it is indeed true that these features affect the washout pattern.

Appendix 1: The computer program "QCPEL" used for the analysis of the washout data by the method of curve-peeling.

```
00010 REM   THIS PROGRAM IS DESIGNED TO ANALYZE WASHOUT DATA
00011 REM  BY THE METHOD OF CURVE PEELING. THE DATA ARE IN FILES
00012 REM  IN THE FORM OF LISTS OF THE VOLUME PERFUSED WHEN A SAMPLE
00013 REM  IS TAKEN AND THE LOGARITHM (BASE 10) OF THE CONCENTRATION.
00014 REM  THE DATA FROM SEVERAL EXPERIMENTS CAN BE ANALYZED INDIVID-
00015 REM  -UALLY OR, AS AN ALTERNATIVE, THE DATA CORRESPOND-
00016 REM  ING TO THE SLOWEST-DECAYING COMPONENT FROM ALL CURVES CAN
00017 REM  BE FITTED TO A COMMON LINE WHICH IS THEN SUBRTRACTED FROM
00018 REM  EACH CURVE AS THE FIRST 'PEEL',AND IS TAKEN AS THE SLOW-
00019 REM  EST-DECAYING COMPONENT OF EACH CURVE. THE PARAMETERS OF
00020 REM  EACH COMPONENT IN ALL THE CURVES ARE TAKEN TOGETHER AND THE
00021 REM  AVERAGE AND STANDARD ERROR OF THE MEAN ARE COMPUTED.
00022 REM
00023 REM
00100 DIMENSION V(27,50),C(4,50),X(500),Y(500),L2(27,50)
00105 DIMENSION N1$(27)
00106 DIMENSION V5(27)
00107 DIMENSION I9(27,5),K(27,5),V8(27,5),S(27,5),F1(27,5),F2(27,5)
00108 DIMENSION W(27)
00110 FILES QREST,NQREST
00111 REM
00112 REM    THE FOLLOWING LINES READ THE DATA
00119 REM
00120 RESTORE #1,2
00130 INPUT #1,P1,K1,N1
00140 FOR I=1 TO N1
00150 INPUT #1,P1,I2,W(I)
00160 INPUT #2,P1,N1$(I)
00165 IF I2=I THEN 190
00170 PRINT "TROUBLE IN READING EXPT.  ";I
00180 STOP
00190 FOR K=1 TO K1
00200 INPUT #1,P1,K2,V(I,K),L2(I,K)
00210 IF K2=K THEN 240
00220 PRINT "TROUBLE IN READIN SAMPLE";K;" IN EXPT.";I
00230 STOP
00240 NEXT K
00250 NEXT I
00360 REM  THE NAMES OF THE EXPERIMENTS AND THE FIRST DATA POINTS ARE
00361 REM  PRINTED FOR VERIFICATION. THE EXECUTION CAN BE STOPPED HERE.
00368 REM
00369 REM
00370 FOR T2=1 TO N1
00380 PRINT N1$(T2),L2(T2,1),L2(T2,2)
00390 NEXT T2
00400 PRINT "OK";
00410 INPUT B5$
```

```
00420 IF B5$="OK" THEN 670
00430 STOP
00610 DATA "INTERCEPTS","DECAY CONSTANTS","HALF-VOLUMES","% TOTAL
FLOW","% TOTAL STORE"
00670 PRINT "NUMBER OF RUNS IS";
00680 PRINT N1
00700 PRINT "EXPERIMENTS-";
00715 FOR M9=1 TO N1
00720 PRINT N1$(M9);"  ";
00730 NEXT M9
00735 LET L3=10
00740 PRINT
00741 REM
00742 REM     THE FOLLOWING LINES COMPUTE THE COMMON
00743 REM     CURVE FOR THE SLOWEST DECAYING COMPONENT, IF
00744 REM     IT IS DESIRED.
00745 REM
00750 PRINT "DO YOU WANT REGRESSION ON POINTS FROM ALL RUNS FOR LAST
COMP.";
00760 INPUT B3$
00770 IF B3$="NO" THEN 1230
00780 PRINT "CUTOFF FOR LAST COMP. IS";
00790 INPUT V6
00800 PRINT "DO NOT INCLUDE THIS IN SUBSEQUENT ENTRIES!"
00820 LET N=0
00830 FOR I=1 TO N1
00840 FOR K=21 TO K1
00850 IF V(I,K)<V6 THEN 900
00851 REM
00852 REM     SAMPLES WHOSE CONCENTRATIONS WERE NOT MEASURED
00853 REM     WERE ENTERED WITH CONC.=0.0. THESE, OF COURSE,
00854 REM     ARE NOT FITTED TO THE COMPONENT CURVES.
00855 REM
00860 IF L2(I,K)<L3-8 THEN 900
00870 LET N=N+1
00880 LET X(N)=V(I,K)
00890 LET Y(N)=(L2(I,K)*2.3025851)
00900 NEXT K
00910 NEXT I
00920 GOSUB 1870
00930 LET I7=EXP(B)
00940 LET B0=B
00950 LET N0=N
00960 LET M0=M
00970 LET S7=I7/-M
00980 LET V7=0.69315/-M
00990 PRINT "INTERCEPT","DECAY","V-1/2","STORE","NUMBER OF PTS."
```

```
01000 PRINT I7,-M,V7,S7,N
01008 REM
01009 REM
01010 REM   THE LINES  1230-1840 DEFINE A LOOP WHICH IS REPEATED FOR
01020 REM   EACH CURVE AND DOES THE ACTUAL CURVE-PEELING
01028 REM
01029 REM
01230 FOR I=1 TO N1
01240 PRINT "WASHOUT ";I;"  ";N1$(I)
01241 REM
01242 REM
01245 REM   THE CUTOFF POINTS ARE INPUT HERE AFTER BEING DETERMINED
01246 REM   BY MANUAL CURVE-PEELING.
01248 REM
01249 REM
01250 PRINT "STARTING POINTS FOR COMPONENTS ARE";
01260 MAT INPUT V9
01270 IF B3$="NO" THEN 1300
01280 LET J0=NUM+1
01285 LET V9(J0)=V6
01290 GO TO 1310
01300 LET J0=NUM
01310 FOR K=1 TO K1
01320 LET C(J0,K)=EXP(L2(I,K)*2.3025851)
01330 NEXT K
01340 PRINT "COMPONENT","INTERCEPT","DECAY","STORE","NUMBER OF PTS."
01341 REM
01342 REM     LINES 1350-1680 DEFINE A LOOP WHICH 'PEELS' A
01343 REM      COMPONENT OFF THE ORIGINAL CURVE ON EACH EXECUTION.
01349 REM
01350 FOR J=J0 TO 1 STEP -1
01360 IF J<J0 THEN 1420
01361 REM
01362 REM
01363 REM      THE FITTING OF THE INDIVIDUAL POINTS OF THE CURVE IS
01364 REM      OMITTED IF THIS HAS ALREADY BEEN DONE FOR THE CURVES
01365 REM      TAKEN TOGETHER.  THE COMPONENT IS SIMPLY  SUBTRACTED
01366 REM      FROM THE CURVE AND THE PEELING CONTINUES WITH THE
01367 REM      NEXT COMPONENT.
01368 REM
01369 REM
01370 IF B3$="NO" THEN 1420
01380 LET M=M0
01390 LET B=B0
01400 LET N=N0
01410 GO TO 1580
01420 LET N=0
```

```
01421 REM
01422 REM
01423 REM      THE LINES 1430-1450 FIT THE POINTS TO A STRAIGHT LINE
01424 REM      BEYOND THE JTH CUTOFF POINT.  THIS IS DONE BY ENTERING
01425 REM      THEIR COORDINATES IN THE ARRAYS X &Y FOR USE BY THE
01426 REM      SUBROUTINE BEGINNING AT LINE 1880. POINTS WITH INSIGNIF-
01427 REM      ICANT CONCENTRATION REMAINING AFTER THE PREVIOUS PEEL
01428 REM      ARE REJECTED.
01429 REM
01430 FOR K=1 TO K1
01440 IF V(I,K)<V9(J) THEN 1550
01450 IF C(J,K)<= EXP(2.3026*(L3-8)) THEN 1550
01460 IF J=J0 THEN 1520
01470 IF V(K)>=V9(J+1) THEN 1550
01520 LET N=N+1
01543 LET X(N)=V(I,K)
01544 LET Y(N)=LOG (C(J,K))
01550 NEXT K
01560 IF N<2 THEN 2440
01570 GOSUB 1880
01571 REM
01572 REM
01573 REM      THE INTERCEPT AND SLOPE(DECAY CONSTANT) OF THE LINE ARE
01574 REM      RETURNED AS VARIABLES "B" AND "M".  THE PARAMETERS
01575 REM      OF THE COMPONENT, (#J OF THE ITH CURVE) ARE STORED IN
01576 REM      ARRAYS AS I9(I,J)=ANTILOG OF THE INTERCEPT,
01577 REM                K(I,J)=DECAY CONSTANT(+VE VALUE)
01578 REM                V8(I,J)=HALF-VOLUME,&S(I,J)=CELL STORE.
01579 REM
01580 LET I9(I,J)=EXP(B)
01590 LET K(I,J)=-M
01600 LET V8(I,J)=0.69315/K(I,J)
01610 LET S(I,J)=I9(I,J)/K(I,J)
01620 PRINT J,I9(I,J),K(I,J),S(I,J),N
01630 IF J=1 THEN 1680
01640 FOR K=1 TO K1
01650 IF V(I,K)>V9(J) THEN 1670
01660 LET C(J-1,K)=C(J,K)-EXP(B+M*V(I,K))
01670 NEXT K
01680 NEXT J
01681 REM
01683 REM      THE VARIABLES I9(I,5) AND S(I,5) STORE THE TOTALS
01684 REM      OF THE INTERCEPTS AND THE AREAS
01685 REM      UNDER THE CURVES, RESPECTIVELY.
01686 REM
01690 LET I9(I,5)=0
01700 LET S(I,5)=0
```

```
01710 FOR K5=1 TO J0
01720 LET I9(I,5)=I9(I,5)+I9(I,K5)
01730 LET S(I,5)=S(I,5)+S(I,K5)
01740 NEXT K5
01741 REM
01742 REM      F1 AND F2 ARE ARRAYS THAT STORE THE FRACTION
01743 REM      OF THE TOTAL INTERCEPT AND THE TOTAL AREA (CELL
01744 REM      STORE) RESPECTIVELY, IN THE JTH COMPONENT OF THE ITH
01745 REM      CURVE.
01746 REM
01749 REM
01750 FOR J2=1 TO J0
01760 LET F1(I,J2)=100*I9(I,J2)/I9(I,5)
01770 LET F2(I,J2)=100*S(I,J2)/S(I,5)
01780 NEXT J2
01785 REM
01790 PRINT "COMPONENT","%FLOW","% STORE","V-1/2"
01800 FOR J2=1 TO J0
01810 PRINT J2,F1(I,J2),F2(I,J2),V8(I,J2)
01820 NEXT J2
01830 PRINT "TOTAL",I9(I,5),S(I,5)
01840 NEXT I
01850 GO TO 2120
01860 STOP
01869 REM
01870 REM  SUBROUTINE FOR CALCULATING THE LINE OF BEST
01871 REM  FIT TO THE ARRAYS X _Y.
01872 REM
01880 LET X1=0
01890 LET X2=0
01900 LET Y1=0
01910 LET Y2=0
01920 LET Z1=0
01930 FOR Q=1 TO N
01940 LET X1=X1+X(Q)
01950 LET X2=X2+X(Q)^2
01960 LET Y1=Y1+Y(Q)
01970 LET Y2=Y2+Y(Q)^2
01980 LET Z1=Z1+X(Q)*Y(Q)
01990 NEXT Q
02070 LET D=N*X2-X1^2
02080 LET D2=N*Y2-Y1^2
02090 LET B=(Y1*X2-X1*Z1)/D
02100 LET M=(N*Z1-X1*Y1)/D
02110 RETURN
02111 REM
02112 REM   THE FOLLOWING LINES COMPUTE THE AVERAGE AND STANDARD ERROR
```

```
02113 REM   OF THE MEAN FOR EACH OF FIVE PARAMETERS
02114 REM            V8=HALF-VOLUME
02115 REM            I9=ANTILOG OF INTERCEPT
02116 REM            K=DECAY CONSTANT
02117 REM            F1=FRACTION OF TOTAL FLOW
02118 REM            F2=FRACTION OF TOTAL STORE
02119 REM
02120 FOR J=1 TO J0
02130 FOR H=1 TO 5
02140 LET A(H,J)=0
02150 LET E(H,J)=0
02160 NEXT H
02170 FOR I=1 TO N1
02180 LET A(1,J)=A(1,J)+I9(I,J)
02190 LET A(2,J)=A(2,J)+K(I,J)
02200 LET A(3,J)=A(3,J)+V8(I,J)
02210 LET A(4,J)=A(4,J)+F1(I,J)
02220 LET A(5,J)=A(5,J)+F2(I,J)
02230 LET E(1,J)=E(1,J)+I9(I,J)^2
02240 LET E(2,J)=E(2,J)+K(I,J)^2
02250 LET E(3,J)=E(3,J)+V8(I,J)^2
02260 LET E(4,J)=E(4,J)+F1(I,J)^2
02270 LET E(5,J)=E(5,J)+F2(I,J)^2
02280 NEXT I
02290 FOR H=1 TO 5
02300 LET A(H,J)=A(H,J)/N1
02305 IF N1<2 THEN 2320
02310 LET D(H,J)=SQR((E(H,J)-N1*A(H,J)^2)/(N1*(N1-1)))
02320 NEXT H
02330 NEXT J
02340 FOR H=1 TO 5
02350 READ H$(H)
02360 PRINT H$(H)
02370 PRINT "COMPONENT","AVERAGE","S.E.M.","CRUDE S. S."
02380 FOR J=1 TO J0
02390 PRINT J,A(H,J),D(H,J),E(H,J)
02400 NEXT J
02410 PRINT
02420 NEXT H
02430 STOP
02431 REM
02432 REM   IN THE CASE THAT MISSING DATA OR AN IMPROPER CHOICE OF
02433 REM   CUTOFF POINTS ALLOWS TOO FEW POINTS FOR THE FITTING TO
02434 REM   A STRAIGHT LINE,THIS PART OF THE PROGRAM ALLOWS
02435 REM   INTERPOLATION OF DATA, AND THEN STARTS THE PEEL
02436 REM  AGAIN AT THE POINT WHERE THE CUTOFF POINTS ARE SELECTED,
02437 REM   SO THAT THEY TOO CAN BE CHANGED. IF NO CHANGES CAN BE MADE
```

```
02438 REM   THE PROGRAM IS STOPPED.
02439 REM
02440 PRINT "LINE";J;" IN WASHOUT";I;" HAS LESS THAN 2 VALID POINTS."
02480 PRINT "DO YOU WANT TO FUDGE DATA";
02490 INPUT B6$
02500 IF B6$="YES" THEN 2520
02510 STOP
02520 PRINT "SAMPLES CONCERNED"
02525 PRINT "N","VOL.","LOG. CONC.","PT. FIT."
02530 FOR P9=1 TO K1
02540 IF V(I,P9)<V9(J) THEN 2580
02550 IF V(I,P9)>V9(J+1) THEN 2580
02551 IF C(J,P9)>0 THEN 2560
02552 PRINT P9,V(I,P9),L2(I,P9)
02553 GO TO 2580
02560 PRINT P9,V(P9),L2(I,P9),LOG(C(J,P9))/2.30258
02580 NEXT P9
02590 PRINT "WHICH POINT DO YOU WISH TO CHANGE";
02600 INPUT P8
02610 PRINT "LOG. CONC.=";L2(I,P8);" CHANGED TO ";
02620 INPUT L2(I,P8)
02630 PRINT "OK";
02640 INPUT B7$
02650 IF B7$="OK" THEN 1240
02655 IF B7$= "STOP" THEN 2670
02660 GO TO 2590
02670 STOP
02680 END
```

APPENDIX 2: The computer program "GEORGE".

```
00010 REM
00011 REM     THIS PROGRAM COMPUTES THE MEAN TRANSIT VOLUME
00012 REM   OBTAINED IN A SET OF RUNS OBTAINED IN ONE EXPER-
00013 REM   IMENT FROM DATA OBTAINED DIRECTLY FROM CELLOSCOPE
00014 REM   COUNTS AND VOLUME MEASUREMENTS. THE  DATA ARE ASSUMED
00015 REM   TO COME FROM A SERIES OF SAMPLES OF EQUAL VOLUME
00016 REM   STARTING AT THE INITIAL INJECTION,FOLLOWED BY A LARGER
00017 REM   SAMPLE, CALLED THE 'TAIL' IN WHICH ALL THE INJECTED
00018 REM   CELLS NOT IN THE SMALL SAMPLES ARE COLLECTED.
00019 REM
00020 REM    A BACKGROUND OF CELLS RELEASED FROM THE MUSCLE DURING
00021 REM  THE TRANSIT OF CELLS IS ASSUMED TO HAVE A CONSTANT VALUE
00023 REM  WHICH IS THE MINIMUM OF THE CONCENTRATIONS IN SAMPLES TAKEN
00024 REM  BEFORE AND AFTER THE RUN.
00025 REM
00026 REM   THE MEAN TRANSIT VOLUME IS CALCULATED BY CALCULATING
00027 REM   APPROXIMATIONS TO THE INTEGRALS-
00028 REM         (               (
00029 REM     I(1)=I C(V) AND I(2)=I C(V)*V
00030 REM         )               )
00031 REM    I(1) IS ESTIMATED BY COMPUTING THE TOTAL NUMBER
00032 REM   OF CELLS IN ALL THE SAMPLES, AND I(2) BY THE TOTAL OF
00033 REM   THE NUMBER OF CELLS IN EACH  SAMPLE TIMES THE VOLUME
00034 REM   PERFUSED AFTER INJECTION WHEN IT WAS TAKEN.
00035 REM   THIS IS POSSIBLE FOR ALL SAMPLES BUT THE TAIL, WHERE
00036 REM   SOME ASSUMPTION OF THE CHANGE IN CONCENTRATION WITH
00037 REM   VOLUME PERFUSED MUST BE MADE, IN ORDER TO COMPUTE ITS
00038 REM   CONTRIBUTION TO I(2). A SINGLE EXPONENTIAL DECAY
00039 REM   OF CELL CONCENTRATION WITH VOLUME PERFUSED IS ASSUMED
00040 REM   AND ITS DECAY CONSTANT AND INTERCEPT ARE CALCULATED
00041 REM   FROM THE NUMBER OF CELLS IN THE TAIL AND THE CONCENTRATION
00042 REM   OF THE PREVIOUS SAMPLE.
00043 REM
00045 REM
00600 DIM O(8,30),F(8,30),C9(8,30),C(8,30),T(8,30),T9(8,30)
00610 DIM X(15),Y(15),V4(30),01(30),02(30),03(30),04(30),05(30),06(30)
00611 REM
00612 REM    THE DATE,D$, AND THE NUMBER OF RUNS, I, IS READ.
00613 REM    THE CELLOSCOPE BACKGROUND, B1,  AND SAMPLE VOLUME,
00614 REM    V2, ARE READ.
00615 REM
00620 READ D$,I,V2,B1
00621 REM
00622 REM    V6 IS THE VOLUME OF DILUENT PLACED IN THE SAMPLE TUBE
00623 REM    BEFORE EACH SAMPLE IS TAKEN.
00624 REM
00630 LET V6=1.0
```

```
00640 FOR I1=1 TO I
00643 REM
00644 REM    T$ IS THE TYPE OF CELLS USED, FOR THE PRINTED RECORD.
00645 REM
00650 READ T$(I1)
00651 REM
00652 REM    THE BACKGROUND BEFORE THE RUN IS COMPUTED.(B1)
00660 READ D2,N2
00670 LET B2(I1)=D2*128*(N2-B1)
00671 REM
00672 REM      STMTS.  680-780 DEFINE A LOOP WHICH READS THE
CELLOSCOPE
00673 REM    COUNTS AND DILUTIONS AND COMPUTES SAMPLE CONCENTRATIONS
00674 REM       C9(I1,J1). IT ALLOWS FOR SKIPPED SAMPLES ,IF THE ARE
00675 REM    NOT IN SEQUENCE.
00676 REM
00680 FOR J1=1 TO 30
00690 IF J1=1 THEN 710
00700 IF T9(I1,J1-1)=1 THEN 750
00710 READ N
00720 IF N=J1 THEN 750
00730 LET T9(I1,J1)=1
00740  GO TO 780
00750 READ D1,N1
00760 LET C9(I1,J1)=((V2+V6)/V2)*D1*128*(N1-B1)
00770 LET T9(I1,J1)=0
00780 NEXT J1
00781 REM
00782 REM    THE LOOP(STMTS.700-820) FINDS THE CONCENTRATIONS OF
00783 REM   SKIPPED SAMPLES BY GEOMETRIC INTERPOLATION.
00784 REM
00790 FOR J2=2 TO 29
00800IF T9(I1,J2)=0 THEN 820
00810 LET C9(I1,J2)=SQR(C9(I1,J2-1)*C9(I1,J2+1))
00820 NEXT J2
00821 REM
00822 REM  T3(I) IS THE TOTAL NUMBER OF CELLS IN THE TAIL SAMPLE.
00823 REM
00830 READ D3,N3,V3(I1)
00840 LET T3(I1)=D3*128*(N3-B1)*V3(I1)
00841 REM
00842 REM   C5(I1) IS THE CONCENTRATION IN THE SAMPLE AFTER THE TAIL.
00843 REM
00850 READ D4,N4
00860 LET C5(I1)=D4*128*(N4-B1)
00861 REM
00862 REM   V5(I1) IS THE VOLUME PERFUSED BETWEEN THE SAMPLE BEFORE
```

```
00863 REM   THE BACKGROUND SAMPLE OF RUN (I1) AND THAT SAMPLE.
00864 REM
00870 READ V5(I1)
00880NEXT I1
00890 PRINT "DATA READ FOR";I;"RUNS ON ";D$
00895 PRINT
00900 LET V9(1)=V5(1)
00905 LET A1$="OK"
00910 FOR I3=1 TO I
00913 IF I3=1 THEN 917
00915 LET V9(I3)=V9(I3-1)+30*V2+V3(I3-1)+V5(I3)
00917 PRINT "RUN";I3;", ";T$(I3);",STARTS AT ";V9(I3);" ML. PERFUSION."
00920 PRINT "CONCENTRATIONS IN CELLS/ML. "
00930 PRINT "BEFORE","FIRST SAMPLE","TAIL","AFTER"
00940 PRINT B2(I3),C9(I3,1),T3(I3)/V3(I3),C5(I3)
00941 REM
00942 REM
00943 REM  STMTS. 950-1078 COMPUTE THE CONSTANT LEVEL OF BACKGROUND
00944 REM  CELLS RELEASED BY THE MUSCLE, AS WELL AS A DECAYING
00945 REM COMPONENT IF THE BACKGROUND SAMPLE BEFORE THE RUN
00946 REM  IS OF HIGHER CONCENTRATION THAN THIS.
00947 REM
00948 REM
00950 LET A=C5(I3)
00960 IF B2(I3)>=C5(I3) THEN 1020
00970 LET A=B2(I3)
00980 IF C9(I3,1)>=B2(I3) THEN 1000
00990 LET A=C9(I3,1)
01000 LET Q$="NO"
01005 PRINT "BACKGROUND ASSUMED CONSTANT AT ";A;"CELLS/ML."
01010 GO TO 1080
01020 IF C9(I3,1)<C5(I3) THEN 990
01030 LET Q$="YES"
01035 PRINT "BACKGROUND ASSUMED CONSTANT AT ";A;"CELLS/ML.,"
01040 IF B2(I3)>C9(I3,1) THEN 1070
01050 LET D9=2.3026
01060 GO TO 1075
01070 LET D9=LOG((1.76*(B2(I3)-A))/(C9(I3,1)-A))/5.5*V2
01071 IF D9<1.151 THEN 1050
01075 PRINT "PLUS INITIAL DECAY FROM ";1.76*B2(I3);" CELLS/ML. AT
";5*V2;"ML.BEFORE START"
01076 PRINT  "DECAY RATE = ";D9/2.3026;" DECADES/ML."
01077 LET J4=INT(6.9/(D9*V2)-4.0)
01078 PRINT "CORRECTION STOPS AT SAMPLE";J4
01079 REM           SUBTRACTION OF BACKGROUND IN STMTS. 1080-1110
01080 FOR J3=1 TO 30
01090 LET C(I3,J3)=C9(I3,J3)-A
```

```
01091 IF Q$="NO" THEN 1100
01092 IF J3>J4 THEN 1100
01093 LET X9=(-D9*V2*(J3+4.5))
01094 LET B8=1.76*(B2(I3)-A)*EXP(X9)
01095 LET C(I3,J3)=C(I3,J3)-B8
01100 NEXT J3
01110 LET T3(I3)=T3(I3)-A*V3(I3)
01120 IF T3(I3)>0 THEN 1190
01130 LET A1$="NO"
01140 PRINT "NEGATIVE TAIL ON RUN"
01190 PRINT
01200 NEXT I3
01205 IF A1$="OK" THEN 1210
01206 STOP
01210 PRINT "OK";
01220 INPUT A1$
01230 IF A1$="OK" THEN 1250
01240 STOP
01241 REM
01242 REM   THE DECAY CONSTANT OF THE SINGLE EXPONENTIAL FUNCTION
01243 REM   WHICH IS ASSUMED TO DESCRIBE THE CONCENTRATION VS. VOLUME
01244 REM   PERFUSED IN THE TAIL IS CALCULATED IN STMTS 1270-1490.
01245 REM     THE FORMULA
01247 REM            K=C/T(1-EXP(-K*V))
01248 REM   IS SOLVED BY ITERATION.
01249 REM
01250 FOR I6=1 TO I
01260 PRINT
01270  PRINT"TAIL DECAY RATE CALCULATION ON RUN";I6
01280 PRINT "AVG. CONC. IN TAIL= ";T3(I6)/V3(I6);" CELLS/ML."
01290 PRINT "VOLUME OF TAIL SAMPLE = ";V3(I6);" ML."
01300 PRINT "COMPONENT","CELLS IN T.","SAMP. _ 30","DECAY","TOT. CELLS"
01310 PRINT "TOTAL",T3(I6),C(I6,30)
01320 LET Q=C(I6,30)/T3(I6)
01330 IF Q*V3(I6)>1 THEN 1380
01340 LET H$(I6)="TAIL CONCENTRATION TOO LARGE"
01350 PRINT H$(I6)
01360 GO TO 1500
01380 LET G1=Q
01390 FOR K=1 TO 20
01400 LET G2=Q*(1-EXP(-G1*V3(I6)))
01410 LET A=ABS((G2-G1)/G1)
01420 IF A<.001 THEN 1460
01430 LET G1=G2
01440 NEXT K
01450 PRINT "CONVERGENCE NOT OBTAINED, ERROR= ";A
01460 LET M3(I6)=G2
```

```
01470 LET C3(I6)=C(I6,30)
01480 LET T4(I6)=C3(I6)/M3(I6)
01490 PRINT "EXP. TAIL",T3(I6),C3(I6),M3(I6)/2.302585,T4(I6)
01500 PRINT "OK ";
01505 INPUT A2$
01510 IF A2$="OK" THEN1770
01520 STOP
01770 NEXT I6
01771 REM
01772 REM THE DATA ARE CLEARED OF POINTS LESS THAN
01773 REM THE BACKGROUND VALUE.
01774 REM
01780 FOR K3= 1 TO I
01790 FOR K4=1 TO 30
01800 IF C(K3,K4)>1 THEN 1820
01810 LET C(K3,K4)=1
01820 NEXT K4
01830 NEXT K3
01831 REM
01832 REM    THE VARIABLES T1(I8) AND T2(I8) ARE USED TO
01833 REM   APPROXIMATE THE INTEGRALS I(1) AND I(2)
01834 REM
01835 REM   THE MEAN TRANSIT VOLUME IS ESTIMATED BY T2/T1.
01836 REM
01840 FOR J8= 1 TO 30
01850 LET V4(J8)=(J8-0.5)*V2
01860 NEXT J8
01870 LET T1(I8)=0
01880 LET T2(I8)=0
01890 FOR I8=1 TO I
01900 PRINT
01910 REM PRINT STMTS.2180-2250,2370-2840
01920 PRINT "RUN  ";I8,T$(I8),D$
01930 FOR J9=1 TO 30
01940 LET T(I8,J9)=C(I8,J9)*V2
01950 LET T1(I8)=T1(I8)+T(I8,J9)
01960 LET T2(I8)=T2(I8)+T(I8,J9)*V4(J9)
01970 NEXT J9
01980 LET T6=T1(I8)
01990 PRINT "ASSUMPTION","AREA-CELLS","2ND-MOM ","M.T.V.-ML."
02000 PRINT "NO TAILS",T1(I8),T2(I8),T2(I8)/T1(I8)
02001 REM
02002 REM    THE CONTRIBUTION OF THE TAIL TO T2,T1, AND THE M.T.V.
02003 REM    IS ADDED.
02004 REM
02010 IF H$(I8)="TAIL CONCENTRATION TOO LARGE" THEN 2060
02020 LET  T1(I8)=T1(I8)+C3(I8)/M3(I8)
```

```
02030 LET T2(I8)=T2(I8)+(C3(I8)/M3(I8))*(1/M3(I8)+30*V2)
02040 PRINT "EXP.TAIL",T1(I8),T2(I8),T2(I8)/T1(I8)
02050 LET M1(I8)=T2(I8)/T1(I8)
02051 REM
02052 REM     THE TAIL CELLS (T3) CAN BE ADDED DIRECTLY TO T1
02053 REM     TO PROVIDE AN ESTIMATE OF THE TOTAL CELLS RECOVERED
02054 REM     WHICH IS INDEPENDENT OF THE ASSUMPTION USED. THIS
02055 REM      IS IMPOSSIBLE FOR T2
02056 REM
02060 LET T1(I8)=T6+T3(I8)
02070 PRINT "COLLECTED TAIL",T1(I8)
02071 REM
02072 REM    THE FRACTIONS OF THE TOTAL AND THE OGIVE (CUMUL-
02073 REM    ATED FRACTIONS) CORRESPONDING TO THE VOLUME PERFUSED
02074 REM    AT EACH SAMPLE ARE COMPUTED.
02075 REM
02080 LET F(I8,1)=T(I8,1)/T1(I8)
02090 LET O(I8,1)=F(I8,1)
02100 FOR K4=1 TO 30
02110 LET F(I8,K4)=T(I8,K4)/T1(I8)
02120 LET O(I8,K4)=O(I8,K4-1)+F(I8,K4)
02130 NEXT K4
02140 PRINT
02150  PRINT "SAMPLE","VOLUME","CONC.","FRACTION","OGIVE"
02160 FOR J=1 TO 30
02170 IF T9(I8,J)=1 THEN 2200
02180 PRINT J,V4(J),C(I8,J),F(I8,J),O(I8,J)
02190 GO TO 2210
02200 PRINT J,V4(J),"N. C.",F(I8,J),O(I8,J)
02210 NEXT J
02211 REM
02212 REM     THE 'TAB ' FUNCTION IS USED TO PROVIDE A ROUGH
02213 REM     GRAPH OF THE FRACTION OF THE TOTAL IN EACH SAMPLE.
02214 REM
02220 PRINT "FRACTION OF TOTAL IN EACH SAMPLE"
02230 PRINT
TAB(17);"0";TAB(27);".05";TAB(37);".10";TAB(47);".15";TAB(57);".20";TAB(6
7);".25"
02240 FOR  G=1 TO 30
02250 LET Q=INT(F(I8,G)*200)+17
02260 PRINT V4(G);TAB(Q);"*"
02270 NEXT G
02280 PRINT
02290 NEXT I8
02291 REM
02292 REM    THE RESULTS ARE PRINTED AGAIN AS A SUMMARY
02293 REM
```

```
02300 PRINT "REVIEW OF RESULTS"
02310 PRINT
02320 PRINT "RUN","TYPE","M.T.V.","CELLS RECOVERED"
02323 FOR I9=1 TO I
02325 PRINT I9,T$(I9),M1(I9),T1(I9)
02330 NEXT I9
02340 STOP
02350 END
```

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