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FRACTIONATION AND COMPOSITIONAL STUDIES OF RABBIT SKELETAL MUSCLE MEMBRANES

A Thesis

Presented to the National University of Ireland

for the Degree of

DOCTOR OF PHILOSOPHY

by

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December 1975

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CORRIGENDA

p.5 line 18 for 'triple-layered' read 'triple-stacked'.

p.7 line 24 for 'Franzini-Armstron' read 'Franzini-Armstrong'.

p.13 line 8-9 should read '..... an ATP-dependent Ca^{2+}-transport system is located ....'.

p.66 line 17 for 'diffuse' read 'volatilize'.

p.66 line 23, 24 for 'N-hexane' read 'n-hexane',

p.73 line 22, 23 after 'The reaction was terminated by the addition of 1 ml of 6% w/w perchloric acid.' insert 'The tubes were then placed in an ice-water bath'.

(Note that the rate of hydrolysis of ATP after deproteinisation is given in line 28 p.73 and line 1 p.74.)

p.162 After line 10 insert 'The use of a 3mM Ca^{2+}-EGTA buffer prevents the solubility product for calcium oxalate being exceeded in the medium. However, when Ca^{2+} ions are transported into a sealed vesicle the solubility product is exceeded in the vesicle and calcium oxalate is deposited'.

p.165 Fig 3.30 insert 'The calcium oxalate loaded pellets shown were photographed in MSE 59209 centrifuge tubes. In each case the pellet is viewed from the bottom of the tube, looking upwards. Magnification is 7X.'

p.172 line 6-7 for '.... the low level of cholesterol .....' read '.... cholesterol found at a low level ....'.

p.297 line 25 for '....enzymes occurred in parallel' read '.... enzymes coincided'.

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"For the last 25 years, I have roamed through living cells, but with the help of a centrifuge rather than a microscope."

Christian de Duve,
Nobel Lecture, Stockholm,

12 December 1974

SECTION 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

The observations of Hooke (1665), Schleiden & Schwann (1839) and Virchow (1855) led to the identification of the cell as the basic structural unit of living material. In the intervening years, it has been firmly established that the chemical processes which underlie the proper functioning, development and reproduction of the organism are cellular activities.

The development of the electron microscope has enabled cell structure to be studied in detail. A picture of the cell as an entity with a complex and highly organised internal structure has emerged from the work of Palade, Porter, Fernandez-Moran and many others. Although cells from different tissues and organisms differ in aspects of their structure and consequently in function, they have several features in common. A retentive membrane encloses a number of cell constituents, which include membrane-enclosed subcellular structures known as organelles. The cells of most tissues also contain a reticulum or system of branching tubules. The interplay of the biochemical activities of these structures enables the cell to function.

Almost thirty years ago, Claude, Palade, Schneider, Hogeboom, de Duve and others set out to analytically fractionate the subcellular components obtained after the fragmentation of liver cells. This approach has become known as *subcellular fractionation*, and signalled a major conceptual breakthrough in biochemistry (reviewed by de Duve, 1964, 1967, 1971). The significance of this breakthrough has been underlined by the award of the 1974 Nobel Prize in Medicine to de Duve,

Palade and Claude.

This thesis is concerned with the application of subcellular fractionation techniques to the separation and characterisation of the membrane systems of the rabbit skeletal muscle cell.

1.2 INTRODUCTION

(a) Muscle tissue

Muscle is found in the meaty parts of the animal body and, in the case of man, constitutes approximately 40% of body weight (Wilkie, 1968). There are three types of muscle: smooth, cardiac and skeletal (Gould, 1973).

Smooth muscle, which is the least specialised of the three types, has a widespread distribution in the body, being found in the intestine, respiratory tract, blood vessels and reproductive organs. It is under the control of the autonomic nervous system and undergoes slow contraction-relaxation cycles.

Cardiac muscle is found only in the heart and is unique in its unceasing rhythmic activity as it pumps blood around the body. It has a high content of mitochondria, fat droplets and glycogen granules.

Skeletal muscle is quantitatively the most important of the three types. There are approximately 150 different skeletal muscles in the human body and almost all are attached by tendons to the skeleton. Skeletal muscle gives the animal the ability to move and interact with its environment, whereas, smooth muscle generally helps to maintain the internal environment of the body.

Skeletal muscle may be subdivided into red, white, and intermediate-type muscle which contains both red and white cells.

White muscle cells have a low content of mitochondria and, consequently, a predominantly anaerobic metabolism (Needham, 1973). They are capable of short, intense bursts of activity.

Red muscle cells, on the other hand, are rich in mitochondria and the oxygen-binding protein, myoglobin, and are capable of slower, more prolonged periods of contraction. Red muscle is particularly suited to the maintenance of posture.

In the research described in this thesis, only white skeletal muscle from rabbit hind leg has been used.

(b) Structure of the skeletal muscle cell

Skeletal muscle tissue is composed of many muscle cells which are also known as muscle fibres. These cells range from 10 - 100 μm in diameter and from 20 μm to several cm in length. Each is composed of three main types of structural components: the internal and external membrane systems, the contractile elements and the subcellular organelles. A diagrammatic representation of a muscle cell is shown in Fig. 1.1.

The cell is enclosed by a triple-layered outer membrane known as the sarcolemma. The innermost layer is the plasmamembrane proper; it is coated with a 100Å thick basement membrane and a network of collagen fibrils (Mauro & Adams, 1961). The overall structure is responsible for some of the mechanical properties of muscle cells.

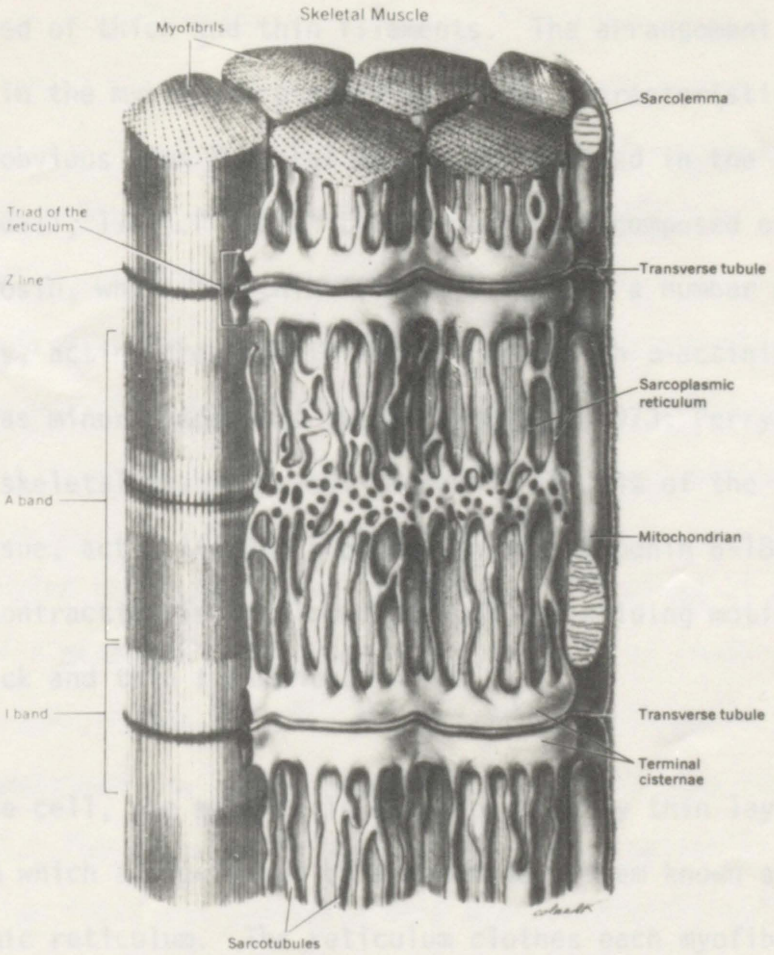


Fig. 1.1. Diagrammatic representation of the skeletal muscle cell (courtesy of Oxford University Press).

The contractile elements occupy most of the cell volume (Franzini-Armstrong, 1973). They are arranged in columns, known as myofibrils, which are in alignment with the long axis of the cell. The myofibrils are composed of thick and thin filaments. The arrangement of the filaments in the myofibril gives rise to the characteristic striations which are obvious when skeletal muscle is examined in the light microscope (Bendall, 1969). The thick filaments are composed of the protein myosin, while the thin filaments contain a number of proteins, principally, actin, tropomyosin and troponin,with α-actinin and β-actinin as minor components (Weber & Murray, 1973; Perry, 1974). In rabbit skeletal muscle, myosin accounts for 34% of the total protein of the tissue, actin 14-20%, tropomyosin and troponin 6-18%. The force of contraction is developed through the sliding motion interaction of the thick and thin filaments.

In the cell, the myofibrils are separated by thin layers of sarcoplasm which are pervaded by a membrane system known as the sarcoplasmic reticulum. The reticulum clothes each myofibril and encloses a space separate from the sarcoplasm. It has a large surface to volume ratio and, consequently, may be expected to have important surface-associated functions.

The sarcoplasmic reticulum can be divided into three distinct regions on the basis of obvious morphological differences which repeat in harmony with the striations in the myofibrils (Porter & Palade, 1957; Peachey, 1965; Franzini-Armstron, 1973). 1. At the level of the A - I junction in frog muscle (Fig. 1.1), the reticulum forms

dilated sacs, known as terminal cisternae. At the Z line two neighbouring terminal cisternae are separated from one another by a tubular membrane system, known as the transverse tubular or T-system. The structure formed by the two terminal cisternae and a T-system tubule is termed a triad and is the site where excitation is coupled to contraction. On either side of the triad, the morphology of the reticulum undergoes a dramatic change. 2. At about the level of the A - I junction, parallel longitudinal tubules form a palisade around the myofibril. 3. At the centre of the A band the longitudinal tubules reform into a cisternum which is characterised by having distinctive pores and is termed the fenestrated collar (Peachey, 1965).

The tubules which abut onto the terminal cisternae at the triad are part of a membrane system known as the T-system (Andersson-Cedergren, 1959). The T-system tubules arise as invaginations of the sarcolemma and run in a transverse direction to the long axis of the cell. The T-system tubules are in communication with the extracellular fluid and penetrate very deeply into the cell along a winding course between the myofibrils, at the level of the triad (Huxley, 1964; Page, 1964; Endo, 1964; Peachey & Schild, 1968).

The relative surface areas of the three membrane systems have been estimated by Peachey (1965). These depend on the cell size, however in a 100 μm diameter cell from frog sartorius muscle the T-system surface area is seven times that of the sarcolemma. The sarcoplasmic reticulum is quantitatively the most important - its surface area being 40-50 times that of the sarcolemma.

The final group of structural components in muscle include nuclei, mitochondria, lysosomes, and ribosomes. Muscle cells are multinucleate since they are derived from the fusion of many cells during development (Fischmann, 1973). The nuclei are generally located beneath the sarcolemma and are elongated in the direction of the long axis of the cell (Gould, 1973; Franzini-Armstrong, 1973).

Mitochondria are found beneath the sarcolemma and interspersed between the myofibrils (Andersson-Cedergren, 1959; Eisenbeg et al., 1974; Bullock et al., 1973). Differences in the respiratory activity of various muscles are due to differences in the mitochondrial content of the cells (de Haan et al., 1973). Lipid droplets are usually found in association with mitochondria in red and heart muscle.

Lysosomes have been detected in skeletal muscle subcellular fractions (Stagni & De Bernard, 1968); Canonico & Bird, 1970; Stauber & Bird, 1974).

Ribosomes are found free in muscle cells and are not attached to membranes as in liver.

(c) The excitation-contraction-relaxation cycle of muscle

An indication that Ca^{2+} ions were involved in contraction came from the work of Ringer in 1883 on frog cardiac muscle. However, it was not until 1947 (Heilbrunn & Wiercinski) that Ca^{2+} ions were shown to be important in the contractile activity of skeletal muscle. Subsequent research has established that Ca^{2+} ions activate the contractile mechanism in all types of muscle.

In resting muscle, Ca^{2+} ions are stored in the sarcoplasmic reticulum (Winegrad, 1968). Depolarisation of the sarcolemma results in the release of stored Ca^{2+} from the sarcoplasmic reticulum into the neighbourhood of the myofibrils. Contraction occurs when the released Ca^{2+} binds to troponin on the thin filaments and initiates the interaction of actin and myosin (Weber & Murray, 1973; Perry, 1974).

Relaxation occurs when the concentration of free Ca^{2+} ions in the sarcoplasm is reduced to less than 10^{-7} M, through the uptake of Ca^{2+} ions by regions of the sarcoplasmic reticulum.

The principal events in the excitation-contraction-relaxation cycle have been identified by Winegrad (1968, 1970). The complete cycle is very rapid, being complete in 0·1 sec in the case of one stimulation of frog skeletal muscle.

The arrival of the nerve impulse at the neuromuscular junction on the sarcolemma results in the rapid spread of depolarisation across the sarcolemma and inwards along the T-system, to the neighbourhood of the sarcoplasmic reticulum. At the triad, excitation is coupled to contraction by the release of Ca^{2+} ions from the terminal cisternae. The mechanism by which excitation-contraction coupling occurs and Ca^{2+} is released is not yet understood, although it is under intensive investigation in several laboratories. Relaxation occurs when the released Ca^{2+} is bound by the longitudinal tubules of the sarcoplasmic reticulum. The bound Ca^{2+} is transported into the reticulum and subsequently stored in the terminal cistenae. The sarcoplasmic reticulum may be divided, therefore, into Ca^{2+}-transporting and Ca^{2+}-storing regions.

(d) The biochemistry of relaxation - historical outlines

In the early 1950s Marsh (1951, 1952) found that a muscle extract contained a component which caused the relaxation of an actomyosin gel in the presence of ATP - in other words, the in vivo process of relaxation could be carried out in vitro. Marsh called this component the relaxing factor. It's presence was quickly confirmed by Bendall (1952, 1953) and Hasselbach & Weber (1953). Later Kumagai et al. (1955) found that the relaxing factor could be precipitated by ammonium acetate and had a lipoprotein composition and Mg^{2+}-activated ATPase* activity.

After Portzehl's demonstration (1957) that the relaxing factor was contained in the particulate fraction of a muscle extract, Ebashi (1958), Bendall (1958), Nagai et al. (1960), Muscatello et al. (1961) and Ebashi & Lipmann (1962) showed that it sedimented between 8000g and 30 000g and consisted of membrane-bound vesicles. At about this time Porter & Palade (1957), Andersson-Cedergren (1959) and others rediscovered the sarcoplasmic reticulum and T-system, described many years earlier by Retzius (1881) and in greater detail by Veratti (1902). A relationship between the relaxing factor found in the high-speed particulate fraction of a muscle extract and the membrane systems observed in the electron microscope seemed possible after the findings of Palade & Siekevitz (1956). These workers found that rounded-off structures, which they termed vesicles, were formed from the membrane systems of liver during homogenisation or tissue disrupture. Vesicles formed in this way, are found in the subcellular fraction prepared by

*Adenosine triphosphatase

centrifugation at gravitational forces greater than 10 000g. This fraction is known as the microsomal fraction (de Duve, 1964, 1967, 1971; Reid, 1967; Dallner & Ernster, 1968). It is important to bear in mind that microsomes are artifacts of homogenisation. They are derived from subcellular entities during the rupturing of the tissue and do not constitute subcellular structures in their own right.

The relaxing factor, as originally isolated by various workers, corresponds to the microsomal fraction (Muscatello et al., 1961). This fraction would be expected to contain fragments of the sarcoplasmic reticulum, T-system and sarcolemma. Depending on the severity of the homogenisation it would also contain some mitochondrial membranes.

Assuming that the sarcolemma, T-system and sarcoplasmic reticulum were equally susceptible to homogenisation and using the data of Peachey (1965), one would expect to find fragments of the sarcolemma, T-system and sarcoplasmic reticulum in the ratio of 1 : 7 : 40-50 in the microsomal fraction.

In this thesis,the fractionation and characterisation of the membrane fragments in a muscle homogenate are described.

In 1959,Weber suggested that the relaxing factor might function by binding Ca^{2+}. Shortly afterwards,Ebashi (1960, 1961) showed that not only was this the case, but that the binding occurred in the

presence of ATP. Hasselbach & Makinose (1961) attributed the binding of Ca^{2+} to its removal from the medium by an ATP-dependent transport system. When vesicles accumulating Ca^{2+} in the presence of oxalate were examined in the electron microscope, calcium oxalate deposits were seen in up to 20% of the vesicles (Hasselbach & Makinose, 1961). These and other results (reviewed by: Martonosi, 1972(a), 1972(b); Inesi, 1972) established that the so-called relaxing factor activity involved of the removal of Ca^{2+} ions from the medium by an ATP-dependent Ca^{2+}-transport system located in at least some of the membranes of the microsomal fraction.

(e) Subcellular Fractionation

The biochemical approach to the study of subcellular components is that of fractionation. De Duve (1967) has divided the fractionation procedure into three obligatory and successive steps: homogenisation, fractionation and analysis.

Homogenisation results in the rupture of the cell and the introduction of a considerable amount of disorder. A degree of order is reintroduced during fractionation when subcellular components are separated into groups on the basis of their physical and/or chemical and/or enzymic properties. Finally, the separated fractions are analysed by physical, morphological, chemical and enzymic methods. The interpretation of the analytical data involves a retracing through the three successive stages. In this way, information on the intracellular localisation of chemical and enzymic constituents may be obtained.

The homogenisation process is an important one (de Duve, 1967, 1971; Reid, 1967, 1971) since it has a critical influence on the separations subsequently obtainable. It will be considered in some detail in this thesis.

The separation of subcellular components in the homogenate can be carried out by a number of techniques, the most important being: centrifugation (Reid, 1971, 1973; Reid & Williamson, 1974; Tolbert, 1974; Cline & Ryel, 1971), free flow electrophoresis (Hannig & Heidrich, 1974); countercurrent distribution (Albertsson, 1971) and immunological techniques (Luzio et al., 1974).

Separation by centrifugation was chosen for the research described in this thesis. This decision was largely influenced by the successful separations obtained by others (for example in: Reid, 1971, 1973, 1974; Amar-Costesec et al., 1974a; Beaufay et al., 1974b; Headon & Duggan, 1973) using centrifugation. It also provides a rapid means of preparing subcellular particles in relatively large amounts.

Centrifugation

Subcellular components are separated by centrifugation on the basis of differences in their sedimentation behaviour.

The rate of movement $(\frac{dr}{dt})$ of an ideal particle in a centrifugal field is described by the equation

$$\frac{dr}{dt} = \frac{a^2 (Dp - Dm) \omega^2 r}{18\eta}$$

where a = particle diameter (cm),

r = distance from centre of rotation (cm),

t = time (sec),

ω = angular velocity (radians/sec),

η = viscosity (poises),

Dp = particle density (g/cm^3),

Dm = density of medium (g/cm^3).

The rate at which a particle moves in a centrifugal field depends, not only,on its distance from the centre of the rotor and the angular velocity, but also, on its density, the square of its diameter and the density and viscosity of the medium. In density gradient centrifugation, the changing density and viscosity considerably influence the rate of sedimentation. When a sedimenting particle reaches the point in a gradient where the density of the medium is equivalent to its own buoyant density, its rate of movement will become zero. The density at this point is termed the isopycnic or equilibrium density of the particle.

Particles may be separated either on the basis of differences in their sedimentation rates or isopycnic densities. Separations in the centrifuge can be divided into three classes:

(i) rate separations either in a homogeneous medium by differential pelleting or in a density gradient by differential banding;

(ii) isopycnic banding in a density gradient;

(iii) combined rate-isopycnic separations.

Subcellular components are not ideal particles since they do not fully satisfy the necessary requirements of being spherical, rigid,

uncharged, unhydrated and osmotically inactive. However, their sedimentation is broadly similar to that expected for an ideal particle.

Differential Pelleting

Prior to the use of density gradient centrifugation, differential pelleting was probably the best available separatory technique in subcellular fractionation. In this method of separation, differences in the sedimentation rates of particles which are initially evenly dispersed throughout a homogeneous medium are exploited. Ideally, these particles should pellet on the basis of their sedimentation rates. However, the initial distribution of particles in the centrifuge tube ensures that the pellet of more rapidly sedimenting particles will always be contaminated by the more slowly sedimenting species present. Since differential pelleting is generally carried out in angle-head rotors, most particles will collide with the wall of the tube during sedimentation. This leads to the adhesion of particles to the tube wall, and in certain situations, to clumping and anomolous sedimentation.

Density Gradient Centrifugation

Many of the problems associated with differential pelleting are considerably reduced by centrifugation in density gradients.

Density gradient separations can be carried out in either swing-out or zonal rotors. The latter have several advantages over swing-out rotors. The sector-shaped compartments in a zonal rotor overcome the

problem of side-wall impactation. Since zonal rotors are loaded while rotating, disturbances to the gradient during loading, unloading, and acceleration and deceleration of the rotor are kept to a minimum. Finally, the larger volume of zonal rotors allows more material to be separated.

The development of zonal rotors by Anderson and co-workers is comprehensively recorded in the *National Cancer Institute, Monograph 21* (1966). The methodology involved in zonal-rotor separations has been reviewed by Cline & Ryel (1971) and many theoretical and practical aspects are dealt with in *Methodological Developments in Biochemistry* (Reid, 1971, 1973a, 1973b, 1974).

Choice of Gradient Material

Factors to be considered in the selection of gradient material have been considered by Cline & Ryel (1971), Hartman et al., (1974) and others.

Sucrose was chosen as the gradient material for this study, since it permitted the desired type of separation without seriously affecting the properties of the components being separated.

Density Perturbation Techniques

The sedimentation behaviour of some subcellular particles can be altered by exploiting the composition or functional activity of the particle in such a way as to cause a change in its density. The density change in some cases may be sufficiently large to allow for the complete isolation of the particle. In other cases, it is small and usually detected by analytical fractionation. This type of

approach has been termed *density perturbation* by Wallach & Winzler (1974).

Several examples of density perturbation may be cited:

(i) the use of triton WR-1339 in the separation of rat liver lysosomes (Leighton et al., 1968);

(ii) the deposition of insoluble formazan derivatives in mitochondria during succinate oxidation (Davis & Bloom, 1973);

(iii) the calcium oxalate loading of vesicles accumulating Ca^{2+} ions (Graeser et al., 1969a);

(iv) the deposition of lead phosphate at sites of phosphatase activity (Leskes et al., 1971b);

(v) the binding of concanavalin A coupled to a high density phage (ρ=1·495) to receptor sites on lymphocyte membranes (Wallach et al., 1972);

(vi) the binding of digitonin to cholesterol-containing membranes (Thines-Sempoux et al., 1969; Amar-Costesec et al., 1974b).

Density perturbation is a promising approach and has considerable scope for development. The term density perturbation, however, does not seem entirely appropriate. It may be wrongly interpreted as implying disorder in the separation.

(f) Analysis of Subcellular Fractions

Subcellular fractions can be characterised by a wide range of physical, chemical and enzymic analyses (de Duve, 1967; Reid, 1967; Beaufay et al., 1974a).

Physical Analyses

Fractionation by density gradient centrifugation enables particle densities and $S_{20.W}$ values to be determined where appropriate. Morphological characterisation is of limited value, unless the preparations retain characteristic structural features during the homogenisation process (examples: intact mitochondria, myofibrils). Generally, different types of microsomes cannot be readily distinguished from one another by morphological examination, although there are exceptions: vesicles accumulating Ca^{2+} can be identified by the calcium oxalate deposits they contain; also membranes containing phosphatase activity can be identified by the lead phosphate deposits at the reaction site (El Aaser, 1971, 1973; Leskes et al., 1971a).

Data on particle size can be obtained by centrifugation, electron microscopy and, to an extent, by filtration studies. Quantitative morphological analysis is difficult but has been attempted by Baudhain in the case of liver microsomes (Baudhain et al., 1967).

Chemical Analyses

Since most subcellular components are either membranous or associated with membranes, a knowledge of the protein and lipid composition of subcellular fractions is of particular value.

The protein constituents are generally released from membranes by treatment with the detergent sodium dodecyl sulphate and separated by electrophoresis in polyacrylamide gels.

While all membranes contain phospholipids, differences in the fatty acid moieties may be informative. Subcellular fractions may also differ in their cholesterol content. Emmelot & Bos (1962) have found that liver plasmamembrane is particularly rich in cholesterol.

Subcellular fractions may also be assayed for ribonucleic acid (e.g. Beaufay et al., 1974a)and sialic acid (e.g. Morre et al., 1974).

Enzymic Analyses

Many enzyme activities are now known to be associated with particular subcellular components and are termed *marker enzymes* (de Duve, 1967, 1971; Reid, 1967; Beaufay et al., 1974b).

De Duve (1967) has laid down two criteria for the selection of marker enzymes:

(i) the enzyme should be located in a single population of subcellular particles;

(ii) relative to their mass, the activity of the enzyme should be the same in all particles forming the population.

According to Jacques (1974), the marker constituent must also be measurable by a specific and quantitative method, be stable throughout the fractionation and be unaffected by the reagents used in the separation.

(g) Marker enzymes for use in the subcellular fractionation of muscle

Sarcolemma

(i) Ouabain-sensitive (Na^+ and K^+)-adenosine triphosphatase*

This enzyme regulates the distribution of Na^+ and K^+ across the plasmamembrane by transporting K^+ ions into the cell and simultaneously extruding Na^+ ions. The transport reaction is ATP dependent and is inhibited by the cardiac glycoside ouabain. It is believed that the enzyme hydrolyses ATP in a stepwise fashion involving the Na^+-dependent phosphorylation of the enzyme and the K^+-dependent hydrolysis of the phosphoprotein. The literature on the enzyme has been reviewed by Baker (1972); Dahl & Hokin (1974) and Hokin & Dahl (1972).

(ii) 5'-nucleotidase (5'-ribonucleotide phosphoydrolase)

Henderson & Patterson (1973) report that there appear to be two general types of 5'-nucleotidases: one with an alkaline pH optimum which hydrolyses AMP most rapidly and one with an acid pH optimum which dephosphorylates IMP, XMP and GMP preferentially; rat liver contains both types. Headon (1975) has found that the acid form predominates in muscle and postulates that this is due to the large amount of IMP produced from AMP after exercise by the action of AMP deaminase (AMP aminohydrolase) and to the lowering of the pH by lactate production. This would account for the virtual absence of 5'-nucleotidase activity in muscle fractions assayed at pH 7·4, with AMP as the substrate.

*abbreviated to ATPase.

Mitochondria

(i) Succinate-INT reductase (Pennington, 1961) is a widely used marker for the inner membrane of the mitochondrion.

(ii) Azide-sensitive ATPase (Fanburg & Gergely, 1965) is also a marker for the inner membrane.

Ca^{2+}-transporting regions of the sarcoplasmic reticulum

Membrane fragments originating from the Ca^{2+}-transporting regions of the sarcoplasmic reticulum can be identified by their ability to transport Ca^{2+} in the presence of ATP. When these membranes are in the form of sealed vesicles with the proper orientation, the transported Ca^{2+} is stored in the vesicle. Ca^{2+}-transport is accompanied by the Ca^{2+}-dependent hydrolysis of ATP and the formation of a phosphoprotein intermediate (recently reviewed by Martonosi, 1972a, 1972b).

K^+ ions are required for maximum activity of Ca^{2+}-transport and Ca^{2+}-dependent ATPase (Duggan, 1967, 1968a, 1971, 1974). The stimulation of the Ca^{2+}-dependent ATPase activity by K^+ ions has been termed the K^+-stimulated ATPase activity.

The Ca^{2+}-dependent ATPase has been referred to as the extra ATPase by some workers (e.g. Hasselbach & Makinose, 1963).

Lysosomes

Acid phosphatase and β-glucuronidase (Stagni & De Bernard, 1968; Canonico & Bird, 1970; Stauber & Bird, 1974).

Nuclei

DNA.

Ribosomes

RNA.

Sarcoplasm

Lactate dehydrogenase.

Miscellaneous

Muscle microsomal preparations contain a Ca^{2+}-independent ATPase which is also known as *basal* ATPase (Weber et al., 1966; Duggan, 1968b, 1971). This enzyme is active in the absence of Ca^{2+}, and is therefore assayed for in the presence of the Ca^{2+} chelator EGTA.*

The hydrolysis of ATP by basal ATPase does not involve a detectable phosphoprotein intermediate (Makinose, 1969; Martonosi, 1969b; Pang & Briggs, 1973).

(h) Biological Membranes

Protein and lipid are the major constituents of biological membranes. Protein accounts for 50-60% of the weight of the membrane, lipid approximately 40% and carbohydrate 0-10%. Phospholipid is the predominant lipid, although in plasmamembrane cholesterol may account for up to 40% of the total lipid.

*EGTA = ethyleneglycolbis-(aminoethylether) tetra-acetic acid.

Membrane Proteins

The true protein composition of a membrane is difficult to determine since many proteins may form loose or trivial associations with membranes, particularly during homogenisation and fractionation. Conversely, proteins loosely associated with membranes in vivo may be lost during the preparation.

In general, membrane proteins may be defined operationally as those proteins which remain associated with membranes unless extraction procedures are employed to remove them. Protein hormones and immunoglobulins which bind to receptor sites on membranes would not be classified as membrane proteins. A distinction must be drawn between the proteins of membranes and those of organelles. In the case of mitochondria,a large proportion of the protein is contained in the mitochondrial matrix and is not located in the membrane. A similar, but less dramatic, distinction can be drawn between the protein constituents in the membrane of a vesicle and those enclosed in the lumen. In the case of liver microsomes, the proteins enclosed in the vesicle are largely the secretory products of the rough endoplasmic reticulum (Kreibich & Sabatini, 1974).

Membrane proteins may be divided into two classes: intrinsic or integral proteins and extrinsic or peripheral proteins.

Intrinsic proteins can only be released from the membrane by hydrophobic bond-breaking agents such as detergents, organic solvents and chaotrophic compounds. They are usually associated with lipids when solubilised and are insoluble in aqueous buffers.

Extrinsic proteins can be freed from the membrane by high ionic strength and metal ion chelators such as EDTA* and EGTA. On release from the membrane, they are soluble in aqueous buffers.

Intrinsic proteins constitute 70-80% of the protein of the membrane. In addition to acting as transport enzymes, hormone receptors etc., they interact with the lipids of the membrane and are involved, to varying degrees, in the maintenance of membrane structure.

The functional activity of many intrinsic proteins is lost when the membrane is exposed to the action of phospholipases (e.g. Martonosi, 1972a, 1972b). When membrane structure is destroyed by treatment with sodium dodecyl sulphate, all enzyme activities associated with the membrane are lost. Treatment with deoxycholate is not so damaging to membrane - bound enzymes and many have been isolated with satisfactory activity as deoxycholate-complexes.

Characterisation of Membrane Proteins

The characterisation of membrane proteins, which has been reviewed by Juliano (1973), Steck (1974) Maddy (1974) and Wallach & Winzler (1974), may be divided into two stages:

(i) the disruption of membrane structure and the release of the protein constituents in soluble monomeric form;

(ii) the subsequent separation of the solubilised proteins.

*EDTA = ethylenediamine tetraacetic acid.

The principal agents which have been used to solubilise membrane proteins are: (1) chaotrophic agents (guanidine hydrochloride, urea); (2) organic solvents (butanol, phenol); (3) non-ionic detergents (triton X-100, lubrol PX), and anionic detergents (sodium cholate, sodium deoxycholate, sodium dodecyl sulphate*, and sodium N-lauryl sarcosinate). Of all these agents, sodium dodecyl sulphate is the most effective. It dissolves membrane proteins and lipids into soluble dodecyl sulphate complexes.

Proteins differ in their affinity for SDS, depending on their charge and the ionic strength of the solubilising medium, however, the average binding ratio is thought to be 1·4 g SDS per gram of protein at an ionic strength of 0·1. Glycoproteins bind considerably less detergent than protein alone.

The charges contributed by the bound SDS considerably outweigh those on the protein, hence charge differences between membrane proteins are abolished on solubilisation with SDS.

SDS-protein complexes can be readily separated on the basis size by electrophoresis in polyacrylamide gels equilibrated with SDS. The molecular weights of proteins, separated in this way, can also be estimated (Weber & Osborn, 1969).

SDS-polyacrylamide gel electrophoresishas become such a powerful tool in the study of membrane proteins, that most of the recent major

*sodium dodecyl sulphate is generally abbreviated to SDS.

advances in the understanding of membrane proteins can be attributed to its use. The technique has been further developed in the studies described in this thesis.

In order to separate membrane proteins on the basis of charge, solubilising agents which do not impose their charge on the proteins must be used. Furthermore, the solubilising agents must have a sufficiently low conductivity not to affect the electrophoretic separation. The most satisfactory system is the phenol-acetic acid-water-urea system of Takayama et al., (1966). 8M urea has also been used to solubilise membrane proteins for isoelectric focusing (Merz et al., 1972).

Information on the location of membrane proteins can be obtained by using specific labelling procedures prior to solubilisation and separation. This will be discussed in more detail later (Section 3.5). Selective proteolysis followed by SDS-electrophoresis has also been used to investigate the disposition of proteins in membranes.

Membrane Lipids

The phospholipids commonly found in the membranes of mammalian cells are phosphatidylcholine, phosphatidyl ethanolamine,sphingomyelin, phosphatidylserine and phosphatidylinositol. In general phosphatidyl-choline and phosphatidylethanolamine are the most abundant, although in the erythrocyte membranes of herbivores phosphatidylcholine is almost totally absent.

The physical state of the fatty alkyl side-chains of the phospholipid has a considerable influence on the functional activity of the membrane. At a certain temperature, known as the critical temperature or transition temperature, the lipids may undergo a phase transition from a gel state to a liquid crystalline state. The temperature at which this transition occurs, depends on the chain length and degree of unsaturation of the fatty alkyl side chains, the cholesterol content and the nature of the protein-lipid interactions in the membrane.

The fluidity of a membrane is influenced by the effect of cholesterol on the packing of the fatty alkyl chains of the membrane lipids (Phillips, 1972; Oldfield & Chapman, 1972). Papahadjopoulos et al.,(1973) have postulated that the high cholesterol content found in plasmamembrane is needed to protect or stabilise the membrane against mechanical stresses. However, since the fluidity of a membrane has an important influence on the functioning of transport enzymes located in it, cholesterol is believed to be excluded from highly functional areas of the membrane, e.g. the neighbourhood of the (Na^+ and K^+)-ATPase (Papahadjopoulos et al., 1973). With the exception of Golgi membranes, intracellular membrane systems generally have a considerably lower content of cholesterol than plasmamembrane. Indeed in the case of liver, the endoplasmic reticulum has been shown to contain little or no cholesterol (Amar-Costesec et al., 1974b).

The studies described in this thesis are concerned with many aspects of subcellular methodology and may be divided into a number of sections:

(i) The initial investigations into the effect of varying homogenisation time on the fragmentation of the membrane systems of the muscle cell.

(ii) The identification of factors to be considered in the choice of media for the density gradient separation of muscle microsomes.

(iii) The comprehensive analysis of microsomal fractions prepared in the form of concentrated suspensions.

(iv) The development of a highly resolving two-dimensional electrophoretic technique for the separation of membrane proteins.

(v) The isolation and characterisation of the Ca^{2+}-transporting membrane of the sarcoplasmic reticulum and a study of the distribution of proteins in the membrane.

(vi) Measurements of the distribution of cholesterol in muscle subcellular fractions. The differentiation of microsomes on the basis of their cholesterol content and the identification of enzyme activities associated with cholesterol-containing membranes.

(vii) The fractionation of the rapidly sedimenting particulate material in a muscle homogenate.

SECTION 2

MATERIALS & METHODS

2.1 TISSUE PREPARATION AND HOMOGENISATION

(a) Tissue Preparation

Young adult New Zealand White rabbits of both sexes, fed on a balanced diet, were killed by cervical dislocation. White skeletal muscle was quickly excised from the hind legs, trimmed of fat and connective tissue, and washed in ice-cold homogenisation medium. The tissue was then minced at full speed in a Kenwood Mincer (A700 Mincer; Kenwood Manufacturing Co., Hants., England.) precooled to 4°.

(b) Homogenisation

A 25% w/v homogenate was formed by homogenising the minced tissue in ice-cold homogenisation medium using a Kenmix Blender (Kenwood Manufacturing Co.,). The homogenisation media and the duration of homogenisation are given in the Results Section. There were no variations in the load placed in the homogeniser from experiment to experiment since the same weight of mince was homogenised on each occasion. Low speed centrifugations were carried out at 4° in the Sorvall RC2 - B (Ivan Sorvall Inc., USA) and the M.S.E. High Speed 18 (M.S.E. Ltd., Sussex, England) centrifuges.

2.2 DENSITY GRADIENT CENTRIFUGATION

(a) Preparation of solutions used in fractionation

10 litre stock solutions of 2·0 M sucrose were prepared from AnalaR grade sucrose (BDH Ltd., Poole, England) and stored at 4°. Other sucrose solutions required for fractionation were freshly prepared by dilution of

the stock solution. Sucrose concentrations were determined by refractive index measurements using an Abbe '60' Refractometer (Bellingham & Stanley Ltd., London) thermostatted at 20^{o}. Interpolation of the data given by Dobrota & Reid (1971) on the refractive index and molarity of sucrose solutions was used to prepare a refractive index to sucrose molarity conversion table to cover the refractive index range 1·3330 to 1·4330 in 0·0002 increments. Sucrose densities at the temperature of centrifugation were calculated by computer using the data of Barber (1966).

(b) Rotors used for density gradient centrifugation

The HS and BXIV zonal rotors and the 3 x 25 ml swinging bucket rotor (all manufactured by MSE Ltd.,) were used for density gradient centrifugation. The HS zonal rotor is constructed from high tensile aluminium and transparent perspex and has a nominal capacity of 695 ml. It is a low speed rotor, having a maximum operating speed of 10000 rev/min (8400g at r_{av} = 7.58 cm), and is described in detail in the manufacturer's technical bulletin (MSE Technical Publication No. 57). The BXIV rotor used has a nominal capacity of 650 ml and a maximum operating speed of 30000 rev/min (58000g at r_{av} = 4·51 cm) in the MSE Superspeed 40 Centrifuge. This type of rotor has been described by Anderson et al. (1967). Technical data is given in the manufacturer's bulletin (MSE Technical Publication No. 49). The 3 x 25 ml swing-out rotor (MSE Rotor No. 59590) was operated at its maximum speed of 30000 rev/min (95000g at r_{av} = 9·38 cm) in the MSE Superspeed 40 Centrifuge.

(c) Zonal rotor separations

For separations in zonal rotors, gradients and samples were dynamically loaded according to the procedure of Anderson (1967). The gradient was pumped through 3 mm bore peristaltic tubing (Silktube; J. G. Franklin & Sons Ltd., High Wycombe, England.) using the Hiloflow peristaltic pump (Metering Pumps Ltd., London.), and cooled by passing through a 3 mm bore stainless steel cooling coil immersed in an ice-water bath. Immediately before entering the rotor the temperature of the gradient was monitored using a themocouple (Zeal Ltd., London.) inserted into the flow line.

The BXIV and HS rotors were loaded while rotating at 3000 and 1500 rev/min respectively. The gradient was pumped to the edge of the rotor, light end first, at a rate of 30 ml/min. The volume of the gradient depended on the requirements of the experiment and is reported in the relevant results section. With the completion of gradient loading, sufficient 2·0 M sucrose was pumped to the rotor edge to move the light end of the gradient to the rotor core and to displace a few ml of it through the centre line. The flow was then reversed and a measured volume of sample loaded through the centre feed line from a 50 ml disposable syringe at the rate of 5 ml/min. When loaded, the sample was displaced radially from the rotor core by loading 100 ml of 0·05 M sucrose through the centre line at the rate of 5 ml/min initially.

The rotor was then accelerated to its maximum operating speed for the required length of time. During centrifugation the temperature was maintained as close as possible to 4^{o}.

The rotor contents were recovered by deceleration of the rotor to the loading speed and displacement of the contents through the centre line by pumping 2.0 M sucrose to the edge. Fractions were collected manually on a volume basis and stored in an ice-water bath.

The progress of separations in the HS zonal rotor was observed using a stroboscopic lamp (Edwards Scientific International Ltd., Yorkshire, England.)

A zonal rotor separation protocol sheet is shown in Fig. 2.1

(d) Preparation of concentrated microsomal suspensions

Headon & Duggan (1973) have described a procedure whereby concentrated microsomal suspensions can be prepared from large volumes using the BXIV rotor. This method avoids the use of sedimentation-resuspension steps which result in extensive aggregration of microsomes (Glaumann & Dallner, 1970).

For much of the work described in this thesis microsomal suspensions were prepared by this method.

The sample preparation is summarised in Fig. 2.2. The use of 1·0 M sucrose in the homogenisation medium reduces the amount of mitochondrial fragmentation (Headon & Duggan, 1973). 450 ml of the $5{\cdot}76 \times 10^5$ g-min supernatant in 0·67 M sucrose, were pumped to the edge wall of a BXIV rotor rotating at 3000 rev/min. This was followed by 100 ml of 1·0 M sucrose - 5 mM imidazole-HCl, pH 7·4 and 2·0 M sucrose-5 mM imidazole-HCl, pH 7·4 to fill the rotor. After centrifugation at 30 000 rev/min for 60 min, the rotor was unloaded at 3000 rev/min by pumping 2.0 M sucrose to the edge and displacing the contents through the centre line. The

ZONAL CENTRIFUGATION

Rotor _____ Sample _____ Z-UCC Run _____

Sample Weight _____g Date _____

Homogenisation Medium _____ Loading and Unloading speed _____

Volume of Homogenate _____ml Rotor Speed _____

Density of Homogenate _____g/ml Time at Speed _____

Preparation _____

_____

_____

Gradient Material _____

Gradient Volume _____ml Density Range _____ to _____

Gradient Shape in Rotor _____ Operating Temperature _____

Gradient Preparation _____

_____

Gradient Loading Rate _____ml/min Gradient Material

Gradient Loading Rate _____ml/min

Sample Volume _____ml.

Sample Loading Rate _____ml/min

Overlay Material _____

Overlay Volume _____ml

Overlay Loading Rate _____ml/min

Gradient Unloading Rate _____ml/min

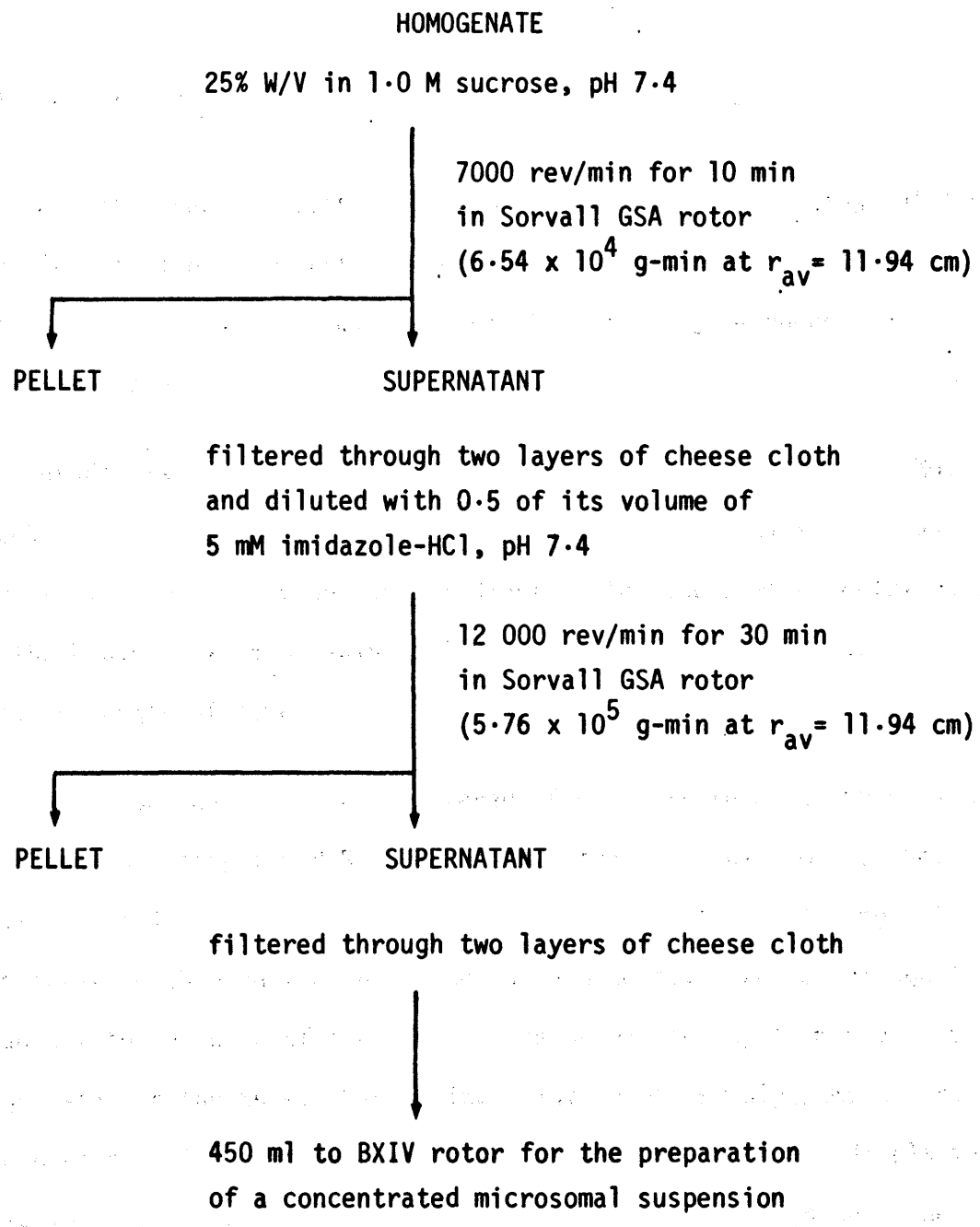
Number of Fractions Collected ____

Volume of Each Fraction _____ml

Fraction Number	Molarity	Density
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		
22		
23		
24		
25		
26		
27		
28		
29		
30		
31		
32		
33		
34		
35		
36		
37		
38		
39		
40		

Fig. 2.1. Example of zonal rotor separation protocol sheet.

Fig. 2.2. The sample preparation procedure for obtaining a concentrated microsomal suspension



first 450 ml were collected as one fraction and the next 150 ml in 25 ml fractions. The microsomal suspension was collected in four fractions over a short gradient corresponding to the original band of 1·0 M sucrose.

(e) Density gradient centrifugation in swing-out tubes

Density gradient centrifugation was carried out in swing-out tubes when insufficient material was available to proceed with a separation in a zonal rotor or when it was required to centrifuge a number of samples simultaneously.

Gradients were formed in 23 ml polycarbonate tubes (M.S.E. No. 59209) as described later in this section. 1 or 2 ml of sample, as required, were layered on each gradient. The tubes were centrifuged in the M.S.E. 3 x 25 ml swing-out rotor at 30 000 rev/min for the desired length of time.

The tube contents were recovered after centrifugation by upward displacement using the M.S.E. Gradient Extraction Unit (M.S.E. No. 42116-101 & 42117-104). A fine needle filled with displacing solution was inserted through the unloading cap and gently lowered through the gradient to the base of the tube. The unloading cap formed a liquid-tight seal on the tube. Displacing solution was slowly pumped through the needle. The gradient was raised upwards ahead of the displacing solution and collected in fractions as it emerged through the side exit of the cap assembly. By pumping the displacing solution from a burette fractions of known volume were collected. In some experiments fractions were collected dropwise using the Gilson Microfractionator

(Gilson Medical Electronics, Villiers-le-Bel, France). Fractions were stored at 4^{o}.

(f) Formation of density gradients for zonal rotors

The basic function of a density gradient in a zonal or swing-out rotor is to separate zones of sedimenting particles. The composition, shape and capacity of the gradient influence the separations that can be obtained.

A gradient maker for use with zonal rotors should, according to Hinton & Dobrota (1969) be able to reproduce a range of gradient shapes which can be predicted mathematically.

The exponential gradient maker of Birnie & Harvey (1968) produces exponential gradients whose shape is determined by the volume and concentration of the solution in the reservoir and the volume and initial concentration of the solution in the mixing vessel. The shape of these gradients can be predicted by the following equation

$$M_{(V)} = M_{(M)} + \left[M_{(R)} - M_{(M)}\right]\left[1 - e^{(-V/V_{(M)})}\right]$$

where

$M_{(V)}$ = molarity at volume V;

$M_{(M)}$ = initial molarity of solution in the mixing vessel;

$M_{(R)}$ = molarity of solution in the reservoir;

V = volume of gradient formed;

$V_{(M)}$ = volume of solution in mixing vessel.

Complex gradient formation requires that $V_{(M)}$, $M_{(M)}$ and $M_{(R)}$ can be varied during gradient formation. This gradient maker was modified to enable these changes to be carried out. The modified gradient maker, while retaining the simplicity of the Birnie & Harvey (1968) model, can produce a wider range of gradient shapes.

Description of gradient maker

A diagram of the gradient maker is shown in Fig. 2.3. The apparatus is so designed that the molarity and/or the volume of the solution in the mixing vessel can be readily changed during a pause in the loading operation.

The reservoir solution is introduced into the centre of the mixing vessel, and the gradient drawn from the outer edge of the vessel floor. Exit lines from the reservoirs to the mixing vessel (lines *a* and *b*) must be sufficiently wide to prevent the viscosity of the gradient material becoming a limiting factor in the flow of heavy solution from the reservoirs. If this flow is limited, pressure will fall in the mixing vessel, and result in a net loss of solution from the vessel. To overcome this problem, exit lines from the reservoirs of 4mm internal diameter have been found suitable for use with sucrose solutions. To achieve efficient mixing in the mixing vessel, the solution should occupy 20-40% of the vessel volume. 3 mm bore peristaltic tubing is suitable for flow lines to the rotor. It is desirable to keep all flow lines as short as possible. Artery clamps have been found particularly suitable for clamping flow lines. T-junctions and three-way stopcocks (Pharmaseal Laboratories, California, USA) are useful accessories. The Hiloflow peristaltic pump gives a satisfactory performance, enabling the pumping

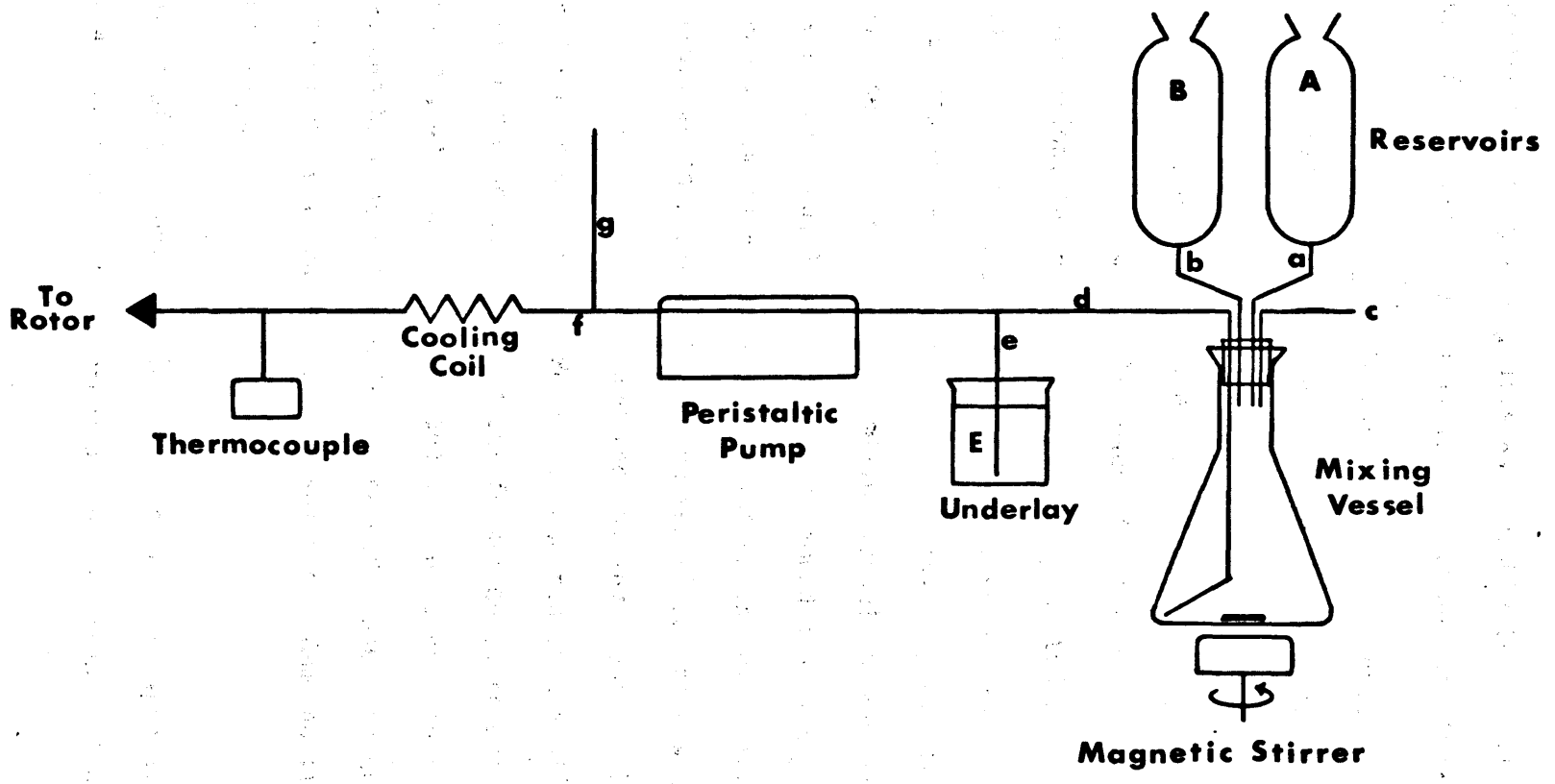


Fig. 2.3. Diagrammatic representation of modified exponential gradient maker.

rate to be varied during gradient loading. Line *c* acts as an air inlet during the partial or complete emptying of the mixing vessel, or as an air outlet during the addition of further solution to the mixing vessel. Line *e* is used to deliver underlay solution after gradient loading. During gradient loading line *g* acts as a bubble trap, and while adjusting the volume of the mixing vessel it is used as an exit line.

Operation of gradient maker. To form a sucrose density gradient, line *a* is clamped, and reservoir A is loaded with the required volume of gradient material of desired concentration. The mixing vessel is loaded with its solution. Lines *b*, *c*, *e* and *g* are clamped, and the system is checked to ensure that it is air-tight. The magnetic stirrer is started. To commence gradient formation, the peristaltic pump is operated and line *a* unclamped simultaneously. During gradient formation, changeover to a second heavy solution, stored in reservoir B, can be achieved by clamping line *a* and unclamping line *b*. To reduce the mixing vessel volume, the lines to the reservoirs are clamped, lines *c* and *g* unclamped and line *f* clamped. The required volume of mixing vessel solution is pumped through line *g*. If it is required to replace the mixing vessel solution with another solution, the vessel is emptied and the pumping stopped. Line *g* is clamped and line *b* unclamped. When the required volume of liquid has been run in from reservoir B, gradient formation is recommended by clamping lines *b* and *c* , unclamping lines *f* and *a*, and operating the pump. These procedures can be repeated as often as needed. When the gradient has been loaded, the underlay is pumped from vessel E by clamping line *d* and unclamping line *e*.

Examples of gradient profiles produced by this apparatus are shown in Fig. 2.4. Details of their preparation are given in Table 2.1.

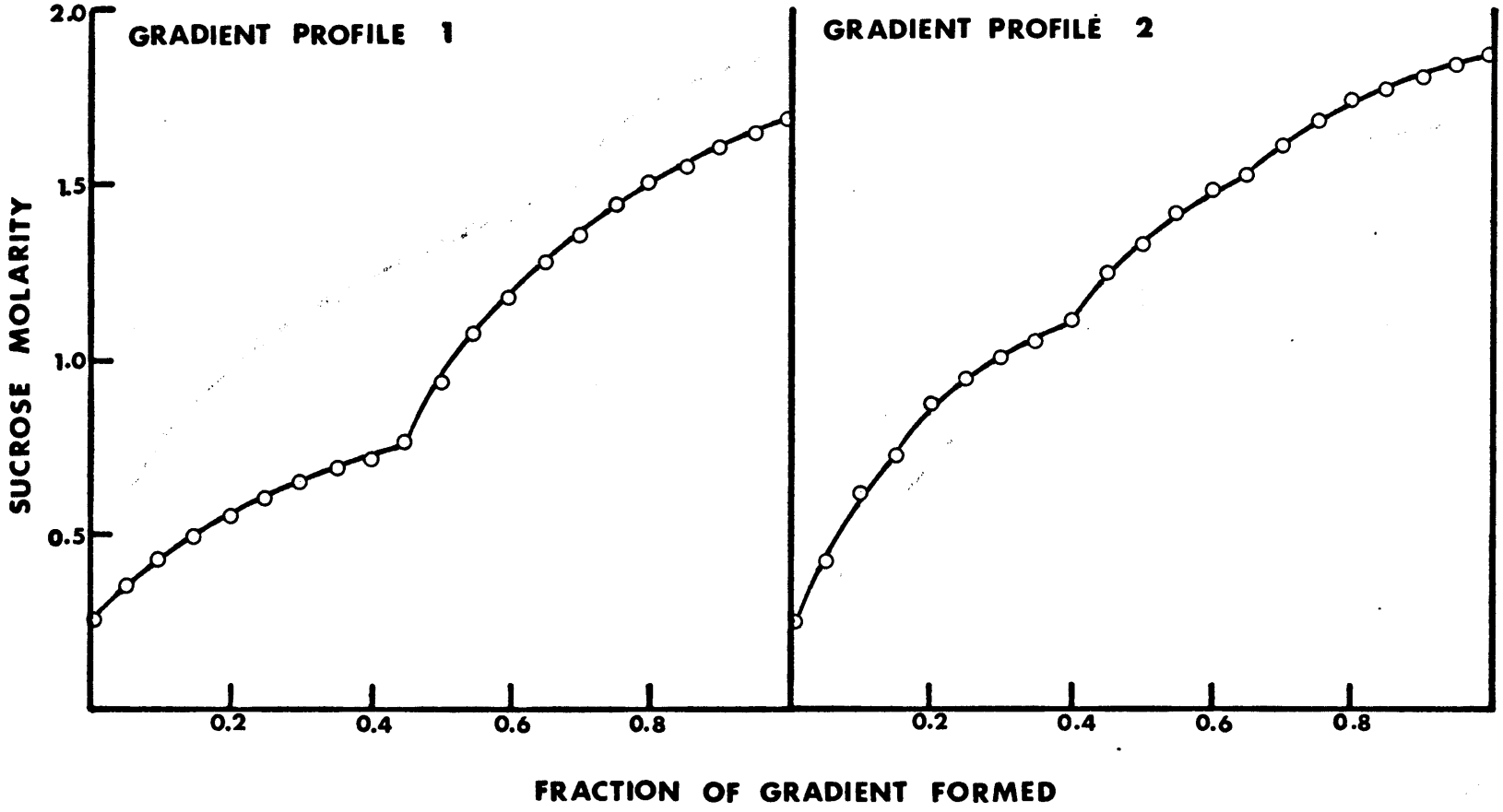


Fig. 2.4. Gradient profiles produced by the gradient maker shown in Fig. 2.3. Details of their preparation are given in Table 2.1. Each gradient had a volume of 400 ml and was collected in 20 ml fractions.

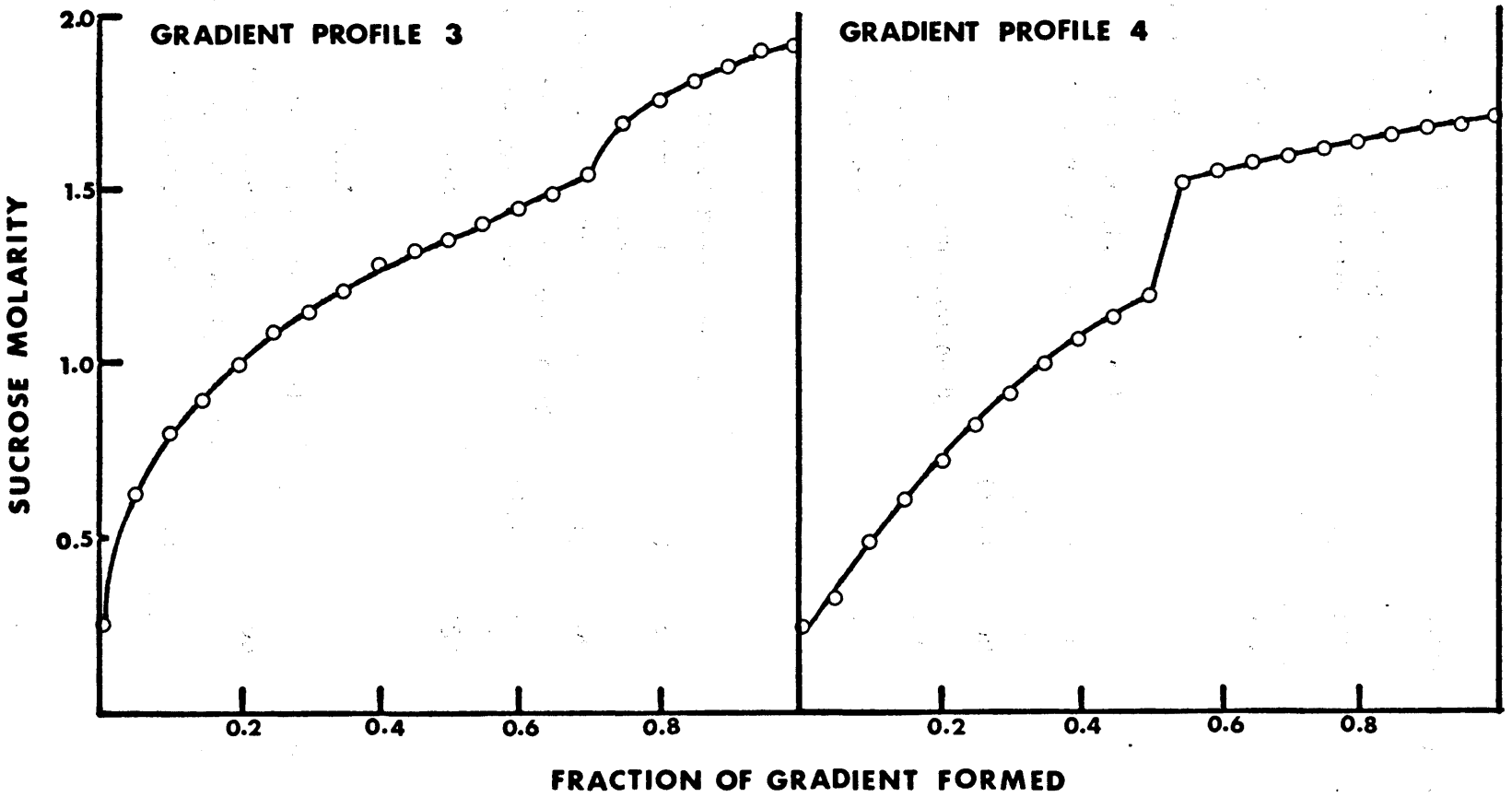


Fig. 2.4. contd. Gradient profiles produced by the gradient maker shown in Fig. 2.3. Details of their preparation are given in Table 2.1. Each gradient had a volume of 400 ml and was collected in 20 ml fractions.

TABLE 2.1

Procedures for the formation of the gradient profiles shown in Fig. 2.4. Volumes are expressed in ml.

Gradient Profile 1		Volume (ml)		Sucrose Molarity
Initial content of mixing vessel			150	0·25
Additions from reservoirs	(i)	170		1·00
	(ii)	230		2·00
Volume of gradient		400		

Gradient Profile 2				
Initial content of mixing vessel			150	0·25
Additions from reservoirs	(i)	50		2·00
	(ii)	90		1·50
	(iii)	90		2·00
Mixing vessel solution reduced by			50	
Addition from reservoir	(iv)	170		2·00
Volume of gradient		400		

Gradient Profile 3				
Initial content of mixing vessel			100	0·25
Addition from reservoir	(i)	50		2·00
Addition to mixing vessel			200	1·00
Addition from reservoir	(ii)	200		2·00
Mixing vessel solution reduced by			200	
Addition from reservoir	(iii)	150		2·00
Volume of gradient		400		

Gradient Profile 4				
Initial content of mixing vessel			250	0·25
Addition from reservoir	(i)	200		2·00
Mixing vessel solution replaced by			400	1·50
Addition from reservoir	(ii)	200		2·00
Volume of gradient		400		

The gradients formed are closely related to the mathematically calculated gradients. The largest deviation from the calculated shape (never exceeding 5%) occurs at the points of changeover from one reservoir to another and is due to the hydrostatic pressure exerted by the solutions in the reservoirs.

(g) Formation of density gradients for swing-out tubes

Gradients for swing-out tubes were formed using the M.S.E. linear gradient maker (M.S.E. No. 36657). This gradient maker consists of two identical 40 ml cylindrical chambers cut in a perspex block. Both chambers are linked at base level and flow from one to the other is regulated by a stop-cock. These chambers act as reservoir and mixing vessel. Three identical gradients were formed simultaneously by withdrawal of liquid at an equal rate from the mixing vessel through three exit lines at the base of the vessel. The flow rate from the mixing vessel was controlled by using 0·073 inch internal diameter standard Technicon pump tubing (Technicon Instruments Corporation, New York.) in the Gilson Minipuls II pump. Lengths of fine glass tubing were attached to the pump tubes for insertion into upright centrifuge tubes as shown in Fig. 2.5. Adequate mixing in the mixing vessel can be achieved through the use of a magnetic stirrer.

Formation of linear gradients. Linear gradients are formed according to the following equation

$$M_{(V)} = M_{(M)} + (M_{(R)} - M_{(M)}) (V / (V_{(M)} + V_{(R)}))$$

where

$M_{(V)}$ = molarity at volume V;

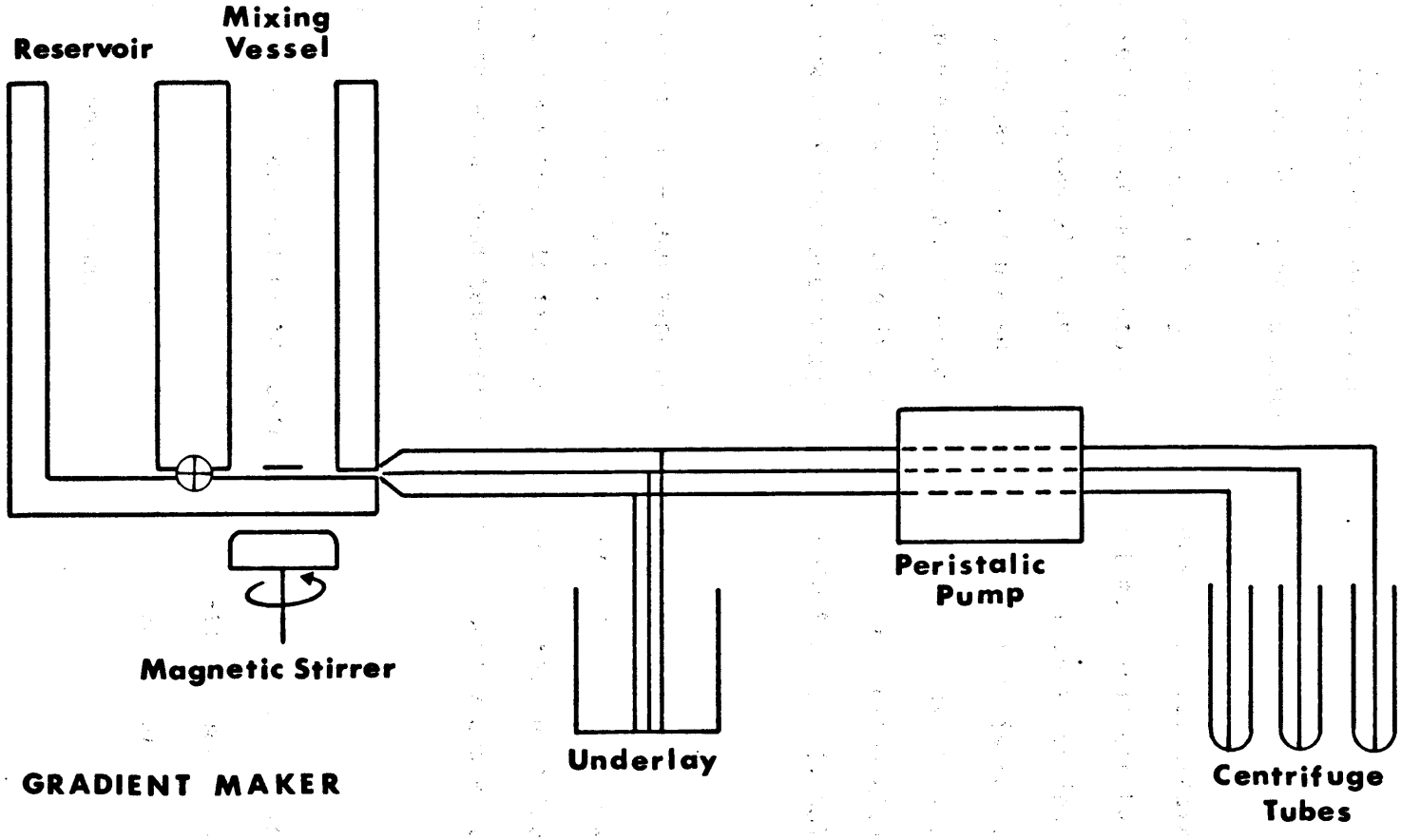


Fig. 2.5. Diagrammatic representation of apparatus used to form density gradients in swing-out tubes.

$M_{(M)}$ = initial molarity of solution in the mixing vessel;

$M_{(R)}$ = molarity of solution in the reservoir;

V = volume of gradient formed;

$V_{(M)}$ = initial volume of solution in the mixing vessel;

$V_{(R)}$ = initial volume of solution in the reservoir.

Formation of exponential gradients. To form an exponential gradient the mixing vessel must be made into a closed system. This can be done by sealing the mixing vessel with a rubber stopper. In inserting the stopper it is important that pressure in the mixing vessel is not increased. Any increase in pressure can be prevented by inserting a hypodermic needle with the stopper and withdrawing it some seconds later. Exponential gradients can now be formed according to the Birnie & Harvey (1968) procedure.

15 ml gradients of either type were formed at the rate of 1-2 ml/min using the Gilson Minipuls II pump. The gradient was displaced upwards by sufficient 2·0 M sucrose to fill the hemispherical region of the tube. Gradients were formed at room temperature and subsequently cooled to 4^{o}.

(h) Gradient design

Gradient shapes were calculated using the computer programs in Appendix A. These programs are written in conversational mode and were used to compute linear and exponential gradients. Samples of the output are given in the appendix also.

(i) Calculation of sedimentation coefficients

A computer program based on that of Bishop (1966) was used to compute sedimentation coefficients. It includes equations, derived by Barber (1966), expressing the density and viscosity of sucrose solutions as functions of concentration and temperature.

The input data consists of the speed of the rotor in revolutions per minute, the centrifugation time in minutes, the volumes in ml of overlay, sample and fractions, the number of fractions collected after the sample, the temperature of centrifugation in ^{o}C and the range of particle densities for which sedimentation coefficients are required.

The program, written in Fortran IV was run on an IBM 370/135 computer. This program, which is entitled 'Sedimentation Job' is listed in Appendix B and a sample of the output is also given.

Equations describing the relationship between radius and volume for the HS and BXIV zonal rotors were required in the calculation of sedimentation coefficients for centrifugation in these rotors. These equations were obtained using the computer program listed in Appendix C. This program enables a first or second degree polynomial equation to be obtained to fit, in a least squares sense, the data given for the particular rotor by the manufacturer. Table 2.2 lists the equations obtained for a number of commonly used zonal rotors. The data points for these rotors and the equations to fit them are plotted in Figure 2.6.

(j) Optimisation of conditions for density gradient separations.

Conditions to suit a particular separation can be 'optimised' by simulating the separation using the computer program in Appendix D.

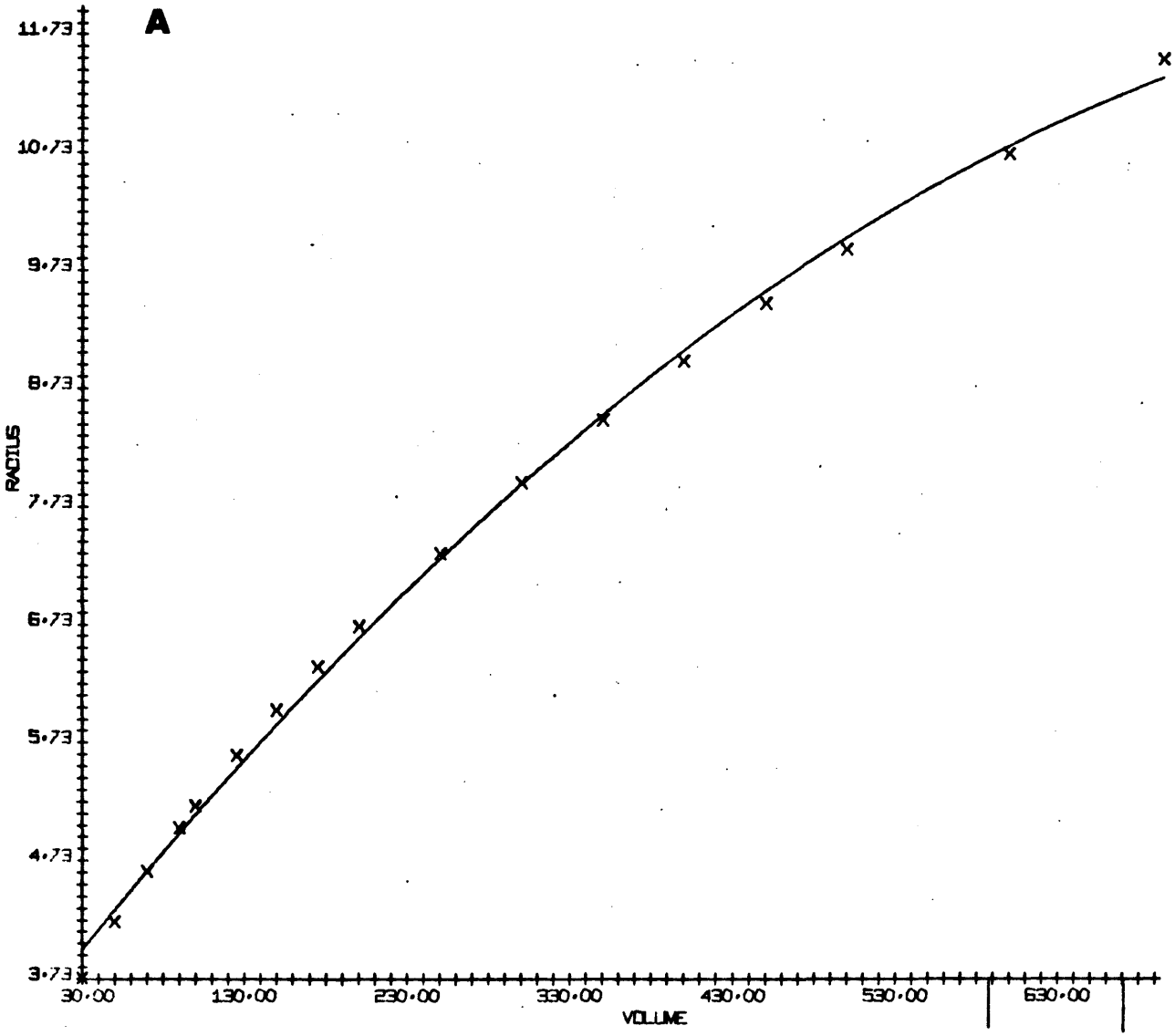
TABLE 2.2.

Equations describing the relationship between radius and volume for a number of zonal rotors

ROTOR	EQUATION
HS	$R = 3{\cdot}466 + (0{\cdot}0175)(V) + (-0{\cdot}0000087)(V)^2$
BXIV	$R = 2{\cdot}262 + (0{\cdot}0100)(V) + (-0{\cdot}0000049)(V)^2$
AXII	$R = 5{\cdot}121 + (0{\cdot}0142)(V) + (-0{\cdot}0000035)(V)^2$
BXV	$R = 2{\cdot}190 + (0{\cdot}0076)(V) + (-0{\cdot}0000028)(V)^2$

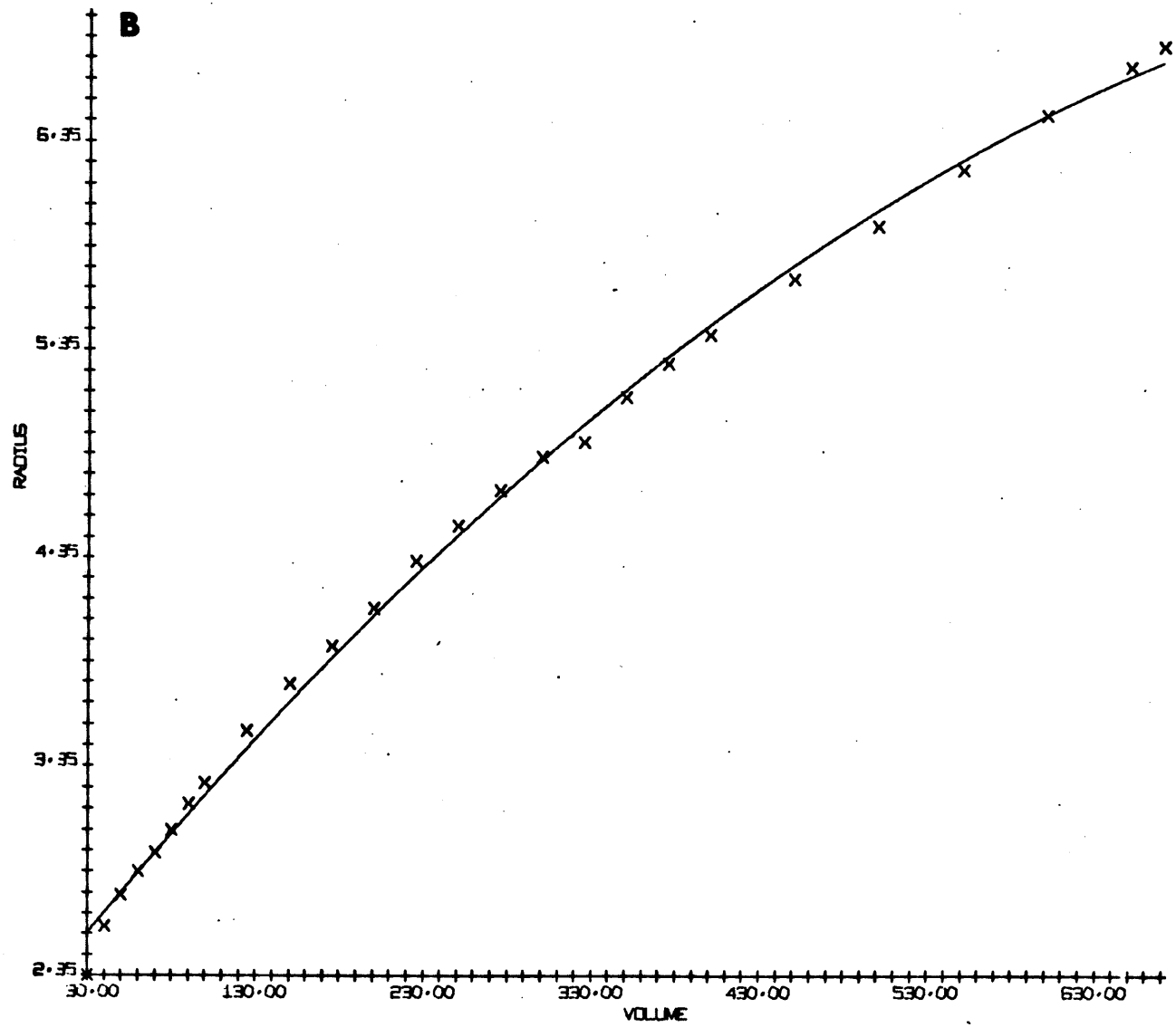
R = radius (cm)
V = volume (ml)

Fig. 2.6. (A - D on the following four pages). Radius to volume relationships for a number of zonal rotors. Equations, computed to best fit volume and radius data for the HS (A), BXIV (B), AXII (C), and BXV (D) rotors are plotted.



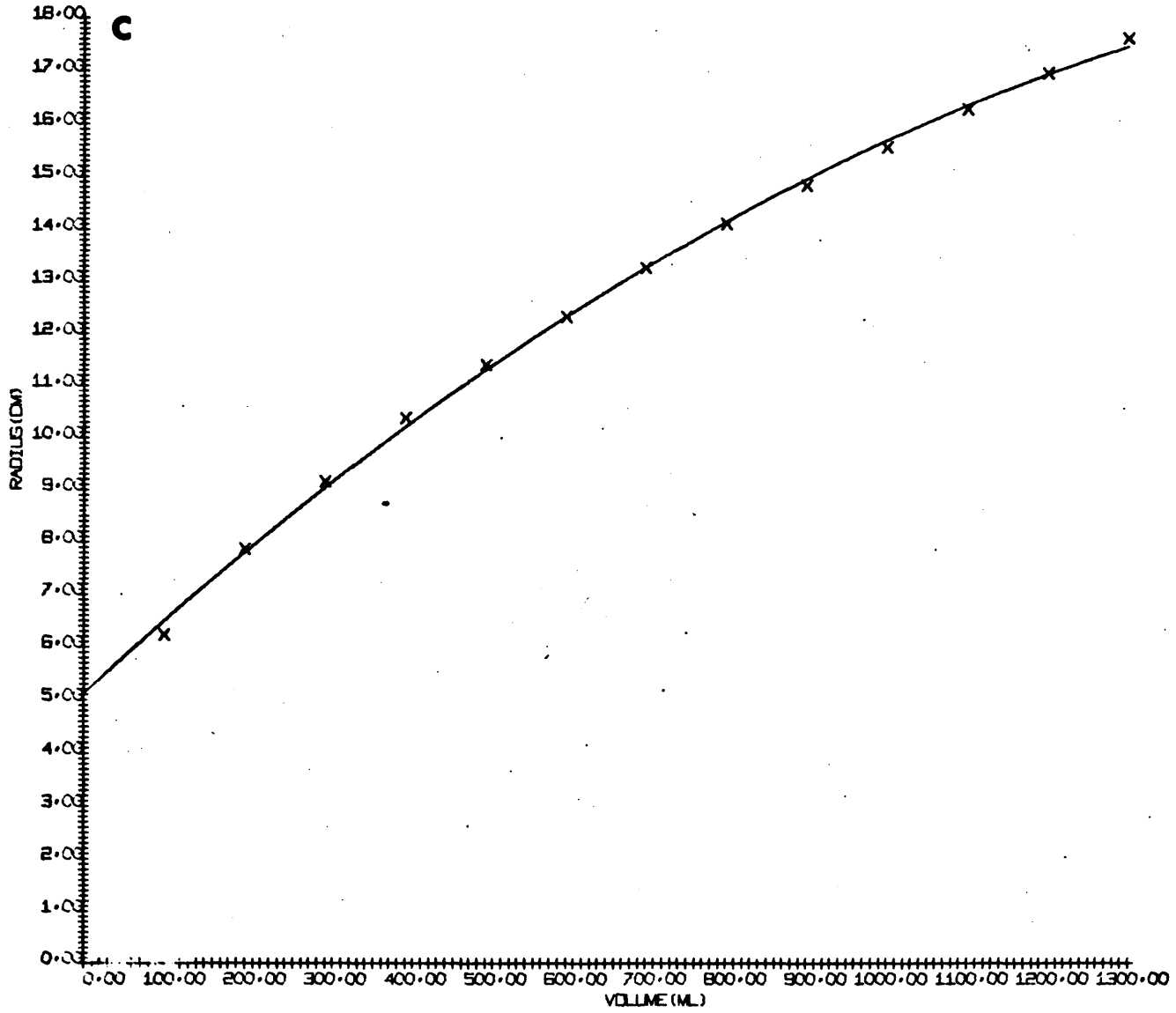
RADIUS = 3.466+ 0.0175*V+-0.0000*V**2

CALIFORNIA COMPUTER PRODUCTS, INC. ANAHEIM, CALIFORNIA CHART NO. 668 MADE IN U.S.A.

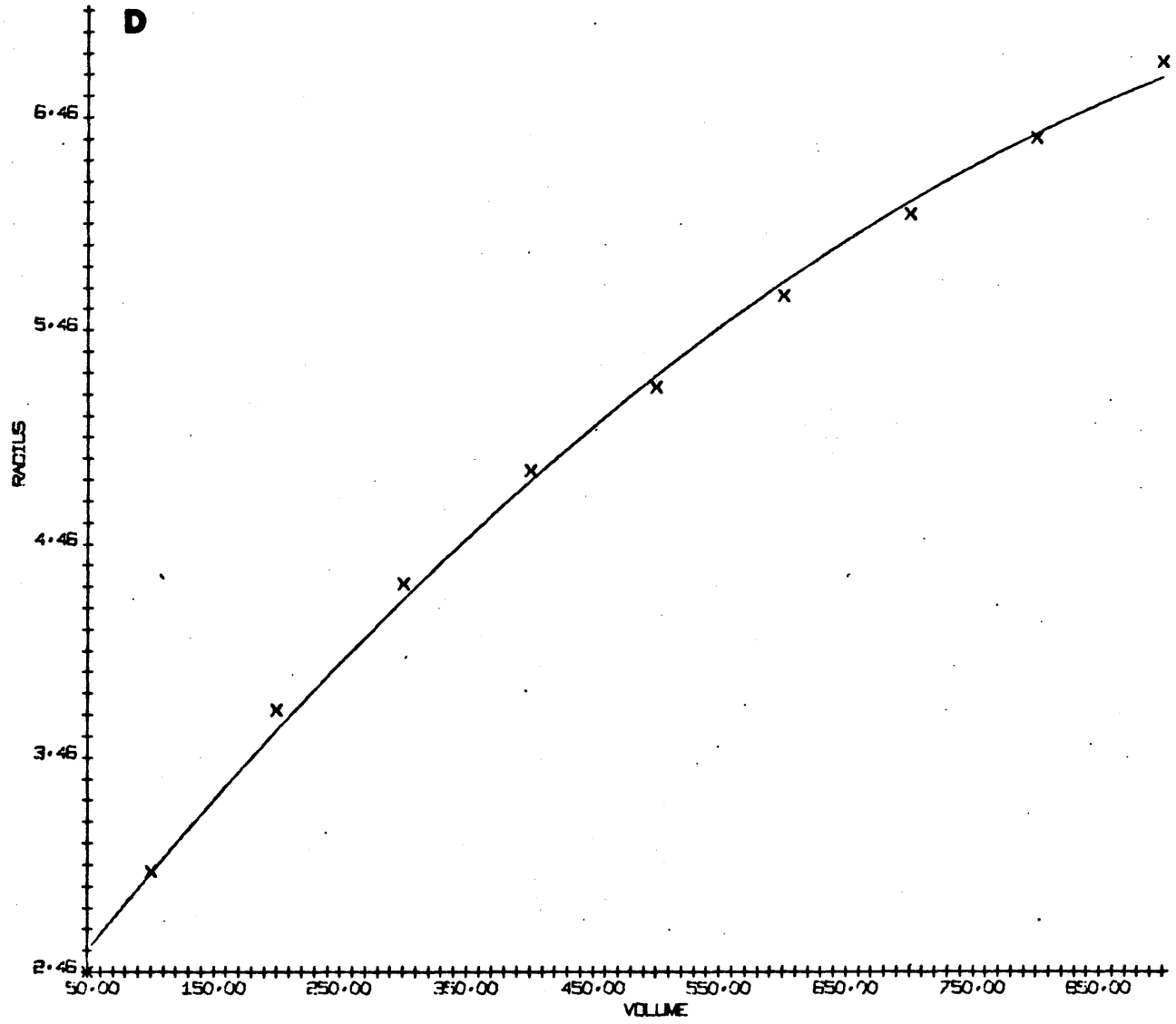


RADIUS = 2.262+ 0.0100*V+-0.0000049*V**2

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RADIUS = 5.121+ 0.0142*V+-0.0000035*V**2



RADIUS = 2.190+ 0.0076*V+-0.0000028*V**2

A looping mechanism in the program allows the operator to hold the gradient, rotor speed, and temperature constant and quickly determine sedimentation coefficients for any number of centrifugation times and particle densities.

2.3 ANALYSES

Density gradient separations in zonal rotors yield a large number of fractions which must be assayed for a number of enzymes and constituents. Without automation considerable time and labour would be involved in these analyses. In this study protein concentration and adenosine triphosphatase(ATPase) activities were measured by automated and semi-automated procedures respectively, using the Technicon Auto Analyzer (Technicon Instruments Corporation, New York, USA.). The system used consisted of the following modules: sampler, proportioning pump, manifolds, heating bath (all manufactured by Technicon) and a spectrophotometer (Model 124 Double-Beam Grating Spectrophotometer; Perkin-Elmer Ltd.)

(a) Determination of protein concentration

Protein concentration was determined by the automated method of Leighton et al. (1968). This method is adapted from the manual procedure of Lowry et al. (1951). The manifold used is illustrated in Fig. 2.7. Samples for analysis were suitably diluted with distilled water. Bovine serum albumin fraction V at a concentration of 100 µg/ml was routinely used as a standard. Fig. 2.8 shows a plot of extinction (E) at 750 nm versus protein concentration over the range 0 to 200 µg/ml.

Sucrose interferes in the assay of protein by the Lowry procedure (Hinton et al., 1969; Hinton & Norris, 1972; Hartman et al., 1974).

Fig. 2.7. (Overleaf) AutoAnalyser manifold for the automated estimation of protein based on that of Leighton et al. (1968).

D1, D2: glass fittings as in Technicon catalogue.

105-83, 105-88, 105-89: refer to Technicon mixing coils.

HB: heating bath at 37^{o}, 80 ft coil, 1·6 mm internal diameter.

S: spectrophotometer, wavelength = 750 nm.

The reagents used had the following composition:

NaOH, 1·0N;

Copper-tartrate, 0·73 mM $CuSO_4$ and 26 mM sodium-potassium tartrate;

Na_2CO_3, 0·69M;

Folin, freshly prepared 12·5 fold aqueous dilution of Folin & Ciocalteu's Phenol reagent (BDH Ltd.);

Standard Technicon pump tubing with the indicated flow rates was used.

Sampling rate: 30 samples/hour.

Sampler wash: 1% W/V NaCl.

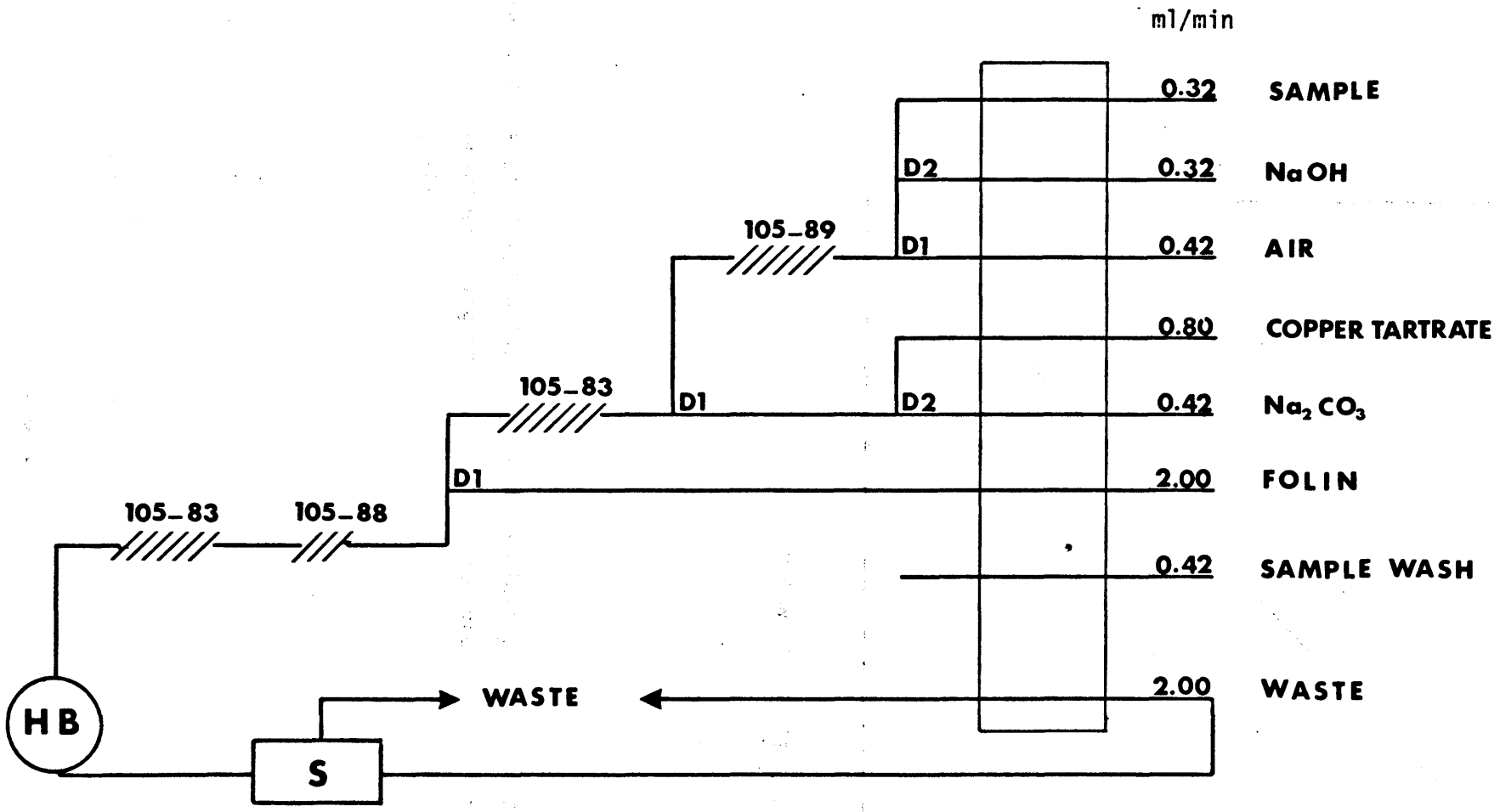


Fig. 2.7. Legend on previous page.

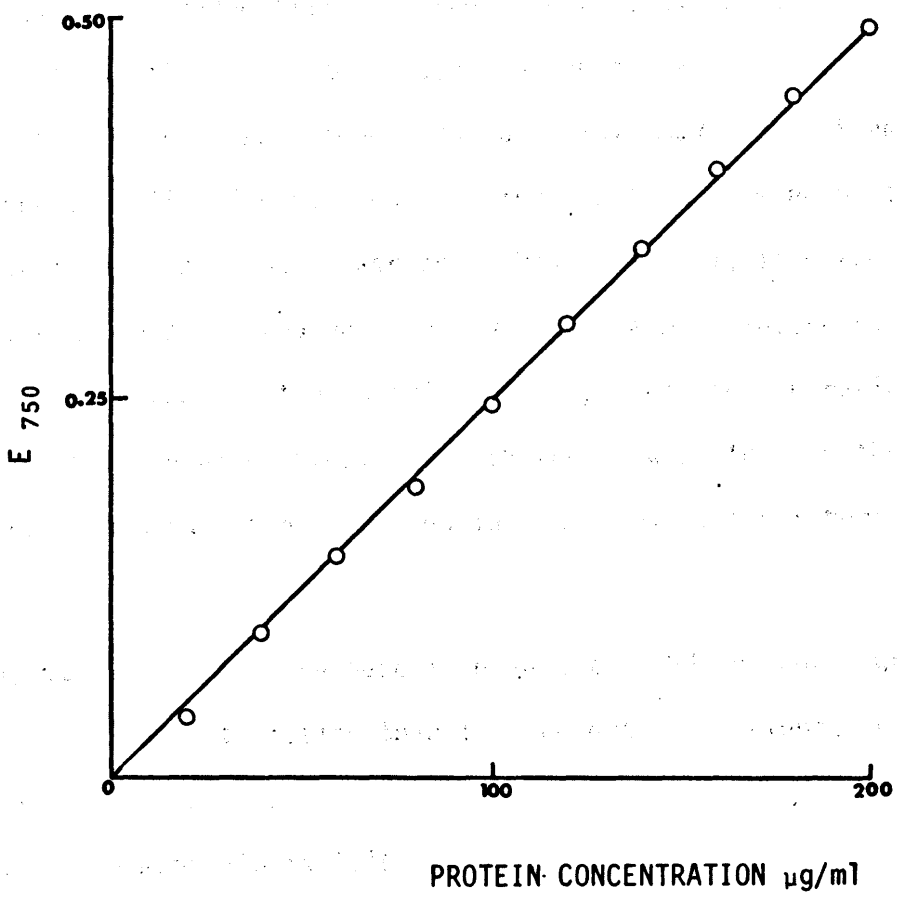


Fig. 2.8. Plot of extinction at 750 nm against protein concentration obtained using the automated procedure of Leighton et al. (1968).

The effect of sucrose on the assay procedure used in this study has been investigated by Headon (1972). The effect is two-fold:
(i) Sucrose reacts with the reagents used in the assay, giving an extinction linear with sucrose concentration. This may vary between sucrose batches. (ii) Sucrose inhibits the reaction between protein and the reagents in a nonlinear fashion. Headon (1972), in correcting for these effects, has derived equations which have been incorporated into a computer program for the calculation of protein concentration (Appendix E). Sucrose also interferes with the flow pattern in the AutoAnalyzer. This has been described in detail by Hinton & Norris (1972).

Beaufay et al. (1974) have described an automated procedure which gives better flow characteristics than that reported by Leighton et al. (1968). This procedure has been used for part of the work described in this thesis. The manifold is illustrated in Fig. 2.9. Sucrose exerts effects on this manifold similar to those described already, however the correction factors are different. These effects are shown in Fig. 2.10 and the equations describing them in Table 2.3.

Without pretreatment, the protein concentration of grossly particulate suspensions, e.g. homogenates cannot be reliably estimated.
These suspensions were 'solubilised' by treatment at 100^{o} in IN NaOH containing 2% W/V Na_2CO_3 prior to analysis.

(b) Analyses for phospholipid and cholesterol

Extraction. Samples for lipid analysis were extracted with chloroform-methanol 2 : 1 (V/V) according to the procedure of Folch et al. (1957) with the following modifications. Phase mixing and extraction were carried out by homogenisation for 30 sec using the Ultra-Turrax homogeniser

Fig. 2.9. (Overleaf) Manifold for the automated estimation of protein based on that of Beaufay et al. (1974a).

AI, CI, DI, GI: glass fittings as in Technicon catalogue.

105-86, 105-88: refer to Technicon mixing coils.

J M. 010: jet mixer, alternate segments of 0·010 and 0·0625 inch internal diameter Tygon tubing.

HB: heating bath at 37^{o}; 40 ft coil, 1·6 mm internal diameter, on each passage through the bath.

S: spectrophotometer, wavelength = 660 nm.

The reagents used had the following composition:

Copper-tartrate, 1·6 mM $CuSO_4$ and 2·83 mM sodium-potassium tartrate; Na_2CO_3 - NaOH, 755 mM Na_2CO_3 and 340 mM NaOH;

NaDOC, 2% W/V in water;

Folin, freshly prepared eight fold aqueous dilution of Folin & Ciocalteu's Phenol reagent (BDH Ltd.);

Sampler wash, water.

Standard Technicon pump tubing with the indicated flow rates was used.

Sampling rate: 20 samples/hour.

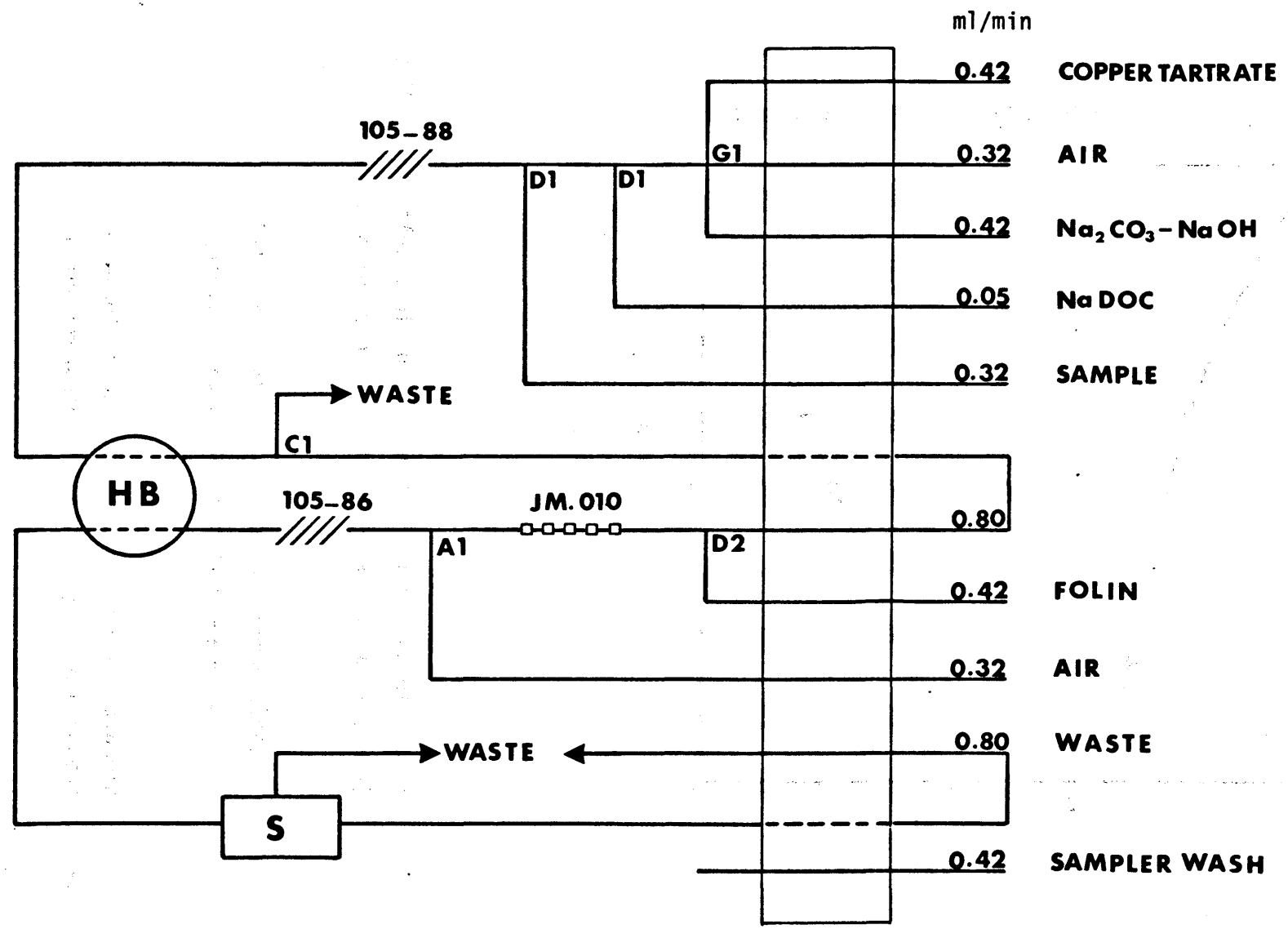


Fig. 2.9. Legend on previous page.

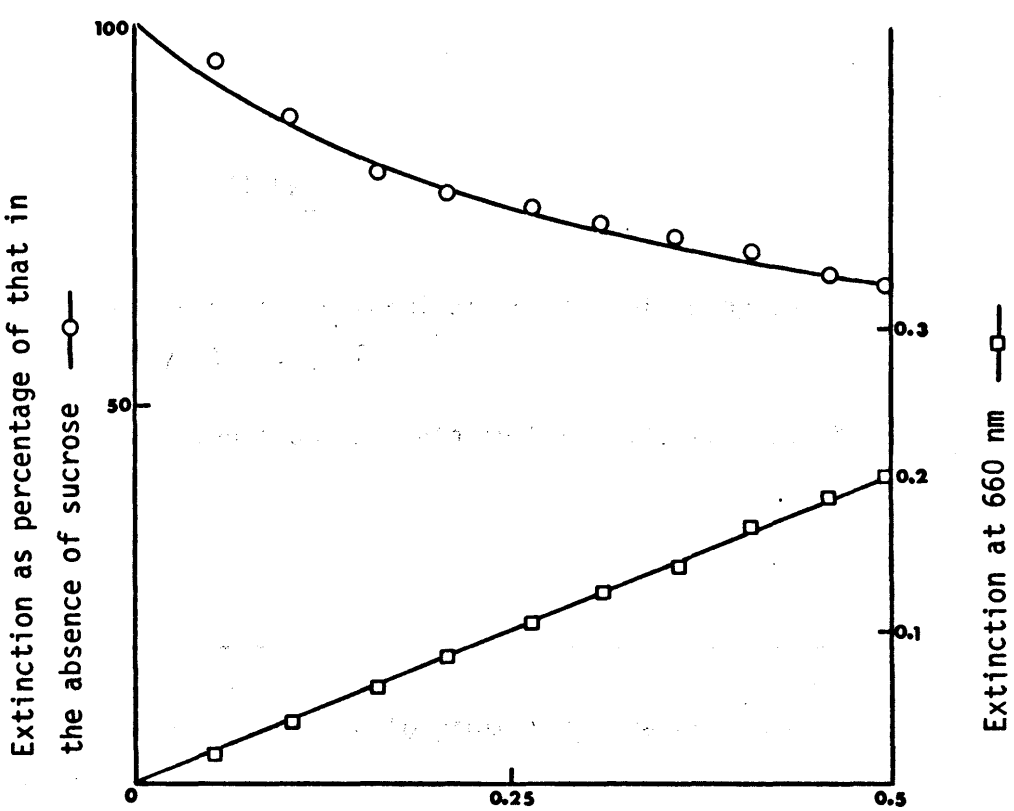


Fig. 2.10. The effects of sucrose on the estimation of protein by the automated procedure of Beaufay et al (1974).

Extinction at 660 nm due to sucrose alone plotted against sucrose concentration —□— .

—○—Colour produced by bovine serum albumin in the presence of increasing sucrose concentration expressed as a percentage of that in the absence of sucrose.

TABLE 2.3.

Effects of sucrose on the determination of protein by the method of Beaufay et al. (1974).

Equations derived from the data in Fig. 2.10 to describe these effects.

(i) Inhibition of colour due to protein in the presence of sucrose

Molarity range	Extinction of protein as % of that in the absence of sucrose
0 to 0·149	[100 - 102·25 (molarity)]
0·150 to 0·500	[87·5 - 42·37 (molarity)]

(ii) Extinction due to sucrose only.

Extinction = (0·406) (molarity)

(Janke & Kunkel; with TPION or TP18N shaft as appropriate to the volume extracted.). The phases were then separated by centrifugation at a speed setting of 5 in the MSE Minor Centrifuge. The lower, lipid containing, phase was removed and taken to dryness under a stream of nitrogen at 40^{o}. The dried extract was carefully redissolved in 1·0 ml of chloroform and stored at -20^{o} in glass vials (G.L.C. vials; Pye-Unicam Ltd., Cambridge, England). Since fatty acid analysis was not being carried out on these extracts precautions were not taken to ensure that they were always under nitrogen.

Estimation of total phospholipid phosphorus. Phospholipid phosphorus was measured according to the procedure of Bartlett (1959). A suitable aliquot of lipid extract was taken to dryness. 0·5 ml of 10N H_2SO_4 were added to the dried extract and the mixture heated at 160^{o} for 3 to 4 hours. Two drops of 30% W/V H_2O_2 (AnalaR Grade) were added and the mixture heated for a further 2 hours to clear the charring and decompose the excess H_2O_2. 4·6 ml of 0·22% W/V ammonium molybdate and 0.2 ml of Fiske & SubbaRow reagent (Bartlett, 1959) were added. After a thorough mixing the solution was heated at 100^{o} for 7 minutes. On cooling the extinction at 810nm was measured. Blanks and standards were analysed simultaneously. To convert µg of phospholipid phosphorus to µg of phospholipid a factor of 25 was used. Fig. 2.11 shows a plot of extinction at 810nm versus µmoles of phospholipid.

Separation of phospholipid classes. Phospholipid classes were separated by one-dimensional thin layer chromatography on 20 x 20 cm precoated plates (Anachem Ltd., Luton, England.). These plates were coated by the manufacturer with silica gel G to a thickness of 250 µm. The volume of sample applied depended on the phospholipid concentration and ranged from

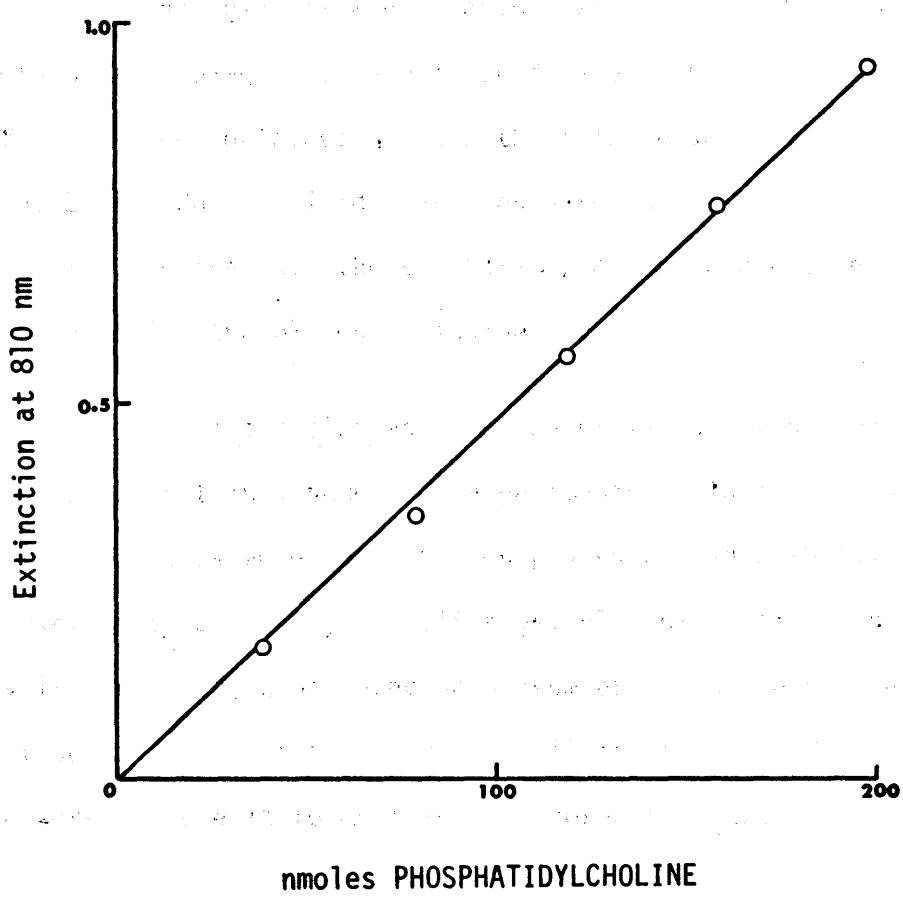


Fig. 2.11. A plot of extinction at 810 nm against nmoles of phosphatidylcholine estimated according to the method of Bartlett (1959).

25 - 100 µl. Either of the following two solvents were used:
(i) chloroform-methanol-glacial acetic acid - water (100 : 60 : 16 : 8),
(ii) chloroform-methanol-glacial acetic acid-water (100 : 20 : 12·5 : 5).
Examples of separations obtained using both of these solvent systems are shown in Fig. 2.12. Phospholipid standards were co-run on the same plate as the tissue extracts. The separated phospholipid classes were visualised by exposing the plates to iodine vapour.

Quantitation of phospholipid classes. The quantitation of phospholipid classes was carried out in a number of experiments. In these experiments the phospholipid classes were separated on plates coated with silica gel HR (Merck, Darmstadt, Germany.). Silica gel HR, unlike silica gel G, is a specially pure grade containing no gypsum or organic binder and is particularly suited to quantitative work. It is less suited to general qualitative work because it forms fragile adsorbent layers.

The phospholipid classes, separated on silica gel HR, were located with iodine vapour. The stained areas were carefully removed from the plate to test tubes and the iodine was allowed to diffuse from the silica. The phospholipid phosphorus content of each area was determined by the procedure of Bartlett (1959). Appropriate blanks and standards were analysed simultaneously.

Analysis of neutral lipids by thin layer chromatography. Neutral lipid classes were separated on 20 x 20 cm silica gel G precoated plates (Anachem Ltd.). Either N-hexane-diethylether-acetic acid (80 : 20 : 1) or chloroform - N-hexane-ethyl acetate - acetic acid(150 : 50 : 25 : 4) were used as solvents. Chromatograms obtained using these solvent systems are shown in Fig. 2.13. The neutral lipid classes were stained with iodine vapour. In addition, cholesterol and cholesterol esters were

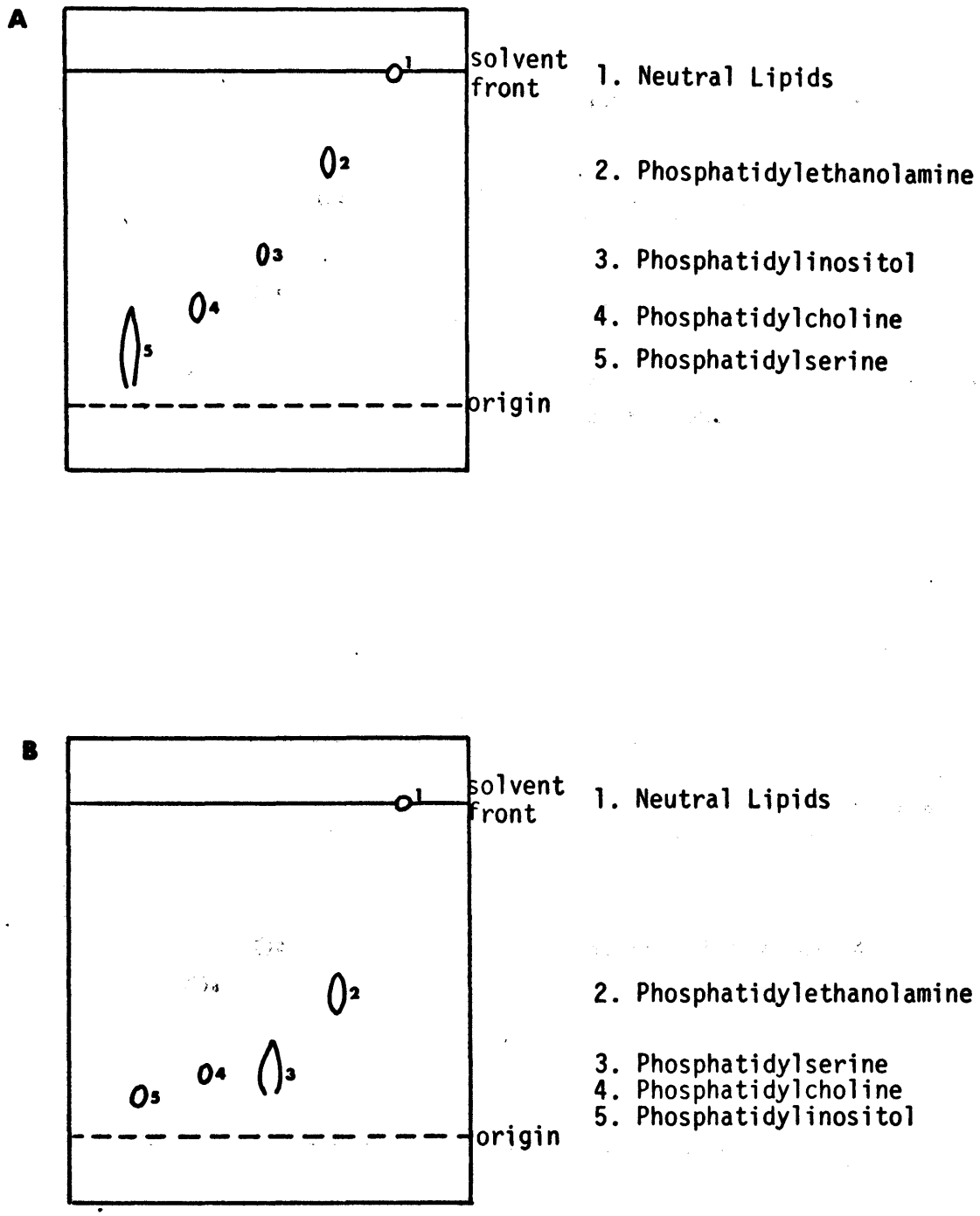


Fig. 2.12. Diagrammatic representation of one-dimensional thin layer chromatograms of phospholipid standards on silica gel G.

The following solvents were used to develop the chromatograms: Chromatogram A: chloroform-methanol-acetic acid-water(100 : 60 : 16 : 8); Chromatogram B: chloroform-methanol-acetic acid-water (100 : 20 : 12·5 : 5).

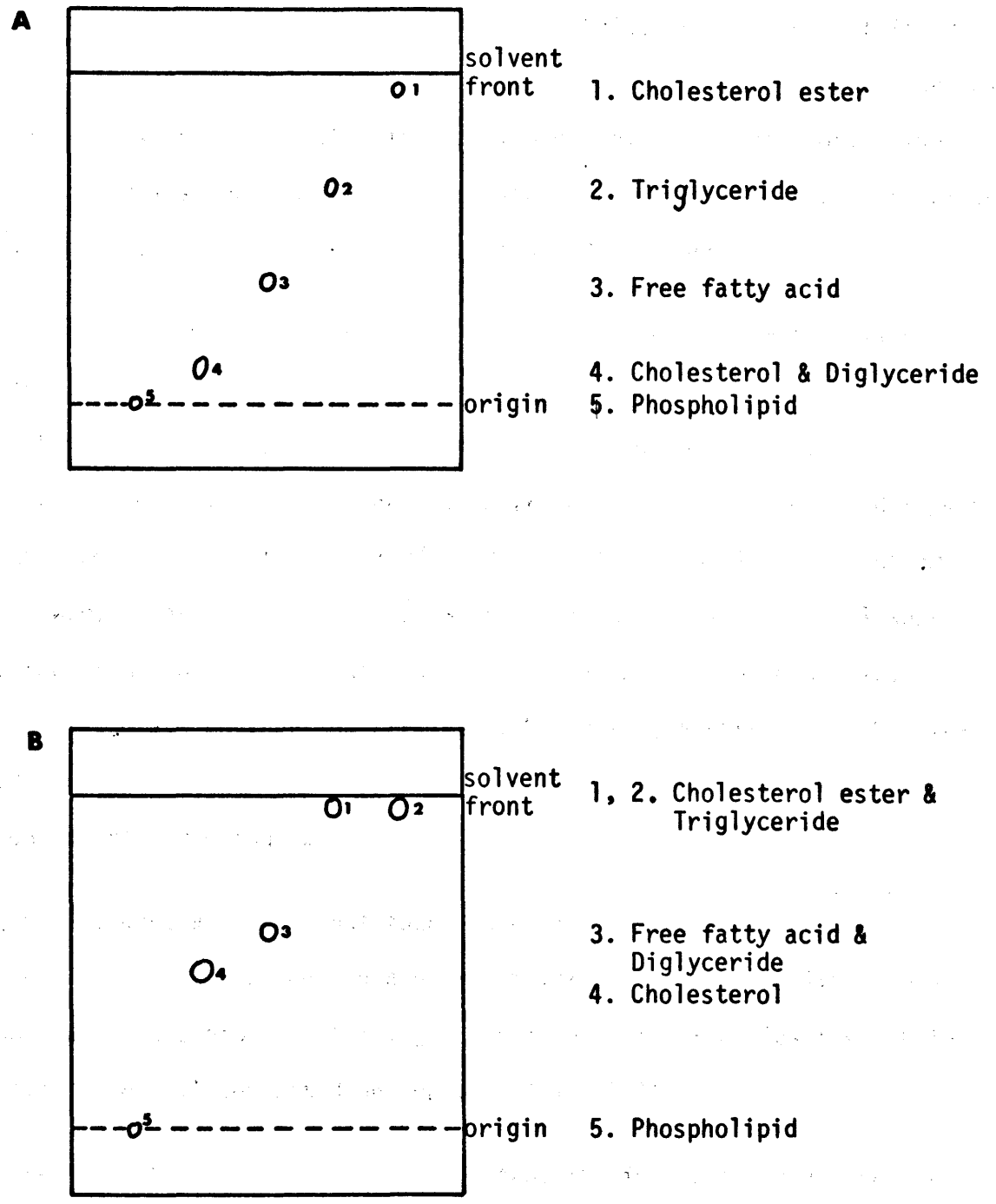


Fig. 2.13. Diagrammatic representation of one-dimensional thin layer chromatograms of neutral lipid standards on silica gel G.

The following solvents were used to develop the chromatograms:

Chromatogram A: N-hexane-diethyl ether-acetic acid(80 : 20 : 1).

Chromatogram B: chloroform-N-hexane-ethyl acetate-acetic acid (150 : 50 : 25 : 4).

detected using the ferric chloride spray of Lowry (1968).

Quantitative analysis for cholesterol. The cholesterol content of lipid extracts was determined by gas liquid chromatography in a Pye-Unicam Series 104 (Model 24) chromatograph with dual flame ionization detector. The chromatograph was coupled to a Servoscribe Is recorder with disc integrating facilities (R.E. 542.20; Smiths Industries Ltd., London.).

Separations were carried out in 5 ft x 6 mm outer diameter glass column packed with 3% methyl silicone gum (OV-1) on 100-120 mesh diatomite CQ. Both the column and support were silanised with dimethyl dichlorosilane. The carrier gas was nitrogen at a flow rate of 50 ml/min and the operating temperature 250^{o}. Gas purifying bottles (Pye-Unicam Ltd.,) were inserted into all the gas feed lines. Samples of 1µl were injected from S.G.E. 1B syringes with either 7 cm or 11·5 cm needles (Scientific Glass Engineering Pty. Ltd., Melbourne, Australia). For the automatic injection of samples the autojector S4 (Pye-Unicam Ltd.,) was linked to the chromatograph.

Cholestane, a steroid not found in animal tissues, was used as an internal standard. Dried lipid extracts for cholesterol analysis were dissolved in chloroform containing a known quantity of cholestane. Both steroids were well separated on analysis (Fig. 2.14).

Peak areas were calculated either from the integrator trace on the recorder, according to the manufacturer's instructions or alternatively, using the method of Carroll (1961). The latter method assumes that the peak area is directly proportional to the product of the peak height and retention time.

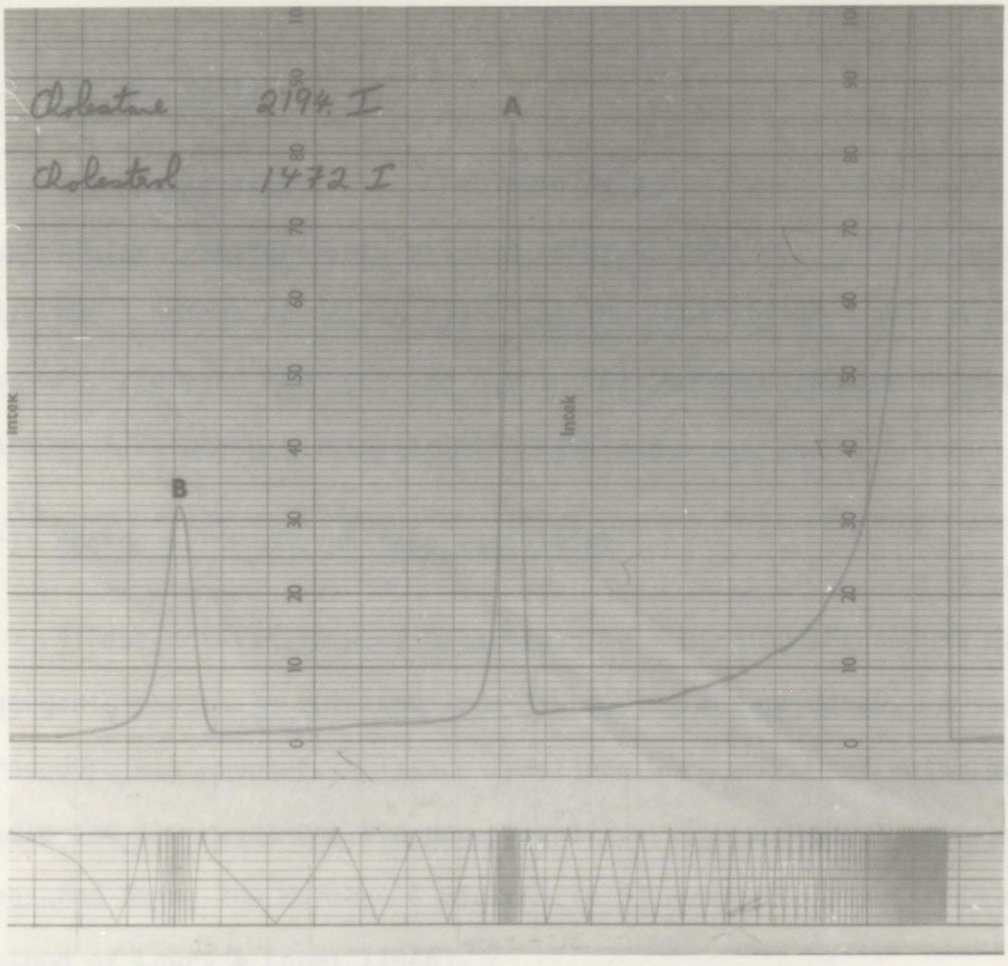


Fig. 2.14. Cholesterol analysis by gas liquid chromatography carried out as described in the text. The separation of cholesterol from the internal standard, cholestane, is shown.

1 µl of sample containing 100 ng of cholesterol and 100 ng cholestane in chloroform was injected. The cholesterol peak has an area of 1472 integration units and a retention time of 16·5 min. The cholestane peak has an area of 2194 integration units and a retention time of 9·5 min.

Conditions: 3% OV-1 column, operating temperature of 250°, carrier gas N_2 at 50 ml/min, attenuator 2 x 10^{-10}A, chart 1 cm/min.

The sensitivity of the detectors was such that 1 ng of cholesterol could be reliably measured.

(c) Enzymic analyses

All enzyme assays were performed at 25°. Where necessary, subcellular fractions were diluted prior to analysis. In accordance with the findings of Headon (1972) on the lability of muscle microsomal enzymes, analyses were carried out in the sequence calcium uptake, the various ATPases and succinate-INT* reductase.

(i) Adenosine triphosphatase activities

ATPase activities were determined by measuring the amount of inorganic phosphate released from ATP during enzyme assay. A semiautomated procedure was adopted. Tissue fractions were incubated with the assay media and the reactions stopped manually. The phosphate released was measured using the Marzban & Hinton (1970) automated adaptation of the method of Lowry & Lopez (1946).

The ATPase activities assayed for in this work differ in their response to certain monovalent and divalent cations. It is therefore necessary that the commercially available disodium salt of ATP be first converted to the imidazole form, in order that the concentration of cations in the various assay media can be regulated independently of the ATP concentration.

Preparation of ATP imidazole. Disodium ATP (product No. A-3127; Sigma Chemical Co., London.) was converted to the imidazole form by treatment with a cation exchange resin in the H^+ form. The resin was prepared by

*INT = 2 -(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride.

successively washing Amberite IR-120 resin (BDH Ltd., Poole, England.) three times with each of the following: 1N HCl, distilled deionized water, 1N NH_4OH, distilled deionized water, and 1N HCl. Finally the resin was washed with distilled deionized water until all the acid was removed.

1g of disodium ATP was dissolved in 5 ml of water. 5g of washed resin were added and the mixture stirred at 4^{o} for 20 min. The resin was removed by filtration and the treatment repeated with a further 5g of resin. The pH of the final filtrate was adjusted to 7·0 with 1M imidazole. The ATP concentration was determined spectrophotometrically using the molar extinction coefficient of $15{\cdot}4 \times 10^3$ at 260 nm (Burton, 1969). The filtrate was diluted with 50 mM imidazole-HCl, pH 7·0 to give a stock solution of 15 mM. The stock solution was divided into suitable aliquots and stored at -20^{o}. Under these conditions the ATP solution was stable for several months.

Assay media. The composition of assay media for the various ATPase activities was as follows:

Mg^{2+} - ATPase

3 mM ATP-imidazole,
3 mM $MgCl_2$,
50 mM imidazole-HCl, pH 7·0

K^{+}-stimulated Mg^{2+}-ATPase

3 mM ATP-imidazole,
3 mM $MgCl_2$,
100 mM KCl,
50 mM imidazole-HCl, pH 7·0.

Basal ATPase/Ca^{2+}-insensitive ATPase

3 mM ATP-imidazole,
3 mM $MgCl_2$,
1 mM *EGTA-imidazole, pH 7·0,
50 mM imidazole-HCl, pH 7·0.

Azide-sensitive ATPase

The inhibition of Mg^{2+}-ATPase by 50 mM NaN_3. When necessary 50 mM NaN_3 was incorporated into the other media also.

Ouabain-sensitive Na^+- and K^+-ATPase

3 mM ATP-imidazole,
3 mM $MgCl_2$,
100 mM NaCl,
10 mM KCl,
50 mM imidazole -HCl, pH 7·0 with and without 1 mM ouabain.

Note: the concentration of ouabain required for maximum inhibition will depend on the protein concentration in the reaction mixture.

Assay procedure.0.1ml of fraction containing 10 to 100 µg of protein was added to 1·0 ml of incubation medium, which had been preincubated at 25° for 10 min. Depending on the activity of the fraction, the incubation time varied from 5 to 20 min. The reaction was terminated by the addition of 1 ml of 6% W/W perchloric acid. The precipitated protein was pelleted by centrifugation at top speed for 5 min in the M.S.E. Minor Centrifuge. The inorganic phosphate concentration in the protein-free supernatant was then measured. Blank determinations were carried out on the incubation media and, where necessary, on the tissue fractions. The rate of hydrolysis of ATP after deproteinisation was found to be

*EGTA = ethyleneglycolbis-(aminoethylether) tetra-acetic acid.

0·015 μmoles per ml per hour. By having initial and final blanks this breakdown was readily corrected for on the recording trace from the AutoAnalyzer.

Estimation of inorganic phosphate. A diagram of the manifold used to estimate inorganic phosphate is shown on Fig. 2·15. A plot of extinction at 700 nm versus phosphate concentration can be seen in Fig. 2·16 and a recorder trace of ATPase activity with increasing reaction time in Fig. 2·17.

Sucrose was found to react with the reagents used and gives an extinction at 700 nm which is linear with increasing sucrose concentration (see Fig. 2·18.). Samples containing high sucrose concentrations trailed a little.

(ii) Calcium uptake activity

ATP-mediated Ca^{2+}-uptake in the presence of oxalate was measured in a medium containing

3 mM ATP-imidazole,
3 mM $MgCl_2$,
100 mM KCl,
3 mM oxalate-imidazole, pH 7·0,
50 mM imidazole-HCl, pH 7·0,
100 μM $CaCl_2$ - consisting of $^{40}CaCl_2$ and the radioactive compound $^{45}CaCl_2$.

Non-specific binding was measured by omitting ATP from the medium and ATP-mediated binding by omitting oxalate. 0·15 ml of fraction containing 15 to 150 μg of protein were added to 1·5 ml of assay medium, which had been preincubated for 10 min at 25°. The reaction was terminated by filtering 1 ml of the reaction medium through 0·45 μm average pore diameter

Fig. 2.15. (Diagram on following page).

Estimation of inorganic phosphate based on the Marzban & Hinton (1970) automated adaptation of the method of Lowry & Lopez (1946).

D1: glass fitting as in Technicon catalogue.

105-86, 105-87: refer to Technicon mixing coils.

HB: heating bath at 25^{o}, 40 ft coil, 1·6 mm internal diameter.

S: Spectrophotometer, wavelength = 700 nm.

The reagents used had the following composition:

Ammonium molybdate, 0·6% W/V ammonium molybdate in 5% W/V perchloric acid, water being used as the initial solvent to aid the dissolution of the molybdate; Ascorbate, aqueous solution, 2mg/ml; Sampler wash, 3% W/W perchloric acid.

Standard Technicon pump tubing with the indicated flow rates was used.

Sampling rate: 50 samples / hour.

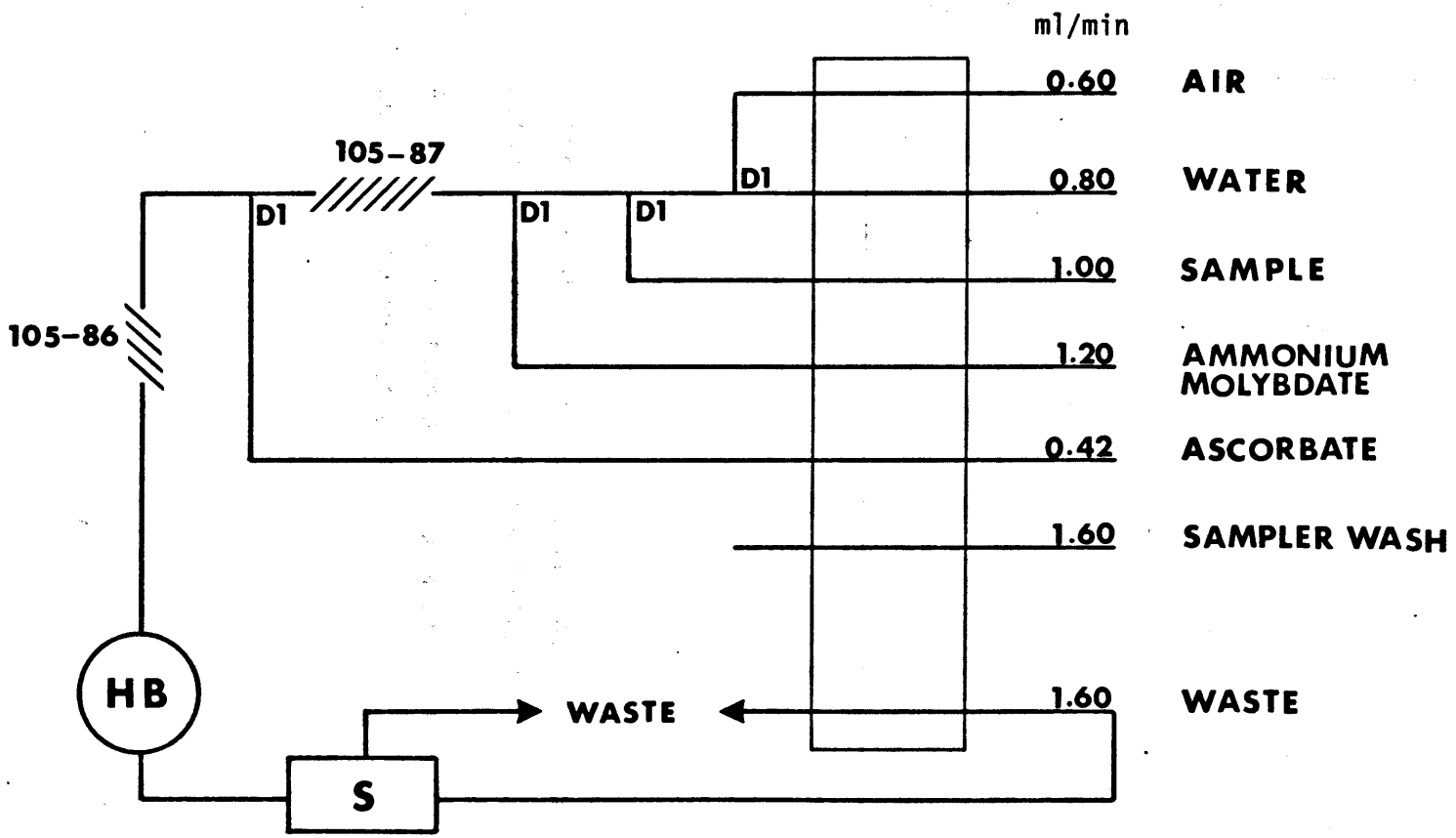


Fig. 2.15. Legend on previous page.

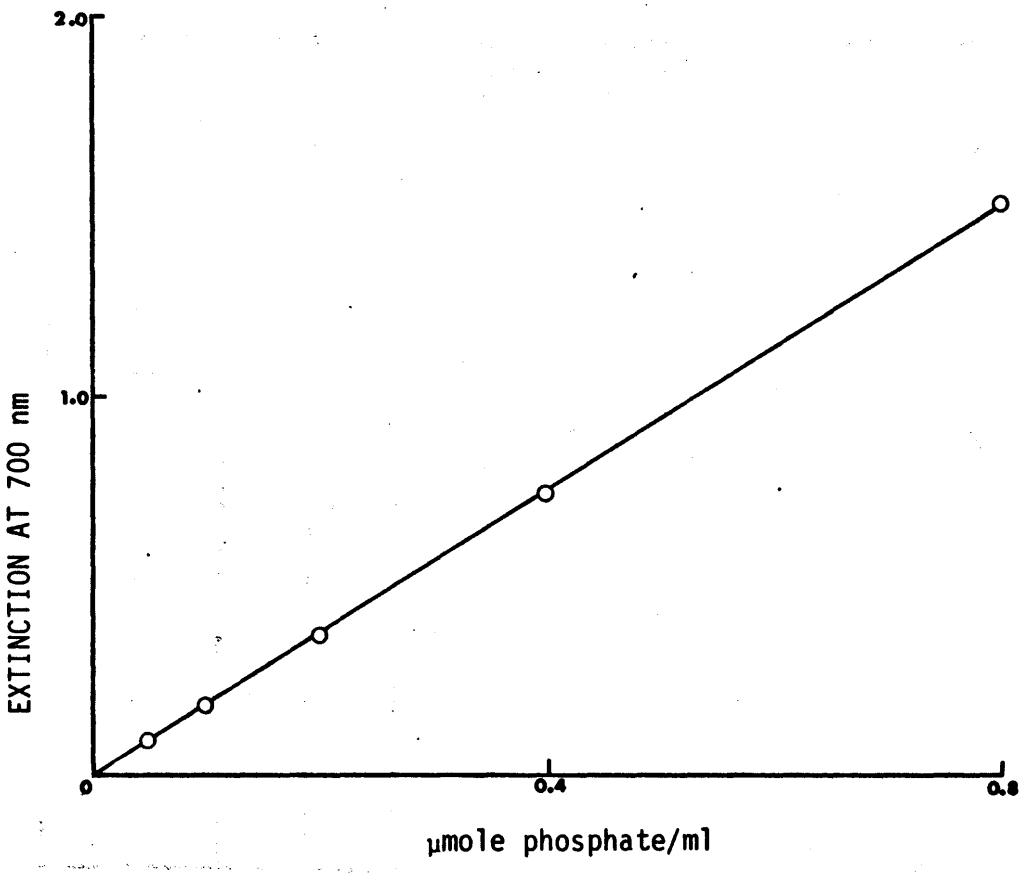


Fig. 2.16. Estimation of inorganic phosphate according to the automated method of Marzban & Hinton (1970).

A plot of extinction at 700 nm against inorganic phosphate concentration.

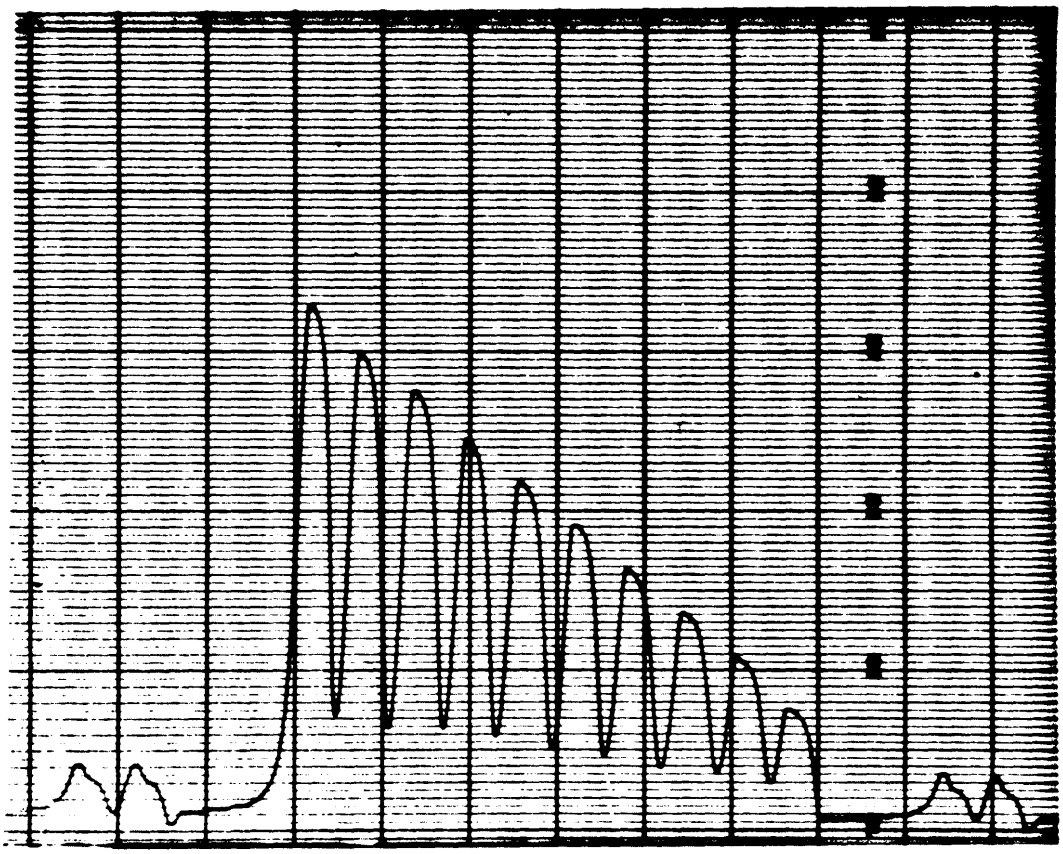


Fig. 2.17. Determination of inorganic phosphate released during ATPase assay.

An ATPase reaction was carried out manually for reaction times of from 1 to 10 minutes. After terminating the assay, the samples were arranged in order of increasing reaction time and analysed for inorganic phosphate, using the manifold shown in Fig. 2.15.

The recorder trace obtained is shown. Reaction time (in min) increased from right to left and extinction at 700 nm is represented on the vertical axis.

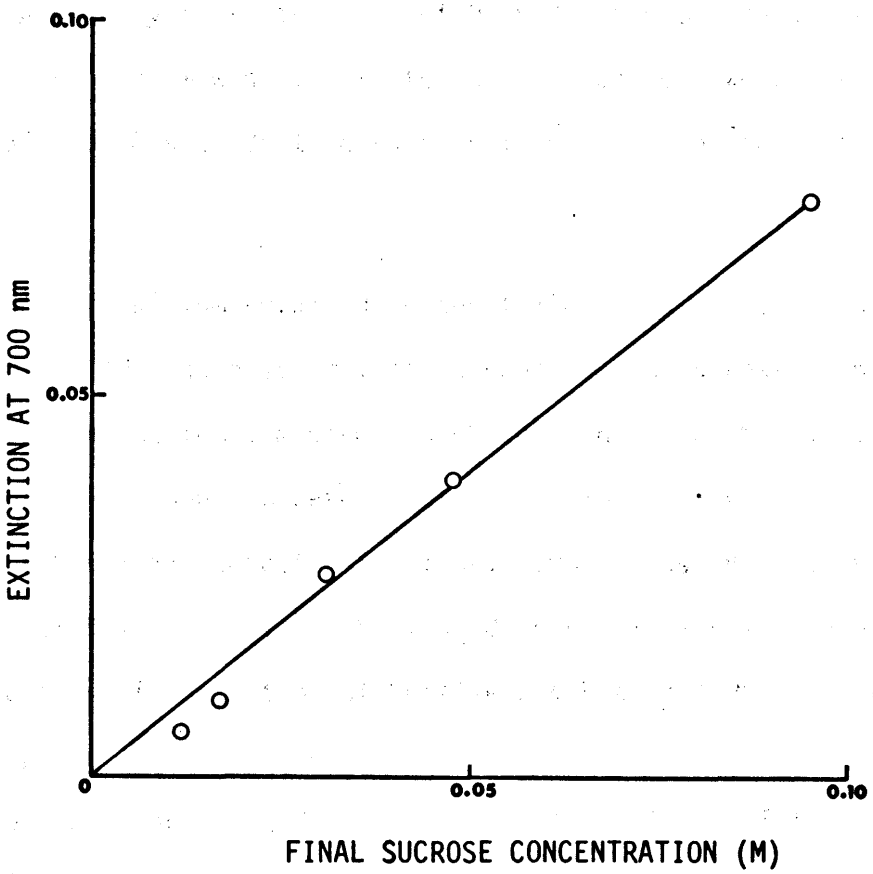


Fig. 2.18. The effect of sucrose on the estimation of inorganic phosphate by the method of Marzban & Hinton (1970).

A 0·1 ml aliquot of a sucrose solution of known concentration was added to 1 ml of water. This was followed by 1 ml of 6% W/W perchloric acid and the inorganic phosphate concentration of the mixture determined. This procedure was repeated for a range of sucrose solutions up to a concentration of 2·0 M. A plot of extinction at 700 nm against final sucrose concentration is shown. The extinction due to sucrose was unchanged whether ATPase medium or water was used in the assay.

Extinction due to sucrose = (0·82) (molarity of fraction/dilution in assay).

filter (type HA; Millipore (U.K.) Ltd., London). The filter was subsequently washed by filtering 1 ml of 0·25 M sucrose through it. As a control, the medium was also filtered. Incubations were carried out for 15, 30 and 60 seconds to ensure that the reaction had not reached completion.

The amount of calcium retained on the filters was measured in either a gas flow or liquid scintillation counter and expressed in terms of that present in the total medium, which contained 100 μM Ca^{2+}. Filters for gas flow counting were counted in a Model 161A Nuclear Chicago counter with a Model 480 detector (voltage: 1200v; Qgas (B.O.C., London.) at a pressure of 8 lb/sq. in). The liquid scintillation method used was a modification of the method of Loftfield & Eigner (1960). Dried filters were placed in vials containing 10 ml of scintillation fluid composed of 5·0 PPO* and 0·5g dimethyl -POPOP** per litre of toluene (all scintillation grade; Koch-Light Laboratories Ltd.,). The vials were counted in the Beckman LS-100-C Liquid Scintillation System (Beckman-RIIC Ltd., Glenrothes, Scotland.)

*PPO = 2, 5-diphenyloxazole

**Dimethyl-POPOP = 1, 4-di-[2-(4-methyl-5-phenyloxazolyl)] benzene.

(iii) Phosphoprotein intermediate of the Ca^{2+}-dependent ATPase

The hydrolysis of ATP by the Ca^{2+}-dependent ATPase occurs through the formation and breakdown of a phosphoprotein intermediate (Martonosi, 1969). The formation of this intermediate is one of the first steps leading to calcium transport (Makinose, 1969). The intermediate is formed very rapidly and reaches a steady state concentration within 2 sec (Meissner & Fleischer, 1971).

Assay procedure. The concentration of intermediate is most readily determined by measuring the ^{32}P-labelled protein formed, using adenosine 5'-$[\gamma$-$^{32}P]$ triphosphate as substrate. 0·45 ml of fraction (containing 0·75 - 1·0 mg protein) was added to 0·5 ml of a medium containing 200 mM KCl, 10 mM $MgCl_2$, 0·2 mM $CaCl_2$ and 10 mM imidazole-HCl, pH 7·0. The mixture was incubated at 0^o for 10 min. Phosphoprotein formation was initiated by the addition of 0·05 ml of 2 mM ATP containing γ-^{32}P-ATP. The reaction was stopped 10 sec later by the addition of 2·0 ml of ice-cold 1·2M perchloric acid. Control determinations were were carried out by adding 2·0 ml of 1·2 M perchloric acid prior to the substrate. Some workers (e.g. Meissner & Fleischer, 1971) have incorporated 0·1 mM ATP and 1 mM phosphate into the perchloric acid solution. However, since Pang & Briggs (1973) have found that these additions do not affect the apparent level of phosphoprotein intermediate they were not made. The denatured protein was pelleted by centrifugation at top speed for 5 min in an MSE Minor Centrifuge. The pellet was washed by resuspension and sedimentation, first in 4 ml of 5% W/V trichloroacetic acid and then three times in 8·0 ml of 2% W/V trichloroacetic acid. The last wash contained negligible radioactivity. All centrifugations and washings were carried out at 4^o.

The washed sediment was finally resuspended in 2% W/V $Na_2 CO_3$ in 0·1N Na OH and heated at 100^0 for 5 min. Aliquots were taken for the determination of protein by the automated method of Leighton et al. (1968), already described and radioactivity by liquid scintillation counting.

Radioactivity was measured by adding a 0·5 ml aliquot to 10 ml of triton-scintillant (prepared by dissolving 5g PPO and 0·5 g dimethyl-POPOP in 1 litre of toluene and diluting with 500 ml of triton X-100).

The amount of radioactivity used was such that 1nmole of ATP corresponded to $2·92 \times 10^4$ counts/min. Phosphoprotein concentration was expressed either as nmoles per mg protein or nmoles per ml of fraction.

(iv) Succinate dehydrogenase.

Succinate dehydrogenase activity was measured as succinate-INT* reductase activity by a modification (Porteous & Clark, 1965) of the method of Pennington (1961). The assay medium, which had a final volume of 1 ml contained:

50 mM sodium succinate,
0·1% W/V INT,
25 mM sucrose,
2 mM EDTA**,
50 mM potassium phosphate buffer, pH 7·4.

The reaction was allowed to proceed until sufficient reduced formazan was formed as evidenced by its red colour. The reaction was terminated by the addition of 1 ml of 10% W/V trichloroacetic acid. The reduced

*INT = 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride.

**EDTA = ethylenediaminetetroacetic acid.

formazan was extracted into 4 ml of ethyl acetate using a vortex mixer. The phases were separated by centrifugation in the M.S.E. Minor Centrifuge at a speed setting of 5. The amount of product formed was determined by spectrophotometric measurement at 490 nm using the molar extinction coefficient of $20{\cdot}1 \times 10^3$ (Pennington, 1961). Blank determinations were also carried out. Sucrose inhibits succinate-INT reductase (Hinton et al., 1969; Headon, 1972). The inhibition is linear with sucrose concentration. The correction factor reported by Headon (1972) has been used in this study.

(d) Electrophoretic analyses

Electrophoresis of proteins in the presence of dodecyl sulphate was carried out in polyacylamide gels.

(i) Electrophoresis in cylindrical 5·2% polyacylamide gels

Gel preparation. Gels were prepared using the Bio-Rad Model 200 gel preparation system (Bio-Rad Laboratories Ltd., Kent, England). In this system gels are formed in an inverted manner in tubes using the polished surface of glass inserts to form a very flat surface for sample application.

Pyrex glass tubes, 5mm ID X 7mm OD and 75 mm long, were cleaned with hot nitric acid. After extensive rinsing with distilled water, the tubes were immersed in 0·5% V/V Photo-flo 200 (Kodak Ltd., London.) and dried in a clean atmosphere. 24 tubes were secured by liquid-tight seals to a circular perspex base. A glass insert, with its polished surface facing upwards, was dropped into each tube. When the insert reached the bottom of the tube it became seated there. A 2 cm length of tygon tubing was attached to the top of each tube. All tubes were flushed thoroughly with

nitrogen and carefully filled with a freshly prepared, degassed solution containing:

5% W/V acrylamide
0·2% W/V N, N'-methylene-bisacrylamide,
0·2% V/V N,N,N',N' - tetramethylethylenadiamine,
0·075% W/V ammonium persulphate,
0·1% W/V sodium dodecyl sulphate,

in 0·1M sodium phosphate buffer, pH 6·0. When polymerisation was complete, after approximately one hour, the tubes were carefully removed from the perspex base and the inserts withdrawn using a twisting-rocking motion, leaving a well defined surface for sample application. The lengths of tygon tubing were removed from the opposite ends and the excess gel cut off leaving a fairly sharp surface to the bottom end of the gel. The gels were stored in 0·1M sodium phosphate buffer, pH 6·0, containing 0·1% W/V sodium dodecyl sulphate (SDS) for 12 - 24 hours before use.

Sample preparation. Samples for electrophoresis using this system were prepared by overnight incubation at 30^{o}, at a protein concentration of 2 mg/ml, in a medium containing:

0·4% W/V SDS,
7% W/V sucrose,
40 mM dithioerythritol,
in 0·1M sodium phosphate buffer, pH 6·0.

After incubation 10 µl of a 0·5% W/V solution of bromophenol blue was added per ml of incubation medium.

Sample application. A maximum of 12 gel tubes were placed in the Bio-Rad Model 150 Electrophoresis Cell. 50 µl of sample were applied to each gel using a micropipette. The samples were overlaid with electrode buffer consisting of 0·1% W/V SDS in 0·1M sodium phosphate buffer, pH 6·0.

The overlaying process was made easy by using a 10 ml syringe with its plunger removed and the leur attached to a 0·5 mm bore teflon catheter. The flow rate was regulated by varying the height of the syringe. The electrode chambers were filled with buffer. Water at 25^{o} was circulated from a water bath through the reservoir jacket.

Electrophoresis. Electrophoresis was carried out with migration towards the anode, initially at 0·5 m A/gel until the sample entered the gel and then at 5 m A/gel until the dye reached the end of the gel (after 2·5 hours approximately). Gel tubes were removed from the apparatus one by one as the tracking dye reached the end of the gel.

Staining and destaining. The gels were removed from the tubes by carefully injecting electrode buffer between the gel and the glass tube. The gels were fixed by soaking in 25% V/V propan-2-ol, 10% V/V acetic acid for 12 hours, followed by 10% V/V propan-2-ol, 10% V/V acetic acid for 8 hours. After fixing the gels were stained by soaking in 0·1% W/V amido black in 10% V/V acetic acid for 6 hours. Gels were destained in the Bio-Rad Model 170 Diffusion Destainer. Destaining was complete in 12 hours.

Gels were stained for carbohydrate according to the periodic acid-Schiff procedure of Fairbanks et al. (1971). After fixing as described, the gels were soaked for a further 12 hours in 10% V/V acetic acid to remove any residual SDS. The gels were then treated with the following reagents: 0·5% W/V periodic acid for 2 hours; 0·5% W/V sodium arsenite, 5% V/V acetic acid for 1 hour; 0·1% W/V sodium arsenite, 5% V/V acetic acid for 30 min and repeated twice; 5% V/V acetic acid for 30 min; Schiff reagent for 8 hours and finally 0·1% W/V sodium metabisulphite in 0·01N HCl for 6 hours. Gels were stored in 10% V/V acetic acid.

(ii) Electrophoresis in polyacrylamide gel slabs

Electrophoresis in polyacrylamide slabs has a number of advantages over electrophoresis in cylindrical gels. A large number of samples can be co-run, side by side, on the same slab under the same conditions. This allows for the accurate comparison of individual bands in different samples. The protein patterns obtained are more easily quantified using conventional densitometers. Slab gels have a greater surface area for efficient cooling during electrophoresis. Finally slab gel electrophoresis permits the use of two dimensional techniques, one of which is described in this thesis.

Assembly of glass cells for polyacrylamide slab gels. Gels were formed in 3 x 72 x 82 mm 1D glass cells. Each cell was assembled from two 82 x 82 mm glass plates, held 3 mm apart by two 3 x 5 x 82 mm glass spacers. To facilitate the removal of the gel, the spacers were attached to one plate only by silicone rubber sealant adhesive (RTV 108, General Electric, USA; supplied by Pierce Chemical Co., Rockford, Illinois, USA). The entire cell was held together by electric tape (No. 1607; Sellotape Ltd., Dublin.). Before the final assembly with electric tape, the glass cell components were soaked in chromic -sulphuric acid and extensively rinsed with distilled water before drying at 80^{0} in a dust-free oven.

Apparatus for the preparation of polyacrylamide slab gels. Slabs were prepared using the Uniscil Gel Preparation Set (Universal Scientific Ltd., London). The apparatus is illustrated in Fig. 2.19. It consists of the following units:

A, The gel forming tower. Fourteen glass cells are held in a vertical position in this tower. Solutions enter the tower from the three-way stop-cock (C) through the opening (B). A length of anodised aluminium

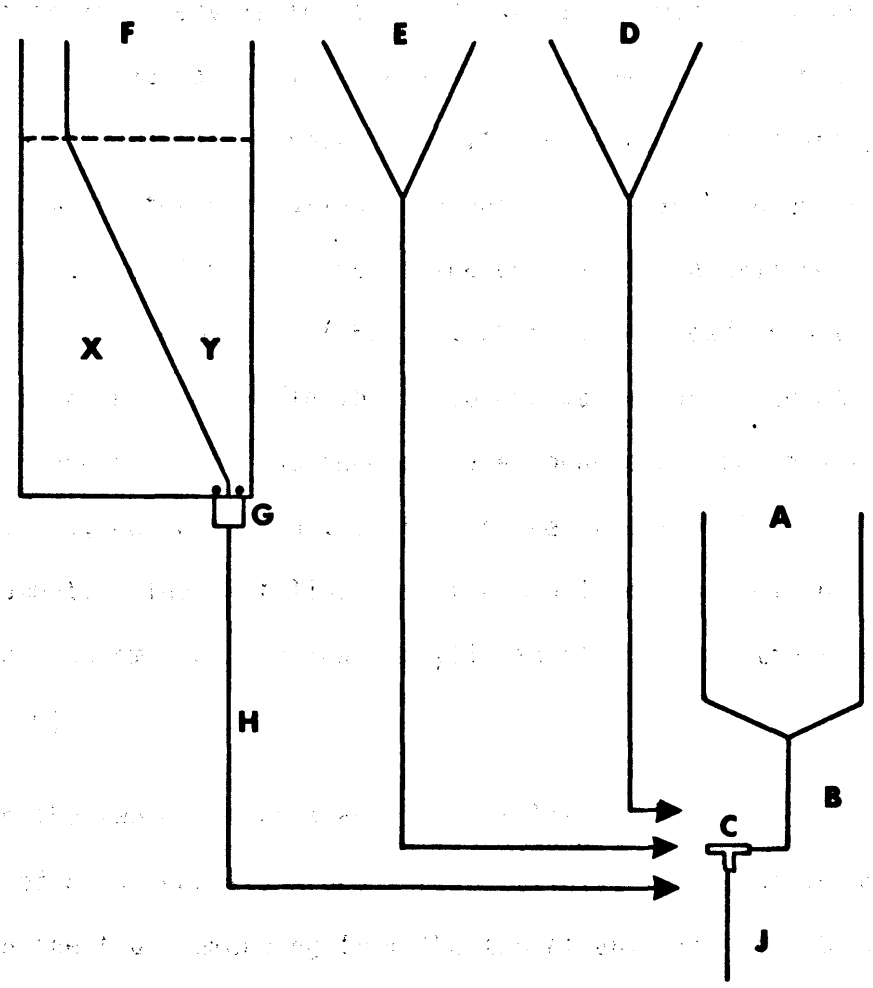


Fig. 2.19. Diagrammatic representation of apparatus used to prepare polyacrylamide slab gels.

A gel forming tower,

B feed line to tower,

C three-way stop-cock,

D & E reservoirs,

F gradient former with two compartments, X & Y,

G exit line from gradient former,

H mixing tube,

J line to waste.

chain and a stainless steel mesh over B act as baffle and bubble trap. Funnel D acts as a reservoir for the overlay and underlay solutions. Funnel E acts as reservoir for the 5·2% W/V acrylamide solution, when homogenous gel slabs are being prepared. Acrylamide gradients are produced by the gradient former, F. It operates on the principle of variable geometry for generating gradients. It has been described in detail by Margolis (1969). The device consists of a tall rectangular compartment with a capacity of 500 ml, which is divided into two compartments X and Y by a flexible neoprene gasket anchored at the lower right-hand corner. The two chambers are connected through a tap system (Rotaflo TF 16; Quickfit Ltd., Staffordshire, England) G, at the base of the instrument. The exit line from the tap leads to a mixing tube (H), containing a 30 cm long helical acrylic inset and onto the three-way stop-cock (C).

When the two chambers X and Y are filled to the same level with solutions of equal density x and y respectively, the relative amount of x and y in the fluid emerging from the tap at any time will be directly proportional to the cross-sectional area of the meniscus in each chamber at that time. When the densities of the two solutions x and y are different, the height of the less dense solution must be increased to give hydrostatic equilibrium before commencing gradient formation.

Formation of polyacrylamide gel slabs. The composition of solutions used in the preparation of polyacrylamide gel slabs is given in Table 2.4. The solutions were incubated at the temperatures stated and degassed before use.

Fourteen fully assembled glass cells were stood vertically in the gel forming tower (A). The tower was slowly filled with overlay solution consisting of 5% V/V methanol in buffer from reservoir D, ensuring that

TABLE 2.4

Compositon of solutions used in the preparation of polyacrylamide gel slabs

---

BUFFER*

88·7 mM Tris, 2·5 mM disodium EDTA and 81·5 mM boric acid, pH 8·3, with 0·02% W/V sodium azide.

OVERLAY

5% V/V methanol in buffer.

UNDERLAY

1·0 M sucrose.

SOLUTION 1

5% W/V acrylamide and 0·2% W/V N,N'-methylene-bisacrylamide in buffer. Degassed and cooled to 4^{o}. TEMED and ammonium persulphate added before use to give final concentrations of 0·2% V/V and 0.075% W/V respectively.

SOLUTION 2

57g acrylamide and 3g N,N'-methylene-bisacrylamide dissolved in buffer to a final volume of 195 ml. Degassed and cooled to 4^{o}. 5 ml of 10% W/V ammonium persulphate and 0·12 ml of 10% W/V DMAPN added before use.

SOLUTION 3

5 ml of 10% W/V ammonium persulphate and 1·2 ml of 10% W/V DMAPN added to 195 ml of degassed buffer

---

Abbreviations:

TEMED N,N,N',N',-tetramethylethylenediamine

DMAPN β-dimethylaminopropionitrile

*Kitchin (1965); Sheridan et al. (1969); Patterson et al. (1971).

no air bubbles were trapped in the system. The reservoir was drained of overlay and filled as far as the closed three-way stop-cock with 1M sucrose. For the preparation of homogenous gels, 275 ml of solution No. 1 were placed in reservoir E. The line from the reservoir was flushed clear of air bubbles by allowing the first 10 - 15 ml of solution out to waste via line J. The flow was then switched to the gel-forming tower. The flow-rate slow at first, was gradually increased to 30 ml/min. The overlay was displaced upwards by the acrylamide solution. When 250 ml of solution had drained from the reservoir, the stop-cock was switched to admit sucrose underlay from reservoir E. Approximately 175 ml of underlay were required to displace the acrylamide solution upwards into the glass cells. The refractive index differences between the overlay, acrylamide and underlay zones enabled their positions to be located.

When polymerisation was complete the feed line to the tower was connected to a water tap. The cells were extruded en bloc by the water pressure. The cells were carefully separated from one another and stored under buffer containing 0·02% W/V azide at 4^{o}. Under these conditions, they remained in excellent condition for several months.

Gradient pore polyacrylamide gels were formed by a similar procedure. However, an acrylamide concentration gradient was fed to the gel forming tower from the gradient maker (F) rather than a homogenous solution from reservoir E. A 250 ml acrylamide gradient was formed from two solutions of extreme concentration, namely a 30% W/V solution (Solution 2) and a diluent (Solution 3). The gradient shape was determined by the profile of the neoprene gasket as discussed earlier. A reverse catalyst concentration gradient was formed, simultaneously with the acrylamide

gradient, ensuring that polymerisation occurred from the top of the cell downwards. The gradient used throughout this work ranged in concentration from 5 - 27% W/V and is illustrated in Fig. 2.20.

Electrophoresis. Electrophoresis was carried out in a four cell electrophoresis unit (Universal Scientific Ltd.,). A centrifugal pump circulated the lower electrode buffer through a cooling coil. Part of the buffer stream was lead to the upper electrode chamber, where it overflowed through a drain tube and returned to the lower chamber. Recirculation in this manner prevented pH and concentration changes between the buffer in both chambers.

Slabs were equilibrated with 0·1% W/V SDS by pre-running in buffer containing 0·1% W/V SDS for one hour at 80 volts.

For one-dimensional electrophoresis samples were applied using a 14 place applicator at the rate of 10 µl per slot. For the first dimension of a two-dimensional run 300 µl of a single sample were applied across the entire top surface of the gel. In both cases the samples were applied under buffer from a micropipette with a 0·5 mm bore catheter attached. Electrophoresis was carried out at 75 volts for the required length of time. A Shandon voKam power pack was used (Shandon Scientific Co. Ltd., London). Migration was towards the anode.

For two-dimensional electrophoresis the sample was run first in a 5·2% W/V slab until the tracker dye reached the end of the gel. A 5 mm wide section was cut from this gel and immediately inserted across the top of a gradient pore slab. Electrophoresis was then carried out in a second dimension. The duration of electrophoresis is given in the appropriate part of the results section.

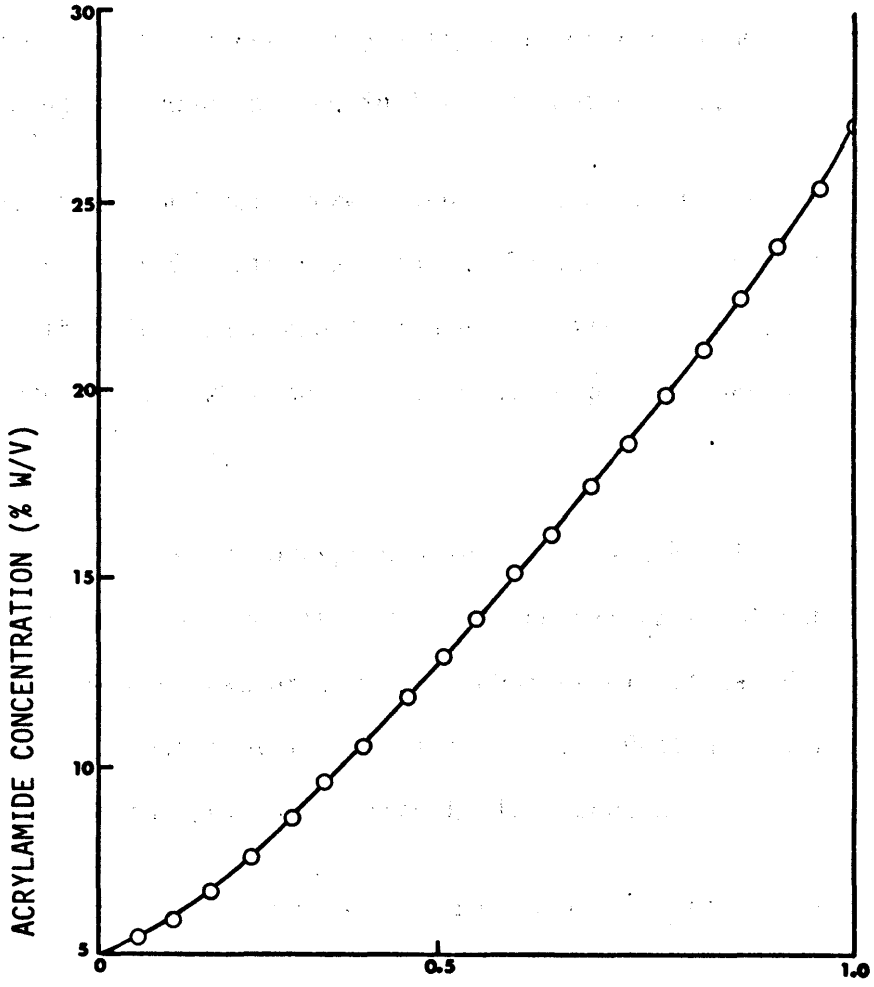


Fig. 2.20. Calculated profile of acrylamide concentration gradient, used to prepare polyacrylamide gel gradients described in the text. The gradient profile was calculated as described by Margolis (1969).

Staining and destaining. Slab gels were fixed and stained according to the procedure described for cylindrical gels. Gels were destained either by diffusion or electrophoretically using a laborarory made apparatus. After destaining gels were stored in 10% V/V acetic acid.

Densitometry. Destained gels were scanned in the Joyce-Loebl Chromoscan MK II (Joyce, Loebl & Co. Ltd., Gateshead, England). A red filter was inserted into the light path and 1 cm wide gel strips were scanned in the reflectance mode. When necessary, scale expansion was used to optimise the peak heights.

Photography. Gels were photographed using Micro-Neg Pan film type B (Ilford Ltd., Essex, England) in a Praktica LB camera (Pentacon, Dresden, DDR.) with a SMC Macro Takumar lens (5871694; Asahi Optical Co., Japan). The gels were illuminated by transmitted light. Photographs were printed on P84 Photographic Paper (Kodak Ltd., London).

Preparation of samples for one- or two-dimensional electrophoresis in slab gels. Samples, having a final protein concentration of 3 mg/ml, were incubated at 100^{o} for 5 min in the presence of

0·6% W/V SDS,
10% W/V sucrose,
40 mM dithioerythritol,
in Tris- disodium EDTA- boric acid buffer pH 8·3.

On cooling, pyronin-Y was added to give a final concentration of 10 µg/ml. Molecular weight markers were treated in the same manner. Identical protein patterns were obtained whether samples were incubated at 100^{o} for 5 min or overnight at 30^{o}.

(d) Miscellaneous

Equipment. SP 800 spectrophotometer (Pye-Unicam Ltd., Cambridge, England).

Acta V spectrophotometer (Beckman - RIIC Ltd., Glenrothes, Scotland) with scattered transmission accessory for the measurement of light scattering.

Radiometer pH meter 28 calibrated at 20^{o} with standard buffer, pH 6·50 (Radiometer Ltd., Copenhagen, Denmark).

Chemicals. (i) General chemicals: AnalaR grade from BDH Ltd., (Poole, England), and Hopkin & Williams Ltd., (Essex, England). Pronalys grade from May & Baker Ltd. (Dagenham, England).
(ii) Organic solvents for lipid analyses: AnalaR and Aristar grades from BDH.
(iii) Bovine serum albumin fraction V, sodium dodecyl sulphate (specially pure grade), manoxol OT (dioctyl sodium sulphosuccinate), mercaptoethanol, ammonium persulphate, and β-dimethylaminopropionitrile from BDH Ltd.

Disodium ATP (Sigma grade), iodonitro-tetrazolium formazan, ouabain octahydrate, dextran sulphate, heparin (sodium salt, grade 1), concanavalin A (grade IV, salt free) and α-methyl-D-glucoside from Sigma Ltd., London.

Lipid standards from Supelco Inc., Bellefonte, Pa. USA.

Acrylamide, N,N'-methylene-bisarcylamide, N,N,N',N'-tetramethylethylenediamine and pyronin-Y from Eastman-Kodak Co., Rochester, N.Y., USA.

Naphthalene Black 10B (=amido black) from Gurr Ltd., London.

Dithioerythritol and molecular weight markers from Schwartz-Mann, N.Y., U.S.A.

Toluene, tritonX-100, PPO, and POPOP (all scintillation grade) and INT from Koch-Light Laboratories Ltd., Bucks., England.

Lactoperoxidase from Boehringer Mannheim GmbH.

^{45}Ca (as $CaCl_2$), ^{131}I (as NaI) and adenosine 5'-[γ-^{32}P] triphosphate (as the ammonium salt) were obtained from The Radiochemical Centre, Amersham, Bucks., England.

SECTION 3

RESULTS

RESULTS

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3.1. The effect of varying homogenisation time on the distribution of a number of constituents in the four classical subcellular fractions

Homogenisation, which is a necessary step to fractionation, is dependent on a number of factors, principally, the type of homogeniser used, the duration of homogenisation, the type of tissue, its connective framework and any pretreatment of the tissue.

The experiments described in this subsection are concerned with an investigation into the effect of varying homogenisation times on the distribution of a number of constituents and enzymes in the four classical subcellular fractions.

Skeletal muscle is a particularly tough tissue due to the presence of a large amount of contractile protein and connective tissue. Contractile proteins alone constitute 60% of the total protein of the tissue (Perry, 1974). A preliminary mincing of the tissue is a useful aid to homogenisation.

Equal weights of minced muscle were homogenised in three volumes of 0·25 M sucrose, 5 mM imidazole-HCl, pH 7·4 at 4^{o} for 30, 60, 90 and 120 sec. Four fractions were prepared from each homogenate according to the procedure summarised in Fig. 3.1. These fractions were:

P1 fraction ($5{\cdot}34 \times 10^{3}$ g-min pellet);

P2 fraction ($2{\cdot}00 \times 10^{5}$ g-min pellet);

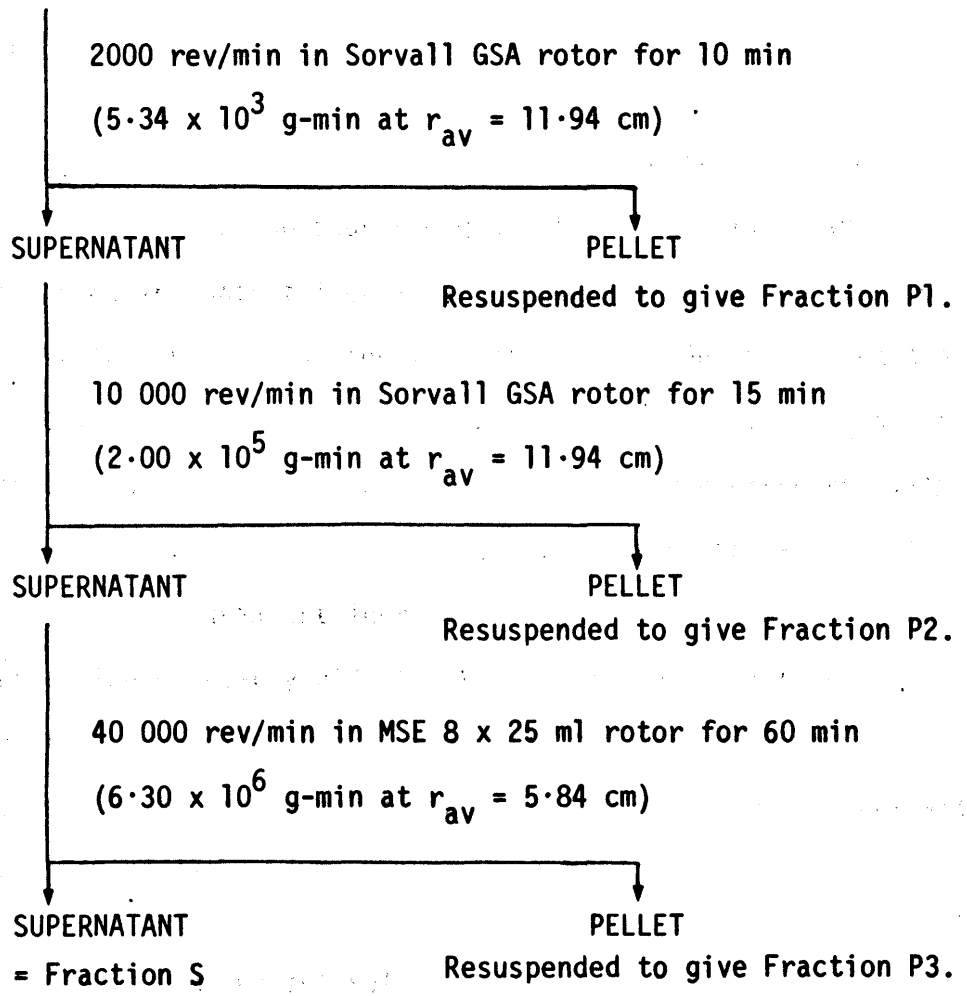
P3 fraction ($6{\cdot}30 \times 10^{6}$ g-min pellet) and the

S fraction ($6{\cdot}30 \times 10^{6}$ g-min supernatant).

These fractions were assayed for protein, phospholipid, cholesterol and succinate-INT reductase activity. The quantities present in each

Fig. 3.1. Procedure used in the preparation of subcellular fractions by differential pelleting.

Pellets were resuspended in the homogenisation medium using a hand-operated Potter-Elvejhem homogeniser.



fraction are expressed as percentages of those in the homogenate.

A plot of the protein, phospholipid and cholesterol content of fraction P1 against homogenisation time is shown in Fig. 3.2. The amount of each of the three constituents in this fraction falls with increasing homogenisation time. For cholesterol and phospholipid the greatest decrease occurs between 60 and 90 sec. However, even after homogenisation for 120 sec over 80% of each of these constituents sediments in P1.

The distribution of these constituents and succinate-INT reductase activity in fraction P2 for the four homogenisation times is shown in Fig. 3.3. The protein content increases gradually with the duration of homogenisation up to a homogenisation time of 90 sec. On increasing the homogenisation time from 90 to 120 sec there is a three-fold increase in the protein content of this fraction. Succinate-INT reductase activity, a marker for the inner membrane of the mitochondrion, also increases with homogenisation time, the greatest increase being a two-fold one between 90 and 120 sec. The greater yield of mitochondria in P2 with longer homogenisation is accompanied by an increase in the fragmentation of mitochondria, leading to increases in the succinate-INT reductase activity of fraction P3 (Fig. 3.4).

The phospholipid and cholesterol contents of P2 follow a different pattern from protein and succinate-INT reductase activity. Both phospholipid and cholesterol peak sharply after 90 sec. Longer homogenisation results in a decrease in the percentage of both these constituents in fraction P2. Skeletal muscle mitochondria have a phospholipid/protein ratio of only 0·14 (Bullock et al., 1973) as compared to 0·60 - 0·70 for microsomal material (results in this thesis). The increased protein content of fraction P2 can

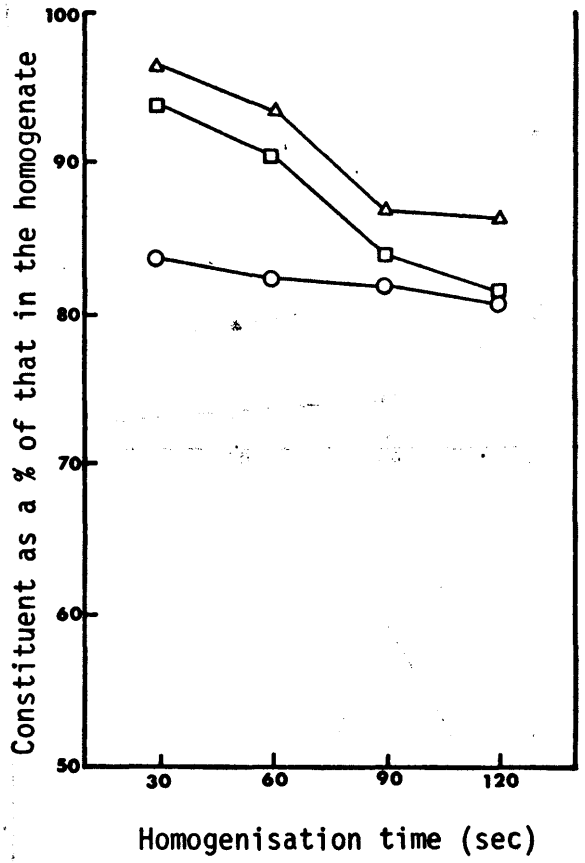


Fig. 3.2. The protein (—○—), phospholipid (—△—), and cholesterol (—□—) content of the Pl fraction prepared from 30, 60, 90 and 120 sec homogenates.

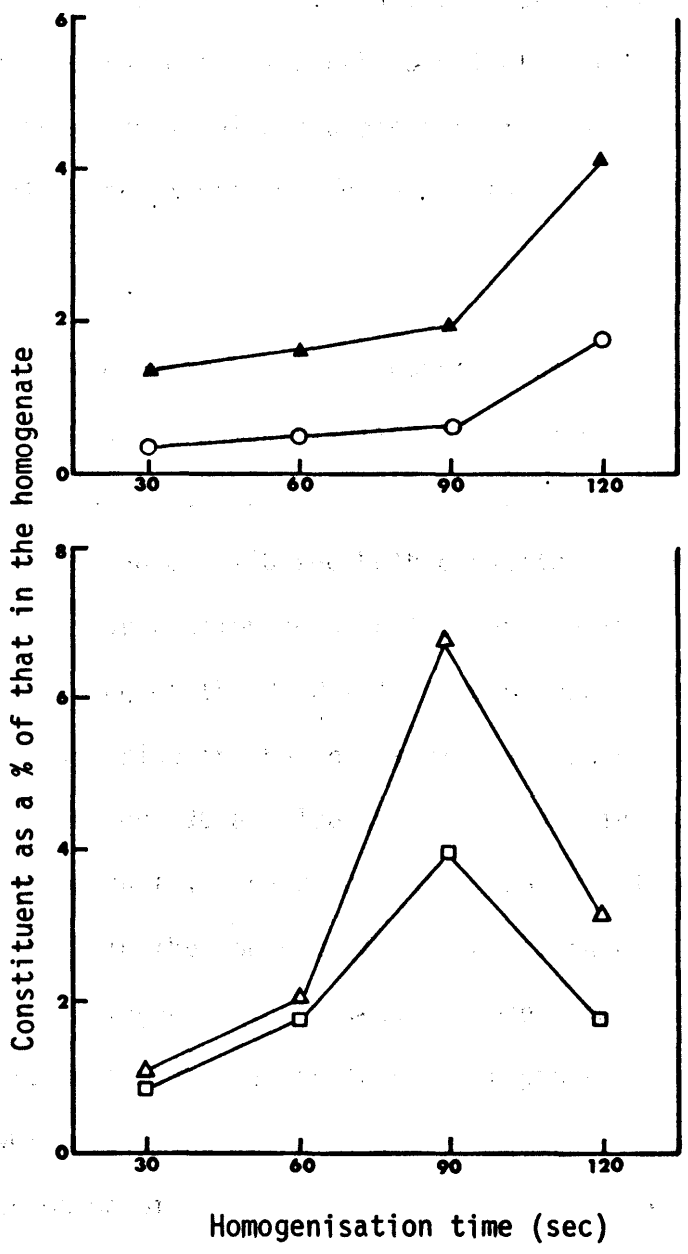


Fig. 3.3. The amount of protein (—○—), phospholipid (—△—), cholesterol (—□—), and succinate-INT reductase activity (—▲—) in fraction P2 prepared from 30, 60, 90 and 120 sec homogenates.

be attributed to the large increase in the mitochondrial content of the fraction, while the decreased phospholipid and cholesterol content is interpreted as being due to the extensive rehomogenisation of large membrane fragments, having a phospholipid/protein ratio five times that of mitochondria, to smaller fragments, which do not then sediment in fraction P2.

In Figs. 3.4 and 3.5 the distribution of constituents and enzymes in fraction P3 is plotted against homogenisation time. The cholesterol content of the pellet increased more slowly than phospholipid over the range 30 to 90 sec. Between 90 and 120 sec both constituents increase sharply. There is a good correlation between the phospholipid content of P3 and the light scattering of the $2{\cdot}00 \times 10^5$ g-min supernatant (i.e. P2 supernatant). The sharp rise in the cholesterol and phospholipid content of this fraction between 90 and 120 sec, expressed in absolute terms or as a percentage of that in the homogenate, can be related in part, at least, to the fall in the content of both constituents in fraction P2, for the same homogenisation times. These findings indicate that parts of the muscle cell membrane systems are ruptured initially into large fragments which sediment in P2. Longer homogenisation leads to a further disruption of these fragments to vesicles or smaller fragments which sediment in P3.

The activities of basal ATPase and K^+-stimulated ATPase in fraction P3 plotted against homogenisation time are shown in Fig. 3.5. Both enzymes were assayed in the presence of 50 mM azide to prevent interference by mitochondrial ATPase (Fanburg & Gergely, 1965). The rate of appearance of K^+-stimulated ATPase decreases with homogenisation times greater than 60 sec. Consequently, homogenisation for longer than 60 sec does not give

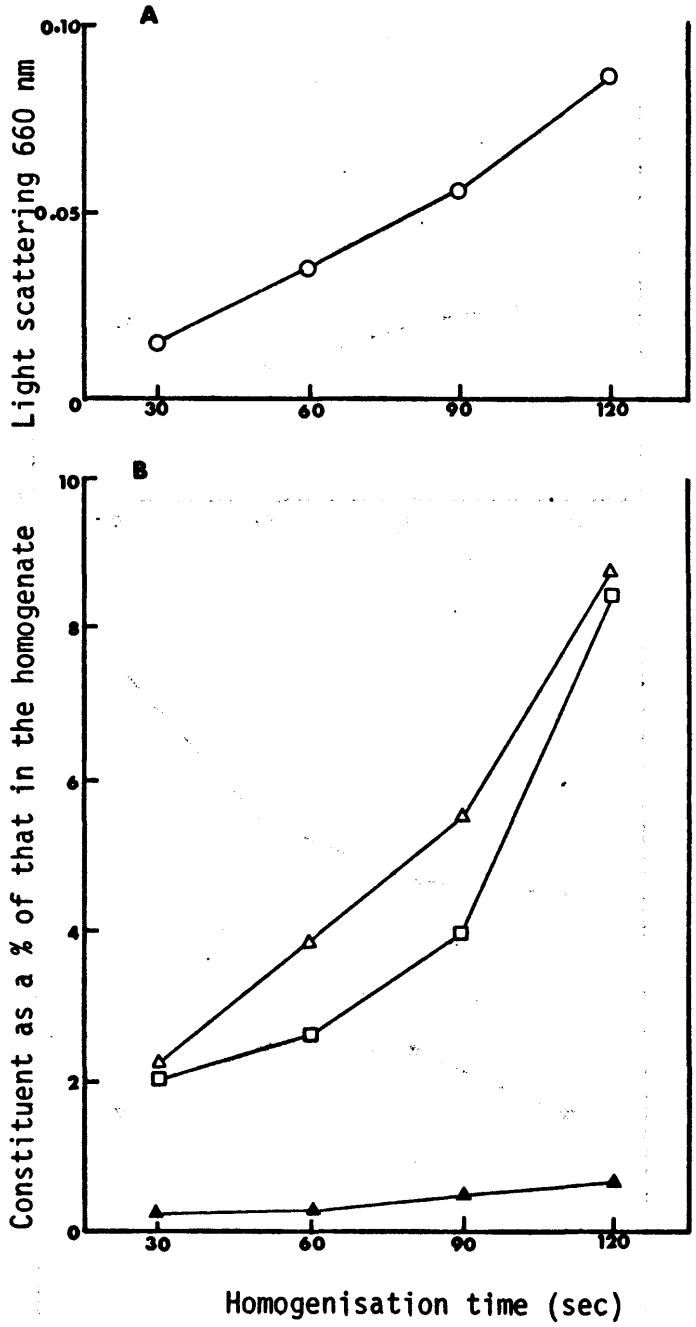


Fig. 3.4. (A) Light scattering (—○—) of the P2 supernatant prepared from 30, 60, 90 and 120 sec homogenates.

(B) The amount of phospholipid (—△—), cholesterol (—□—), and succinate-INT reductase activity (—▲—) in fraction P3 prepared from 30, 60, 90 and 120 sec homogenates.

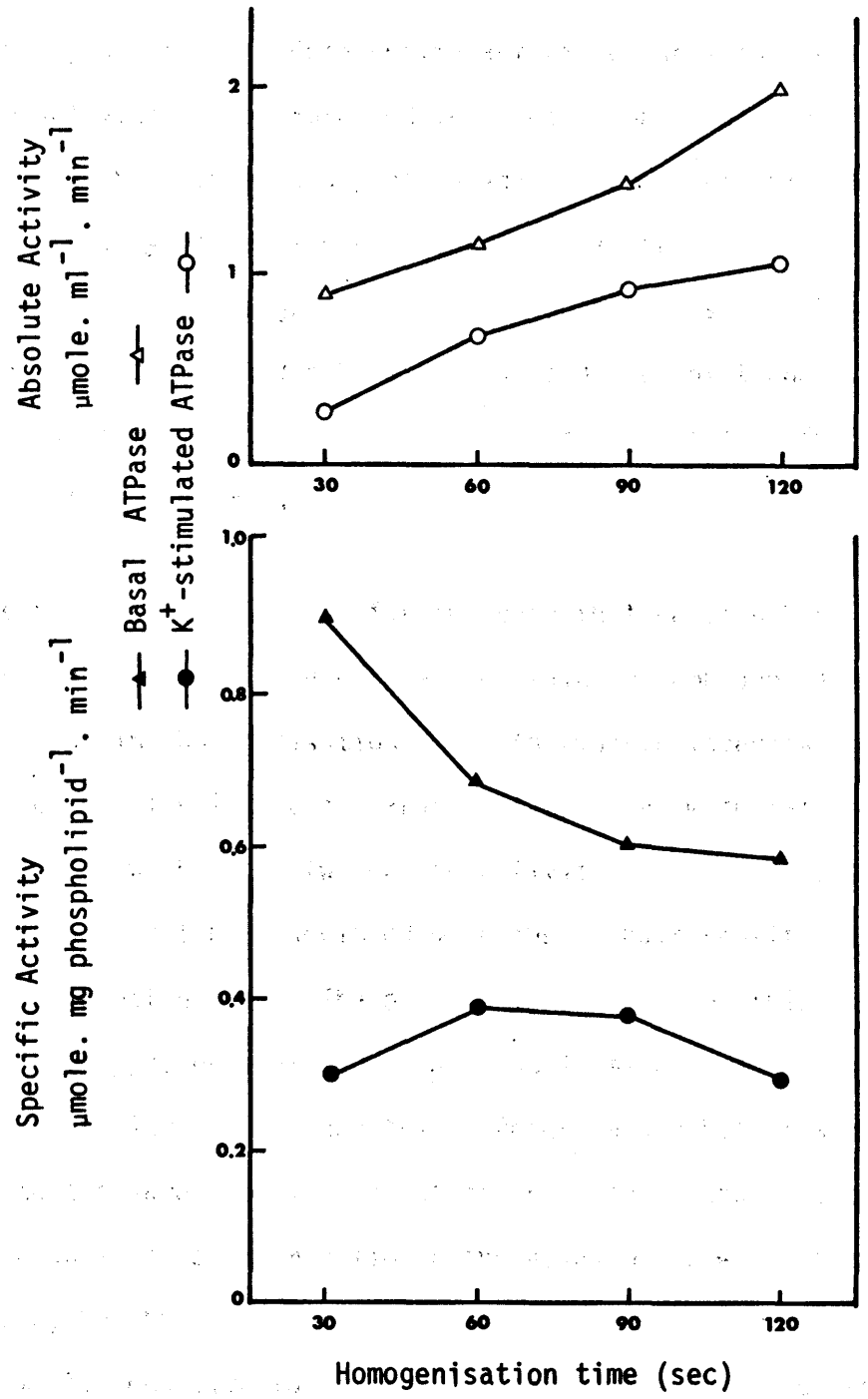


Fig. 3.5. The absolute and specific activities of K^+-stimulated ATPase and basal ATPase in fraction P3 prepared from 30, 60, 90 and 120 sec homogenates.

a proportionately higher yield of membranes containing this enzyme. In contrast, the rate of appearance of basal ATPase does not decrease with longer homogenisation. Both enzyme activities show further differences when the activities are expressed in terms of phospholipid. When expressed in this manner and plotted against homogenisation time, the specific activity of basal ATPase decreases after 30 sec. The fall in specific activity is greatest between 30 and 60 sec. The specific activity of K^+-stimulated ATPase follows a different trend by peaking at a homogenisation time of 60 sec. These findings will be further considered in the light of other results.

The results described so far indicate that basal ATPase - containing membranes and K^+-stimulated ATPase - containing membranes are differentially fragmented during homogenisation. Furthermore, homogenisation for longer than 90 sec results in the appearance of membrane material in fraction P3 not associated with these two enzyme activities. The possibility that this material might be associated with the ouabain sensitive (Na^+ and K^+)-ATPase was investigated. The ouabain sensitivity of this enzyme activity, assayed in the presence of 100 mM Na^+ and 10 mM K^+, was measured in fraction P3 from 30 and 120 sec homogenates. Before assay both fractions were diluted to the same protein concentration. The ouabain concentration and the ouabain to protein ratio in the assay mixture were similar to that reported by Sulakhe et al. (1973). At these concentrations the activity was maximally sensitive to ouabain. The results obtained are shown in Table 3.1. The activity of this enzyme in fraction P3 increases with increasing homogenisation time, however its contribution to the overall ATPase activity decreases from 15·6% in fraction P3 from a 30 sec homogenate to 11·6% in P3 from a 120 sec homogenate. These results suggest that this

TABLE 3.1

The activity of (Na^+ - K^+) - ATPase in fraction P3 from 30 and 120 sec homogenates. Activities were measured as described in Section 2. The ouabain to protein ratio in the assay mixture was 1·8 μmoles ouabain per mg protein, which is equivalent to 1·05 mg ouabain per mg protein.

Homogenisation Time (sec)	Activity(μmoles per fraction per min)		Inhibition[+]
	Control*	Ouabain Treated*	
30	1·3898 ± 0·0533(8)	1·1735 ± 0·0615(8)	0·2163 (15·6%)
120	2·3748 ± 0·1198(7)	2·1000 ± 0·1635(7)	0·2748 (11·6%)

*Values are given as means ± standard deviation. The number of determinations is given in parentheses.

[+]Ouabain inhibition is expressed as μmoles per fraction per minute and as a percentage of the activity in the control.

enzyme is not associated with the sharp increase in the cholesterol and phospholipid content of fraction P3 after homogenisation for 120 sec.

In an interpretation of the results of these experiments consideration must be given to the possible denaturation of enzymes with increasing homogenisation time. The activity of succinate-INT reductase was unaffected over the range of homogenisation times used. The activities of K^+-stimulated ATPase, Ca^{2+}-dependent ATPase, basal ATPase and (Na^+ and K^+)-ATPase cannot be accurately determined in the homogenate due to the interference of a variety of phosphatase activities. As an alternative, the homogenisation conditions were simulated by treating a microsomal suspension with a Waring blender. The results indicate that the ATPase activities should be stable under the homogenisation conditions used.

The protein composition of membranes in fraction P3 for each of the four homogenisation times were separated by SDS-polyacrylamide gel electrophoresis in gradient pore polyacrylamide gels. A photograph of the protein patterns obtained is shown in Fig. 3.6. Densitometer traces of the stained gels are shown in Fig. 3.7. The protein peak marked with an asterisk on the densitometer traces is the principal protein component in many muscle microsomal preparations as will be seen in later parts of this section. Co-electrophoresis of these fractions with other microsomal preparations and molecular weight markers has shown that this protein has a molecular weight of approximately 100 000 and is associated with Ca^{2+}-transporting membranes.

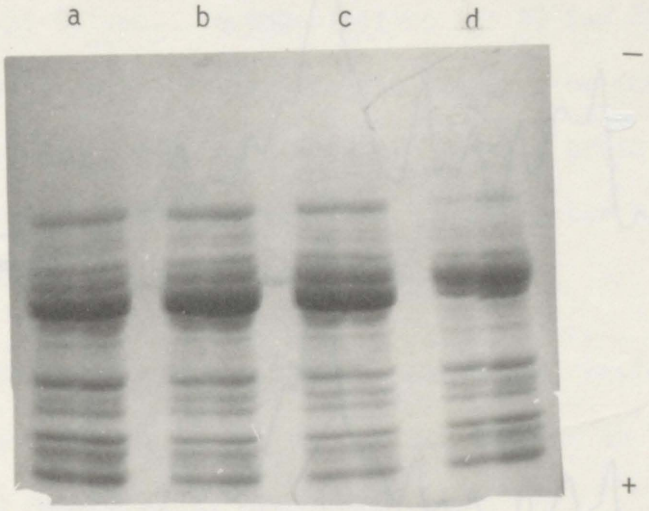


Fig. 3.6. The electrophoretic separation of protein components in the P3 fraction from (a) 30 sec, (b) 60 sec, (c) 90 sec and (d) 120 sec homogenates.

Electrophoresis was carried out in 6-27% polyacrylamide gel gradients containing 0·1% W/V SDS.

Migration was towards the anode, i.e. from top to bottom in the illustration.

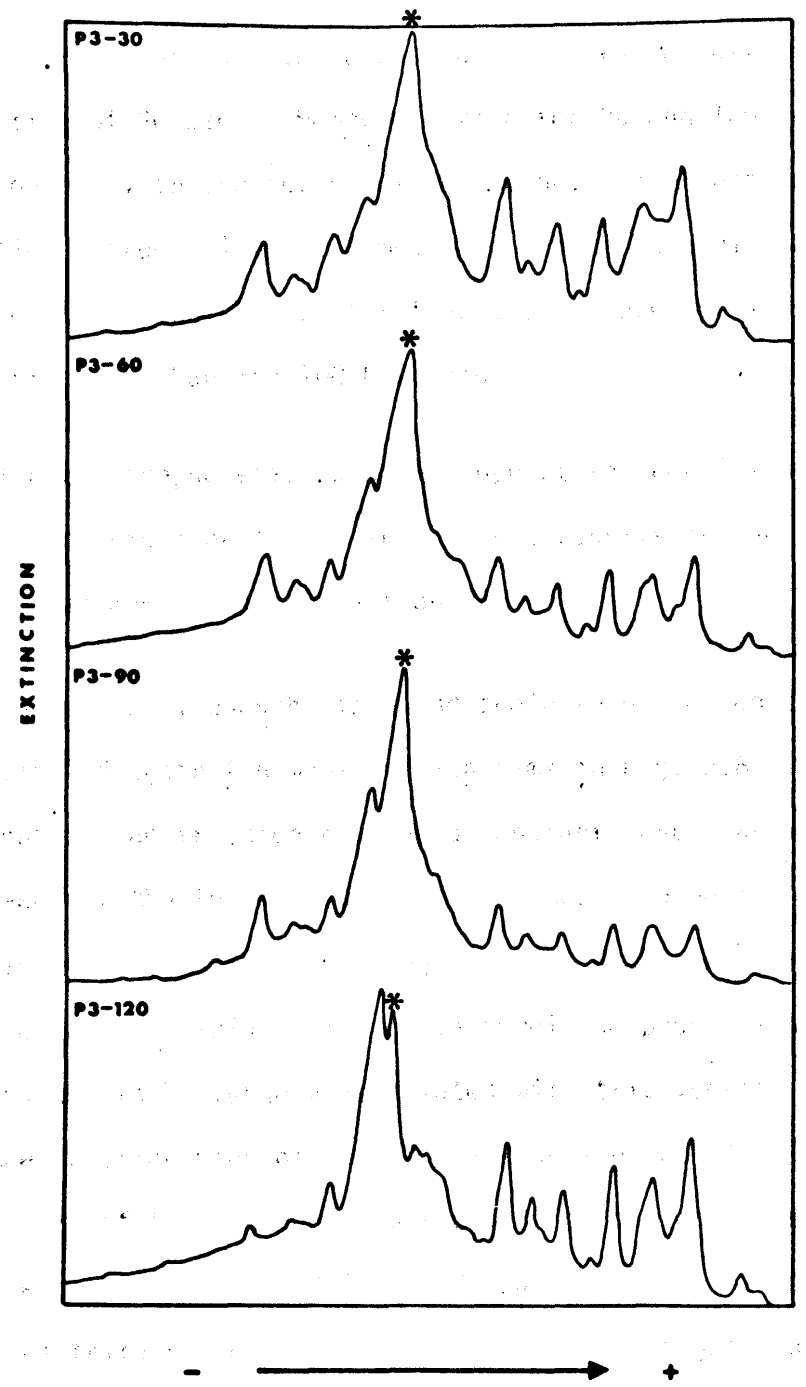


Fig. 3.7. Densitometer traces of the electrophoretic separations shown in Fig. 3.6. The protein component marked with an asterisk is found in association with Ca^{2+}-transporting membranes and has a molecular weight of 100 000.

In fraction P3 from a 30 sec homogenate, proteins of lower molecular weight make a greater contribution to the overall pattern, relative to the 100 000 molecular weight band, than they do in the corresponding fractions from 60 and 90 sec homogenates. Conversely, the 100 000 molecular weight band increases in concentration with homogenisation up to a time of 90 sec. Homogenisation for 90 and 120 sec leads to heterogeneity in the region of this band. The densitometer trace of fraction 3 from a 120 sec homogenate shows a protein band corresponding to a molecular weight greater than 100 000 present in slightly greater concentration than the 100 000 band.

The electrophoretic patterns support the conclusion already made that homogenisation for 90 and 120 sec results in the appearance of additional membranes in fraction P3.

The protein, phospholipid and cholesterol contents of the S fraction are plotted against homogenisation time in Fig. 3.8. The protein content of the fraction reaches a constant level early in homogenisation. For each of the four homogenisation times studied the amount of cholesterol not sedimenting after centrifugation for $6{\cdot}30 \times 10^6$ g-min is greater than that of phospholipid. The cholesterol and phospholipid in the S fraction are thought to be largely associated with triglyceride or fat particles. In the fractionation of liver, Amar-Costesec et al. (1974) have found that 7·8% of the phospholipid and 8·3% of the cholesterol of the homogenate was recovered in the S fraction. These workers also found that centrifugation for longer times did not significantly reduce the amount of both of these constituents in the supernatant fraction.

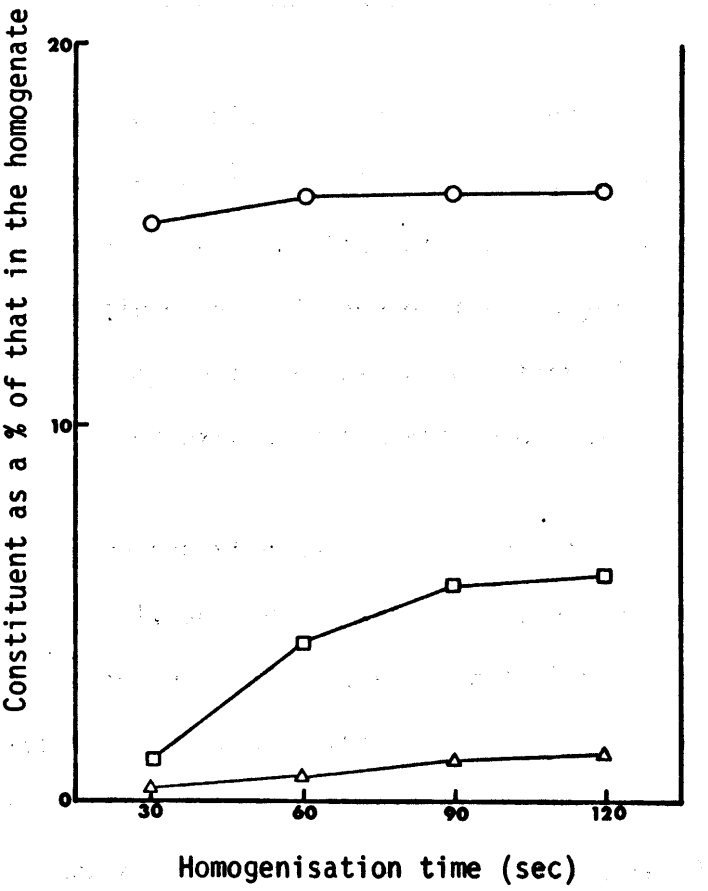


Fig. 3.8. The protein (-○-), phospholipid (-△-) and cholesterol (-□-) content of the S fraction prepared from 30, 60, 90 and 120 sec homogenates.

3.2. The choice of media for density gradient centrifugation

The choice of media for homogenisation and density gradient centrifugation not only have a critical influence on the separations obtainable but may also affect the properties of the separated components. The media used can exert their effects in any of three ways: in the extraction of myosin and actomyosin; in the aggregration of subcellular components; and in the loss of extrinsic membrane proteins.

Uchida et al. (1965) have reported that the contractile protein myosin and, to a lesser extent, actomyosin were extracted by ionic media. The extracted proteins became adsorbed to the microsomal material resulting in a contaminated microsomal fraction. When a sucrose medium was used for homogenisation, these effects were not evident. The incorporation of ionic constituents, such as 10 mM $MgCl_2$ and 15 mM $CaCl_2$, results in the extensive aggregration of subcellular components with an increase in the rate of sedimentation. (Dallner & Nilsson, 1966; Kamath & Narayan, 1972). Similarly, the use of unbuffered sucrose causes clumping of microsomal material (Headon & Duggan, 1972). Aggregration can be reversed by the incorporation of polyanions, such as heparin (Birbeck & Reid, 1956; Wheeldon & Gan, 1971; Headon & Duggan, 1973). Low concentrations of the detergent dioctyl sodium sulphosuccinate (abbreviated DOSSS) are also reported as being useful in overcoming aggregration (Cline & Ryel, 1971).

The effect of a range of media compositions on the aggregration of muscle microsomes was investigated using the technique of ultrafiltration.

Fraction 2 of a concentrated microsomal suspension, prepared as described in Section 2, was supplemented with constituents that have been variously used in homogenisation and density gradient media. 1 ml aliquots, containing 100 μg protein, were filtered through a range of Millipore filters (Millipore (U.K.) Ltd., London) of known average pore size. The protein contents of the samples and the various filtrates were measured. The amount of protein filtered (100 μg) was found to be sufficiently low to ensure that clogging of the filter did not occur.

An examination of muscle microsomes in the electron microscope has shown that the average diameter of vesicles is in the range 0·1 - 0.3 μm (Headon & Duggan, 1973; Meissner & Fleischer, 1971). While the relationship between filtration size and the dimensions evident from electron micrographs was not investigated, the electron microscope data was used as a rough guide and filters having average pore diameters of 0·22, 0·30, 0·45, 0·80 and 1·20 μm were used.

Results are expressed as percentage protein passing the filter.

In Fig. 3.9 the effects of buffering sucrose with low concentrations of imidazole are shown. The incorporation of 1 mM imidazole produces a large increase in the percentage of protein passing the filter over the range of pore sizes. When this medium was adjusted to pH 5·5 with HCl, the amount of protein passing the filter for each pore size decreases and the results resemble those for the sucrose - only medium. These results show that, when the pH of a sucrose - only medium is increased to approximately 7·0 by the addition of 1 mM imidazole, microsomal aggregration is reduced. This effect can be largely reversed by adjusting the pH to 5·5 with HCl.

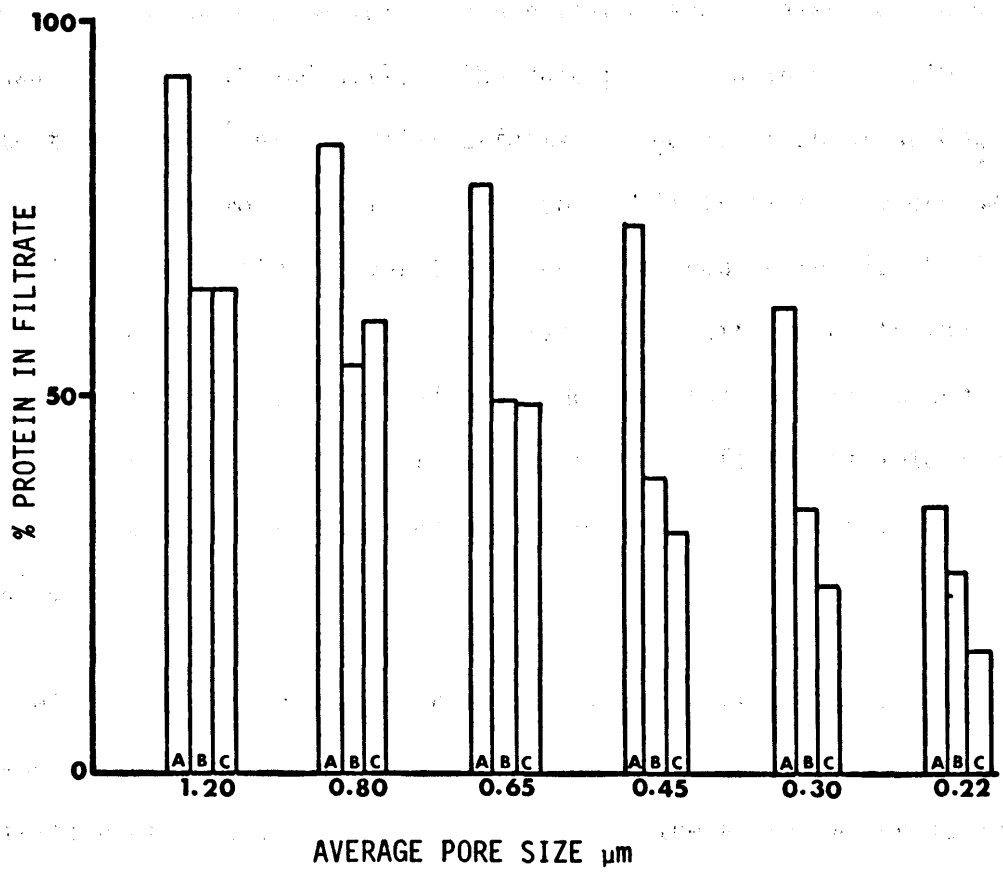


Fig. 3.9. The effect of low concentrations of imidazole on the filtration of a microsomal suspension in unbuffered sucrose.

The media A-C contained 100 µg protein/ml in 0·15 M sucrose. In addition, medium A contained 1 mM imidazole while medium B contained 1 mM imidazole-HCl pH 5·5. Medium C, which was used as a control, contained sucrose and protein only.

The protein content of each filtrate is expressed as a percentage of that in the unfiltered medium.

The effects on the aggregration of microsomes by supplementing a sucrose-1 mM imidazole medium with monovalent and divalent cations are shown in Fig. 3.10 and 3.11. The data presented in Fig. 3.10 shows that Na^+ and K^+ have similar effects. The addition of $NaHCO_3$ to the medium has a more complex effect on the filtration of microsomes through Millipore filters. Over the range of average pore size 0·65 - 1·2 μm, the addition of 15 mM $NaHCO_3$ makes little difference to the percentage protein passing the filter. However for the 0·3 μm and 0·45 μm filters, the amount of protein passing the filter is intermediate between that found for sucrose-imidazole and sucrose-imidazole supplemented with Na^+ or K^+.

The effects of incorporating divalent cations are shown in Fig. 3.11. 15 mM $MgCl_2$ causes more extensive aggregration than an equivalent concentration of $CaCl_2$, although in both cases the aggregration is less dramatic than in 100 mM NaCl or 100 mM KCl. 50% of the protein passes the 0·22 μm filter in the presence of 15 mM $CaCl_2$ as opposed to 36% in a buffered sucrose medium. A possible explanation for this unexpected finding may be the release of some protein from membrane material in the presence of Ca^{2+}.

The results obtained on supplementing a sucrose medium with the polyanion heparin are presented in Fig. 3.12.At a concentration of 50 IU heparin/ml (IU = international units), the amount of protein passing the filter is similar to that found for the buffered sucrose medium. However, when heparin and imidazole are both present, the amount of protein passing the filter is increased. 84% of the protein passes the 0·3 μm filter in the presence of heparin and imidazole, as opposed to 62% for imidazole only.

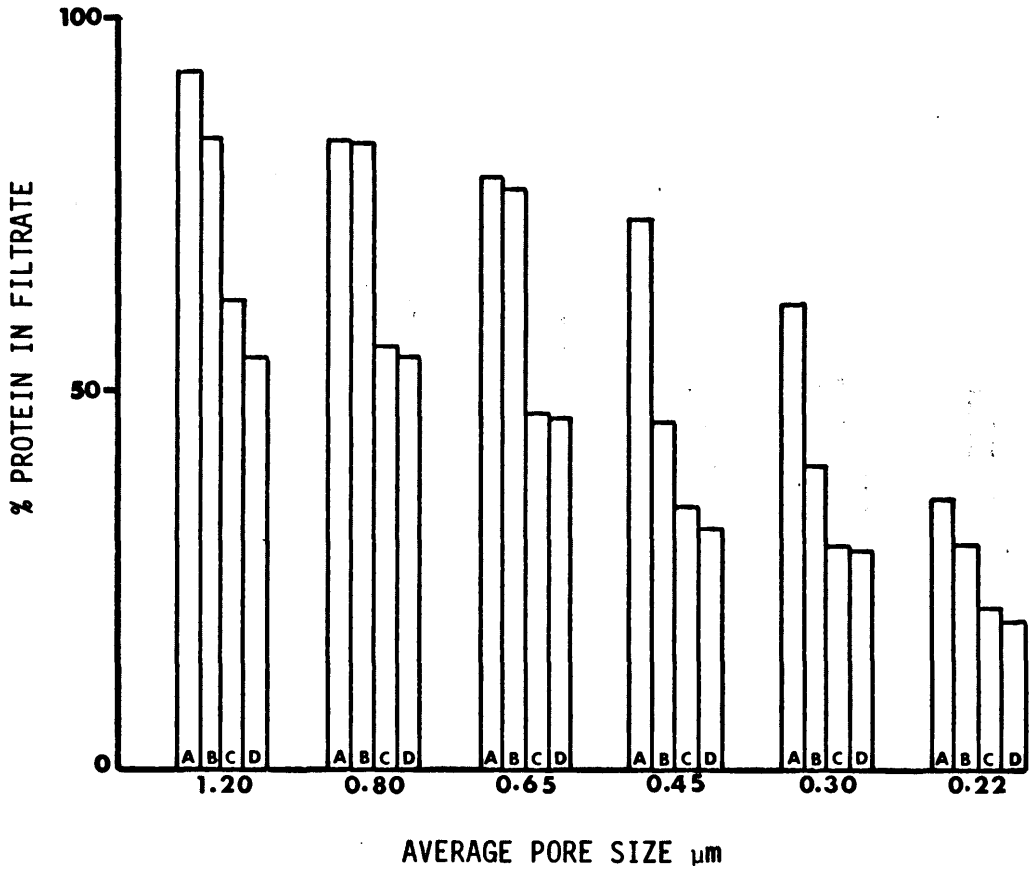


Fig. 3.10. The effects of a number of monovalent cations on the filtration of a microsomal suspension.

The media, A-D, contained 100 μg protein/ml in 0·15 M sucrose, with the following additions:

A 1 mM imidazole;

B 1 mM imidazole + 15 mM Na HCO_3;

C 1 mM imidazole + 100 mM KCl;

D 1 mM imidazole + 100 mM NaCl.

The protein content of each filtrate is expressed as a percentage of that in the unfiltered medium.

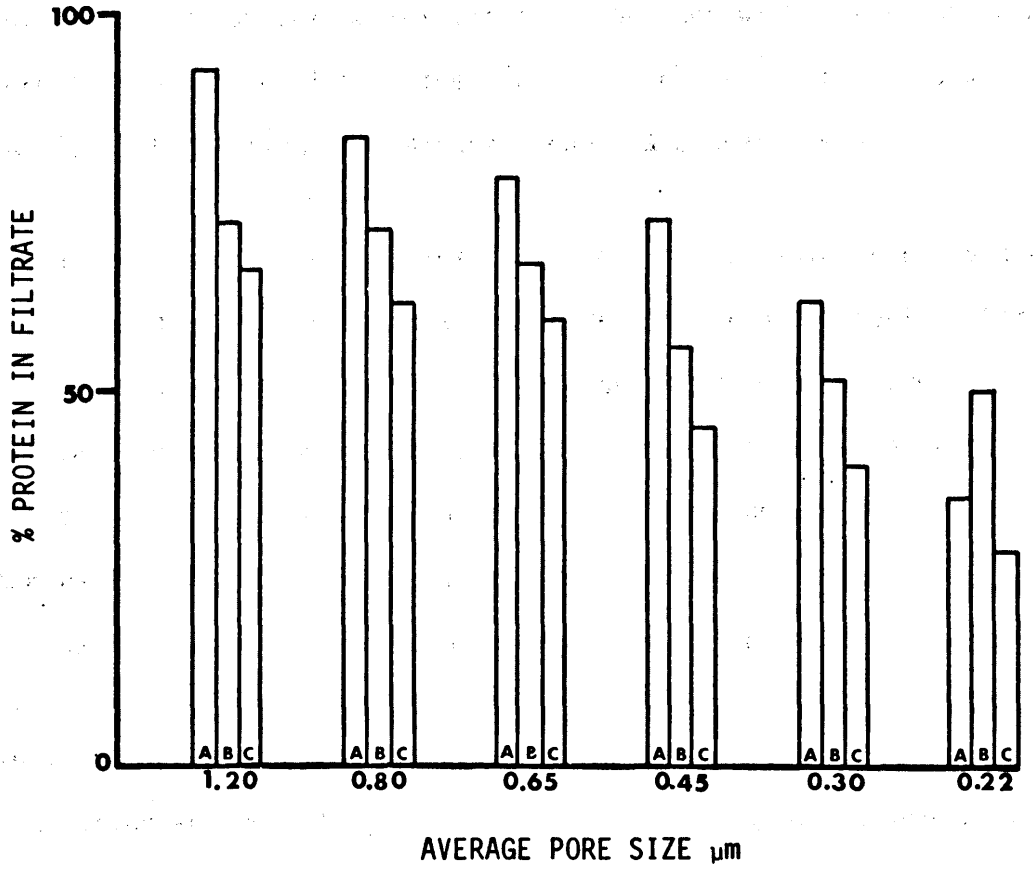


Fig. 3.11. The effect of divalent cations on the filtration of a microsomal suspension.

The media, A-C, contained 100 µg protein/ml in 0·15 M sucrose with the following additions:

A 1 mM imidazole;

B 1 mM imidazole + 15 mM $CaCl_2$;

C 1 mM imidazole + 15 mM $MgCl_2$.

The amount of protein in each filtrate is expressed as a percentage of that in the unfiltered medium.

The usefulness of heparin as a disaggregrating agent is limited by its adverse effect on enzyme activities associated with muscle membranes. Exposure to heparin results in the rapid loss of Ca^{2+}-uptake and a gradual loss of K^{+}-stimulated ATPase (Headon & Duggan, 1973).

The aggregration of microsomes in sucrose-ionic media is reflected in an increased rate of sedimentation in density gradient centrifugation (Dallner & Nilsson, 1966). This is because the rate of movement in a centrifugal field is governed by particle size and to a lesser extent by particle density. Kamath & Narayan (1972) have developed a method for the rapid isolation of rat liver microsomes by low-speed centrifugation after Ca^{2+}-induced aggregration of the membrane material.

In the light of the results obtained in this investigation, homogenisation and density gradient media consisting of sucrose solutions buffered with 5 mM imidazole-HCl at pH 7·4 were used for the work described in this thesis.

In addition to the effects already described, media constituents may interact with membranes, resulting in the loss of extrinsic or loosely associated membrane proteins. Duggan & Martonosi (1970) report that treatment of a muscle microsomal preparation with 1 mM EDTA or EGTA at pH 8·0 results in the loss of 51000 and 63000 molecular weight proteins. Meissner & Fleischer (1972) have found that the treatment of a microsomal preparation with 0·5 M Li Br leads to a 20% increase in the phospholipid-phosphorus/ protein ratio. Similarly, protein is extracted from a microsomal pellet on treatment with 0·6 M KCl (Louis & Shooter, 1972; Martonosi, 1968).

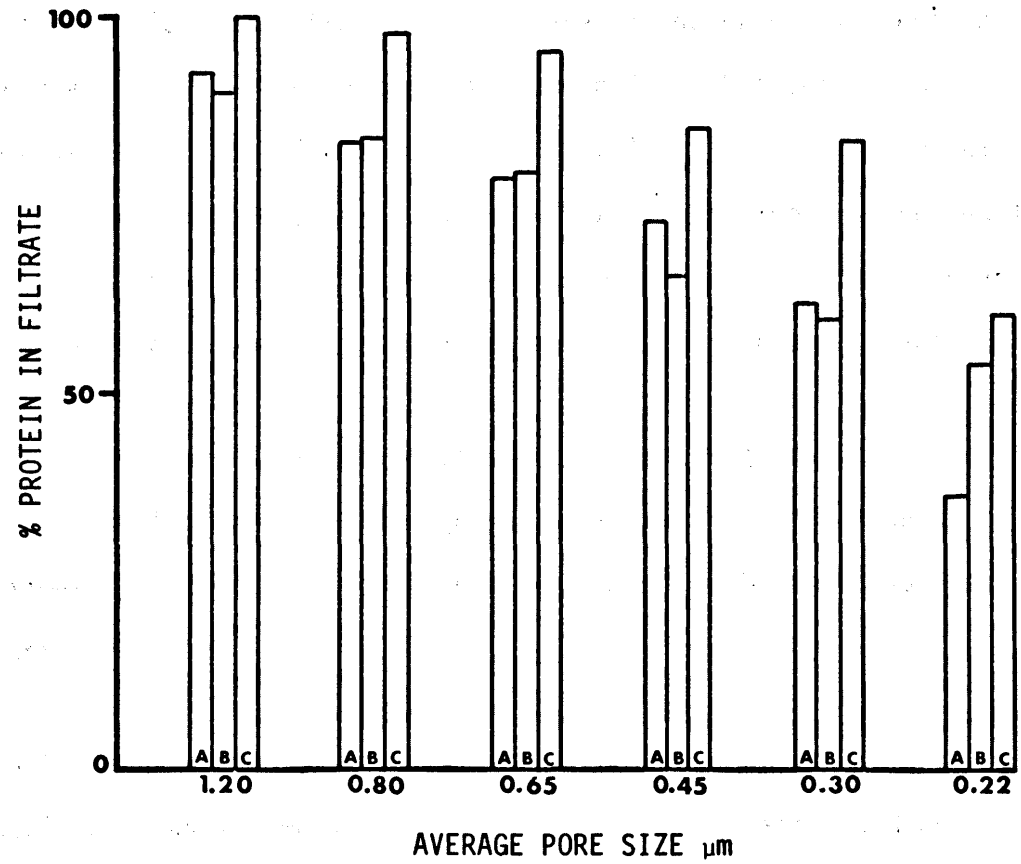


Fig. 3.12. The effect of heparin on the filtration of a microsomal suspension.

In addition to 100 µg protein/ml in 0·15 M sucrose, the media A-C contained:

A 1 mM imidazole;

B 50 IU heparin/ml (IU=international unit);

C 1 mM imidazole + 50 IU heparin/ml.

The protein content of each filtrate is expressed as a percentage of the protein in the unfiltered medium.

The amount of protein extracted by 100 mM KCl in each of the four concentrated microsomal fractions, prepared as described in Section 2, was determined. The percentage of protein in each fraction not sedimenting after centrifugation for $6{\cdot}30 \times 10^6$ g-min (r_{av} = 5·84 cm) in the presence and absence of 100 mM KCl was determined. The results are given in Table 3.2. In the presence of 100 mM KCl, there is an increase in the amount of non-sedimentable protein in each fraction.

The use of dextran sulphate in sucrose density gradients, described in a later part of this section, results in the solubilisation of approximately 30% of the protein sedimentable in a buffered sucrose gradient.

In the light of these results sucrose buffered at pH 7·4 with 5 mM imidazole-HCl was the medium of choice for homogenisation and density gradient centrifugation.

TABLE 3.2

The extraction of membrane-associated protein from concentrated microsomal suspensions (prepared as described in Section 2) on treatment with 100 mM KCl.

The percentage protein remaining in the supernatant after centrifugation at 100 000g_{av} for 60 min in the presence and absence of 100 mM KCl is given.

The amount of protein extracted by KCl treatment was calculated from this data.

FRACTION	% PROTEIN SOLUBLE		Protein extracted by 100 mM KCl (as percentage of total protein in fraction)
	Centrifugation medium		
	Sucrose-imidazole -HCl, pH 7·4	Sucrose-imidazole -HCl, pH 7·4 + 100 mM KCl	
1	79·25	86·62	7·37
2	42·40	55·04	12·64
3	17·86	22·94	5·08
4	29·62	40·26	10·64

3.3. The characterisation of microsomal fractions prepared in the form of concentrated suspensions.

A complete characterisation of muscle microsomes, prepared in the form of a concentrated suspension according to the method of Headon & Duggan (1973) and described in Section 2, was undertaken. This method of preparation involves the sedimentation of microsomal material from 450 ml of a $5{\cdot}76 \times 10^5$ g-min (r_{av}= 11·94 cm) supernatant, containing 0·66 M sucrose-5 mM imidazole-HCl, pH 7·4, into a band of 1·0 M sucrose, also buffered at pH 7·4. Sedimentation beyond this band is prevented by a cushion of 2·0 M sucrose, pH 7·4. After centrifugation for 60 min at 30 000 rev/min ($2{\cdot}61 \times 10^6$ g-min at the volumetric centre of the sample), a concentrated microsomal suspension was collected in six 25 ml fractions over a short gradient corresponding to the original band of 1 M sucrose.

The overall recoveries and the distribution of a number of constituents and enzymes at various stages of the preparation are given in Table 3.3. This data is shown graphically in Fig. 3.13. As found in the investigation on the effect of homogenisation time (Section 3.1), 80-90% of the constituents studied sedimented in the low-speed pellet (P1). 3·5% of the total phospholipid and 2·1% of the phosphoprotein intermediate of the Ca^{2+}-dependent ATPase were recovered in the concentrated suspension.

Fig. 3.14 shows the distribution of protein and phospholipid between the concentrated microsomal suspension collected in six 25 ml fractions and the zonal supernatant. The protein concentration falls sharply between the zonal supernatant, which contains most of the soluble protein of the homogenate, and fraction 1. The six fractions cover

TABLE 3.3

Distributions and recoveries for a number of constituents in the preparation of a concentrated microsomal suspension.

Constituent	Overall Recovery (%)	Subcellular fraction			
		P1	P2	CMS	ZS
Protein	102·9	86·1	0·4	0·4	16·0
Phospholipid	97·6	87·1	3·4	3·5	3·6
Cholesterol	98·9	90·7	1·6	1·7	4·9
Succinate dehydrogenase	101·8	85·2	11·7	1·5	3·4
Phosphoprotein intermediate of Ca^{2+}-dependent ATPase	90·5	85·6	1·1	2·1	1·7

The amount of each constituent in the various fractions is expressed as a percentage of that in the homogenate. Subcellular fractions are abbreviated as follows:

P1 = $6{\cdot}54 \times 10^4$g-min pellet;

P2 = $5{\cdot}76 \times 10^5$g-min pellet;

CMS = concentrated microsomal suspension;

ZS = zonal supernatant obtained in the preparation of the concentrated suspension.

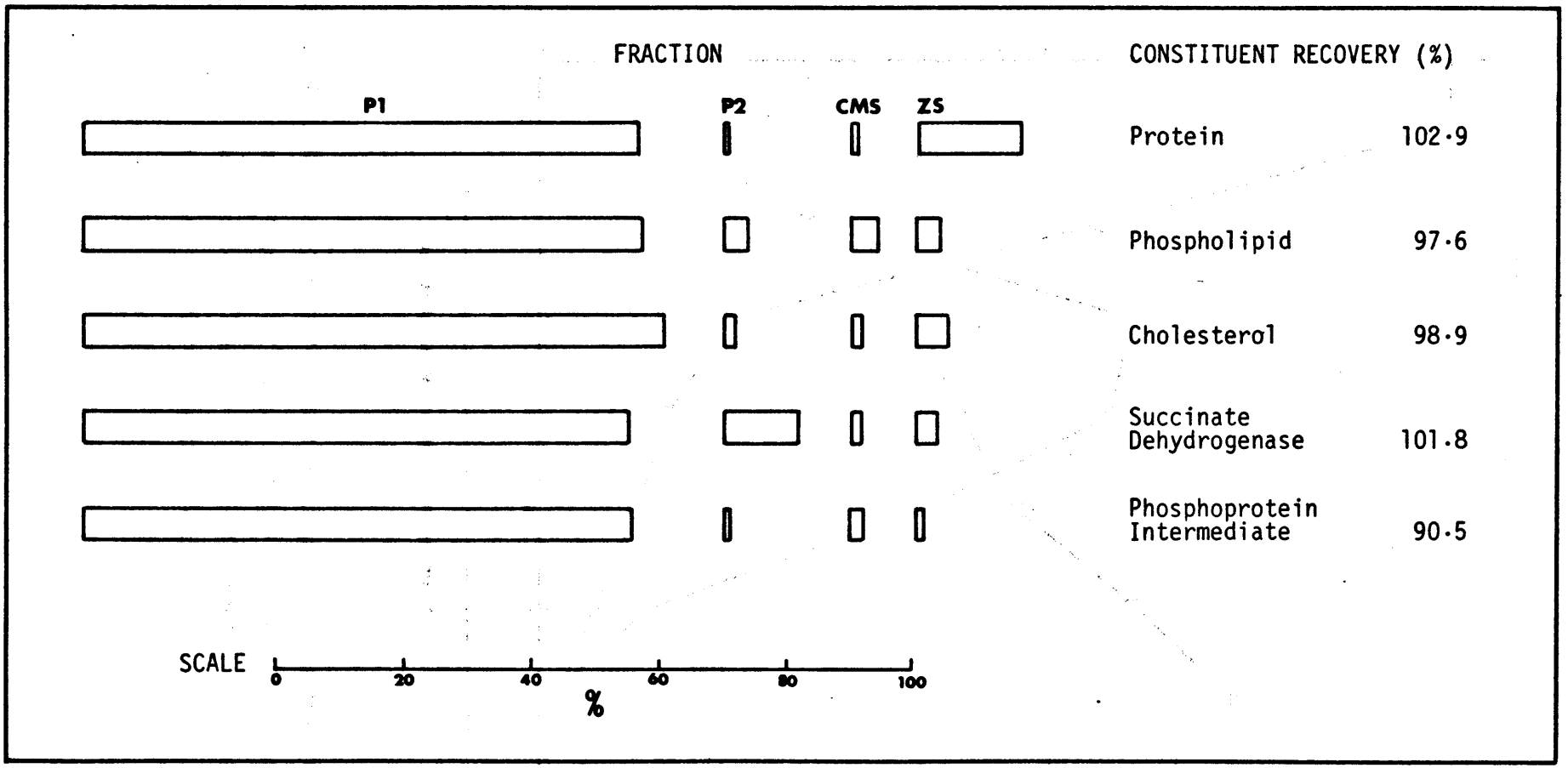


Fig. 3.13. Diagrammatic presentation of the distribution data given in Table 3.3 for a number of constituents in the preparation of a concentrated microsomal suspension. Abbreviations are as given in the legend to Table 3.3.

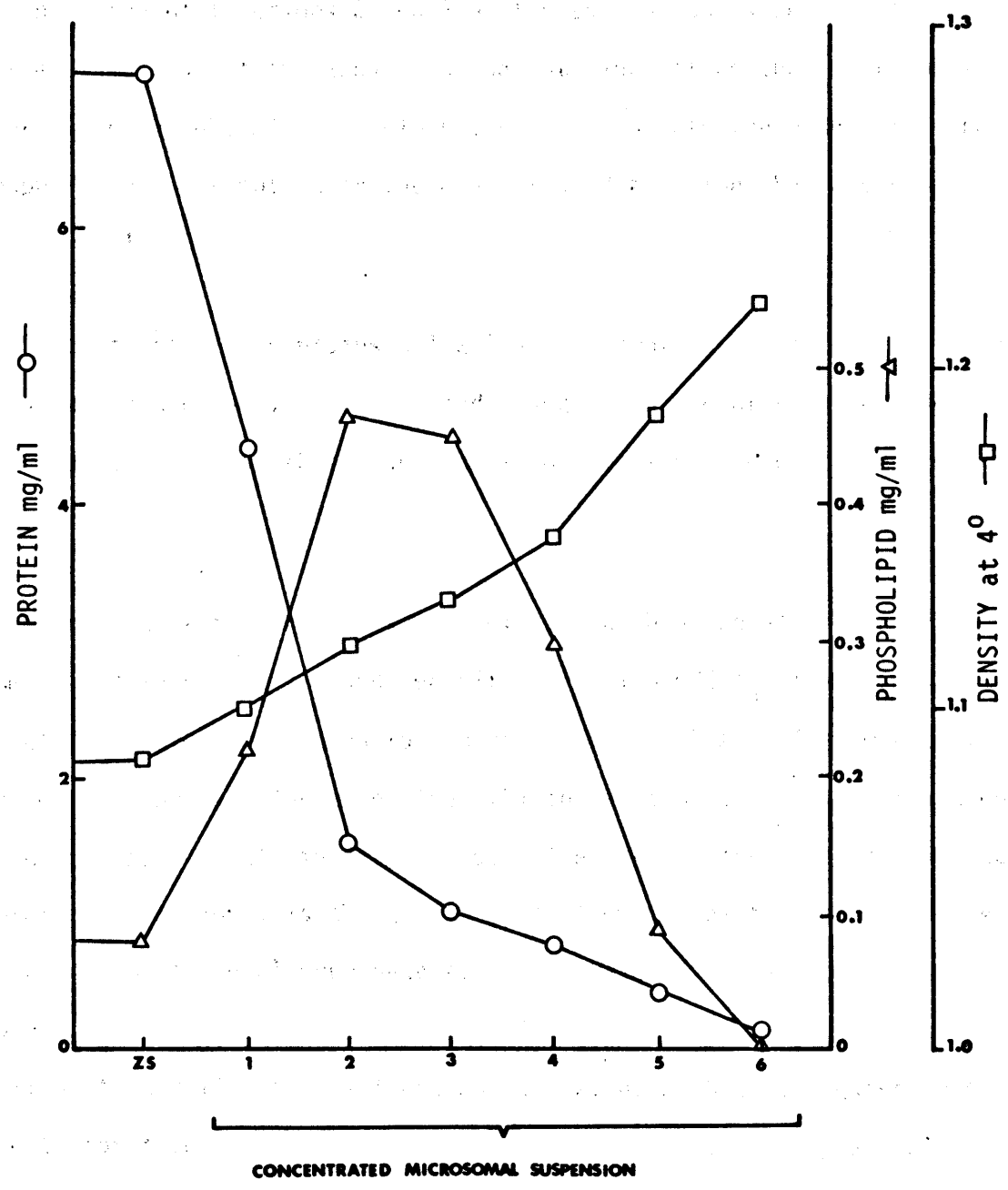


Fig. 3.14. The distribution of protein, and phospholipid in the zonal supernatant (ZS) and the concentrated microsomal suspension collected as 6 25 ml fractions. The density gradient profile is also shown.

the density range 1·1 - 1·2 g/ml approximately. The phospholipid concentration of fractions 2 and 3 of the suspension is several times greater than that of the zonal supernatant, indicating that microsomal material in the 450 ml of sample has sedimented into the gradient. The phospholipid distribution in the concentrated fractions has a median density of 1·120.

Since fraction 6 contains a low level of protein and other constituents, it was not extensively characterised. Regarding the other five fractions, the results will show that fractions 1 to 4 inclusive are of particular interest.

When each of the fractions were diluted with an equal volume of 5 mM imidazole-HCl pH 7·4 and centrifuged at 100 000 g_{av} for 90 min, not all of the protein was found to sediment (Fig. 3.15 A). The amount of sedimentable protein increased from 15% in fraction 1 to 70% in fraction 4 and decreased to 31% in fraction 5. The high content of nonsedimentable protein in fraction 1 is thought to arise from the diffusion of soluble protein from the zonal supernatant. The presence of 69% nonsedimentable protein in fraction 5 is more difficult to explain. The phospholipid/total protein ratio of this fraction is low, being 0·05 as against 0·40 to 0·45 for fractions 2, 3 and 4. This indicates that only a small quantity of the protein in fraction 5 is associated with membranes.

The amount of sedimentable protein in each fraction parallels the phospholipid content of the fraction as would be expected (Fig. 3.15 B).

Fractions 2 and 3 of a concentrated microsomal suspension have high calcium uptake activity. Specific activities of 2·0 - 2·5 μmoles per mg

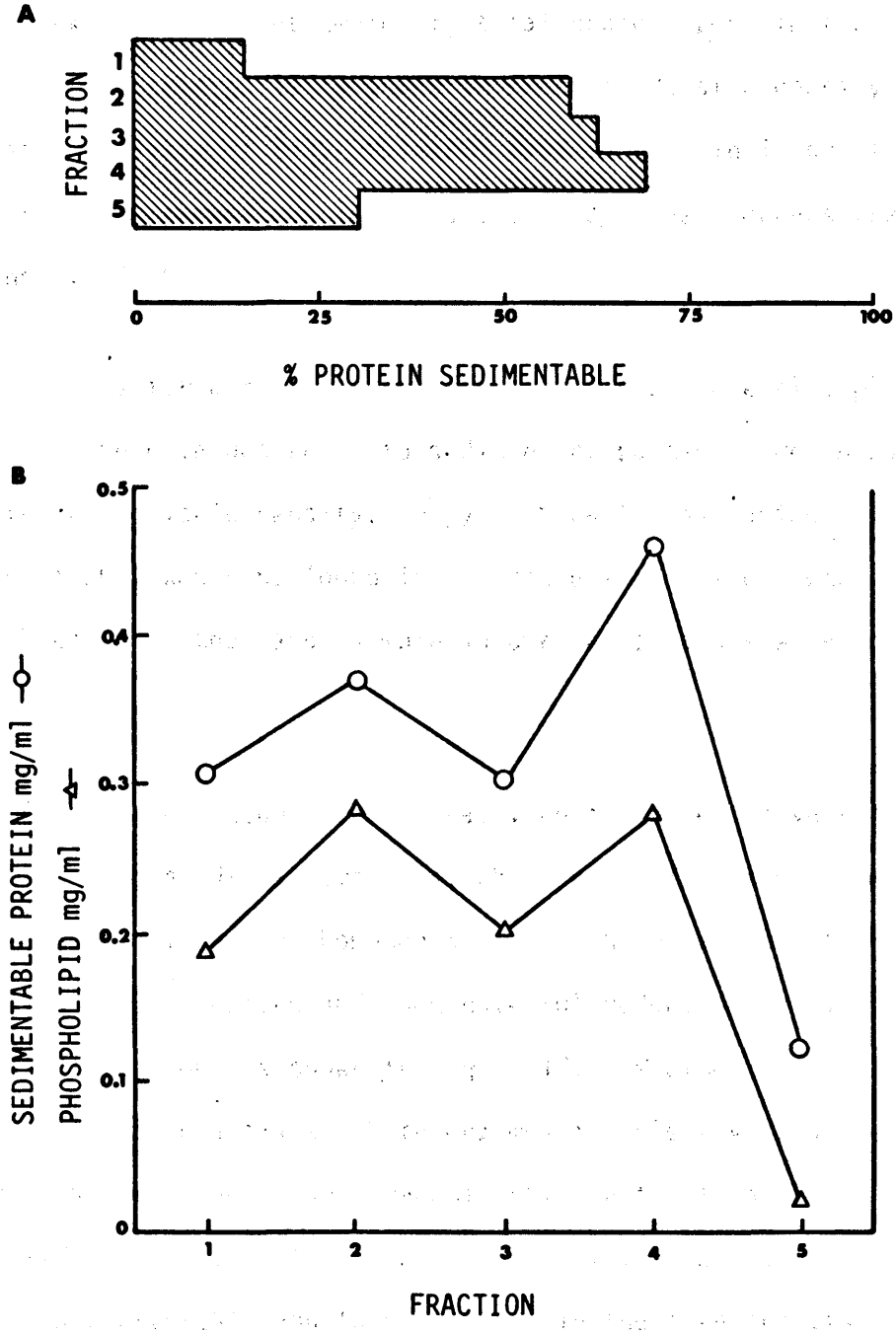


Fig. 3.15. (A) The percentage protein in each of the diluted concentrated microsomal fractions, 1-5, sedimenting after centrifugation at 100 000 g_{av} for 90 min.

(B) The distribution of sedimentable protein and phospholipid in the five concentrated microsomal fractions.

total protein per min were obtained. Fig. 3.16 shows the distribution of Ca^{2+}-uptake expressed as μmoles/mg total protein per min for fractions 1 to 5. Ca^{2+}-uptake activity peaks in fraction 3, at a density of 1·151. The specific activity of 2·39 μmoles/mg protein per min is greater than that reported for rabbit muscle preparations by other workers (review by Martonosi, 1972b).

$S_{20.W}$ values for a particle density of 1·2 g/cm^3 are also plotted in Fig. 3.16. These values refer to sedimenting particles which have not reached their isopycnic density. Only such particles having $S_{20.W}$ values in excess of 600, would be found in the concentrated microsomal suspension. However, because of the large sample volume these values were only used as a guide.

Microsomal preparations are generally contaminated to varying degrees with mitochondrial membranes, which arise from the fragmentation of some mitochondria during homogenisation. A comparison of the distribution of Ca^{2+}-uptake and succinate-INT reductase activity in the concentrated fractions is shown in Fig. 3.17. Whether expressed as absolute or specific activity, both enzymes are clearly separated. Ca^{2+}-uptake activity was found predominantly in fractions 2 and 3 and succinate-INT reductase activity predominantly in fraction 4. 49% of the total succinate-INT reductase activity in the five fractions was found in fraction 4 as against 68% of the total Ca^{2+}-uptake activity in fractions 2 and 3.

The results of a determination of the amount of sedimentable protein in each of the concentrated fractions have been given. A similar experiment was carried out to measure the percentage of the various

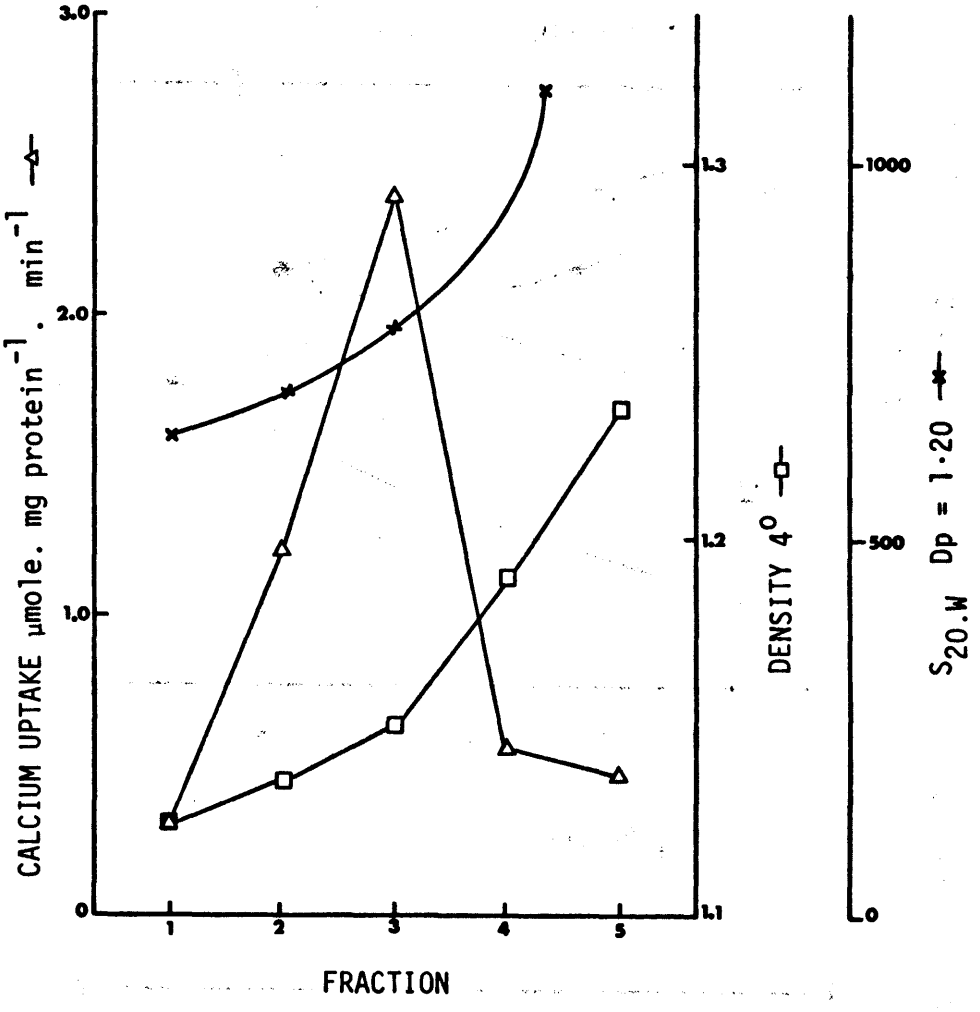


Fig. 3.16. The distribution of Ca^{2+}-uptake activity in the concentrated microsomal fractions. The sucrose density profile and $S_{20.W}$ values for a particle density of 1·20 g/cm^3 are also shown.

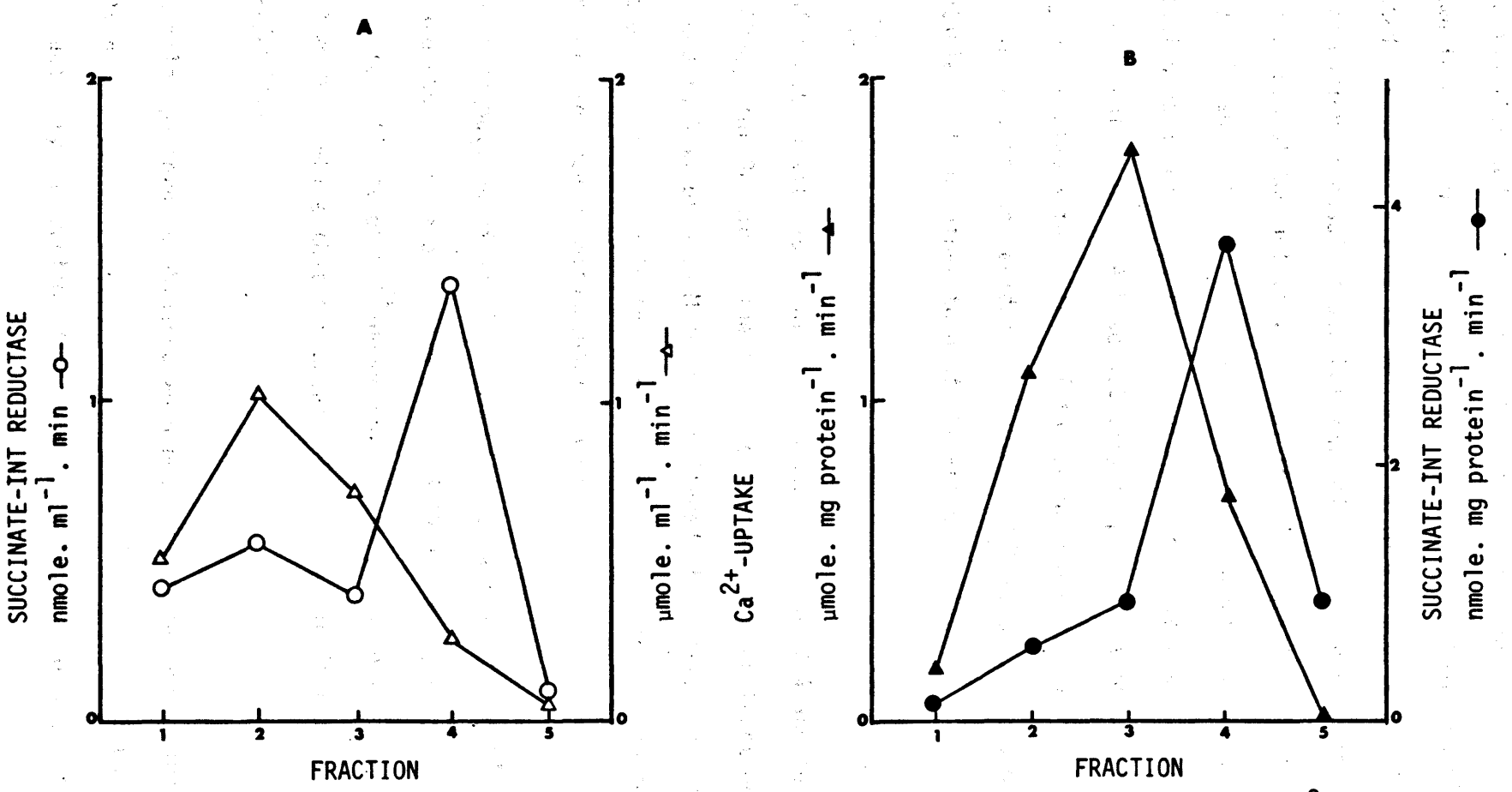


Fig. 3.17. The distribution of absolute activity (part A) and specific activity (part B) of Ca^{2+}-uptake and succinate-INT reductase in the concentrated microsomal fractions.

ATPase activities sedimenting under the same conditions. The results are shown in Fig. 3.18. 100% of the Ca^{2+}-dependent ATPase and K^{+}-stimulated ATPase activities is sedimentable in all four fractions. The distribution of basal ATPase between pellet and supernatant is different. 53% of the activity remains in the supernatant in fraction 1 and 13%, 1% and 14% in fractions 2, 3 and 4 respectively. This distribution parallels that for nonsedimentable protein and implies that nonsedimenting basal ATPase activity is probably a phosphatase activity associated with protein of the soluble phase. In the light of this finding, it was decided to study the ATPase activities of sedimentable protein rather than of the whole fraction.

The sedimentable protein of each fraction was prepared by diluting 20 ml of fraction with 16 ml of 5 mM imidazole-HCl pH 7·4 and centrifuging at 100 000g_{av} for 90 min. The pellet obtained for each fraction was resuspended in 3 ml of 0·25 M sucrose buffered at pH 7·4. ATPase activities were measured in the presence of 50 mM sodium azide to prevent interference by mitochondrial ATPase.

The distributions of absolute and specific activities of Ca^{2+}-dependent ATPase and basal ATPase are shown in Fig 3.19 and 3.20, respectively. The absolute activity of Ca^{2+}-dependent ATPase peaks in fraction 2 (2>3>1>4>5), while that for basal ATPase peaks in fractions 1 and 4 (4>1>2>3>5).

A similar pattern is obtained when both activities are expressed as specific activity, although, in the case of Ca^{2+}-dependent ATPase the specific activity in fraction 3 is slightly greater than that in fraction 2.

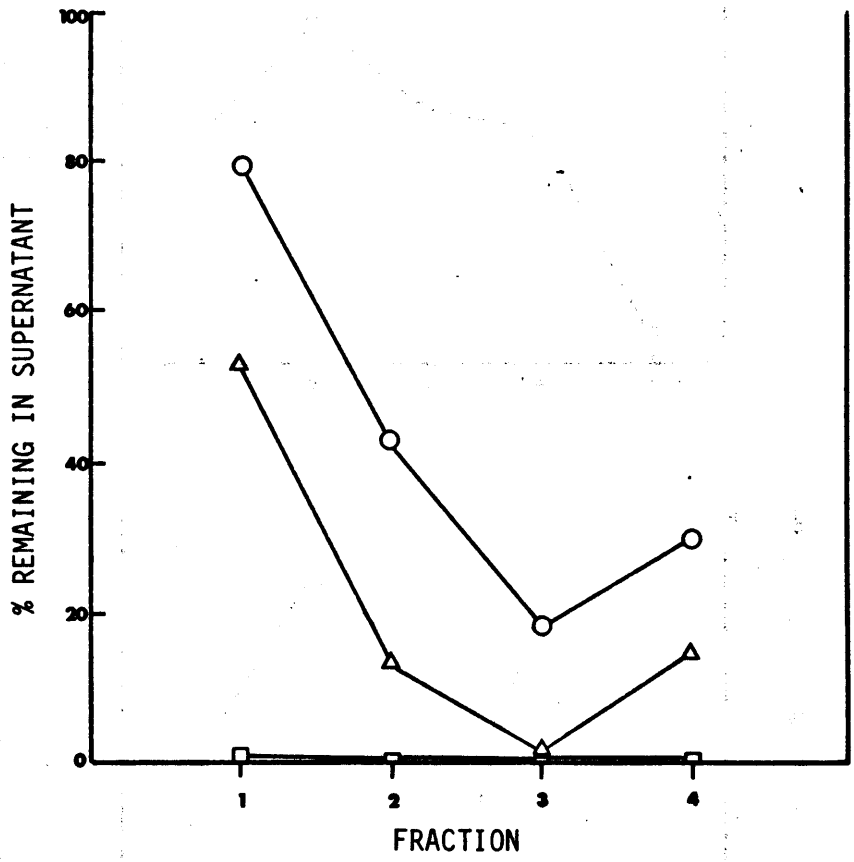


Fig. 3.18. The percentage protein (—○—), basal ATPase activity (—△—), K^+-stimulated ATPase activity (—□—) and Ca^{2+}-dependent ATPase activity (also —□—) remaining in the supernatant after centrifugation of the diluted concentrated microsomal fractions at 100 000 g_{av} for 90 min.

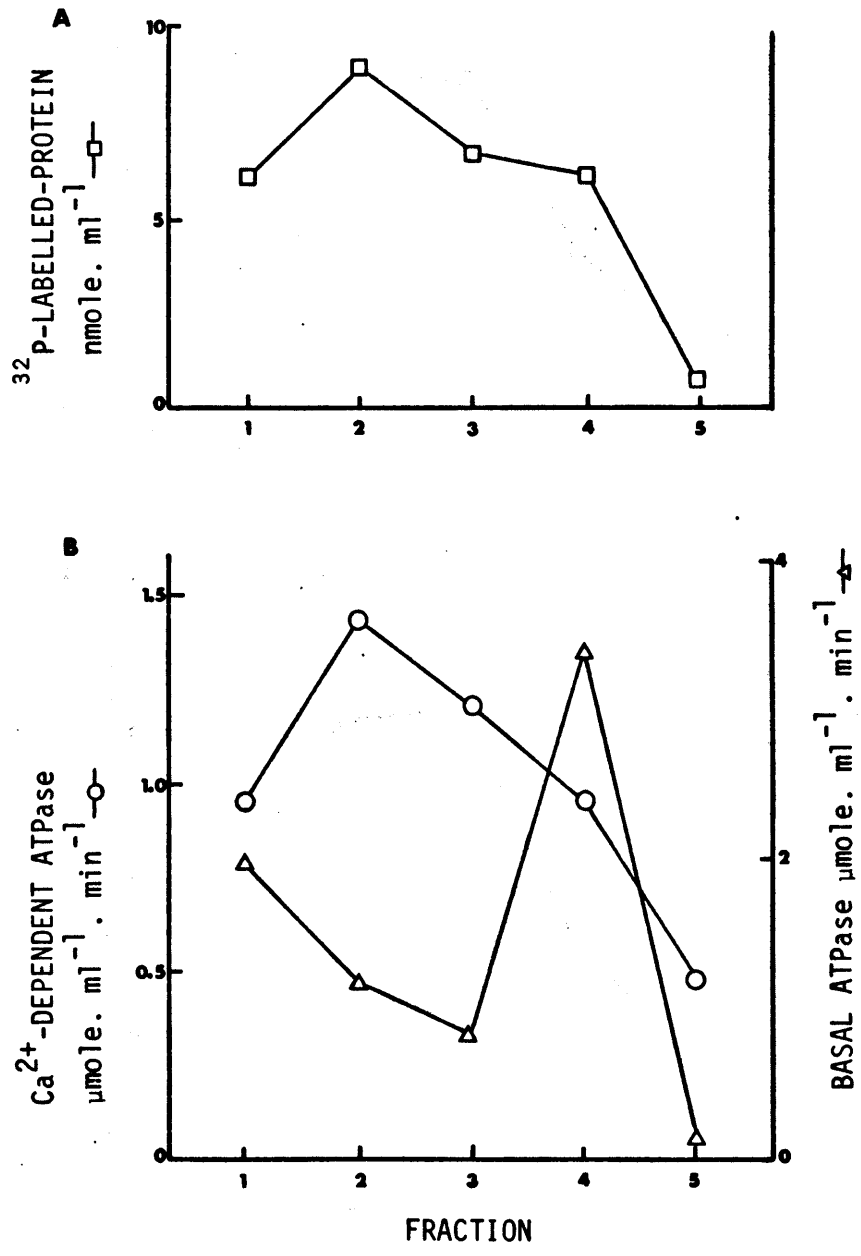


Fig. 3.19. (A) The steady state concentration (nmoles/ml) of the ^{32}P-labelled phosphoprotein intermediate of the Ca^{2+}-dependent ATPase in the concentrated microsomal fractions.

(B) The absolute activities of Ca^{2+}-dependent ATPase and basal ATPase in the concentrated microsomal fractions.

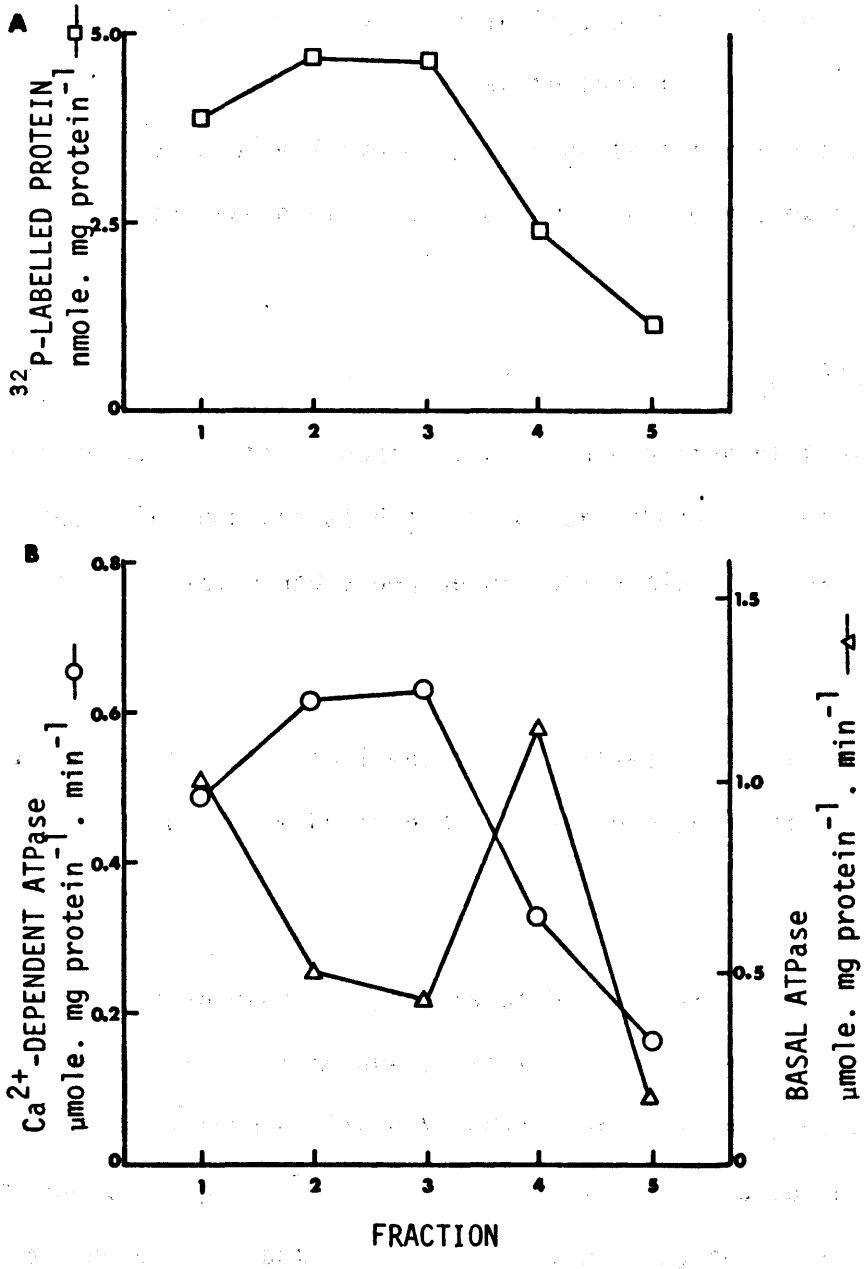


Fig. 3.20. (A) The steady state concentration (nmoles/mg protein) of the ^{32}P-labelled phosphoprotein intermediate of the Ca^{2+}-dependent ATPase in the concentrated microsomal fractions.

(B) The specific activities of Ca^{2+}-dependent ATPase and basal ATPase in the concentrated microsomal fractions.

The steady state concentrations of ^{32}P-labelled protein formed by the Ca^{2+}-dependent ATPase are also shown in Fig. 3.19 and 3.20. The distribution of the intermediate is similar to that for the Ca^{2+}-dependent ATPase. The peak value of 4·7 nmoles per mg protein for fraction 3 is close to the top of the range of values reported in the review by Martonosi (1972a).

In summary, Ca^{2+}-dependent ATPase, Ca^{2+}-transport and the ^{32}P-labelled intermediate have similar distributions, which are different from that of basal ATPase. The concentrated fractions are therefore heterogenous in their enzyme composition and a degree of subfractionation has been achieved.

Having described the principal enzyme activities found in these fractions, attention will now be turned to their phospholipid and cholesterol content.

Phospholipids are inherent components of biological membranes and therefore are indicators of membrane content. Since they constitute 35-40% of the weight [=(phospholipid X 100)/(protein + phospholipid)] of most membranes, the phospholipid/protein ratio may be used to obtain information on the purity, but of course not the homogeneity of a membrane preparation. A very low ratio results from the presence of a large amount of non-membrane protein,while a very high ratio may indicate the loss of native membrane protein during the preparation (see Section 3.2, page 119).

The phospholipid/protein ratios for sedimentable material in fractions 1 to 5 are given in Table 3.4 and Fig. 3.21. Fractions 1 to 4 have ratios

TABLE 3.4

The protein, phospholipid and cholesterol content of sedimentable material in the five fractions of a concentrated microsomal suspension.

Sedimentable material from each fraction was obtained by diluting 20 ml of fraction with 16 ml of 5 mM imidazole-HCl, pH 7·4 and centrifuging for $6{\cdot}3 \times 10^6$ g-min. The pellets obtained for each fraction were carefully resuspended in 3 ml of 0·25 M sucrose, 5mM imidazole-HCl, pH 7·4.

The data given here on the phospholipid to protein, cholesterol to protein, and cholesterol to phospholipid ratios are presented in a diagrammatic form in Fig. 3.21.

	FRACTION				
	1	2	3	4	5
Protein mg/ml	1·933	2·333	1·899	2·899	0·766
Phospholipid mg/ml	1·178	1·777	1·261	1·757	0·124
Phospholipid/protein ratio mg/mg	0·609	0·762	0·664	0·606	0·162
Cholesterol µg/ml	57·38	42·62	31·15	106·56	8·20
Cholesterol/protein ratio µg/mg	29·68	18·27	16·40	36·76	10.70
Cholesterol/Phospholipid ratio µg/mg	48·71	23·98	24·70	60·65	66·13
(moles cholesterol x 100)/ (moles cholesterol + moles phospholipid)	8·887	4·582	4·714	10·830	11·688

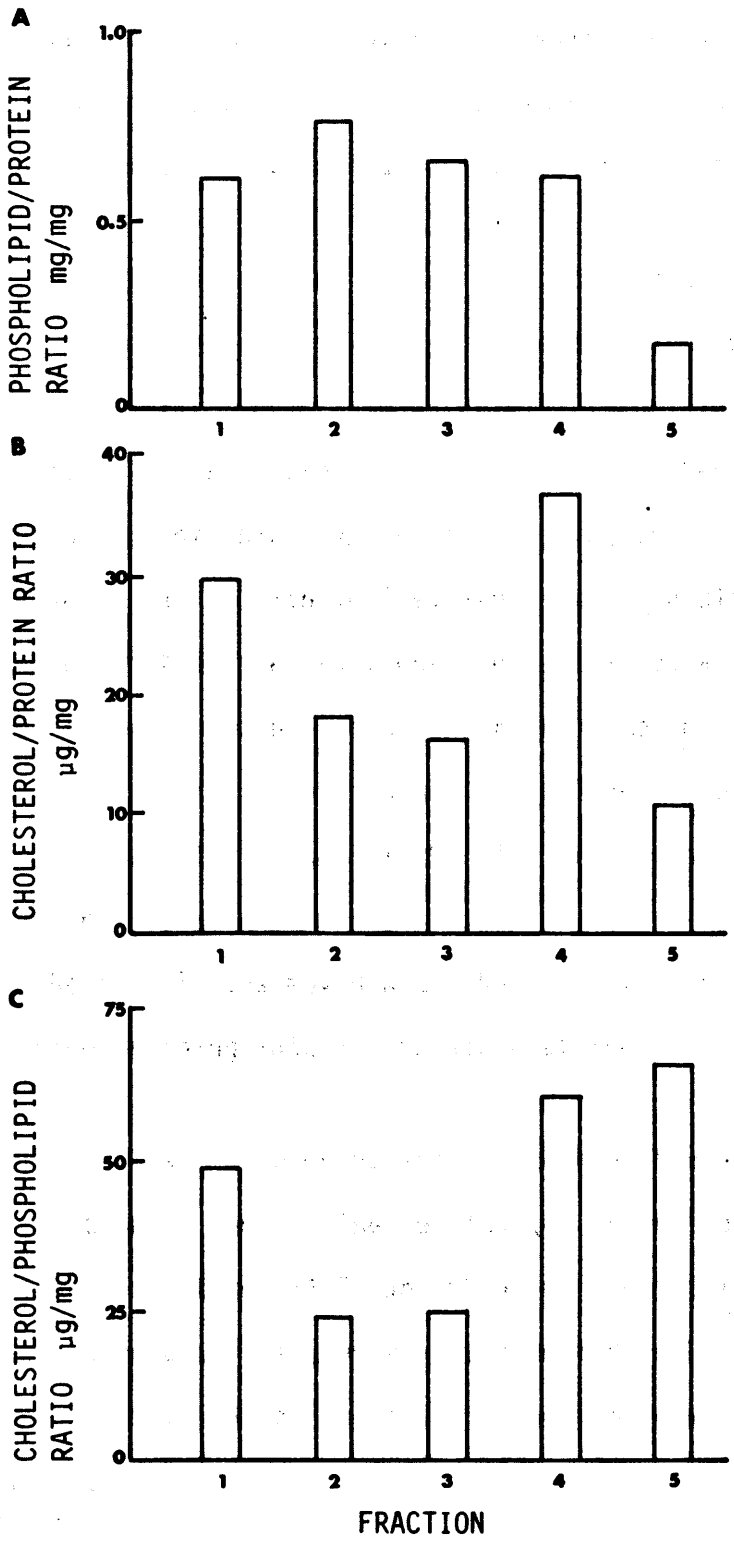


Fig. 3.21. The phospholipid/protein (part A), cholesterol/protein (part B), and cholesterol/phospholipid (part C) ratios in the concentrated microsomal fractions.

in excess of 0·6. The highest ratio, which is one of 0·762, is found in fraction 2, followed by fractions 3, 1 and 4 with ratios of 0·664, 0·609 and 0·606 respectively. The highest phospholipid/protein ratio coincides with the peak of activities associated with Ca^{2+}-transport.

The phospholipid classes present in the lipid extracts of each fraction were separated by one-dimensional thin layer chromatography using chloroform-methanol-glacial acetic acid-water (100 : 20 : 12·5 : 5) as the solvent system. The procedure used is described in Section 2. In Fig. 3.22 photographs of thin layer plates showing the separated phospholipid classes, stained with iodine, are shown. In plate A, the phospholipid classes in the five fractions are separated, and in plate B, the separation of standard phospholipids under the same conditions is shown. The spot corresponding to phosphatidylcholine and phosphatidylserine stains most intensely in each of the five fractions. Each fraction also contains phosphatidylethanolamine, phosphatidylinositol and possibly sphingomyelin. Neutral lipids moved with the solvent front in each case and some iodine staining material remained at the origin.

A quantitative analysis of phospholipid in each of the classes was carried out for fractions 2 and 3. The results are presented in Fig. 3.23. Phosphatidylcholine constitutes 64·7% and 65·9% of the total phospholipid in fractions 2 and 3 respectively, whereas phosphatidylethanolamine accounts for approximately 13% of the total. The remainder of the phospholipid is distributed amongst the other classes.

The cholesterol distribution shown in Table 3.4 is different from that of phospholipid. A bimodal distribution was found with peaks in fractions 1 and 4 and lower concentrations in fractions 2 and 3. The cholesterol/protein ratio is lowest in fraction 3 with 16·4 µg per mg protein. This value is 50-75% of the concentration generally found

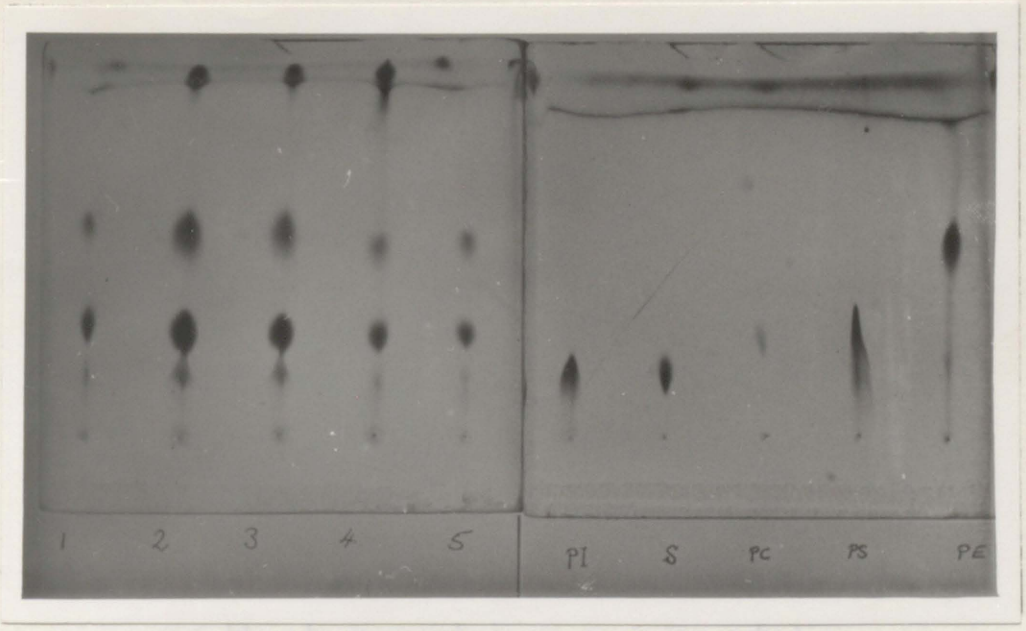


Fig. 3.22. The separation of phospholipid classes in lipid extracts of the concentrated microsomal fractions by thin layer chromatography. The separations were carried out on thin layer plates coated with silica gel G, using chloroform-methanol-glacial acetic acid-water (100 : 20 : 12·5 : 5) as the solvent system. The separated classes were visualised by staining with iodine vapour.

Plate A. The phospholipid classes in the concentrated microsomal fractions 1 to 5.

Plate B. The separation of phospholipid standards.

PI = phosphatidylinositol
S = sphingomyelin
PC = phosphatidylcholine
PS = phosphatidylserine
PE = phosphatidylethanolamine

The origin or site of application is indicated by O and SF indicates the distance travelled by the solvent.

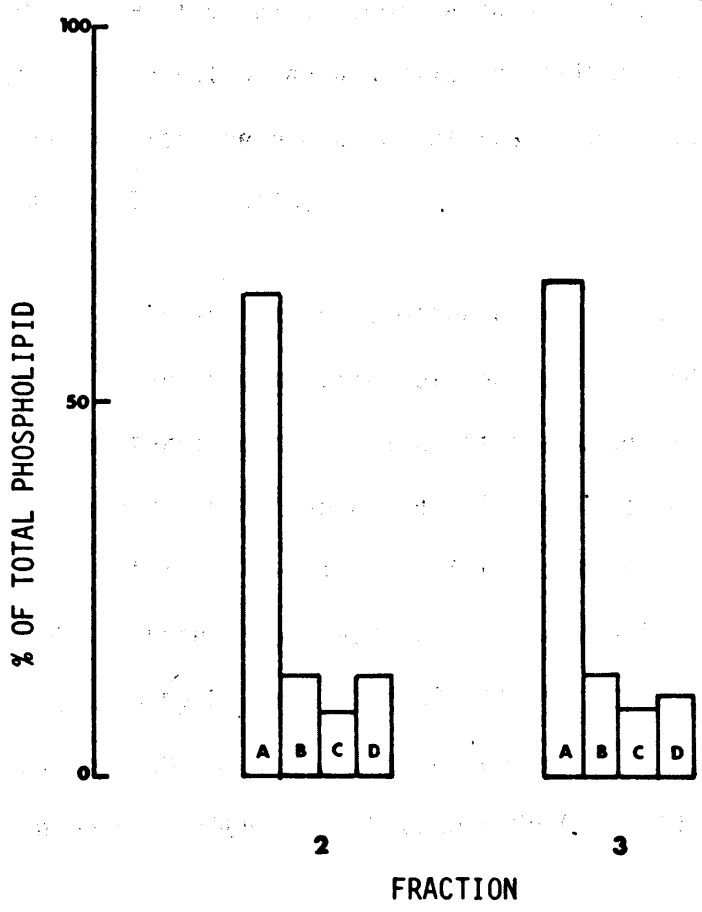


Fig. 2.23. The distribution of phospholipid between the phospholipid classes in fractions 2 and 3 of a concentrated microsomal suspension.

A = phosphatidylcholine
B = phosphatidylethanolamine
C = phosphatidylinositol + sphingomyelin
D = phosphate-containing material remaining at the origin after thin layer chromatography.

in skeletal muscle microsomes prepared by differential pelleting (Martonosi, 1972a; Drabikowski et al., 1966; Sarzala et al., 1975). The cholesterol/phospholipid ratio has a similar distribution to the cholesterol/protein ratio with the exception of fraction 5, which has a high cholesterol/phospholipid ratio.

The molar cholesterol concentration is often expressed as a percentage of the total molar concentration of cholesterol and phospholipid. The cholesterol concentrations of fractions 1 to 5, expressed in this way, are given in Table 3.4. The values range from 8·9 moles% for fraction 1 through 4·7 moles % for fraction 3 to 10·8 moles % for fraction 4. These values are in the lower half of the range 5 - 16 moles % quoted by De Kruyff et al. (1974) for microsomes and considerably less than the 50 moles % reported for erythrocytes and myelin membranes (Van Deenen, 1965; Van Deenen & De Gier, 1974).

Because of the differences in the cholesterol/phospholipid ratios for the five fractions and in an effort to determine if there was a possible association between cholesterol and any of the ATPase activities, cholesterol/enzyme ratios were calculated for the ATPase activities in each fraction. The results are plotted in Fig. 3.24. The cholesterol/ Ca^{2+}-dependent ATPase ratio differs widely between the four fractions. A very similar variation was obtained for the cholesterol/K^{+}-stimulated ATPase, and the cholesterol/^{32}P-intermediate ratios. In contrast,the cholesterol/basal ATPase ratio does not change very greatly between the four fractions.

These results show that cholesterol and basal ATPase have similar distribution patterns which differ from those of activities associated

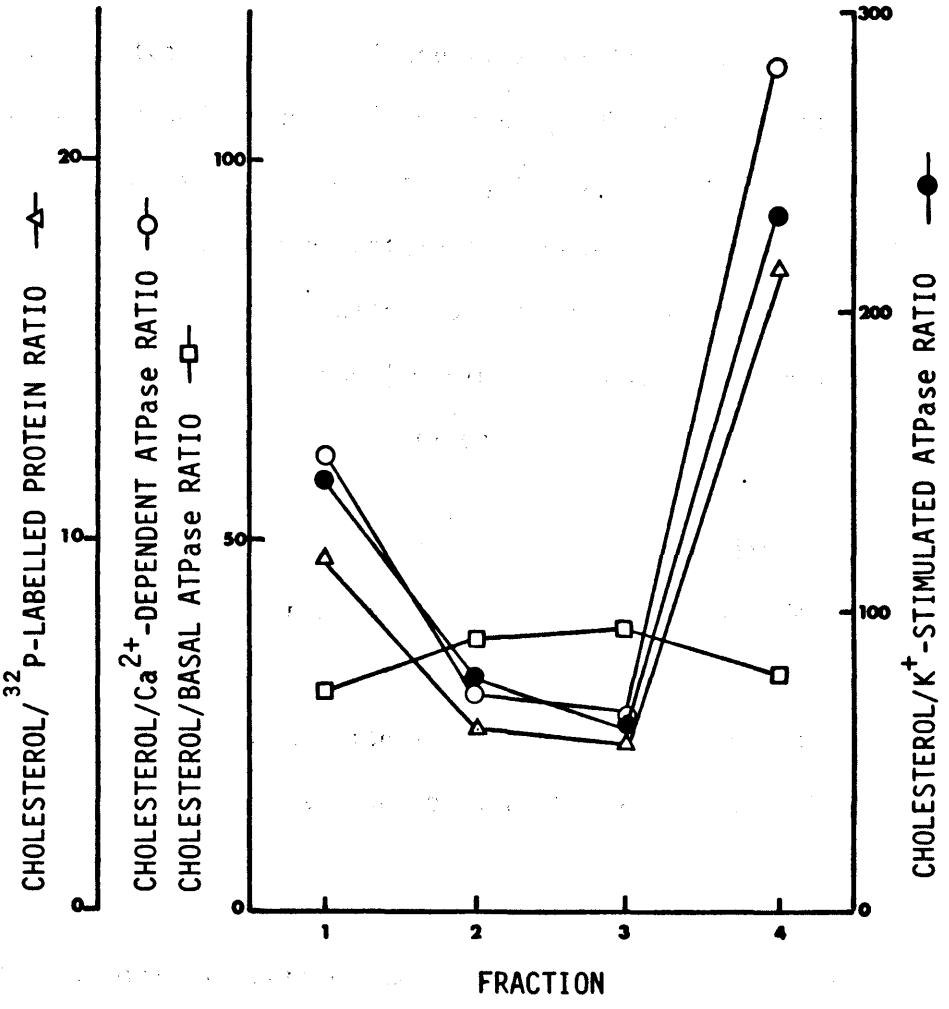


Fig. 3.24. The distribution of cholesterol/enzyme ratios in the concentrated microsomal fractions, 1 - 4. The ratios are expressed in the following units:

cholesterol/^{32}P-labelled intermediate	µg/nmole;
cholesterol/Ca^{2+}-dependent ATPase	µg.minute/µmole;
cholesterol/K^{+}-stimulated ATPase	µg.minute/µmole;
cholesterol/basal ATPase	µg.minute/µmole.

with Ca^{2+}-transport.

Membrane proteins in each of the five fractions were solubilised by treatment with sodium dodecyl sulphate (=SDS) and separated by polyacrylamide gel electrophoresis in gels equilibrated with SDS. The molecular weights of the protein components were determined by coelectrophoresis of samples with proteins, whose molecular weight in the presence of SDS is known (Y-globulin, bovine serum albumin, chymotrypsinogen and cytochrome C) (Weber & Osborn 1969).

Material for electrophoresis was prepared by centrifugation of each fraction at 100 000 g_{av} for 90 min after dilution with an equal volume of 5 mM imidazole-HCl, pH 7·4. The pellets obtained were washed twice by resuspension and sedimentation in 0·25 M sucrose, pH 7·4. After the final washing, each pellet was solubilised by treatment with SDS as described in Section 2.

Electrophoretic separations were initially carried out in 5·2% polyacrylamide gel rods, containing 0·1% W/V SDS. After electrophoresis and fixation, the gels were stained for protein and carbohydrate by treatment with amido black and the periodic acid - Schiff reagent respectively. The patterns obtained for fractions 1 to 4 are shown diagrammatically in Fig. 3.25. While several bands appeared after staining for protein, only one band stained for carbohydrate.

A strong band, corresponding to a molecular weight of approximately 100 000 and marked with an asterisk in Fig. 3.25, was present in all four fractions. In addition, a band of slightly lower molecular weight was present in fraction 1 but not clearly discernable in the other fractions.

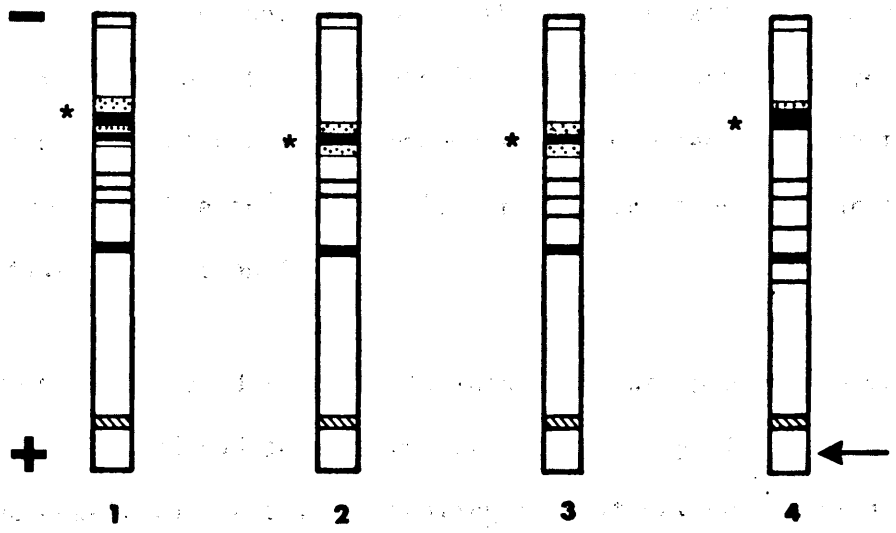


Fig. 3.25. Diagrammatic representation of the amido black and periodic acid-Schiff staining bands observed after the one-dimensional electrophoretic separation of sedimentable material in the concentrated microsomal fractions following solubilisation with SDS.

Electrophoresis was carried out in 5·2% polyacrylamide gel rods containing 0·1% W/V SDS.

The bands represented as ▨ stained with the periodic acid-Schiff stain. The other bands appeared after staining with amido black.

The protein band corresponding to a molecular weight of 100 000 is indicated by an asterisk.

→ indicates the distance travelled by the tracker dye.

The lower molecular weight components differ in intensity between the fractions and their contribution to the overall pattern appears to be greater in fractions 1 and 4 than in fractions 2 and 3. There are four lower molecular weight components in the molecular weight range 15000-80000 visible in fraction 1, three in fraction 2, four in fraction 3 and five in fraction 4.

The periodic acid - Schiff staining band was present in each of the fractions and had an electrophoretic mobility of 0·90 - 0·94. It stained weakly for protein indicating that it may be either a glycoprotein or proteolipid rather than a glycolipid.

The broadness of many of the protein bands indicated the possible presence of a number of proteins of similar mobility. To investigate this possibility, a more highly resolving electrophoretic technique was used.

The procedure chosen was a two-dimensional one, consisting of electrophoresis in the first dimension in 5·2% polyacrylamide gels followed by electrophoresis in a second dimension in gels consisting of a 6 - 27% gel gradient. Electrophoresis in polyacrylamide gel gradients has been successfully used in the separation of multicomponent mixtures (Margolis, 1973; Margolis & Kenrick, 1969). The electrophoresis of proteins, incompletely separated in the first dimension, in a second-dimension gradient gel should enhance the resolution obtainable.

The first-dimension separation was carried out in 5·2% gel slabs at 75 V until the tracker dye reached the end of the gel. A number of experiments were carried out to determine the optimum electrophoretic

conditions for the second-dimension separation. It was found that electrophoresis for 12 hours at 75 V gave the best separation without the loss of low molecular weight proteins except the periodic acid - Schiff staining band.

Molecular weights were determined by coelectrophoresis of molecular weight markers with samples in 5·2% gels. A plot of relative mobility versus molecular weight on a logarithmetic scale is shown in Fig. 3.26.

Photographs of the second-dimension protein patterns for fractions 1 to 6 are shown in Fig. 3·27 A-C. In parts D to F of Fig. 3.27 densitometer traces of the first-dimension separation are superimposed on drawings of the second-dimension separation. The 100 000 component, already referred to, is marked with an asterisk on the densitometer traces.

Densitometer traces of the first-dimension separations show a limited number of peaks similar to those found for separations in 5·2% gel rods. However in the second dimension these peaks are resolved into as many as 2-5 protein components. The separations, which were run in triplicate, were readily reproducible.

The molecular weight ranges corresponding to the protein peaks on the densitometer traces of the first-dimension separation are given in Table 3.5. In fraction 1 the protein bands corresponding to the molecular weight ranges 70 000 - 90 000 and 25 000 - 30 000 are the principal components. These are closely followed by the 100 000 - 120 000 molecular weight band. In fractions 2 - 4 the 100 000 - 120 000 band is the predominant band and is present in greatest concentration in fraction 3. The pattern is changed in fractions 5 and 6, where the 25 000 - 30 000

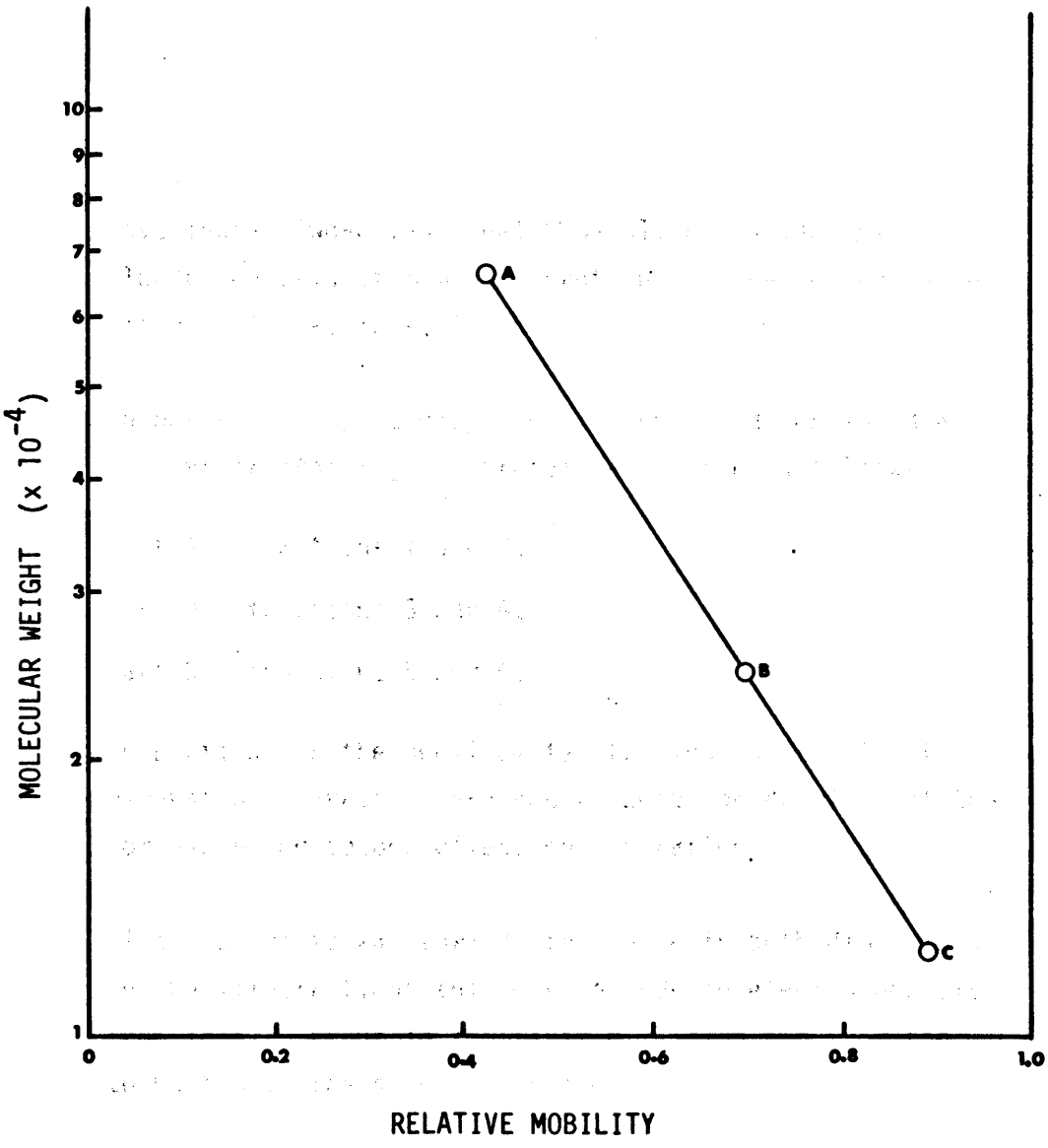


Fig. 3.26. Semilogarithmetic plot of molecular weight versus mobility relative to the tracker dye for the following proteins:

A bovine serum albumin;

B chymotrypsinogen;

C cytochrome C.

The proteins were treated as described in Section 2 and electrophoresis was carried out at 75 V in 5·2% W/V polyacrylamide gel slabs in the presence of 0·1% W/V SDS.

Fig. 3.27. (overleaf) Two-dimensional SDS-polyacrylamide gel electrophoresis of membrane proteins in the concentrated microsomal fractions, 1 - 6.

In parts A - C photographs of the second-dimension gradient gels after staining for protein are shown as follows:

part A fractions 1 and 2;

part B fractions 3 and 4;

part C fractions 5 and 6.

In parts D - F the densitometer tracings of the first-dimension separations are superimposed on drawings of the corresponding second-dimension separation.

Electrophoresis was towards the anode in both dimensions. In the illustrations this corresponds to electrophoresis from left to right in the first dimension and from top to bottom in the second dimension.



Fraction 1



Fraction 2

Fig. 3.27 (A)

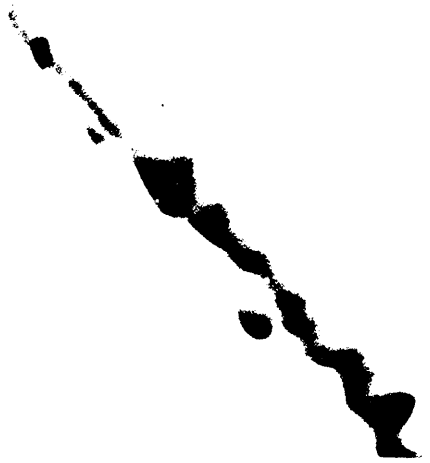


Fraction 3

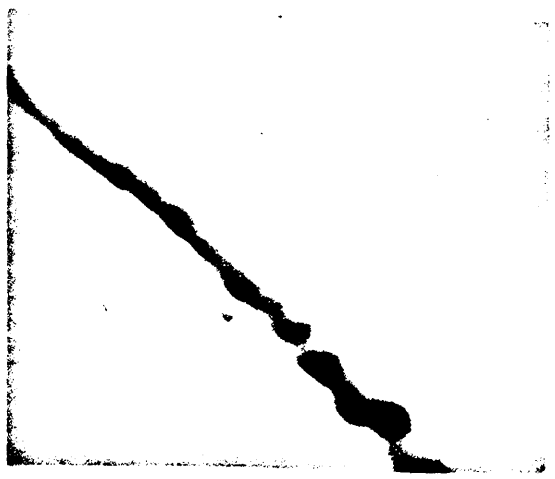


Fraction 4

Fig. 3.27 (B)



Fraction 5



Fraction 6

Fig. 3.27 (C)

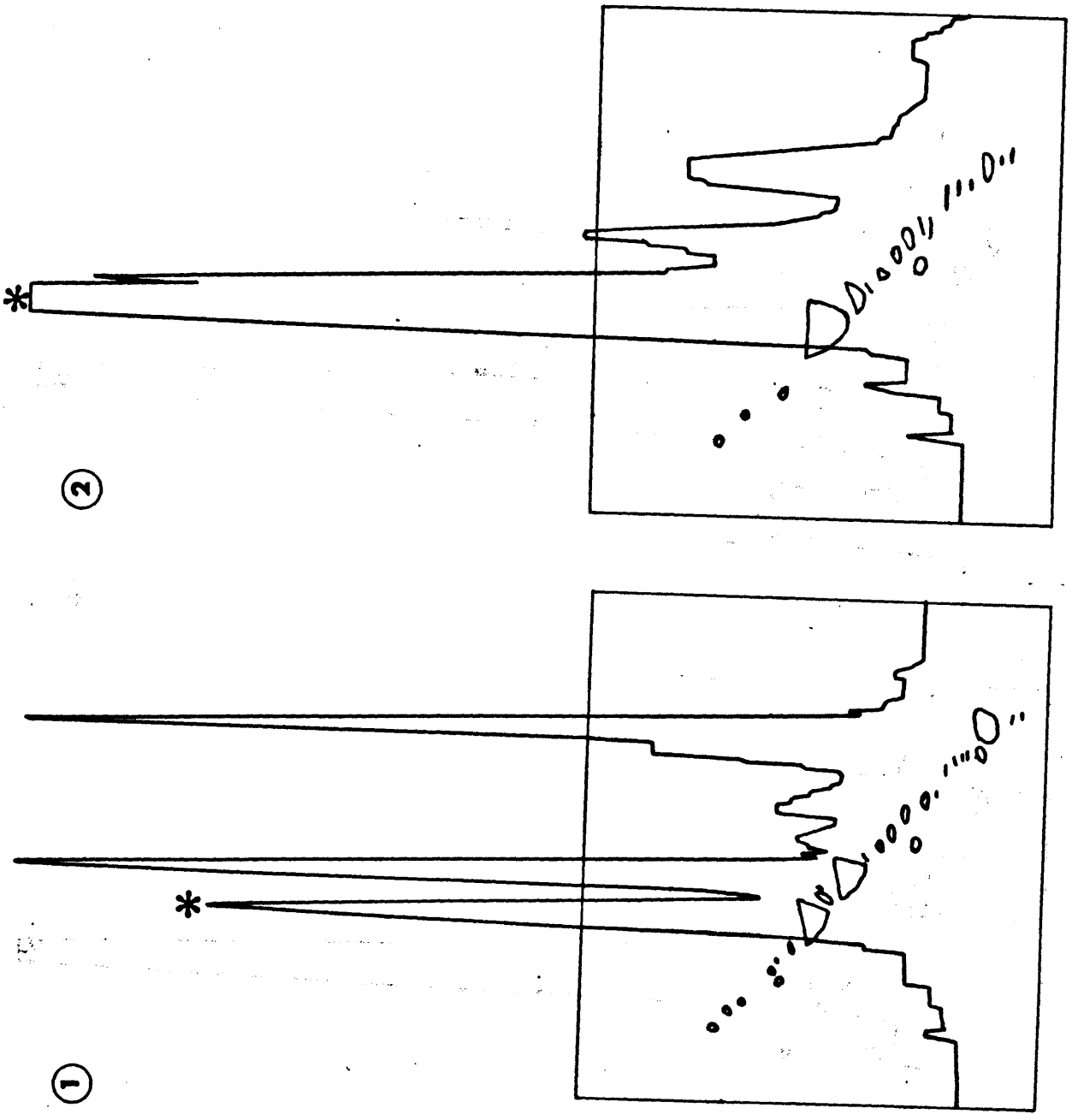


Fig. 3.27 (D)

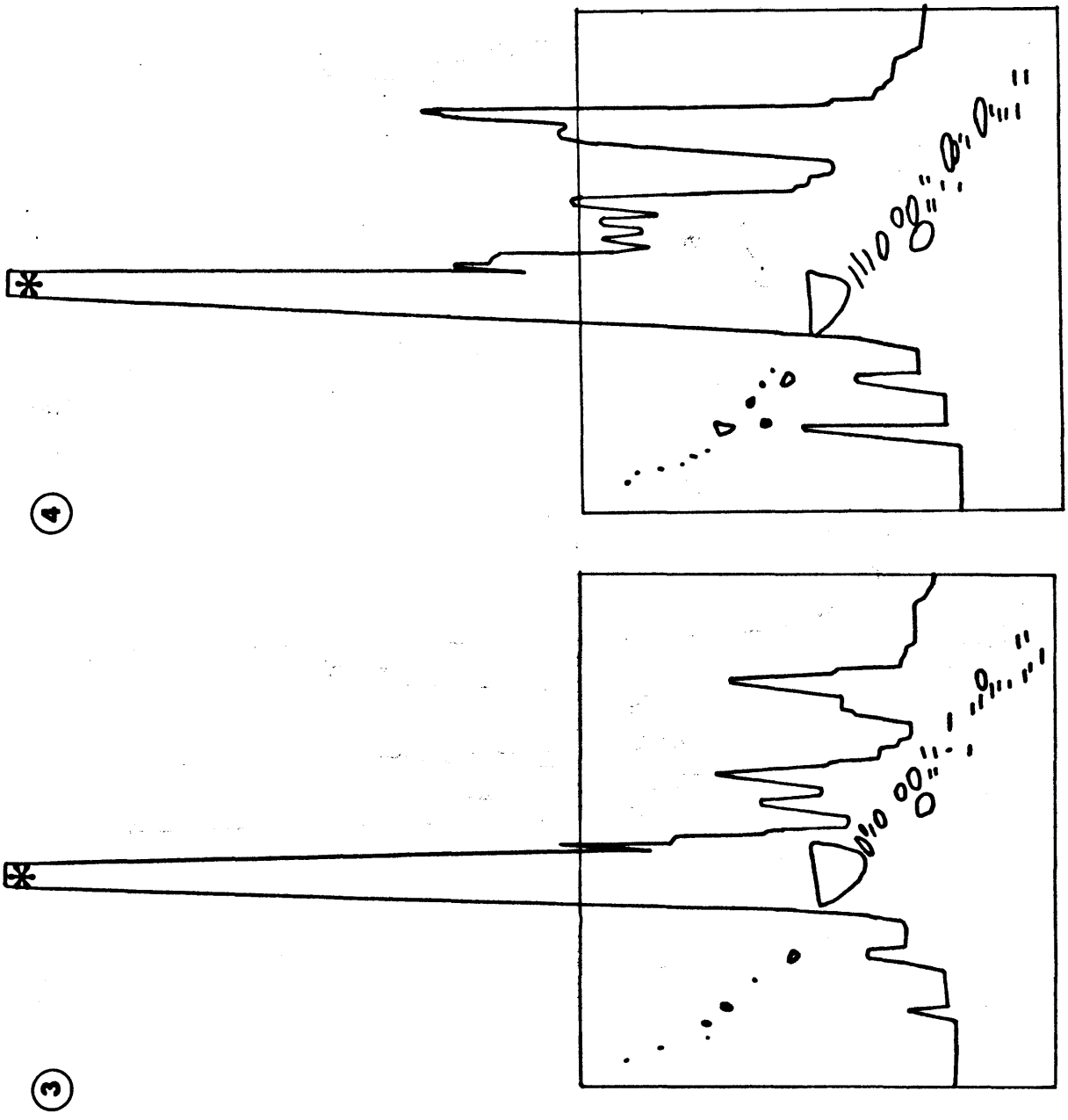


Fig. 3.27 (E)

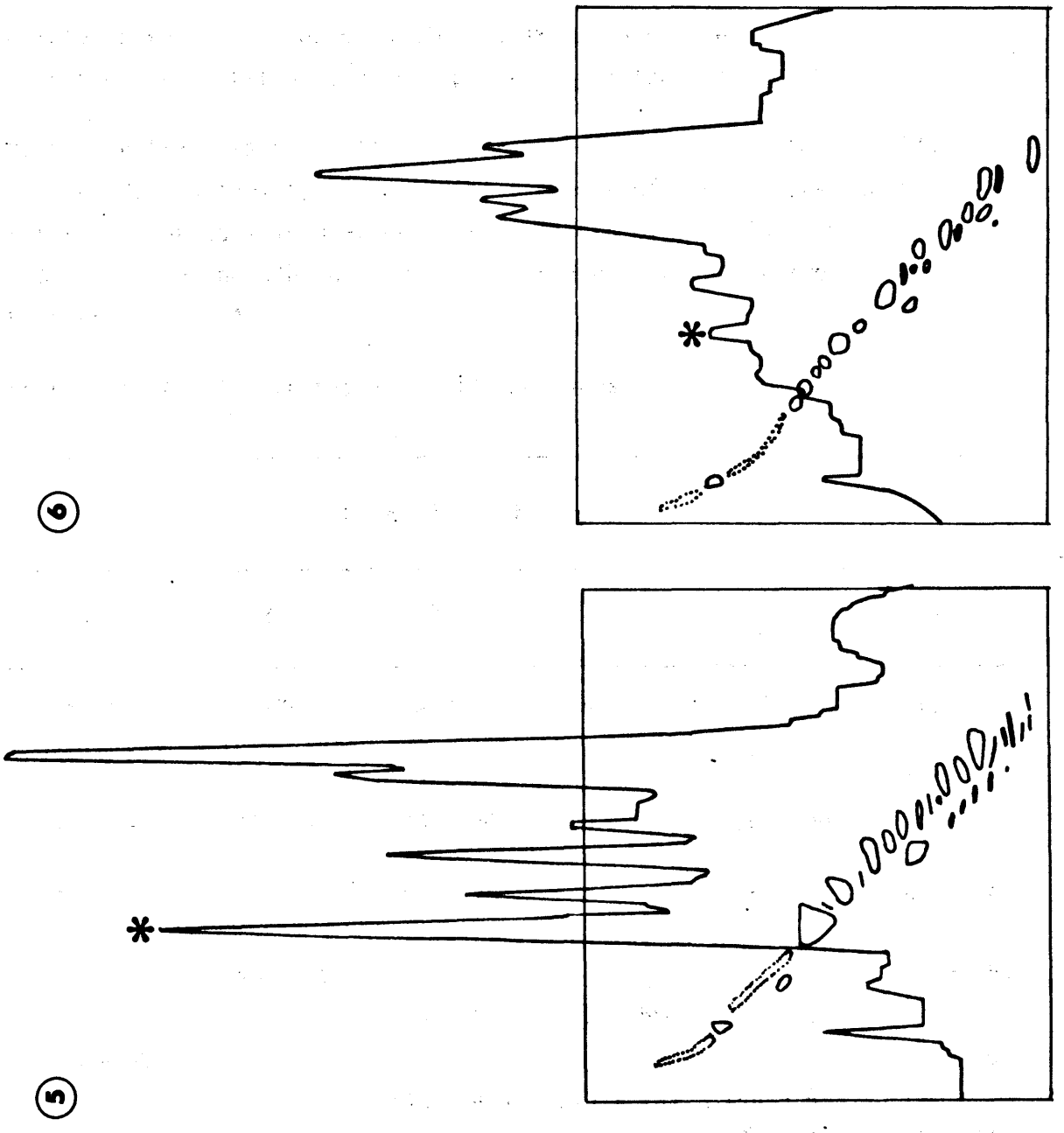


Fig. 3.27 (F)

TABLE 3.5

Molecular weight ranges of protein peaks seen on the densitometer traces of the first dimension separations of fraction 1 - 6.

Values in brackets refer to shoulders as distinct from peaks. >120 is used as a symbol to indicate the presence of one or more bands corresponding to molecular weights in excess of 120 000. In each separation the band/bands having the greatest intensity are labelled with an asterisk*.

Molecular weights are expressed in thousands.

F R A C T I O N					
1	2	3	4	5	6
>120	>120	>120	>120	>120	>120
100 - 120	100 - 120*	100 - 120*	100 - 120*	100 - 120	
					75 - 100
70 - 90*	70 - 90	70 - 90	70 - 90	70 - 90	
			65 - 70		
55 - 70		55 - 70		55 - 70	55 - 75
			55 - 65		
	40 - 70				(50 - 55)
		45 - 55	45 - 55	45 - 55	
40 - 55					40 - 50
		(40 - 45)	(40 - 45)	(40 - 45)	
(30 - 40)		(30 - 40)	30 - 40	30 - 40	35 - 40
	25 - 40				
25 - 30*		25 - 30	25 - 30	25 - 30*	25 - 35*
		(20 - 25)	(20 - 25)	(20 - 25)	20 - 25

band is the principal one.

In Table 3.6 the number of protein components detected in selected molecular weight ranges after the second-dimension separation are given. In the 100 000 - 120 000 range only one component was seen in fractions 1 - 5, and two in fraction 6. Differences in the number of components present in the six fractions are greatest in the 25 000 - 39 000 and 40 000 - 54 000 molecular weight ranges.

The dense bands in the 70 000 - 90 000 and 25 000 - 30 000 molecular weight ranges seen in fraction 1, are considerably weaker in fraction 2. In both fractions 2 and 3, the 100 000 - 120 000 molecular weight band constitutes the bulk of the protein. The minor bands in both of these fractions are resolved into a number of components in the second dimension. In the case of bands in the intermediate molecular weight range, spots of 45000 - 50 000 and 60 000 - 65 000 molecular weight contribute most to the intensity of the bands.

These results show that there are differences in the protein composition of membranes in fractions 1 - 6 as would be expected from the findings already given for the enzyme and cholesterol content of these fractions.

The non-sedimentable protein material in the fractions was briefly examined. The protein patterns obtained after SDS-polyacrylamide gel electrophoresis in 5·2% gel rods are shown diagrammatically in Fig. 3.28.

The separation for fraction 1 shows a considerable number of proteins covering the molecular weight range 10 000 - 200 000. Since approximately

TABLE 3.6

The number of protein components in selected molecular weight ranges seen after the two-dimensional electrophoretic separation of membrane proteins in fractions 1 - 6

Molecular weight range ($\times 10^{-3}$)	FRACTION					
	1	2	3	4	5	6
>120	7	3	7	12	4+	6+
100 - 120	1	1	1	1	1	2
70 - 99	3	2	3	3	3	3
55 - 69	4	3	2	2	3	3
40 - 54	3	3	8	8	6	4
25 - 39	8	6	10	9	10	4
20 - 25	-	-	2	2	1	1
TOTAL:	26	18	33	37	28+	23+

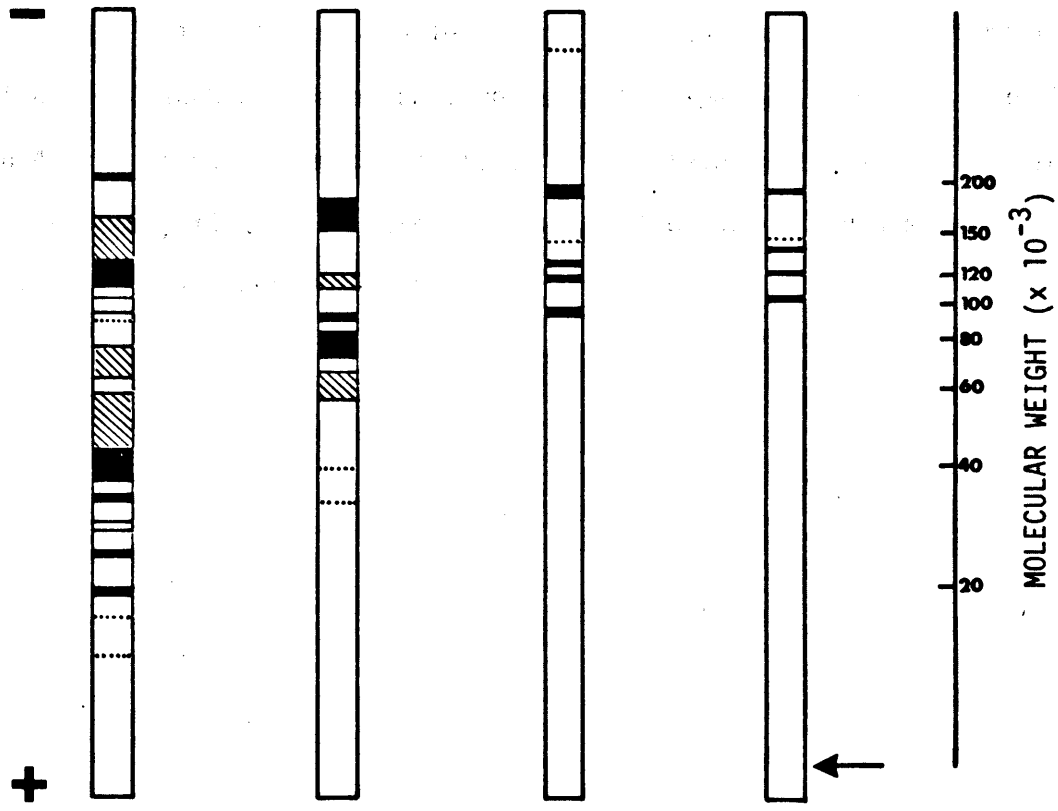


Fig. 3.28. Diagrammatic representation of the protein patterns obtained after the electrophoretic analysis of non-sedimentable protein in fractions 1-4.

Protein remaining in the supernatant after centrifugation of each fraction at 100 000 g_{av} for 90 min was concentrated by freeze-drying and solubilised as described in Section 2. Electrophoresis was carried out in 5·2% polyacrylamide gel rods at 5 mA/gel for 4 hours. Migration was towards the anode.

→ indicates the distance travelled by the tracker dye.

The following symbols were used to indicate the intensity of staining:

■ strong;
▨ medium;
···· weak;

80% of the total protein in fraction 1 is non-sedimentable and originates from the diffusion of the soluble protein from the zonal supernatant into the fraction, such a complex pattern is to be expected. Between fractions 1 and 4 the intensity and diversity of the protein bands decrease. In fraction 4 only five bands were observed, all of which had a molecular weight between 100 000 - 200 000.

3.4. The preparation and characterisation of calcium oxalate loaded vesicles.

The characterisation of the concentrated microsomal fractions has shown that, while cholesterol is present in all of the fractions, its distribution is not uniform. A bimodal distribution was found with peaks in fractions 1 and 4. A consideration of the results led to the conclusion that cholesterol might not be a constituent of Ca^{2+}-accumulating vesicles. In addition, cholesterol and basal ATPase had similar distribution patterns.

In order to characterise the regions of the sarcoplasmic reticulum transporting Ca^{2+} and to measure their cholesterol content, Ca^{2+}-accumulating vesicles were isolated by a density perturbation procedure.

Ca^{2+}-transport by muscle microsomes is increased several fold in the presence of Ca^{2+}-precipitating anions such as oxalate, pyrophosphate or fluoride. The mechanism of this stimulation is known. In the case of the most commonly used anion - oxalate, which freely penetrates the microsomal membrane, the stimulation is due to the precipitation of transported Ca^{2+}, as calcium oxalate, within the sealed vesicles. Since calcium oxalate has a density of 2·2, its deposition within vesicles will increase their density. By exploiting this increase in density, calcium oxalate loaded vesicles can be separated by density gradient centrifugation. This forms the basis of the density perturbation procedure used.

For the initial experiments in this series, 20 ml of the peak Ca^{2+}-uptake fraction, i.e. fraction 3, were diluted with 10 ml of 5 mM

imidazole-HCl, pH 7·4 and centrifuged at 100 000 g_{av} for 60 min. The pellets obtained were resuspended in 4 ml of 0·25 M sucrose, 5 mM imidazole-HCl, pH 7·4 and added to 50 ml of calcium oxalate loading medium consisting of:

5 mM ATP-imidazole, pH 7·4;
5 mM $MgCl_2$;
100 mM KCl;
3 mM Ca^{2+}-EGTA-imidazole, pH 7·0;
5 mM oxalate-imidazole, pH 7·0; and
5 mM imidazole-HCl pH 7·0.

The mixture was incubated at 25^{o} for 30 min. During the incubation there was a considerable increase in the turbidity of the medium. After incubation the medium was centrifuged at 100 000 g_{av} for 60 min and the resulting pellets were very carefully resuspended in 8 ml of 0·25 M sucrose, pH 7·4 using a tightly fitting, hand-operated Potter-Elvejhem homogeniser.

2 ml of the suspension were layered on a 15 ml exponential sucrose density gradient in a 23 ml centrifuge tube (MSE 59209). The gradient consisted of sucrose buffered at pH 7·4 and ranged in density (4^{o}) from 1·089 to 1·226. 3 ml of 2·0 M sucrose (density 4^{o} = 1·255) was used as a cushion. The tube contents were centrifuged at 30 000 rev/min for 3 hours in an MSE 3 x 25 ml swing-out rotor (MSE No. 59590).

The tube contents, as seen after centrifugation, are shown diagrammatically in Fig. 3.29. The sample which had been incubated with the calcium oxalate loading medium separated into a single, broad band in the gradient and a pellet at the bottom of the tube. In the control separation, using material from fraction 3, which had not been incubated

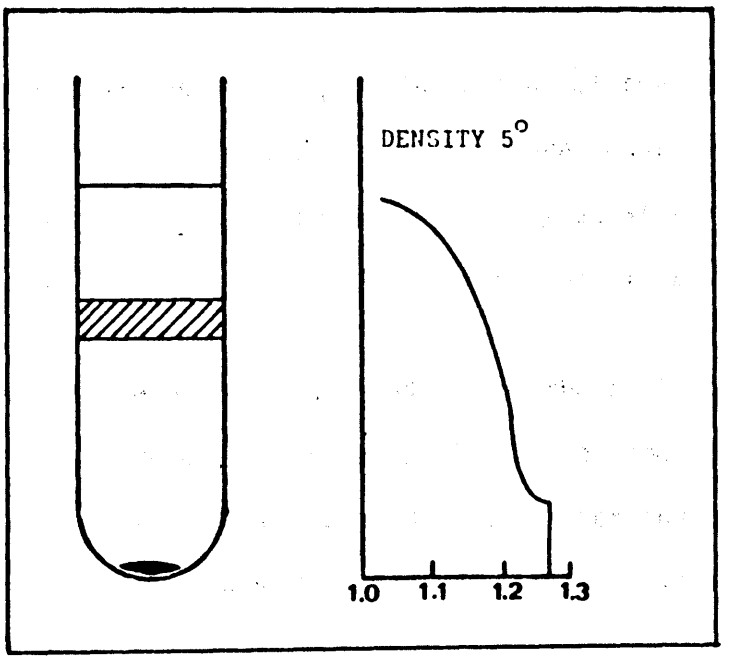


Fig. 3.29. The preparation of calcium oxalate loaded vesicles by density gradient centrifugation.

Diagrammatic representation of the density gradient after centrifugation at 30 000 rev/min for 3 hours. A single, diffuse protein band was observed in the gradient and a pellet containing calcium oxalate loaded vesicles was found at the bottom of the tube.

in the calcium oxalate loading medium, all of the applied sample banded in the gradient.

Determination of the protein content of the gradient and pellet was not possible because of the interference of calcium oxalate in the assay for protein by the method of Lowry et al (1951) and the biuret method of Gornall et al. (1949). Phospholipid was measured instead.

30% of the phospholipid and 15% of the cholesterol applied to the gradient was recovered in the pellet. The remainder banded in the gradient. This was interpreted as a further indication that cholesterol might not be a component of Ca^{2+}-accumulating vesicles.

Further experiments yielded results which were unsatisfactory for two reasons: (i) the amount of phospholipid and cholesterol in the pellets varied with the quantity of sample applied to the gradient; (ii) pellets obtained after the application of different sample loads were contaminated to varying degrees with non-loaded vesicles. As loaded and non-loaded material differed in colour, the presence of contaminating non-loaded material in the pellet could be seen with the naked eye. Using electron microscopy several groups of workers (e.g. Meissner & Fleischer, 1971; Boland et al., 1974; Graeser et al., 1969a) have found that calcium oxalate loaded pellets prepared by density gradient centrifugation were extensively contaminated with non-loaded vesicles.

Photographs of the pellets obtained over a three fold range in sample concentration are shown in Fig. 3.30 A - C. With increasing sample load, the contaminating material can be seen with the unaided eye.



Fig. 3.30. Photographs of calcium oxalate loaded pellets (A, B and C) prepared by density gradient centrifugation over a three fold range in sample concentration. The sample concentrations were such that the ratios of A : B : C were 1 : 2 : 3. The presence of what appears to be nonloaded material in B and C is indicated by an arrow.

Instability of the sample zone (Meuwissen, 1973) or the entrapment of non-loaded vesicles by the rapidly sedimenting calcium oxalate loaded vesicles during centrifugation, are possible explanations of these findings.

To determine the extent of the problem, the amount of sample material applied to the gradient was varied over a six fold range in concentration and the distribution of phospholipid and cholesterol between the gradient and pellet was measured.

16 ml from each of fractions 2 and 3 from two zonal preparations (i.e. a total of 64 ml) were diluted with 32 ml of 5 mM imidazole-HCl, pH 7·4 and centrifuged at 100 000 g_{av} for 60 min. The pellets obtained were resuspended in 10 ml of 0·25 M sucrose buffered at pH 7·4, and added to 100 ml of calcium oxalate loading medium. The mixture was incubated at 25^{o} for 30 min and then centrifuged at 100 000 g_{av} for 60 min. The pellets were carefully resuspended in 25 ml of 0·25 M sucrose, pH 7·4 and allowed to stand overnight at 4^{o}. Samples covering a six-fold range in concentration were prepared. Samples 1 to 5 were prepared by diluting 0·5, 1·0, 1·5, 2·0 and 2·5 ml of suspension respectively, to 3·0 ml with 0·25 M sucrose, pH 7·4. The sixth sample consisted of undiluted suspension. Samples were layered on 15 ml exponential sucrose density gradients, which ranged in density (4^{o}) from 1·089 to 1·226. After centrifugation at 30 000 rev/min for 3 hours, the gradient material was collected by decanting the tube contents and each pellet was resuspended in 3·0 ml of 0·25 M sucrose, pH 7·4.

The percentage of cholesterol and phospholipid sedimenting through

the gradient and cushion for each of the six sample concentrations is given in Fig. 3.31. and Table 3.7. The results are also plotted in histogram form in Fig. 3.32.

For the purpose of the discussion, the sample concentrations 1 - 6 will be referred to as load factors 1 - 6 respectively.

If calcium oxalate loaded vesicles were the only particles sedimenting through the gradient to the pellet at the bottom of the tube, the phospholipid and cholesterol content of the pellet, expressed as a percentage of that in the sample, should not vary with sample concentration. The results obtained from these experiments show that the cholesterol and phospholipid contents of the calcium oxalate loaded pellet are load dependent.

At a load factor of 1,20·5% of the phospholipid and 7·5% of the cholesterol applied to the gradient were recovered in the pellet. With increasing sample concentration, the percentage of both constituents sedimenting to the pellet increased and reached 32·2% for phospholipid and 17·0% for cholesterol at a load factor of 6. The cholesterol to phospholipid ratio of the sample was 33.1 µg cholesterol per mg phospholipid. The ratio was considerably lower for the calcium oxalate loaded pellet, being 12·03 µg/mg at a load factor of 1 and increasing to 17·5 µg/mg at a load factor of 6. With decreasing load factor the material remaining in the gradient during centrifugation became enriched in cholesterol, with a corresponding decrease in the cholesterol content of the calcium oxalate loaded pellet.

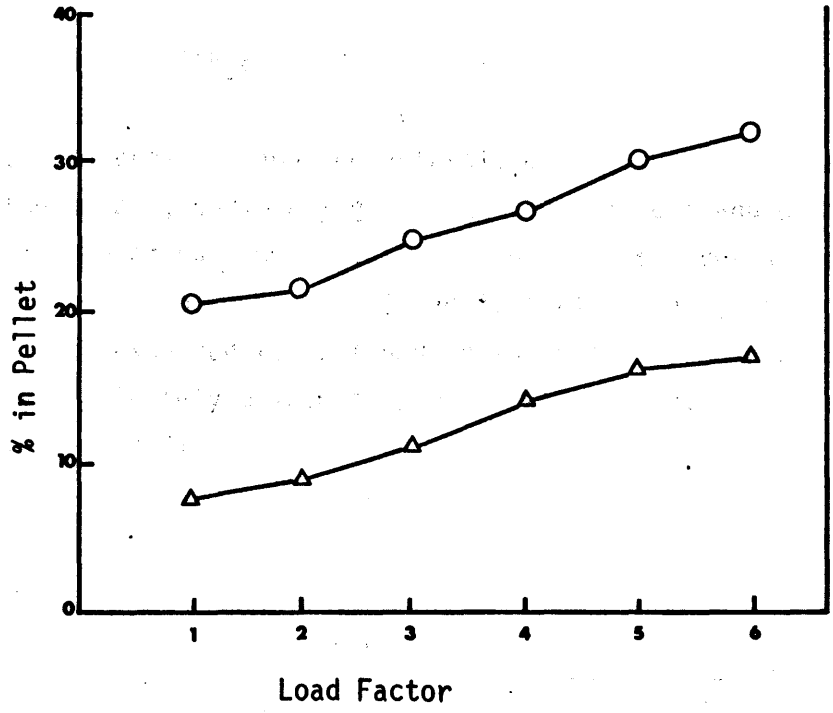


Fig. 3.31. The amount of phospholipid (—○—) and cholesterol (—△—), expressed as a percentage of that in the sample, sedimenting through the gradient and cushion in the preparation of calcium oxalate loaded vesicles over a six fold range in sample concentration (load factor).

TABLE 3.7

The amount of phospholipid and cholesterol, expressed as a percentage of that in the sample, sedimenting through the gradient and cushion in the preparation of calcium oxalate loaded vesicles over a six fold range in sample concentration (load factor). The amounts of both constituents expected to be found in the calcium oxalate loaded pellet from an infinitely dilute sample are also given. This data is plotted in Fig. 3.31.

	LOAD FACTOR						Infinitely dilute sample
	1	2	3	4	5	6	
% Phospholipid in Pellet	20·5	21·7	24·7	26·8	30·2	32·2	18
% Cholesterol in Pellet	7·5	9·0	11·2	14·1	16·0	17·0	6

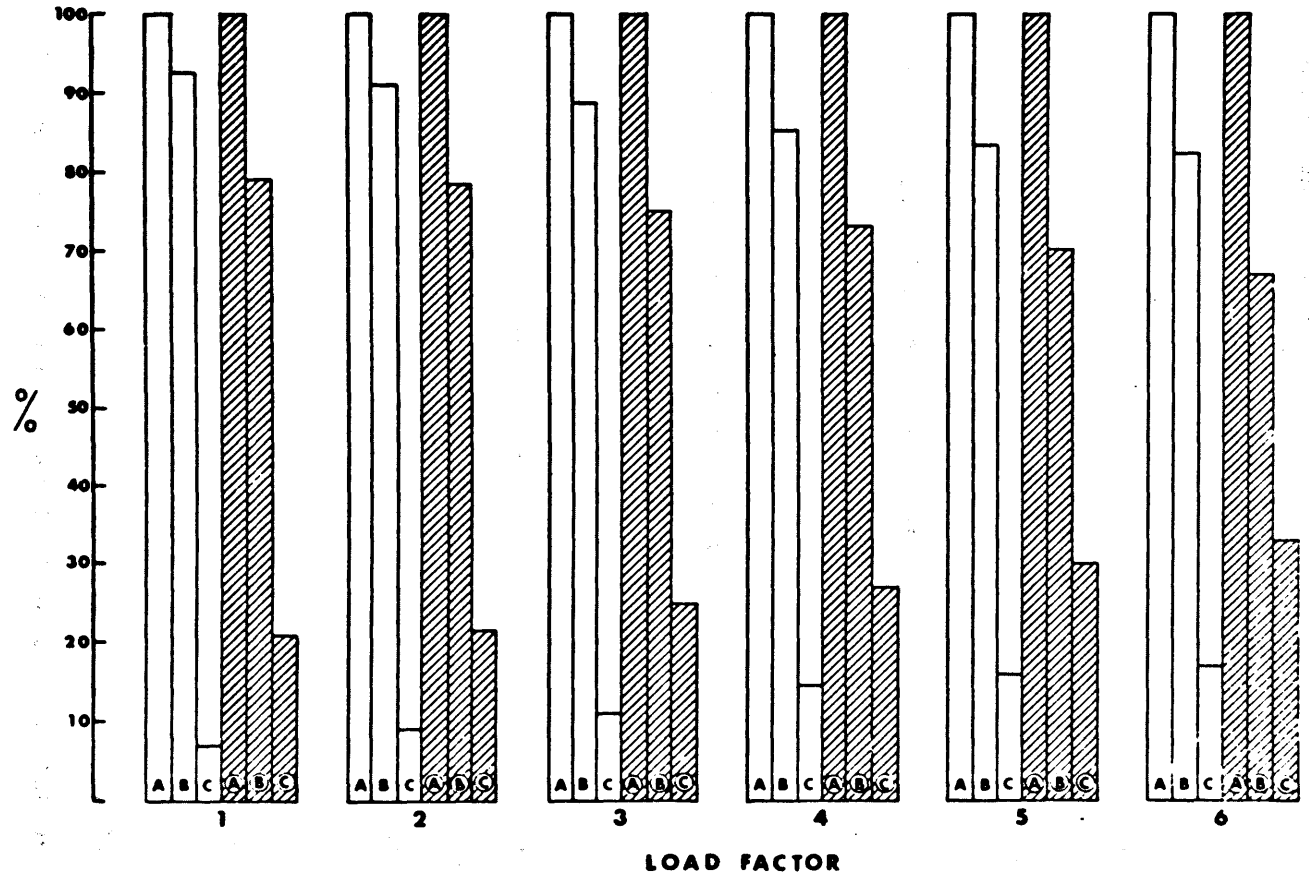


Fig. 3.32. The effect of increasing sample concentration on the distribution of cholesterol and phospholipid in the gradient and pellet during the preparation of calcium oxalate loaded vesicles by density gradient centrifugation. The distribution of both constituents was determined over a six fold range in load factor. Values are expressed as percentage of those in the sample.

Shaded areas represent phospholipid distribution and clear areas represent cholesterol distribution. A refers to the sample, B to the gradient, and C to the pellet.

Extrapolating from the amount of phospholipid loaded at each of the six load factors to an infinitely dilute sample, 18% of the phospholipid and 6% of the cholesterol would be recovered in the pellet. This means that only 18% of the phospholipid in the Ca^{2+}-uptake rich fractions of a concentrated microsomal suspension is associated with vesicles transporting and storing Ca^{2+}.

Two conclusions can be drawn from the calculated presence of cholesterol in the pellet obtained from an infinitely dilute sample:

(i) cholesterol is a component of the Ca^{2+}-transporting regions of the sarcoplasmic reticulum and is present in a lower concentration there than it is in the other membranes of the sample;

(ii) alternatively, the cholesterol of the pellet is found in membranes other than those of the sarcoplasmic reticulum but which also accumulate Ca^{2+}.

The Ca^{2+}-transporting regions of the sarcoplasmic reticulum are not the only membranes in the muscle cell known to contain a Ca^{2+}-transport ATPase. Like the plasmamembrane of other cells, the outer membrane or sarcolemma of the muscle cell also contains a Ca^{2+}-transport system. Sulakhe et al. (1973) have found that the specific activity of Ca^{2+}-uptake by a sarcolemma preparation was 8% of that of a microsomal pellet.

If intact vesicles derived from the sarcolemma were present in fractions 2 and 3 of the concentrated microsomal suspension, they could load sufficient calcium oxalate over the 30 min incubation period to increase their density in a manner similar to that for vesicles from the Ca^{2+}-transporting membranes of the sarcoplasmic reticulum. Plasmamembranes are known to contain a high level of cholesterol (Emmelot et al., 1964), therefore plasmamembrane vesicles loading calcium oxalate could account

for the cholesterol content of the calcium oxalate loaded pellet from an infinitely dilute sample. Taking the cholesterol content of plasmamembrane to be 650 μg cholesterol per mg phospholipid (Papahadjopoulos et al., 1973), then all of the cholesterol in the calcium oxalate loaded pellet would be accounted for if 1·70% of the total phospholipid in the pellet originated from sarcolemma. It is therefore possible, that the low level of cholesterol in the pellet from an infinitely dilute sample may be due to the presence of a small amount of loaded plasmamembrane vesicles.

Sulakhe et al (1973) have found that the Ca^{2+}-transporting systems of sarcoplasmic reticulum and sarcolemma are very similar. The similarity in properties makes it difficult to choose loading conditions which would distinguish between vesicles from both membrane systems loading calcium oxalate. The most readily exploitable difference between the two types of vesicle is their differing rate of Ca^{2+}-transport. Although it was not investigated, it is possible that sarcolemma vesicles may not load sufficient calcium oxalate during a short incubation period to cause a significant increase in particle density.

Regarding the possibility of mitochondrial fragments loading calcium oxalate, Headon (1972) has found that mitochondria do not accumulate Ca^{2+} under the conditions used to measure Ca^{2+} accumulation by sarcoplasmic reticulum vesicles.

Following the preparation of calcium oxalate loaded vesicles, an effort was made to obtain information on vesicles not loading calcium oxalate. An evaluation of the distribution of K^{+}-stimulated ATPase, Ca^{2+}-dependent ATPase and basal ATPase activities between the gradient and pellet was made difficult by the lack of information on the effect of calcium oxalate deposits on the activity of the Ca^{2+}-transport system.

While the true activities of these enzymes could not be determined for the calcium oxalate loaded pellet, this was not the case for membrane material banding in the gradient, i.e. vesicles not loading calcium oxalate. The latter were found to have Ca^{2+}-dependent ATPase, K^{+}-stimulated ATPase, and basal ATPase activities. These results show that at least some of the vesicles, which do not load calcium oxalate, contain the Ca^{2+}-transport enzyme.

The separation of calcium oxalate loaded vesicles from non-loaded vesicles is a partial subfractionation of the parent fractions (i.e. fractions 2 and 3 of the concentrated microsomal suspension). Membranes, which contain 82% of the phospholipidof the sample, do not contain calcium oxalate deposits. These could consist of:

(i) vesicles unable to retain transported calcium because of leakiness;

(ii) vesicles unable to accumulate calcium because of 'inside-out' folding;

(iii) vesicles containing an inactive calcium transport system;

(iv) membranes not containing a Ca^{2+}-transporting enzyme and originating from other regions of the internal membrane systems, or alternatively, membranes derived from the external membrane systems.

Since 98-100% of vesicles loaded with calcium oxalate arise from the fragmentation of Ca^{2+}-transporting regions of the sarcoplasmic reticulum, a study of aspects of their composition should yield information on the composition of this specialised region of the intracellular membrane systems.

Protein composition: The protein composition of calcium oxalate loaded vesicles and vesicles not accumulating Ca^{2+} was investigated using two-dimensional SDS-polyacrylamide gel electrophoresis.

A sufficient quantity of calcium oxalate loaded material was obtained by pooling the pellets collected from twelve density gradient centrifugations at a load factor of 0·5. The combined pellets were resuspended in 2 ml of solubilisation medium containing 5% W/V SDS, 40 mM mercaptoethanol and 0·02% W/V sodium azide in Tris-boric acid-EDTA buffer, pH 8·3. The mixture was heated at 100^{o} for 5 min. The high Ca^{2+} content of loaded vesicles presented difficulties in solubilisation by SDS treatment. The difficulties arise from the precipitation of dodecyl sulphate in the presence of Ca^{2+} ions. By using sufficiently high concentrations of SDS, i.e. 5% W/V, solubilisation could be achieved. Graeser et al. (1969a)have reported unsuccessful attempts to solubilise loaded vesicles with cholate and deoxycholate. These workers ascribed the failure to achieve solubilisation either to the increased resistance of calcium oxalate - containing vesicles to solubilisation or to technical problems.

After solubilisation the mixture was dialysed for 12 hours against 5 litres of 0.1% W/V SDS, 40 mM mercaptoethanol and 0·02% W/V sodium azide in Tris-boric acid-EDTA buffer, pH 8·3. Dialysis was used to reduce the free SDS concentration to 0·1% W/V. Calcium dodecyl sulphate precipitates and calcium oxalate crystals were removed from the dialysed mixture by centrifugation at top speed in an MSE Minor centrifuge.

Sucrose, to a final concentration of 10% W/V, and the tracker dye, pyronin-Y, were added. Electrophoresis was carried out as described for the concentrated microsomal fractions in Section 3.3.

The sample used in the density gradient separation of loaded vesicles

from non-loaded vesicles was solubilised and prepared for electrophoresis simultaneously with the pellet, using the same procedure.

Photographs of the second-dimension separations obtained for the sample applied to the density gradient and the pellet are shown in Fig. 3.33 and drawings of the separations are given in Fig. 3.34.

Proteins in the molecular weight range 50 000 - 70 000 are present in lower concentration in the sample applied to the gradient than in the concentrated microsomal fraction from which it was prepared. Duggan & Martonosi (1970) have found that proteins in this molecular weight range were released from the membrane on treatment with 1 mM EDTA or EGTA at alkaline pH. During calcium oxalate loading, the sample was exposed to 3 mM EGTA at 25^{o} for 30 min and this may account for the reduction in the content of intermediate molecular weight proteins.

In both the sample and pellet the 100 000 component, which is marked with an asterisk on the drawings in Fig. 3.34, is the principal protein. There is considerable trailing of protein in the pattern obtained for the pellet - this is probably due to interference by Ca^{2+} in the solubilisation process. While there are no outstanding differences in the patterns obtained for the sample and pellet, protein components with a molecular weight less than 100 000 are weaker in the pellet, relative to the 100 000 component, than they are in the sample.

Microsomes banding in the gradient during the isolation of loaded vesicles were also prepared for electrophoretic analysis.

Gradients from twelve density gradient centrifugations at a load factor of 0·5 were pooled. Microsomal material in the combined gradients

Fig. 3.33. (overleaf) The protein patterns obtained after the two-dimensional electrophoretic separation of membrane proteins in the sample (part A) and calcium oxalate loaded pellet (part B) following solubilisation with SDS.

After the first-dimension separation in 5·2% polyacrylamide gels, the second-dimension separation was carried out in 6-27% polyacrylamide gel gradients. Migration was towards the anode in both dimensions. In the illustrations this corresponds to a migration from left to right in the first-dimension and from top to bottom in the second-dimension.



Fig. 3.33 (A)

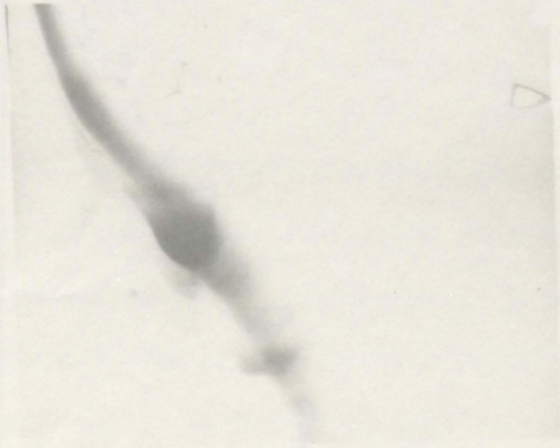
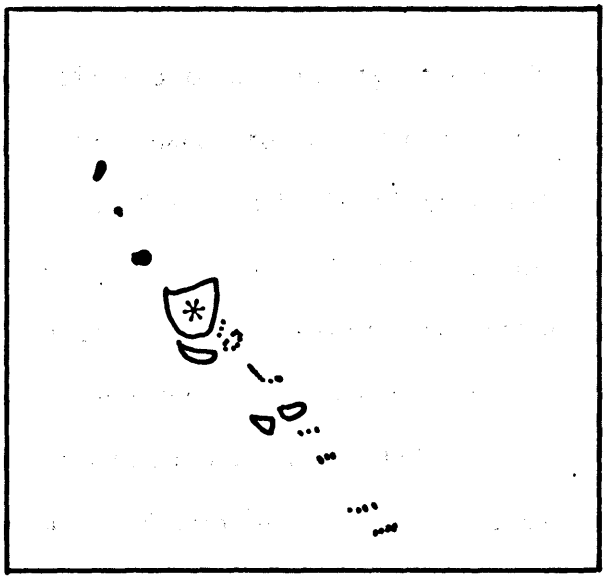
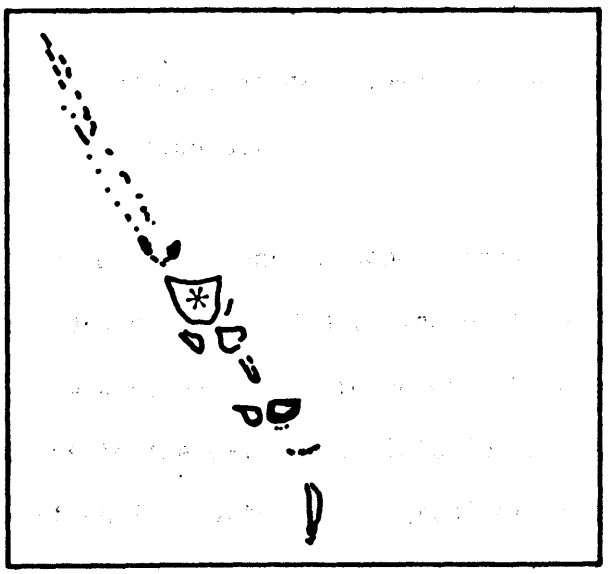


Fig. 3.33 (B)



SAMPLE



CALCIUM OXALATE
LOADED PELLET

Fig. 3.34. Diagrammatic representation of the two-dimensional electrophoretic separations shown in Fig. 3.33.

The 100 000 molecular weight component is indicated with an asterisk.

For further details of the separations, see the legend to Fig. 3.33 and the text.

was concentrated by zonal centrifugation in a manner similar to that for the preparation of the concentrated microsomal fractions. The pooled gradients were diluted to a molarity of 0·67 M with 5 mM imidazole-HCl, pH 7·4 and 500 ml was loaded to the edge of a BXIV rotor rotating at 3000 rev/min. This was followed by 50 ml of 1·0 M sucrose, pH 7·4, and sufficient 2·0 M sucrose pH 7·4 to fill the rotor. After centrifugation for 3 hours at 30 000 rev/min the rotor speed was reduced to 3000 rev/min and the contents were displaced through the centre line. The first 470 ml were discarded. The microsomal material was collected in two 40 ml fractions corresponding to the volume ranges 470 - 509 ml and 510 - 550 ml (See Fig. 3.35). These fractions were termed gradient fraction 1 and gradient fraction 2 and correspond to the density ranges 1·11 - 1·16 g/ml and 1·16 - 1·21 g/ml respectively. Both fractions were diluted with an equal volume of 5 mM imidazole-HCl, pH 7·4 and centrifuged at 100 000 g_{av} for 60 min. The pellets obtained were treated with SDS as described in Section 2. Two-dimensional electrophoresis was carried out, as described for the concentrated microsomal fractions in Section 3.3.

Photographs of the second-dimension separations are shown in Fig. 3.36 and the gels are drawn in Fig. 3.37. The protein patterns obtained for both fractions show a number of differences. Gradient fraction 2 shows more similarities to the calcium oxalate loaded pellet than it does to gradient fraction 1. Furthermore, gradient fraction 1 contains a greater number of proteins and the pattern resembles that for sedimentable material in fraction 1 of the concentrated microsomal fractions. The 100 000 molecular weight component, which is marked with an asterisk

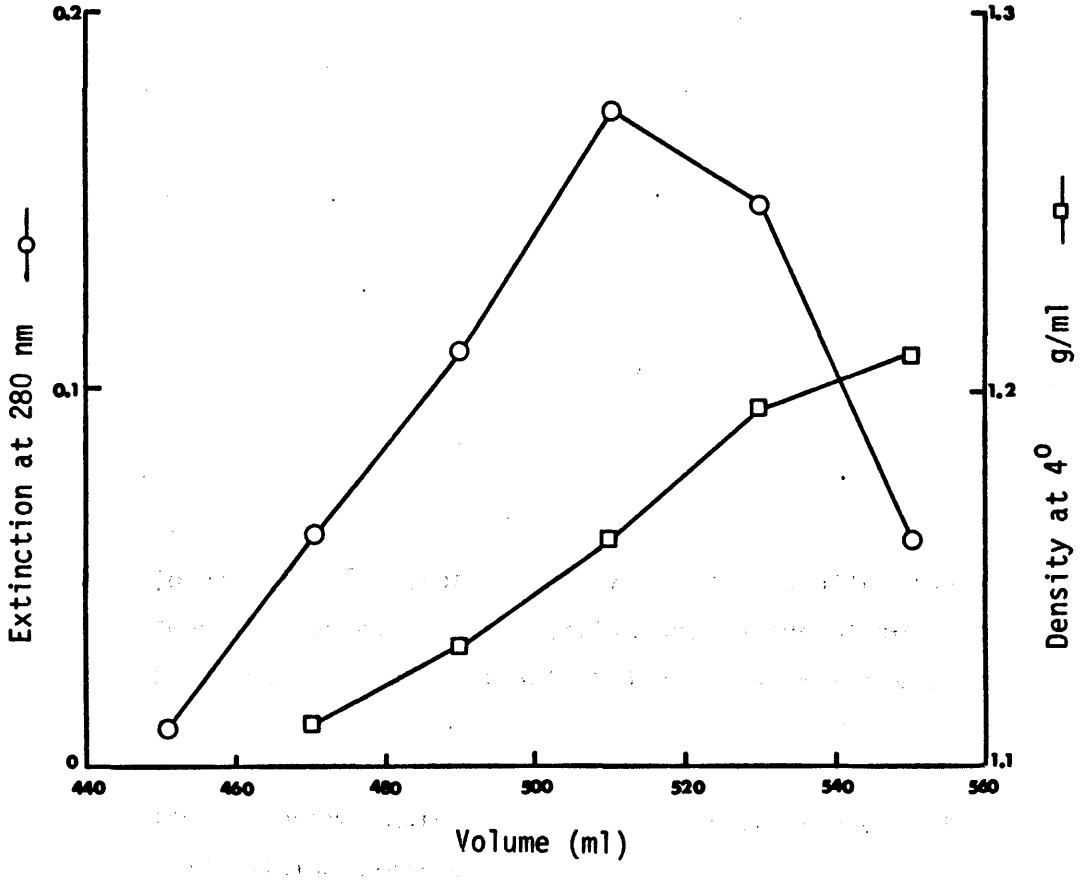


Fig. 3.35. The concentration of membrane material which bands in the gradient during the preparation of calcium oxalate loaded vesicles by density gradient centrifugation.

Microsomes present in 500 ml of diluted gradients were prepared in the form of a concentrated suspension by zonal centrifugation. Details of the preparation are given in the text.

In this figure the density gradient profile and extinction at 280 nm are plotted against rotor volume in the region of the concentrated microsomal suspension.

Fig. 3.36. (overleaf) The protein patterns obtained after the two-dimensional electrophoretic separation of membrane proteins in gradient fraction 1 and gradient fraction 2.

The microsomal material in these fractions does not load calcium oxalate.

The separation for gradient fraction 1 is shown in photograph A and that for gradient fraction 2 in photograph B.

The conditions of electrophoresis are given in the legend to Fig. 3.33.

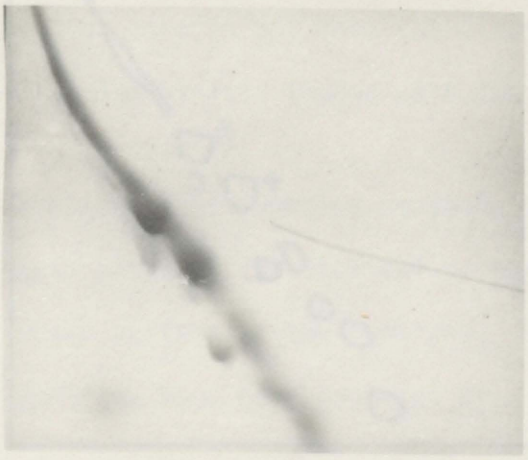
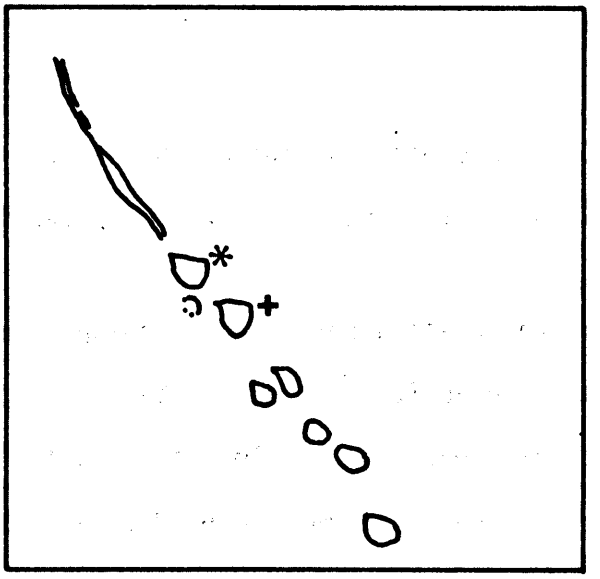


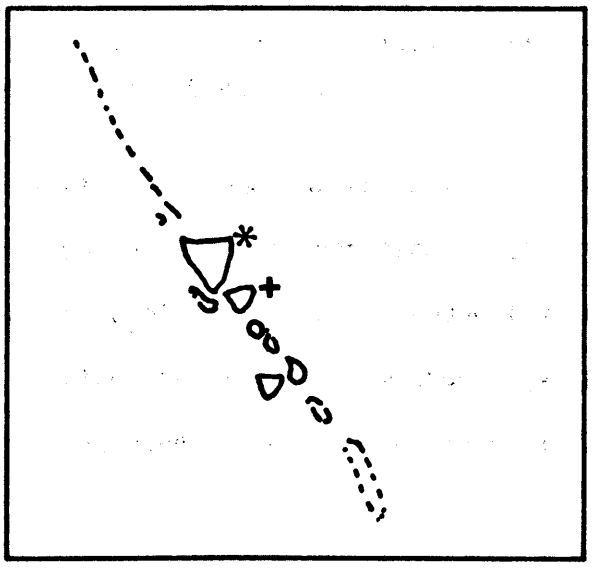
Fig. 3.36 (A)



Fig. 3.36 (B)



GRADIENT FRACTION 1



GRADIENT FRACTION 2

Fig. 3.37. Diagrammatic representation of the electrophoretic separations shown in Fig. 3.36.

The asterisk indicates the 100 000 molecular weight component, while the 70 000 - 90 000 molecular weight component is indicated with a cross.

on the drawing, is not the principal protein in gradient fraction 1 - instead a component which has a molecular weight of 70 000 - 90 000 and marked with a cross (+) is present in somewhat greater concentration.

Next an investigation carried out into the disposition of proteins in the membranes of calcium oxalate loaded vesicles will be described.

The vectorial arrangement of membrane proteins can be studied by a variety of techniques based on the chemical or enzymatic modification of proteins on the outer surface of the membrane. The external proteins of the erythrocyte membrane have been modified by:

(a) limited digestion with pronase, trypsin, chymotrypsin or papain (Steck et al., 1971);

(b) lactoperoxidase iodination of tyrosine and histidine residues (Phillips & Morrison, 1970);

(c) protein labels such as formylmethionyl $[^{35}S]$ sulphone methyl phosphate (Bretscher, 1971a) diazo $[^{35}S]$ sulphanilic acid (Berg, 1969); or 4,4^{1}-di-isothiocyano-2, 2^{1}-ditritiostilbene-disulphonate (Cabantchik & Rothstein, 1974).

It is essential that the enzymes or reagents used have size or solubility properties which prevent them from penetrating the membrane. The labelled or modified proteins can be subsequently identified by SDS-polyacrylamide gel electrophoresis. The methodology involved in this type of investigation has been reviewed by Juliano (1973) and Wallach & Winzler (1974).

As regards muscle microsomal preparations, calcium oxalate loaded vesicles are ideally suited to a study of the arrangement of membrane proteins. Loaded vesicles, which are prepared by virtue of their

ability to transport and retain Ca^{2+}, have defined permeability properties. In addition, these vesicles have a defined orientation in that they all transport Ca^{2+} inwards. The proteins on the outer surface of the vesicles are those which face the extra-reticular fluid in the intact sarcoplasmic reticulum.

The externally facing proteins of calcium oxalate loaded vesicles were labelled by a lactoperoxidase-catalysed iodination. The reaction was carried out in the presence of iodide ion, containing the radioactive isotope ^{131}I and involved the iodination of tyrosine and histidine residues according to the following equation (Phillips & Morrison, 1970):

$$\text{Protein} + H_2O_2 + {}^{131}I^- \xrightarrow{\text{Lactoperoxidase}} \text{Protein}\text{—}{}^{131}I^-$$

Loaded vesicles were prepared by density gradient centrifugation at a load factor of 0·5. Vesicles containing 2 mg phospholipid were iodinated by incubation at 37° in a medium having a final volume of 10 ml and containing 10 μM KI, 1 mCi $^{131}I^-$ and 1 μM lactoperoxidase (78 μg/ml) in 0·25 M sucrose, 50 mM imidazole-HCl, pH 7·4. The reaction was initiated by the addition of 10 μl of 8 mM H_2O_2. Further additions were made after 5 and 10 min. The stepwise addition of H_2O_2 in this manner, reduced membrane oxidation (Phillips & Morrison, 1970). After 15 min the reaction was terminated by layering the reaction mixture on 8 ml of 2·0 M sucrose in a 23 ml centrifuge tube (MSE No. 59209) and centrifuging at 30 000 rev/min for 2 hours in an MSE 3 x 25 ml swing-out rotor. The labelled vesicles sedimented through the 2·0 M sucrose and were further washed by resuspension and sedimentation in 0·25 M sucrose, pH 7·4. The labelled vesicles were found to contain 16% of the

radioactivity in the labelling medium, the remainder being soluble. In addition, they retained their calcium oxalate deposits as evidenced by their sedimentation through 2·0 M sucrose.

Vesicles labelled in this way were solubilised for electrophoretic analysis by the same procedure as that already given for the unlabelled vesicles.

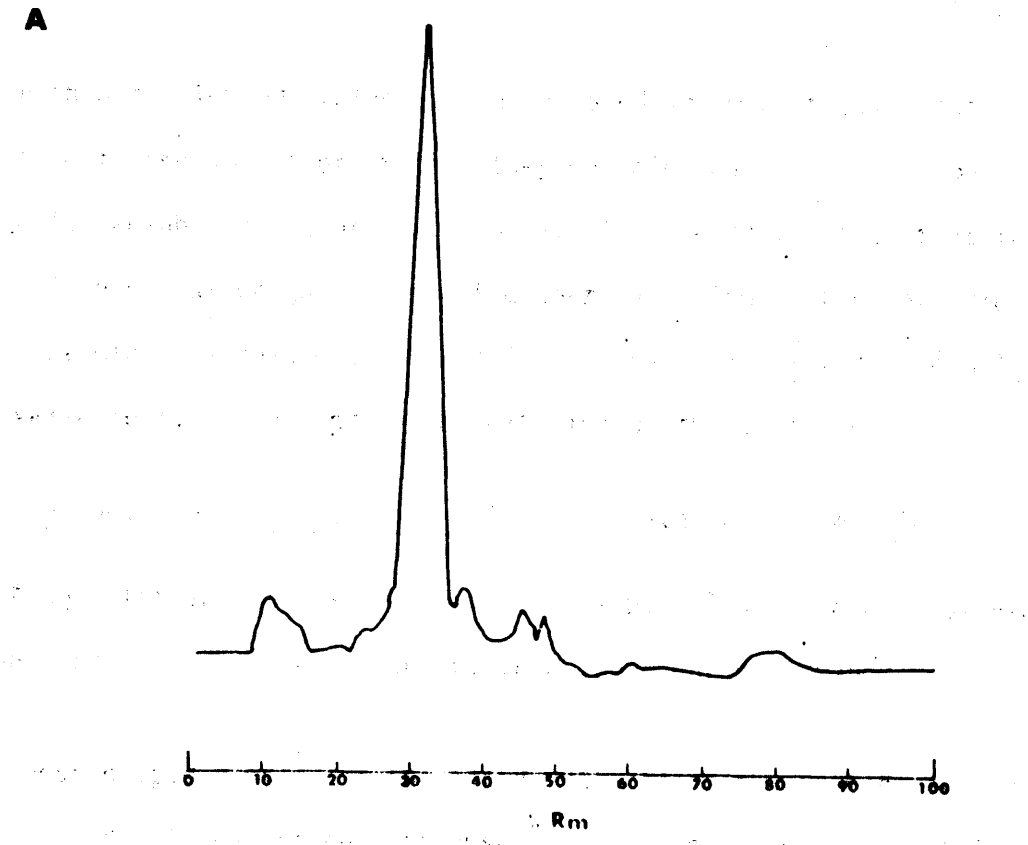
Electrophoresis, in one dimension only, was carried out at 75 V in 6 - 27% polyacrylamide gel gradients. Electrophoresis was stopped when the dye reached the end of the gel.

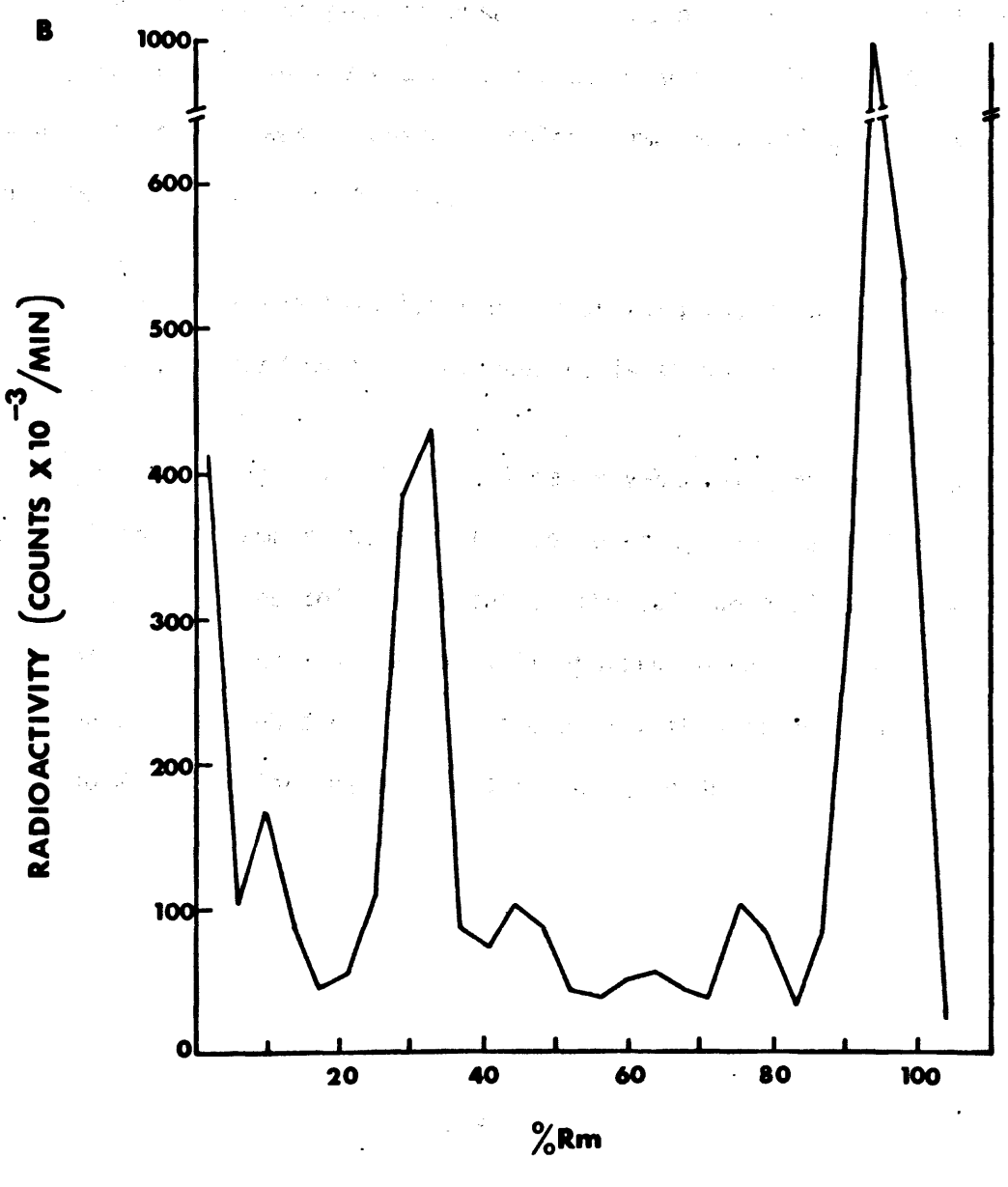
Immediately after electrophoresis part of the gel was removed for protein staining and the remaining portion was cut into 2 mm wide slices for the measurement of radioactivity. Each of the 26 slices, corresponding to the 5·2 cm migrated by the dye, was placed in a scintillation vial containing 10 ml of triton scintillant (composition given in Section 2) and the radioactivity measured.

In Fig. 3.38B the results are presented as a plot of radioactivity versus relative mobility. In part A of Fig. 3.38 the densitometer trace of the amido black-stained gel is shown. The 100 000 molecular weight protein band has a relative mobility of 30% and accounts for 75·4% of the protein in the gel (Table 3.8). The distribution of ^{131}I also has a strong peak corresponding to this mobility. A large peak of radioactivity remained on the top surface of the gel, probably due to incomplete solubilisation in the presence of calcium oxalate. Free ^{131}I moved with the tracker dye (relative mobility = 95%). Protein bands

Fig. 3.38. (overleaf) Polyacrylamide gel electrophoresis of membrane proteins from calcium oxalate loaded vesicles after iodination of proteins on the external surface of the vesicles.

A densitometer trace of the gel after staining for protein is shown in part A. In part B the distribution of ^{131}I is plotted against relative mobility.


A
0
10
20
30
40
50
60
70
80
90
100
Rm


B
1000
600
500
400
300
200
100
0
RADIOACTIVITY (COUNTS X 10 -3 /MIN)
20
40
60
80
100
%Rm

other than the 100 000 molecular weight band are more heavily labelled relative to the amount of protein they contain than is the 100 000 molecular weight component (Table 3.8). This would indicate that the 100 000 molecular weight component either has a low content of tyrosine and histidine residues or, alternatively, that only part of the molecule is available for labelling on the exterior of the vesicles.

The phospholipid composition of calcium oxalate loaded vesicles.

A qualitative analysis of the phospholipid classes in calcium oxalate loaded vesicles was carried out by thin layer chromatography.

Phospholipid classes in the lipid extracts of the sample applied to the gradient and the pellets obtained at each of the six load factors were separated on thin layer plates precoated with silica gel G. The solvent system used was composed of chloroform -methanol-glacial acetic acid-water (100 : 20 : 12·5 : 5).

A photograph of the thin layer plate showing the separated phospholipid classes, stained with iodine vapour, is shown in Fig. 3.39.

Phosphatidylcholine is the most intensely-staining phospholipid class in both the sample and pellets, followed by phosphatidylethanolamine. While phosphatidylinositol is present in the calcium oxalate loaded pellets at each of the load factors, it appears to constitute a lower percentage of total phospholipid in the pellet than it does in the sample and may therefore be enriched in the gradient.

TABLE 3.8

The distribution of protein stain and ^{131}I in selected regions of the polyacrylamide gels illustrated in Fig. 3.38.

Protein was measured from the peak areas on the densitometer trace.

The amount of each constituent in the various gel sections is expressed as a percentage of the total amount in the relative mobility range 3.9 to 84·6% (i.e. excluding free ^{131}I and material on the top surface of the gel).

Gel Section (% Relative mobility range)	(a) Protein % fractional concentration	(b) Protein Bound ^{131}I % fractional concentration	^{131}I/Protein Ratio (=b/a)
3·9 - 15·4	8·2	16·9	2·06
15.4 - 38·5	75·4	57·0	0·76
38·5 - 53·9	9·7	12·0	1·24
53·9 - 69·2	2·3	4·9	2·13
69·2 - 84·6	4·4	8·6	1·95

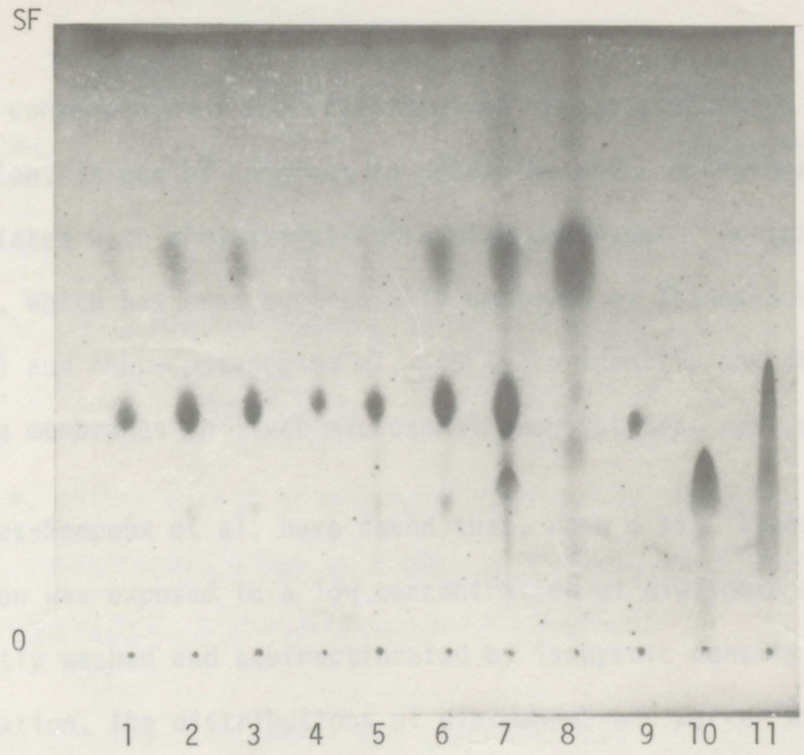


Fig. 3.39. The separation of phospholipid classes in the lipid extracts of calcium oxalate loaded vesicles prepared by density gradient centrifugation.

The separation was carried out on thin layer plates coated with silica gel G using the solvent system chloroform-methanol-glacial acetic acid-water (100 : 20 : 12·5 : 5).

The origin or site of application is indicated by O and the solvent front by SF.

Samples were applied from left to right as follows:

1 to 6 extracts from pellets obtained at load factors of from 1 to 6 respectively;

7 sample applied to the gradient;

8 phosphatidylethanolamine;

9 phosphatidylcholine;

10 phosphatidylinositol;

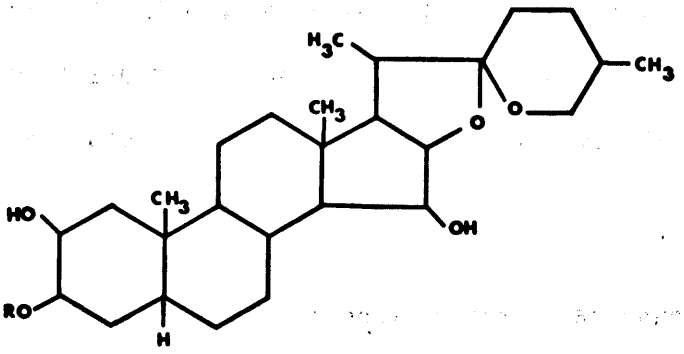
11 phosphatidylserine.

3.5. Cholesterol in rabbit skeletal muscle membrane preparations

As a consequence of the results given in the preceeding part of this section, it was of interest to determine which microsomal enzymes are associated with cholesterol-containing membranes. To this end, digitonin, which has been successfully employed by Thines-Sempoux et al. (1969) and Amar-Costesec et al. (1974b)to identify the cholesterol-containing membranes in liver microsomal preparations, was used.

Thines-Sempoux et al. have found that, when a rat liver microsomal preparation was exposed to a low concentration of digitonin and subsequently washed and subfractionated by isopycnic density gradient centrifugation, the distributions of plasmamembrane marker enzymes and cholesterol were selectively shifted to a region of higher density. The distribution of marker enzymes for endoplasmic reticulum was not similarly affected. The increase in equilibrium density of cholesterol-containing membranes in a sucrose density gradient, after treatment with digitonin, has been termed the digitonin shift by de Duve (1971). The increase in particle density has been attributed to the binding of digitonin to membrane cholesterol (Thines-Sempoux et al., 1969; Amar-Costesec et al., 1974b).

Digitonin is a complex glycoside occurring in digitalis plants (The Merck Index, 8th Edition, 1968). It forms equimolar insoluble complexes with steroids having a 3β-hydroxyl group.



R = 2 galactose + 2 glucose + 1 xylose

DIGITONIN

Studies of the effect of digitonin on the distribution of muscle microsomal constituents given here were carried out as a joint investigation with J. O'Flaherty and D. P. Bradley.

Muscle microsomal material was prepared according to the procedure summarised in Fig. 3.1. The microsomal (P3) pellet was resuspended in 0·25 M sucrose, pH 7·4. The suspension was divided into two equal portions. One portion was diluted with an equal volume of 0·25 M sucrose, pH 7·4 and processed as a control. An equal volume of freshly prepared, ice-cold 0·5% W/V digitonin in 0·25 M sucrose, pH 7·4 was added to the second portion, which was then incubated at 0^{o} for 15 min. The molar ratio of cholesterol to digitonin in the incubation medium was 0·3. After incubation both suspensions were centrifuged at 100 000 g_{av} for 60 min. The pellets obtained were resuspended in 0·25 M sucrose to a known protein concentration in the range 2-3 mg/ml.

2 ml samples of control and digitonin treated microsomes were layered on 15 ml exponential sucrose density gradients, ranging in density (4^{o}) from 1·04 to 1·21 g/ml, with a cushion of 3 ml of 2·0 M sucrose, pH 7·4.

Three 15 ml gradients were formed as described in Section 2, using 45 ml 2·0 M sucrose, pH 7·4 and 30 ml of 0·5 M sucrose, pH 7·4 as heavy and light solutions respectively. The gradient profiles are shown in Fig. 3.40.

Density gradient equilibration was carried out by centrifugation at 30 000 rev/min for 600 min. The temperature of centrifugation was 5^{o}. After centrifugation 20 1 ml fractions were collected manually.

Digitonin, at the concentrations used, was found to affect a number of the properties of the microsomal preparation. These effects are listed in Table 3.9. The detergent properties of digitonin (Helenius & Simons, 1975) are primarily responsible for these effects. The solubilisation of 5-8% of the membrane-associated protein is thought to result in leakiness of the vesicles with the loss of Ca^{2+}-storage. Leakiness of Ca^{2+}-accumulating vesicles, in turn, results in stimulation of the Ca^{2+}-dependent ATPase.

Protein and cholesterol concentrations and Ca^{2+}-dependent ATPase and basal ATPase activities were measured in each of the fractions collected after density gradient equilibration of control and digitonin-treated microsomes. The results are presented in Figs. 3.41 and 3.42. The presentation is in the form of frequency histograms, according to the method of de Duve (1967). Density is plotted along the abscissa and divided into 20 sections corresponding to the density increments over the collected fractions. The ordinate gives the unit frequency within the density increments. Frequency is defined as $(Q.100)/(\Sigma Q.\Delta\rho)$,

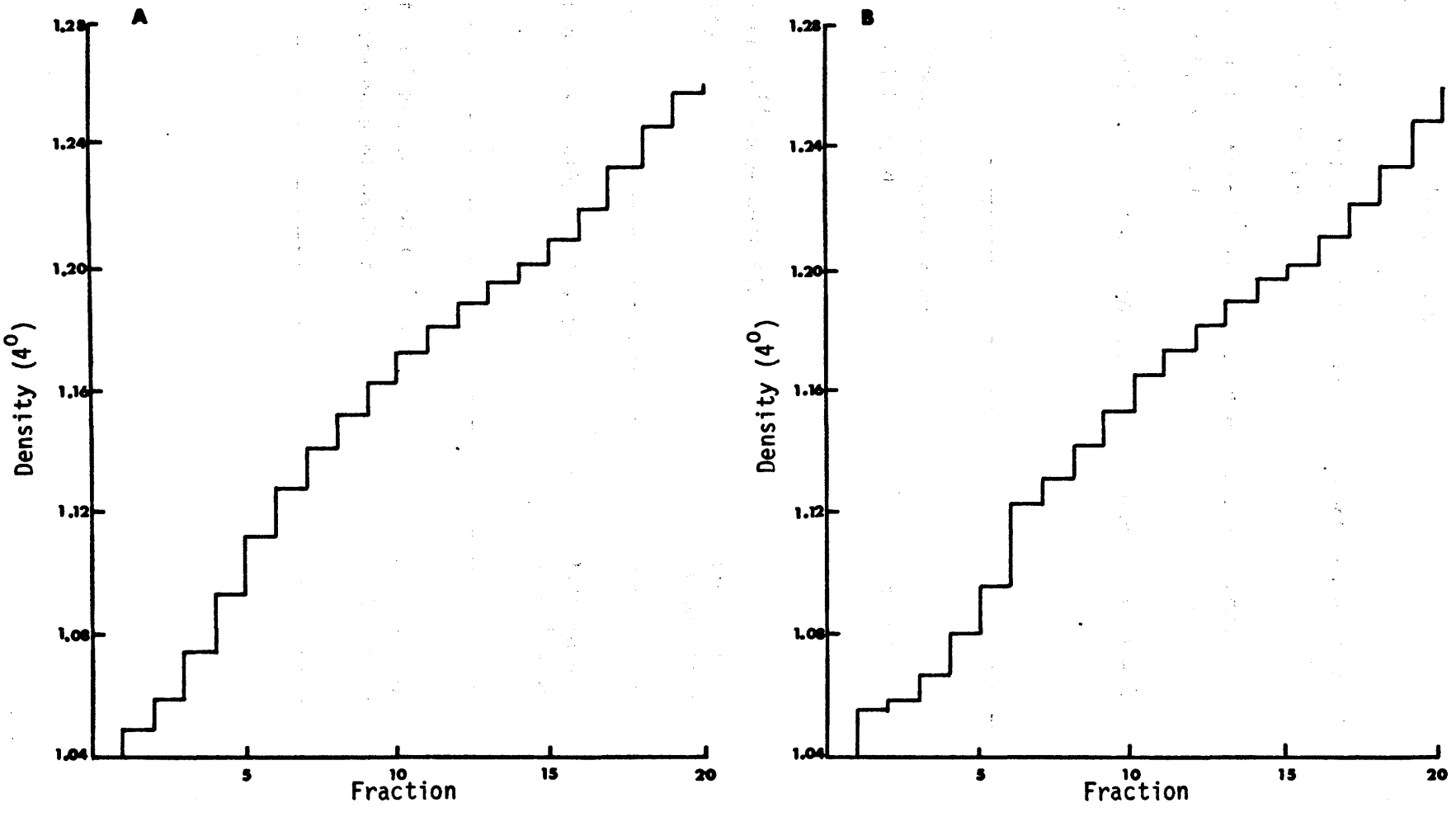


Fig. 3.40. Profiles of sucrose density gradients after the isopycnic equilibration of untreated (A) and digitonin-treated microsomes (B).

TABLE 3.9

The effects of digitonin treatment on the properties of a muscle microsomal preparation.

Muscle microsomes were prepared according to the procedure summarised in Fig. 3.1.

The molar ratio of digitonin to cholesterol, during treatment, was 3·3.

Amount of membrane-associated protein solubilised	5 - 8%
Effect on Ca^{2+}-dependent ATPase activity	1·66 fold stimulation
Effect on basal ATPase activity	1·19 fold stimulation
Effect on ATP-dependent Ca^{2+}-uptake in the presence of oxalate	Complete loss of Ca^{2+}-storage

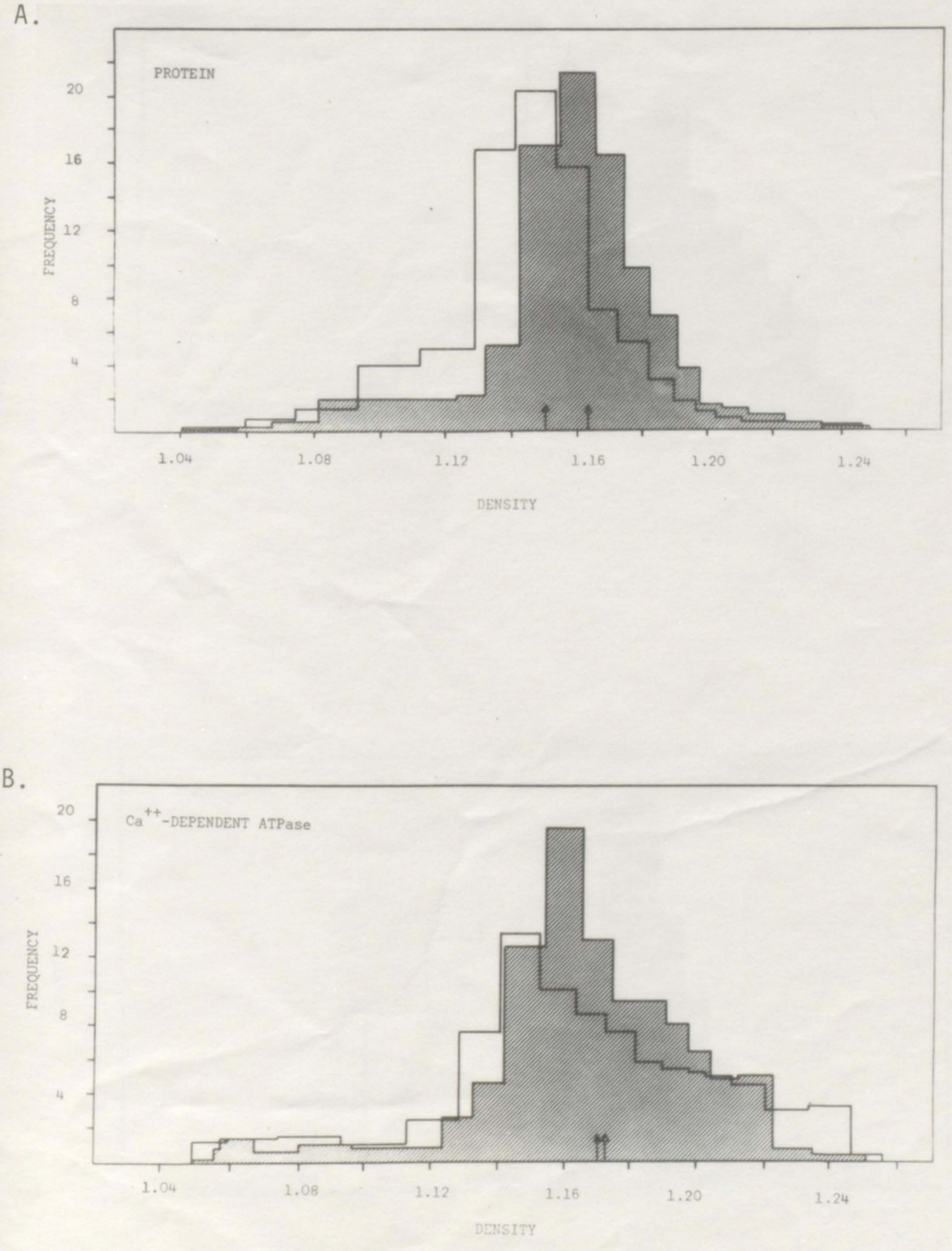


Fig. 3.41. The effect of treatment with digitonin on the equilibrium density distribution of protein (A) and Ca^{2+}-dependent ATPase (B). The shaded area represents the distribution after treatment with digitonin and the unshaded distribution is that of the control. The dark and white arrows indicate the median density values of the digitonin-treated and control distributions, respectively.

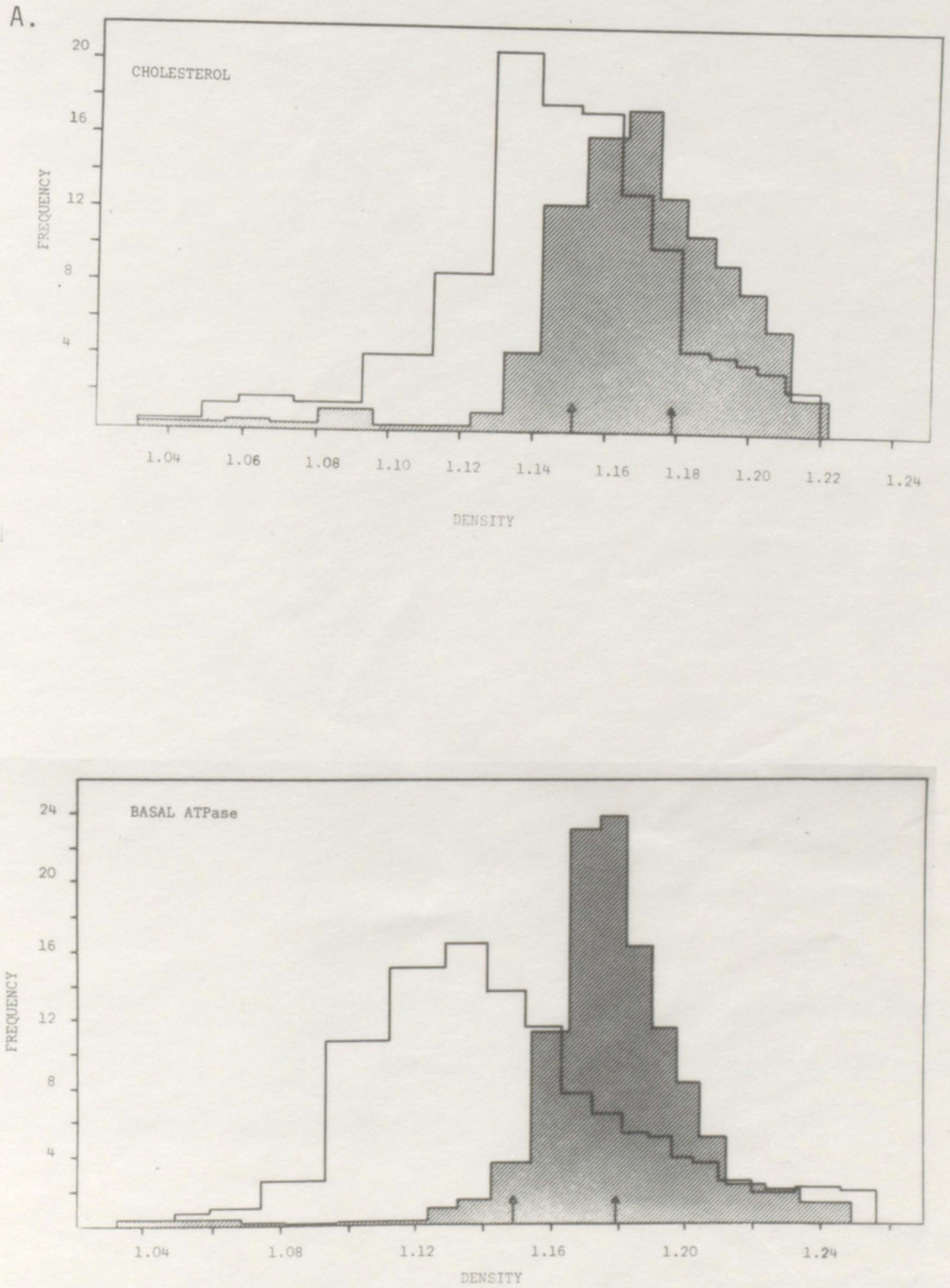


Fig. 3.42. The effect of digitonin treatment on the equilibrium density distribution of cholesterol (A) and basal ATPase (B). The shaded area represents the distribution after treatment with digitonin and the unshaded distribution is that of the control. The dark and white arrows indicate the median density values of the digitonin-treated and control microsomes respectively.

where Q is the concentration or activity found in the fraction corresponding to the density increment $\Delta\rho$, and ΣQ is the total concentration or activity in the gradient. In order to quantify differences in the equilibrium distribution of constituents and enzymes in control and digitonin-treated microsomes, the median density of each distribution was calculated and these are given in Table 3.10.

The results presented in Fig. 3.41 and 3.42 show that Ca^{2+}-dependent ATPase activity (median density = 1·172) and basal ATPase activity (median density = 1·149) in an untreated microsomal preparation can be clearly distinguished by isopycnic density gradient centrifugation.

Furthermore, the similarity in the median densities of basal ATPase (median density = 1·149) and cholesterol (median density = 1·151) in the control separation indicate a possible association of cholesterol with membranes containing basal ATPase.

Treatment with digitonin increases the median density of the cholesterol distribution by 0·028 density units. This is shown in Fig. 3.42 A.

The median density of protein shows a smaller increase after digitonin treatment, the increase being 0·013 density units, indicating that only a portion of the vesicles in the preparation contain cholesterol.

The distribution of basal ATPase in treated and untreated microsomes is shown in Fig. 3.42 B. The median density in the treated microsomes is 0·030 density units greater than that in the untreated microsomes.

TABLE 3.10

Median densities of constituents in control and digitonin-treated microsomes equilibrated in sucrose density gradients.

Constituent	MEDIAN DENSITY (5^{o})		Excess of digitonin-treated over control ($\times 10^{4}$)
	Control	Digitonin-treated	
Protein	1·150	1·163	130
Cholesterol	1·151	1·179	280
Ca^{2+}-dependent ATPase	1·172	1·170	-20
Basal ATPase	1.149	1·179	300

The increase in the median density is close to the value of 0·028 density units found for cholesterol.

Clearly, digitonin treatment increases the equilibrium density distribution of cholesterol and basal ATPase in an almost identical manner. This leads to the conclusion that cholesterol is associated with membranes containing basal ATPase.

Distribution of membranes containing the Ca^{2+}-dependent ATPase is not significantly affected on treatment with digitonin. The median density in this case shows a slight negative shift of 0·002 density units. From this it can be inferred that if membranes containing the Ca^{2+}-dependent ATPase contain cholesterol it is present in such low concentration that treatment with digitonin does not cause a detectable increase in particle density.

The results obtained in Section 3.1 showed that the membrane systems of muscle were differentially fragmented during homogenisation. The specific activity of basal ATPase was highest in the microsomal fraction from a 30 sec homogenate, while that for the Ca^{2+}-transport ATPase was highest in the microsomal fraction from a 60 sec homogenate. In addition, membranes not associated with either of these two ATPases nor with the (Na^+ and K^+) - ATPase were found in the microsomal fractions from 90 and 120 sec homogenates.

In view of the association found between basal ATPase and cholesterol-containing membranes a study of the appearance of cholesterol in the microsomal (or P3) fraction with varying homogenisation time seemed

appropriate. The results are presented in Fig. 3.43.

The highest specific activity of K^+-stimulated ATPase (=Ca^{2+}-transport ATPase) in the fraction coincides with the lowest cholesterol/phospholipid ratio for the four homogenisation times studied. This correlates well with the previous finding that sarcoplasmic reticulum membranes, which transport Ca^{2+}, contain little or no cholesterol.

The cholesterol/phospholipid ratio of the fraction closely parallels the specific activity of basal ATPase up to and including a homogenisation time of 60 sec. Since basal ATPase has been shown to be located in a cholesterol-containing membrane this close association is to be expected. However, between 90 and 120 sec the cholesterol/phospholipid ratio increases while the specific activity of basal ATPase continues to decrease. This finding indicates the presence of two types of cholesterol-containing membranes: one contains basal ATPase and sediments in the microsomal fraction after a short homogenisation time; the other is not associated with the enzyme activities assayed for and is only ruptured into fragments which sediment in the microsomal fraction after longer (90 and 120 sec) homogenisation.

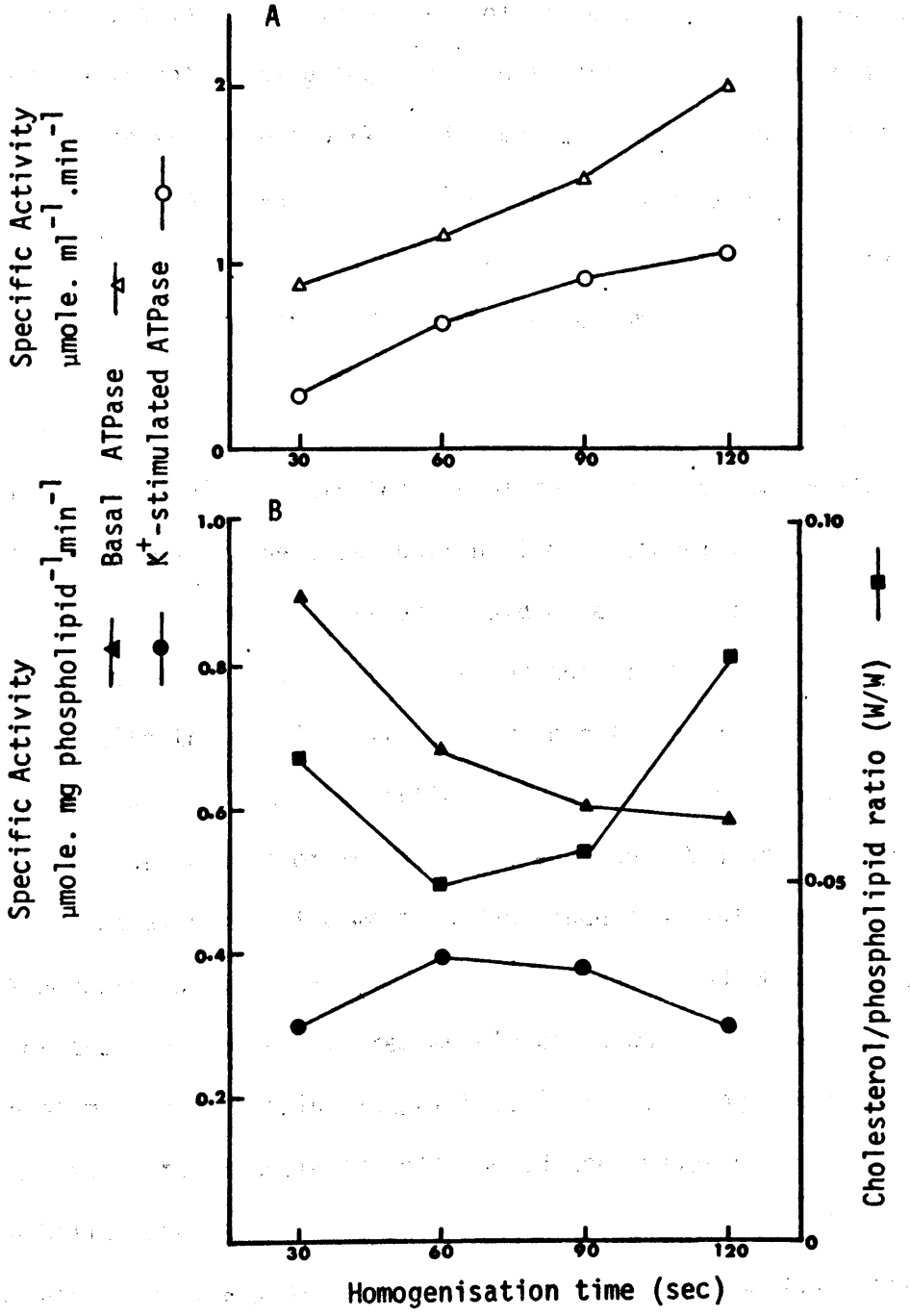


Fig. 3.43. The cholesterol/phospholipid ratio and the absolute and specific activities of K^+-stimulated ATPase and basal ATPase in fraction P3 prepared from 30, 60, 90 and 120 sec homogenates.

Since Emmelot & Bos (1962) concluded that plasmamembranes were characterised by a very high level of cholesterol, significant advances have been made in determining the subcellular localisation of this constituent. The subsequent work of de Duve's group has shown that the cholesterol of the liver cell is located exclusively in the plasmamembrane, Golgi apparatus, endocytotic vesicles and secondary lysosomes and absent from the endoplasmic reticulum.

In the muscle cell, two membrane systems are in contact with the extracellular fluid, namely the sarcolemma and T-system (Huxley, 1964). On the basis of the distribution of cholesterol in liver, one or both of these membrane systems would be expected to contain high levels of cholesterol. Whether or not this is the case could be determined with the availability of a method to specifically label both membrane systems. A particularly suited method would involve the tissue culture of muscle cells and the labelling of the surface proteins by one of the methods described and evaluated by Juliano & Behar-Bannelier (1975). An alternative method which could be used for the preparation of free muscle cells would involve the perfusion of muscle tissue, in situ, with cell dissociating agents such as collagenase. The dissociated cells would then have to be separated from endothelial and other cells prior to harvesting. Evans (1974) has used this type of approach to prepare hepatocytes from liver for the labelling of cell-surface proteins. In view of the technical skills required to carry out either of these types of experiments, a simpler but less satisfactory approach was briefly investigated. The method used involved the infusion of muscle tissue in situ with a

lactoperoxidase iodinating medium. After allowing sufficient time for labelling, the tissue was washed and fractionated. The distribution of iodinated protein was then determined.

Before describing the experiment, consideration will be given to the circulatory system in skeletal muscle and to the accessibility of the iodinating reagents to the sarcolemma and T-system of the muscle cells.

The blood supply to skeletal muscle cells is through a fine network of capillaries which are derived from branches of neighbouring arteries (Gould, 1973). In both skeletal and cardiac muscle, the capillaries are said to belong to the continuous type, in that they have a continuous endothelium and basement membrane. In soleus, a red muscle, the surface area of the muscle cell is seven times that of the blood vessels (calculated from the data of Eisenberg et al., 1974).

The organisation of blood capillaries in skeletal muscle has been described in considerable detail by Bruns & Palade (1968a). The capillary wall is composed of three concentric layers of cellular and extracellular elements. The inner layer faces the blood-front and is composed of a single continuous layer of flattened cells, known as endothelial cells. The tissue-front of the endothelial cells is covered with a 500Å thick basement membrane composed of fibrillar material embedded in an amorphous matrix. Finally, the outermost layer (i.e. the layer which faces the interstitial space) consists of varying proportions of connective tissue and ground substance, in

addition to macrophages and fibrils. Endothelial cells are bounded by a typical plasmamembrane and contain the usual complement of cell organelles. During infusion, the surface proteins on the blood front of the endothelial plasmamembrane would be extensively labelled. In order that the surface proteins of the muscle cells be labelled, the iodination reagents must penetrate the capillary wall and gain access to the interstitial space. Small molecules, such as H_2O_2 and $^{127}I^-$, which are required for iodination would freely traverse the wall, however, the lactoperoxidase molecule (molecular weight = 78 000) is too large to cross by simple diffusion.

The permeability properties of the capillary wall are such that it behaves as though it possessed a set of small and large pores (Pappenheimer, 1953; Landis & Pappenheimer, 1963; Karnovsky, 1970). This has led to the formulation of the so-called Pore Model of capillary permeability. According to this model, skeletal muscle capillaries are penetrated by channels with an approximate diameter of 90Å. Molecules with molecular weights less than 10 000 would exchange rapidly through these pores. A high degree of molecular sieving would occur for molecules with molecular weights between 10 000 and 90 000 - those with a molecular weight of 90 000 being almost completely excluded from passing through the pores. The structural identity and location of these small pores is still under investigation. Karnovsky (1968) has presented evidence that the endothelial intercellular junctions are the morphological equivalents of the small pore system. Palade and co-workers (Bruns & Palade, 1968a; Simionescu et al., 1973; Simionescu et al., 1975) believe that plasmalemmal vesicles are the structural equivalents of both the small and large pore systems.

The passage of large molecules, such as large proteins, destrans and ferritin across the capillary wall has been attributed to the large pore system (Grotte, 1956). These pores have an apparent diameter of 250 - 700Å and are regarded as the only passageway for molecules with a diameter greater than 80Å.

Palade and co-workers have presented considerable evidence in favour of vesicular transport being the mechanism for the transport of large molecules across the endothelium. Using the electron microscope large molecules, such as ferritin (molecular weight = 500 000; diameter 110Å) (Bruns & Palade, 1968a; Wissig, 1958) and colloidal particles (Palade, 1961; Jennings et al., 1962), were observed in sequential stages of transfer across the endothelium while enclosed in vesicles. The vesicles are considered to be the structural equivalent of the large pore system postulated in the Pore Model.

As regards the in vivo situation, the capillaries are somewhat permeable to plasma proteins (Field et al., 1934; Bach & Lewis, 1973; Landis & Pappenheimer, 1963), indeed 24 - 50% of the total plasma protein pool in mammals is found in the interstitial space (review by Wiederhielm, 1968).

It is not possible to decide on the extent to which lactoperoxidase would penetrate the capillary wall during infusion. However, since the infusion medium contained a high concentration of lactoperoxidase, the surface proteins of at least some muscle cells would probably be labelled.

The experiment described in the following pages can be divided into two stages:

(a) the infusion of the tissue with the iodinating reagents;

(b) the fractionation of the infused tissue.

The operative procedure and infusion were skilfully carried out by Dr. B. M. Buckley, whose generous help and advice is appreciated.

Operative Procedure

A 2·5 kg female rabbit was anaesthetised by the slow infusion of 30 mg nembutal in 0·9% NaCl through an ear vein, followed by the inhalation of diethyl ether from a face mask. 1000 IU heparin were injected through a vein in the other ear. The medial aspect of one thigh was skinned. Dissection through the deep fascia exposed the femoral vessels at their origins distal to the inguinal ligament. The femoral artery was ligatured as close to this as possible, and a small cut was made in it a few mm distally, using an iridectomy scissors. A cannula made of 0·9 mm I.D. polyethylene tubing, which was continuous with the arterial line of the infusion apparatus (Fig. 3.44) and filled with infusion medium, was introduced through this cut and passed about 1 cm distally along the artery. This was secured using two ligatures. The femoral vein was cannulated in a similar fashion, except that the cannula was not attached to any part of the apparatus. The flow of infusion medium through the limb was started as soon as the cannulae were in position. About two minutes elapsed between the arterial occlusion and the commencement of infusion. The rabbit was then killed by the injection of 0·1M EDTA into the heart through the thoracic wall.

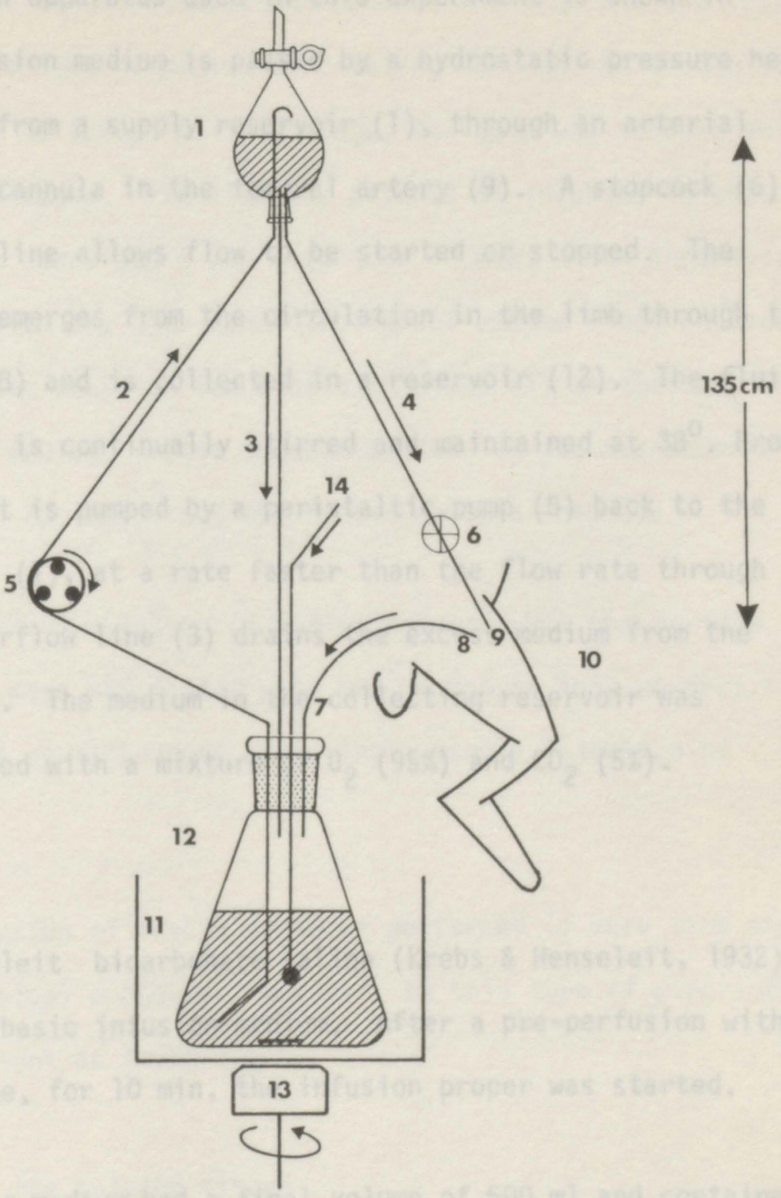


Fig. 3.44. Diagrammatic representation of infusion apparatus.

1. supply reservoir;
2. perfusate return line;
3. overflow line;
4. arterial line;
5. peristaltic pump;
6. stopcock;
7. venous line;
8. venous cannula;
9. arterial cannula;
10. hindlimb;
11. water bath (at 38^{o});
12. collecting reservoir;
13. magnetic stirrer;
14. gas line (O_2 + CO_2)

Infusion Apparatus

The infusion apparatus used in this experiment is shown in Fig. 3.44. Infusion medium is passed by a hydrostatic pressure head of 135 cm water from a supply reservoir (1), through an arterial line (4) to the cannula in the femoral artery (9). A stopcock (6) in the arterial line allows flow to be started or stopped. The infusion medium emerges from the circulation in the limb through the venous cannula (8) and is collected in a reservoir (12). The fluid in the reservoir is continually stirred and maintained at 38^{o}. From this reservoir it is pumped by a peristaltic pump (5) back to the supply reservoir (1), at a rate faster than the flow rate through the limb, an overflow line (3) drains the excess medium from the supply reservoir. The medium in the collecting reservoir was continually gassed with a mixture of O_2 (95%) and CO_2 (5%).

Infusion Medium

Krebs-Henseleit bicarbonate saline (Krebs & Henseleit, 1932) was used as the basic infusion medium. After a pre-perfusion with this medium alone, for 10 min, the infusion proper was started.

The infusing medium had a final volume of 500 ml and contained 20 mg lactoperoxidase, 10 µM KI, 10 mCi ^{131}I (as Na I), 10 mM glucose, 0·2 mM sodium pyruvate and 8 µM H_2O_2 in Krebs-Henseleit bicarbonate saline. Two further additions of H_2O_2, to a final concentration of 8 µM, were made during the infusion.

Progress of the infusion

Due to patent venous connections between the limb and the rest of the body, the volume of medium circulating through the system fell over about 30 min to zero. At this stage, the pump was stopped and the venous line clamped. The limb was allowed to incubate in this condition at room temperature for a further 15 min. This incubation period, coupled with the high concentration of enzyme used, ensured that the iodination reaction had ceased on using all of the H_2O_2 available in the medium. After incubation, the leg was disconnected from the infusion apparatus and the muscle removed from it.

The initial recirculation of medium ensured that the maximum amount of muscle mass was filled with medium and, in this respect, it is an improvement on techniques employing a single injection of medium.

A true perfusion of muscle could be performed if more time was given to the surgical procedure, however, in this type of experiment it may not represent an improvement.

Fractionation of the infused tissue

After removal from the animal, the muscle was minced by cutting into small pieces and then washed by stirring for 1 hour at 4^{o} in Krebs-Henseleit saline containing 5 mM β-mercaptoethanol. The wash medium was changed at 10 min intervals. Mercaptoethanol was incorporated into the wash medium to scavenge any residual H_2O_2.

The chopped tissue was drained of wash medium and homogenised in 3 volumes of 1 M sucrose, pH 7·4 for 60 sec. The P1 and P2 fractions were prepared according to the fractionation scheme shown in Fig. 2.2. Microsomes in the P2 supernatant were pelleted by centrifugation at 100 000 g_{av} for 90 min and termed the P3 fraction. All pellets were extensively washed by resuspension and sedimentation in 0·25 M sucrose, pH 7·4, to remove soluble ^{131}I. Particulate material in the combined washes of P1 was concentrated by centrifugation at 100 000 g_{av} for 90 min.

The distribution of particulate-bound ^{131}I between the P1, P2 and P3 fractions, which is given in Table 3.11, is similar to that for phospholipid and cholesterol. Only 4·3% of the bound I was recovered in the microsomal or P3 fraction as opposed to 94% in the P1 fraction. Also, 16·8% of the total particulate-bound ^{131}I of the homogenate could be removed from the P1 fraction by washing.

The P3 fraction was equilibrated in a sucrose density gradient to determine the isopycnic distribution of membrane-bound ^{131}I in the microsomal fraction.

The washed P3 pellet was carefully resuspended to a protein concentration of 2 mg/ml in 0·25 M sucrose, pH 7·4, using a hand-operated Potter-Elvejhem homogeniser. 15 ml sucrose density gradients were formed in 23 ml centrifuge tubes (MSE 59209). The gradients were linear with volume and ranged in density from 1·05 to 1·25 g/ml. A cushion consisting of 3 ml 2·5 M sucrose, pH 7·4 was used. 2·0 ml of sample were layered on the gradient and density gradient equilibration

TABLE 3.11

Distribution of particulate-bound ^{131}I in the P1,P2 and P3 fractions. The fractions were prepared as described in the text from skeletal muscle which had been infused with an iodination medium.

As a comparison, data on the distribution of cholesterol and phospholipid in comparable fractions has been taken from Table 3.3.

Constituent	Subcellular Fraction		
	P1	P2	P3
Particulate-bound ^{131}I	94·02	1·72	4·26
Phospholipid	87·1	3·4	3·5
Cholesterol	90·7	1·6	1·7

was carried out at 4^{o} by centrifugation at 30 000 rev/min for 10 hours in an MSE 3 x 25 ml swing-out rotor. After centrifugation, 21 fractions of 0·8 ml volume were collected manually.

The equilibrium distribution of ^{131}I is plotted in the form of a frequency histogram in Fig. 3.45. With the exception of a small peak in the region of the sample zone (density 1·03 - 1·06), the distribution consists of a sharp peak with a modal density (4^{o}) of 1·146. The median density (4^{o}) of the overall distribution is 1·142. Both values are reasonably close to the values of 1·151 and 1·149 obtained for cholesterol and basal ATPase in an earlier experiment (Table 3.10). The median density of the ^{131}I distribution is distinct from that of Ca^{2+}-dependent ATPase (P = 1·172; Table 3.10) and succinate-INT reductase (P = 1·179; Table 3.14). Indeed 81% of the ^{131}I in the gradient was recovered at densities lower than 1·170.

The protein components of membranes in the P3 fraction were solubilised by treatment with SDS and separated by one-dimensional electrophoresis in 6 - 27% polyacrylamide gel gradients. After electrophoresis the gels were stained for protein with amido black. Portion of the stained gels were sliced into 28 2 mm wide slices, corresponding to the 5·6 cm migrated by the tracker dye. The ^{131}I content of each slice was measured as described in Section 3.4. The distributions of protein and ^{131}I in the gel are plotted in Fig. 3.46 A and B. The peaks of ^{131}I on the upper surface of the gel (0-10% Rm) and at the dye front (90 - 100% Rm) are thought to represent incompletely solubilised labelled-protein and free ^{131}I, respectively.

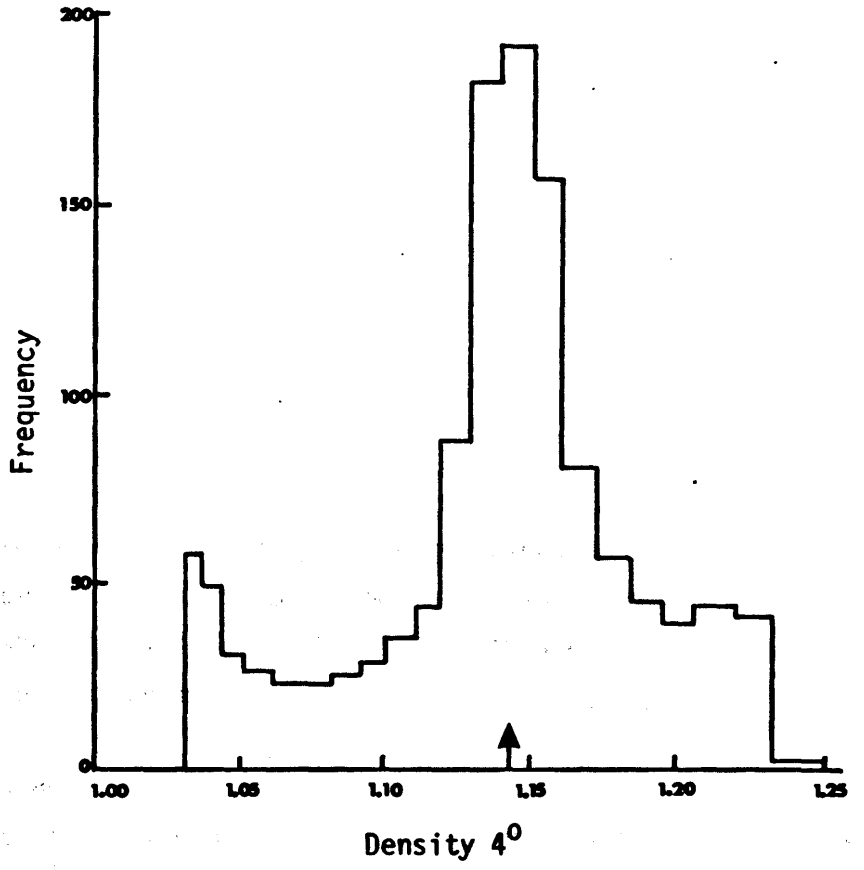
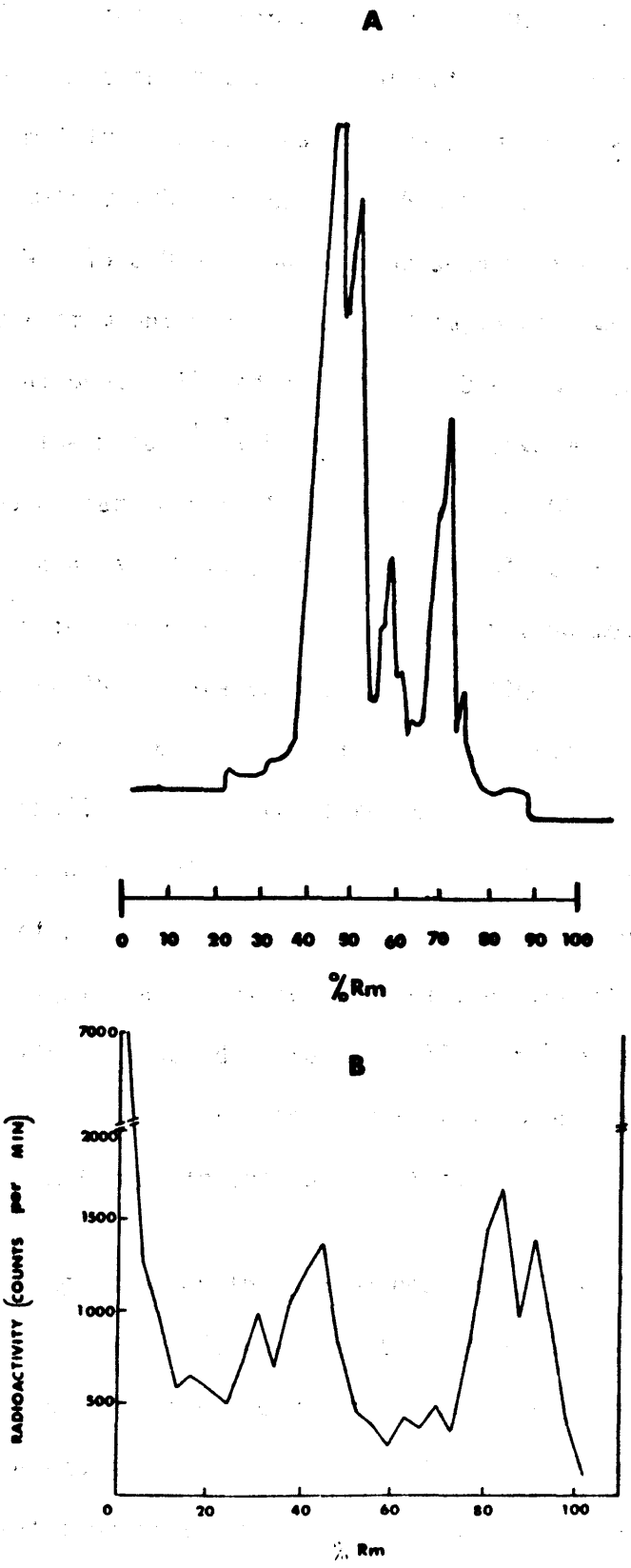


Fig. 3.45. The distribution of ^{131}I after the isopycnic equilibration of the microsomal fraction prepared from skeletal muscle which had been infused with iodination medium.

The median density of the distribution is indicated with an arrow.

Fig. 3.46. (overleaf) Polyacrylamide gel electrophoresis of proteins in the microsomal fraction prepared from skeletal muscle which had been infused with an iodination medium.

A densitometer trace of the gel after staining for protein is shown in part A. In part B the distribution of ^{131}I is plotted against relative mobility.


A
0 10 20 30 40 50 60 70 80 90 100
%Rm
B
RADIOACTIVITY (COUNTS per MIN)
7000
2000
1500
1000
500
0 20 40 60 80 100
% Rm

The protein pattern obtained for the P3 fraction corresponds to the summation of the patterns obtained for the concentrated microsomal fractions. Peaks corresponding to molecular weights of approximately 100 000, 85 000, 65 000, 60 000, 50 000, 38 000, 31 000, 28 000 and 15 000 - 20 000 can be seen on the densitometer trace. In order of magnitude, the 100 000 molecular weight band is the principal one, followed by the 85 000 and 31 000 molecular weight bands. The major ^{131}I band has a relative mobility of 84% corresponding to a molecular weight of approximately 20 000 and is closely followed by a peak at a mobility of 44% and a molecular weight of 100 000. Proteins in the 50 - 75% Rm range and having molecular weights between 25 000 and 75 000 are very poorly labelled. In comparison to the 15 000 - 20 000 molecular weight protein (which, although it represents a very small amount of the total protein, is extensively labelled), the 100 000 molecular weight band is poorly labelled relative to the amount of protein it contains. The distribution of ^{131}I label in the gel is significantly different from the overall distribution of protein. This difference would correlate well with the labelling of a group of membranes which constitute only a small proportion of the total membrane material in the P3 fraction.

During infusion the plasmamembrane of endothelial cells would be extensively labelled, however, the extent to which the sarcolemma and T-system of muscle cells would be iodinated is not known. If the relative amount of labelling of both types of cells was known, the interpretation of the results would be less difficult.

The median density of microsomes from the labelled membranes, after equilibration in a sucrose density gradient, is similar to that for cholesterol and basal ATPase in muscle and plasmamembrane marker enzymes in liver (Beaufay et al., 1974b). This result is compatible with the labelling of an external or pericellular membrane.

The results of these experiments show that, irrespective of the extent of labelling of the sarcolemma and T-system, plasmamembrane fragments from endothelial cells are found in the microsomal fraction of a muscle homogenate. Furthermore, they raise the possibility that activities such as basal ATPase, which are thought to be associated with some muscle membranes, may be located in the contaminating endothelial membranes.

3.6. The fractionation of membrane material present in the low-speed sediment from a muscle homogenate.

The results given in Section 3.1 have shown that 80-90% of the protein, phospholipid and cholesterol of a muscle homogenate sediments in fraction P1 even after extensive homogenisation. Furthermore, muscle microsomal material prepared either as a differential pellet or a concentrated suspension contains only 2-5% of the total membrane material of the homogenate. The concentrated microsomal fractions, for example, account for only 0·4% of the total protein, 3·5% of the phospholipid, 1·7% of the cholesterol and 2% of the sarcoplasmic reticulum membranes transporting Ca^{2+} measured as ^{32}P-labelled phosphoprotein intermediate, (see Table 3.1). Clearly these preparations do not represent a random sample of the total microsomal material present in the muscle homogenate. This contrasts with the distribution pattern obtained on the fractionation of a liver homogenate, when 18-28% of the principal constituents are recovered in fraction P1 and 70-75% of the endoplasmic reticulum membranes sediment in the microsomal fraction (Amar-Costesec et al., 1974a). A fractionation of muscle microsomes is incomplete without a consideration of membrane material present in fraction P1.

In the succeeding pages the fractionation of P1 is described. In these experiments an emphasis was placed on determining differences and similarities between membrane material sedimenting in fraction P1 and that found in fraction P3 or the concentrated microsomal suspension. In the initial experiments the fractionation of P1 was attempted by

combined rate-isopycnic centrifugation in the HS zonal rotor.

P1 was prepared according to the scheme shown in Fig. 3.1 and resuspended in homogenisation medium with six complete strokes in the Potter-Elvehjem homogeniser (No. 54300 teflon plunger in No. 54260 60 ml glass barrel; manufacturer: B. Braun, West Germany).

The suspension was filtered through two layers of cheese cloth to remove unfragmented tissue and other particulate material, which would block the feed lines to the rotor.

As an exploratory separation 40 ml of sample, containing P1 material from 7·5 g of minced muscle, were loaded on a 445 ml exponential density gradient ranging in density from 1·06 to 1·19 g/ml. The gradient was formed using an unmodified Birnie & Harvey (1968) exponential gradient maker. At the commencement of gradient formation the mixing vessel contained 250 ml of 0·5 M sucrose and the reservoir 445 ml of 2·0 M sucrose. An overlay,consisting of 100 ml of 0·05 M sucrose,and a cushion of 2·0 M sucrose were used. Centrifugation was carried out at 9500 rev/min for 50 min at a temperature of approximately 4^{o}. After centrifugation, the rotor contents were collected in 35 20 ml fractions. The results of this fractionation are shown in Fig 3.47 and 3.48.

Three protein peaks were detected as follows: peak 1 at a density of 1·04 and found in fractions 4-7 in the region of the sample zone; peak 2 at a density of 1·09 in fractions 9-10; and finally a large peak extending from fraction 30 to the rotor wall and covering the density range 1·19 - 1·22. Selected $S_{20.W}$ values for particle densities

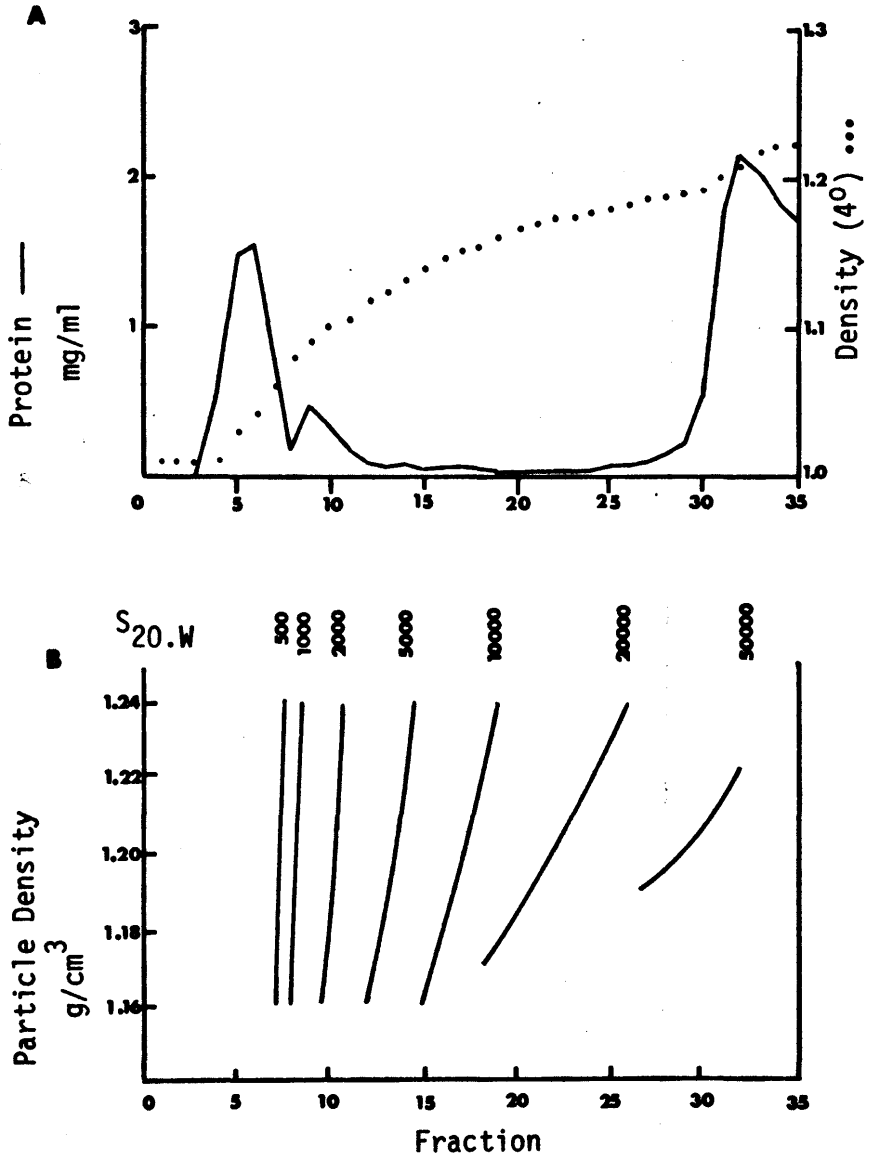


Fig. 3.47. Density gradient centrifugation of fraction P1 in the HS zonal rotor.

(A) The distribution of protein and sucrose density.

(B) The distribution of selected $S_{20.W}$ values for particle densities between 1·16 and 1·24 g/cm^3.

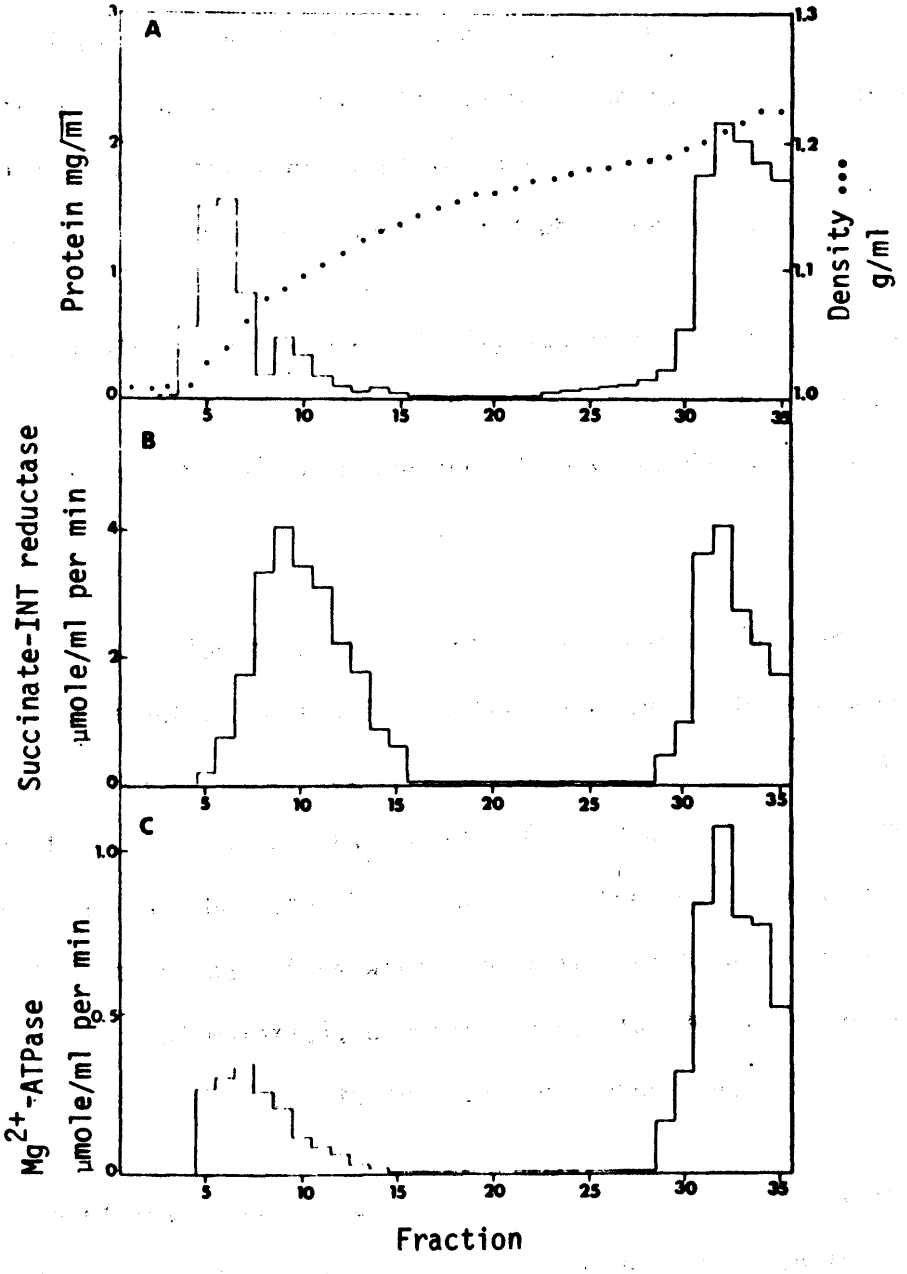


Fig. 3.48. The distribution of protein, succinate-INT reductase activity and Mg^{2+}-ATPase activity after the density gradient centrifugation of fraction P1 shown in Fig. 3.47.

ranging from 1·16 - 1·24 are plotted against fraction number in Fig. 3.47 B. Particles having densities between 1·16 and 1·24 and $S_{20.w}$ values between 500 and 2000 would be found in protein peak 2. Protein peak 3 contained material sedimenting to a density greater than 1·19. When fractions containing this material were examined in the light microscope myofibrils and whole cells could be seen.

Succinate-INT reductase activity was found in two peaks at densities of 1·087 and 1·211. The distribution of Mg^{2+}-ATPase assayed in the absence of azide showed peaks at densities of 1·061 and 1·211.

The separation obtained in this fractionation is poor. The density gradient was not sufficiently steep in the higher density region to prevent particles from sedimenting to the rotor wall. In addition, a large central region of the gradient was unused in the separation. Despite these drawbacks, the results obtained show that P1 contains particles with widely different rates of sedimentation. For this type of material, complex exponential density gradients with high load-carrying capacity are required (Anderson, 1967).

The effect of changing the gradient shape on the distribution of protein and enzymes is shown in Fig. 3.49 and 3.50. In this case, a 400 ml complex exponential gradient ranging in density from 1·07 - 1·26 was used. The gradient was steep over the density ranges 1·12 - 1·18 and 1·20 - 1·24 with a point of inflection at 1·19. In their work, Cline and coworkers (Cline & Ryel, 1971; Cline et al., 1974) have extensively used a series of gradients (termed 'inner gradients') within a gradient to increase resolution and gradient capacity.

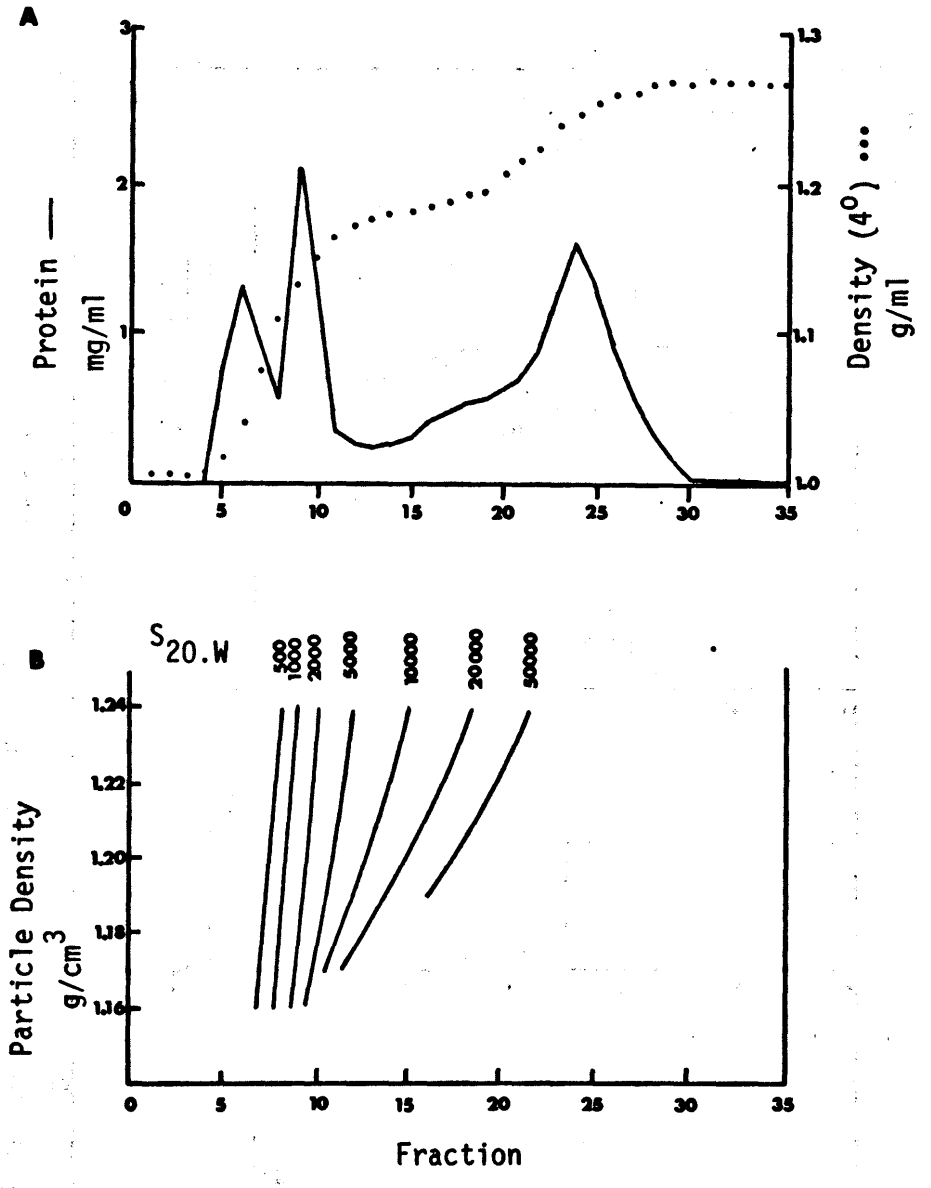


Fig. 3.49. (A) The distribution of protein and sucrose density after density gradient centrifugation of fraction P1 in the HS zonal rotor.

(B) The distribution of selected $S_{20.W}$ values for particles having densities between 1·16 and 1·24 g/cm^3.

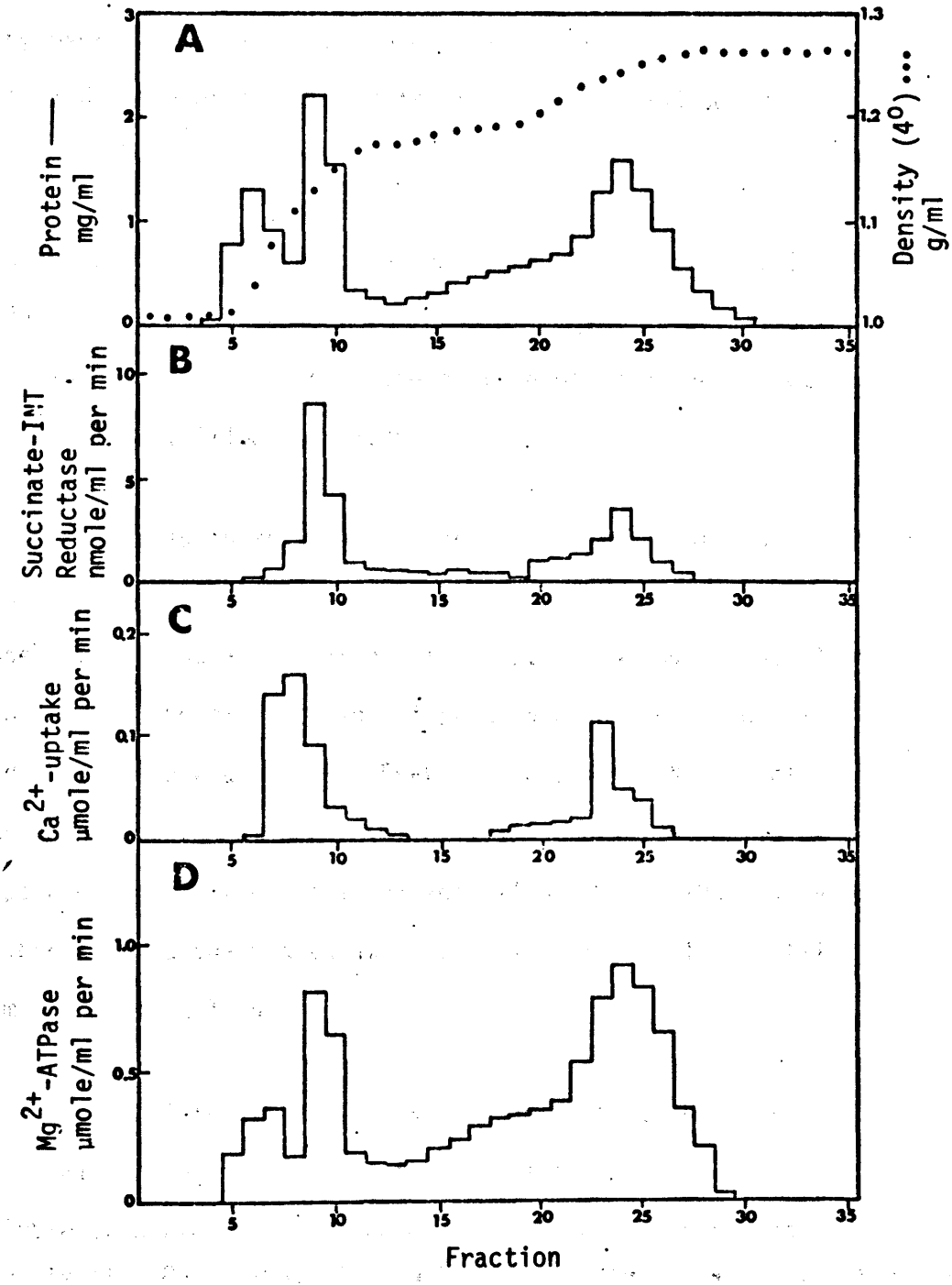


Fig. 3.50. The distribution of protein, and succinate-INT reductase, Ca^{2+}-uptake, and Mg^{2+}-ATPase activities after the density gradient centrifugation of fraction P1 shown in Fig. 3.49.

Centrifugation was carried out at 9500 rev/min for 100 min. Protein was distributed over three peaks: peak 1 at a density of 1·039 and extending over fractions 5-8 which correspond to the sample region; peak 2 at a density of 1·132 over fractions 8-10; and peak 3 at a density of 1·246.

Succinate-INT reductase activity peaked at densities of 1·132 and 1·246. Ca^{2+}-uptake peaks were found at densities of 1·110 and 1·240 and Mg^{2+}-ATPase assayed in the absence of azide peaked at densities of 1·074, 1·132 and 1·246.

Further changes in the gradient shape, giving greater utilisation of the gradient, are shown in Fig. 3.51 and 3.52. Protein peaks, in this case, were found at densities of 1·032, 1·124 and 1·189. There is a shoulder in the distribution of protein at a density of 1·22 - 1·24 and a minor peak at 1·152. Peaks of succinate-INT reductase activity were observed at densities of 1·124 and 1·189, while Ca^{2+}-uptake peaked at densities of 1·097 and 1·183. Mg^{2+}-ATPase assayed in the absence of azide peaked at densities of 1·058, 1·124, 1·189 with a minor peak at 1·152.

As a result of the three separations described, the material present in P1 can be divided into four classes:

(i) Soluble or nonsedimenting protein, which was recovered at a density of 1·03 - 1·04. This material absorbed at 415 nm, which is compatible with the presence of the soluble protein myoglobin. The material also hydrolysed ATP in the presence of Mg^{2+}. This activity is thought to be non-specific phosphatase activity associated with soluble protein.

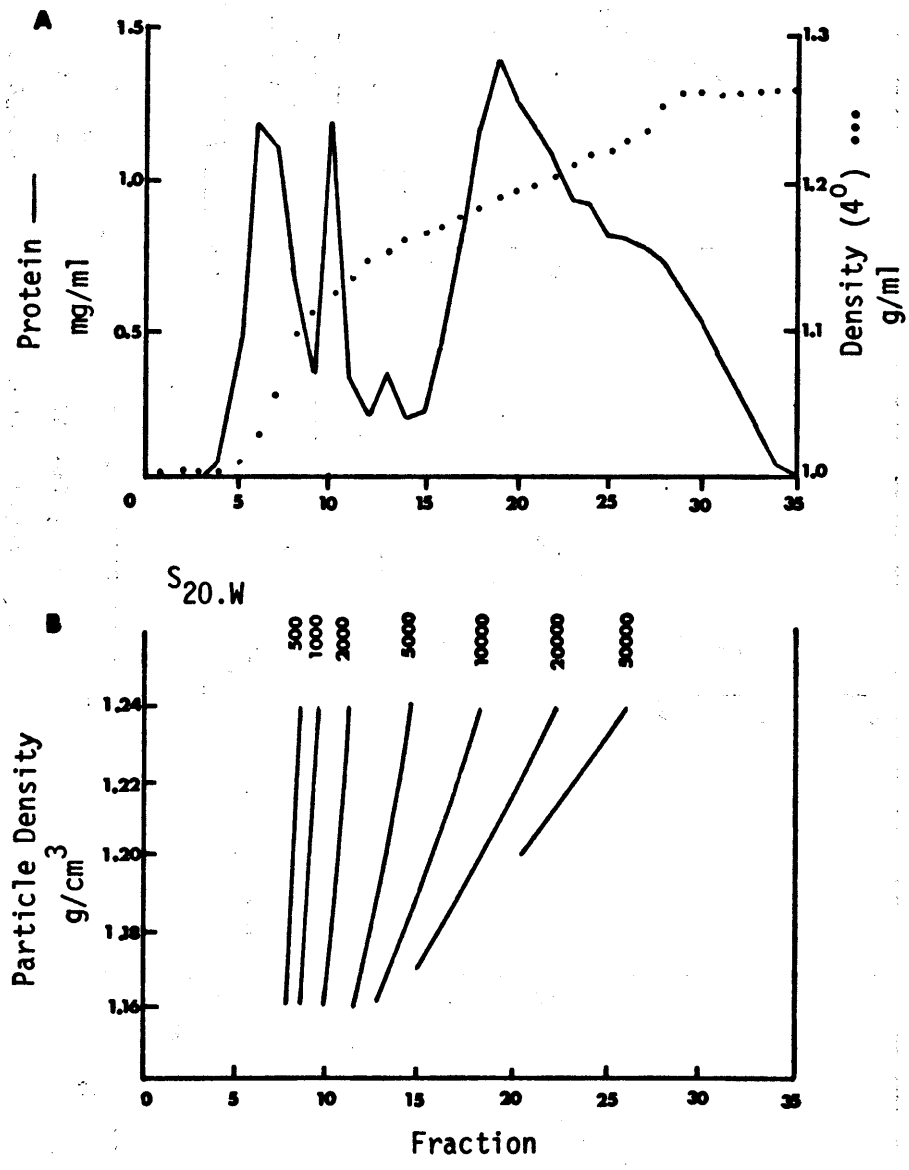


Fig. 3.51. The distribution of protein and sucrose density after density gradient centrifugation of fraction P1 in the HS zonal rotor (Part A of figure).

The distribution of selected $S_{20.W}$ values for particles having densities between 1·16 and 1·24 g/cm^3 is given in part B.

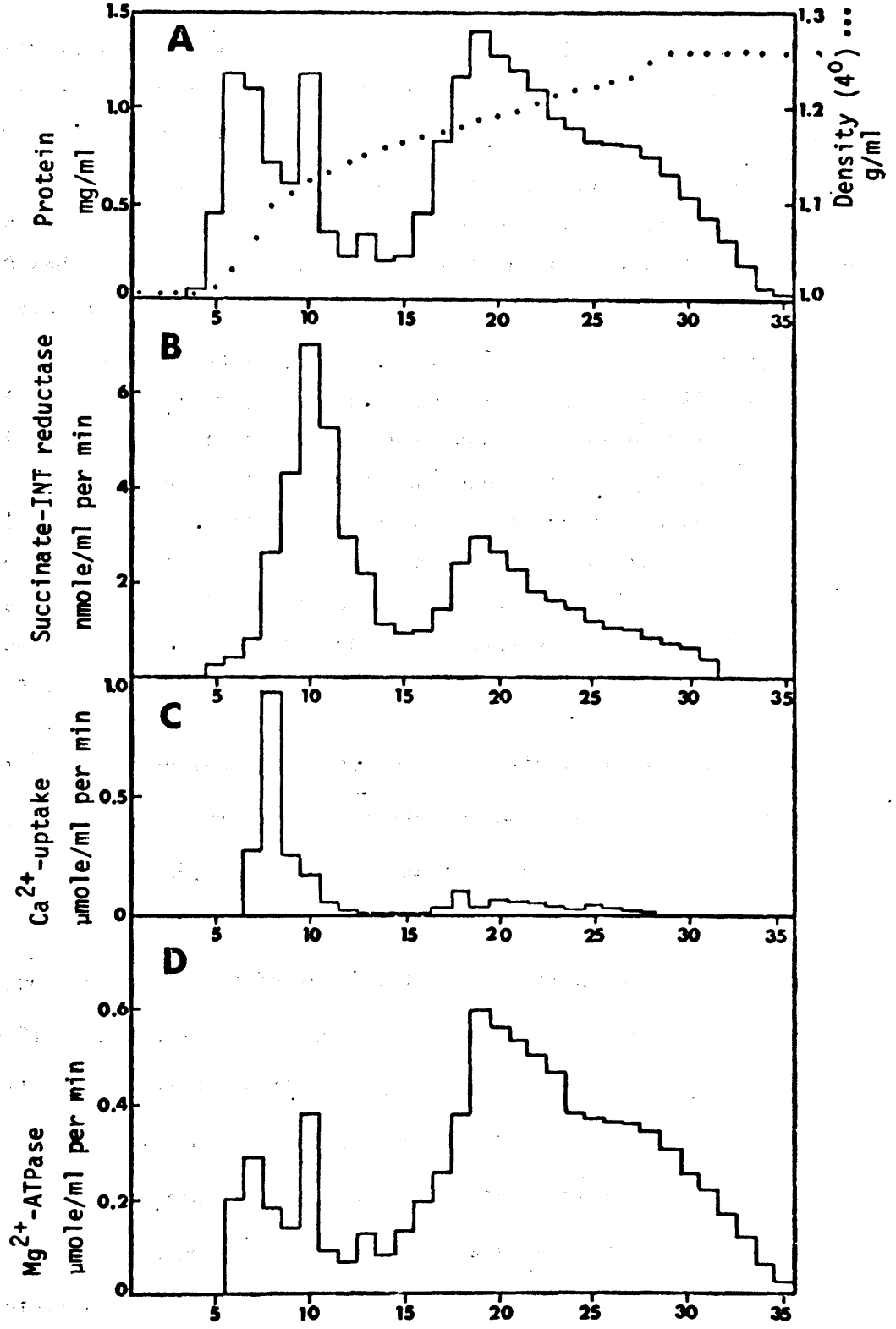


Fig. 3.52. The distribution of protein, and succinate-INT reductase, Ca^{2+}-uptake and Mg^{2+}-ATPase activities after the density gradient centrifugation of fraction P1 shown in Fig. 3.51.

(ii) Slowly sedimenting material having Ca^{2+}-uptake and K^{+}-stimulated ATPase activity and recovered at a density of 1·10 in the experiments described.

(iii) Material having succinate-INT reductase activity and recovered at densities of 1·132 and 1·124 in the separations shown in Fig. 3.50 and 3.52 respectively. These peaks of succinate-INT reductase activity are thought to represent intact mitochondria.

(iv) Material sedimenting to a density of 1·20 - 1·24. This contains myofibrillar protein as evidenced by K^{+}-inhibition of ATPase activity, similar to that which has been reported by Martonosi (1968a). When this material was examined in the light microscope, whole cells, myofibrils, connective tissue and debris could be seen. Fractions containing this material also contained Ca^{2+}-uptake and succinate-INT reductase activities, which are thought to be associated with whole cells, partially fragmented cells, and entrapped mitochondria and vesicles.

Particles sedimenting in P1 may do so by either of two modes of sedimentation: either normal sedimentation or positional sedimentation. In normal sedimentation, particles sediment at their respective sedimentation rates, whereas in positional sedimentation, particles are entrapped in the pellet because of their position in the centrifuge tube. In P1 prepared according to the scheme in Fig. 2.2, the volume of the pellet corresponds to 43% of the volume of the homogenate, consequently, many of the particles found in the pellet are there because of their position in the tube rather than their rate of sedimentation. It is possible to differentiate between the two types of sedimentation by repeated resuspension and sedimentation. The extensively washed pellet would contain particles normally sedimenting under the given conditions, while the pooled washes would contain material which was originally found in the pellet as a result of positional sedimentation.

This approach was chosen to distinguish between particles sedimenting in P1 by the two modes of sedimentation.

Fraction P1 was prepared from 125 ml of homogenate as described in Fig. 2.2. The pellet, which had a volume of 55 ml, was resuspended with 125 ml of 0·66 M sucrose, pH 7·4 and stirred at 4^{o} for 5 min. The suspension was centrifuged at 6000 g for 10 min. The pellet obtained was washed by two further resuspension and sedimentation steps using 125 ml of 0·66 M sucrose, pH 7·4 for each wash. The combined washes were diluted from 375 ml to 450 ml with 0·66 M sucrose, pH 7·4 for concentration by zonal centrifugation.

The amounts of protein, phospholipid and cholesterol removed from P1 by washing are given in Table 3.12 and presented in diagrammatic form in Fig. 3.53. When expressed as a percentage of the total in the homogenate, 18·1% of the cholesterol, 12·7% of the phospholipid and 9·3% of the protein were removed by the washing procedure.

Particulate material in the combined washes was concentrated by zonal centrifugation according to procedure described in Section 2.2, paragraph (d). This enabled particles sedimenting in P1 by positional sedimentation to be concentrated by the same procedure as that used for microsomal particles in the $5{\cdot}76 \times 10^{5}$ g-min supernatant (Fig. 2.2).

The concentrated microsomal suspension, prepared in this way, was collected in 6 x 25 ml fractions, which were analysed in detail.

In Fig. 3.54 the density profile and distribution of the three major constituents in the six fractions are plotted. Protein, phospho-

TABLE 3.12

The amount of protein, phospholipid and cholesterol, expressed as a percentage of that in the homogenate, recovered in fractions P1 and S1. Both fractions were prepared according to the scheme shown in Fig. 2.2. P1 represents the pellet and S1 the supernatant, obtained after centrifugation of the homogenate for $6{\cdot}54 \times 10^4$ g-min.

The amount of each constituent removed from P1, by the washing procedure described in the text, is also given.

Part of this data is given in diagrammatic form in Fig. 3.53.

	Constituent as % of that in the homogenate		
Fraction	Protein	Phospholipid	Cholesterol
Homogenate	100·0	100·0	100·0
Fraction P1	86·1	87·1	90·7
Fraction S1	16·8	10·5	8·2
Washes of Fraction P1	9·3	12·7	18·1
Fraction P1 after washing	74·3	76·8	74·2

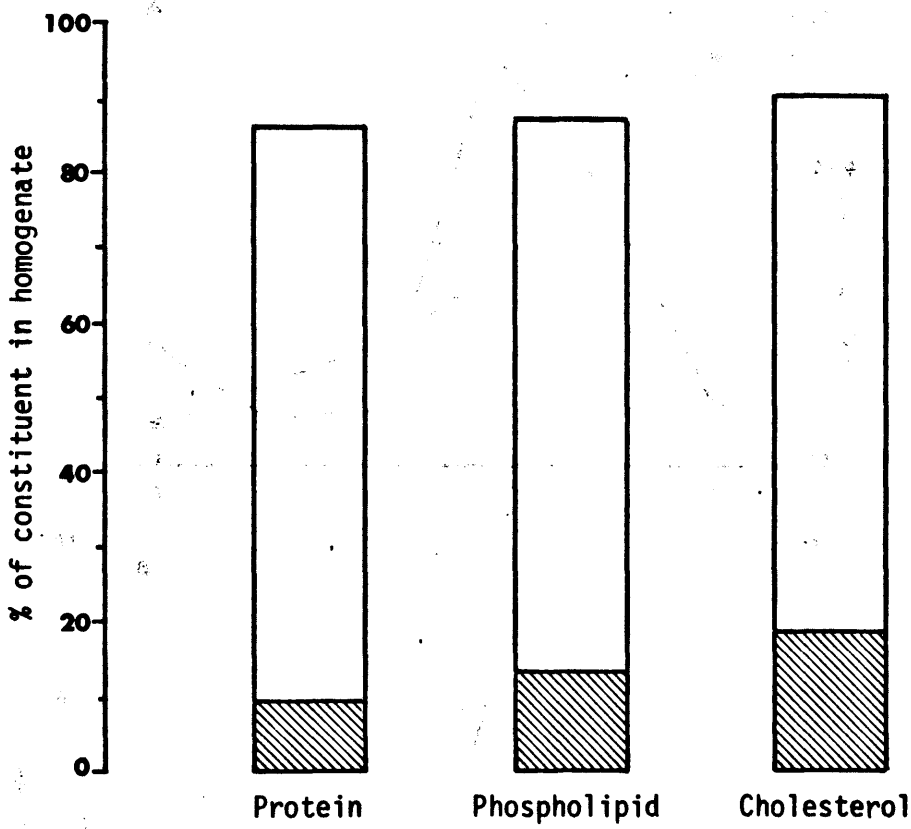


Fig. 3.53. The percentage protein, phospholipid and cholesterol found in fraction P1 prepared according to the procedure in Fig. 2.2. The amount of each of the three constituents, which was removed from P1 by the washing procedure described in the text, is represented by the shaded area.

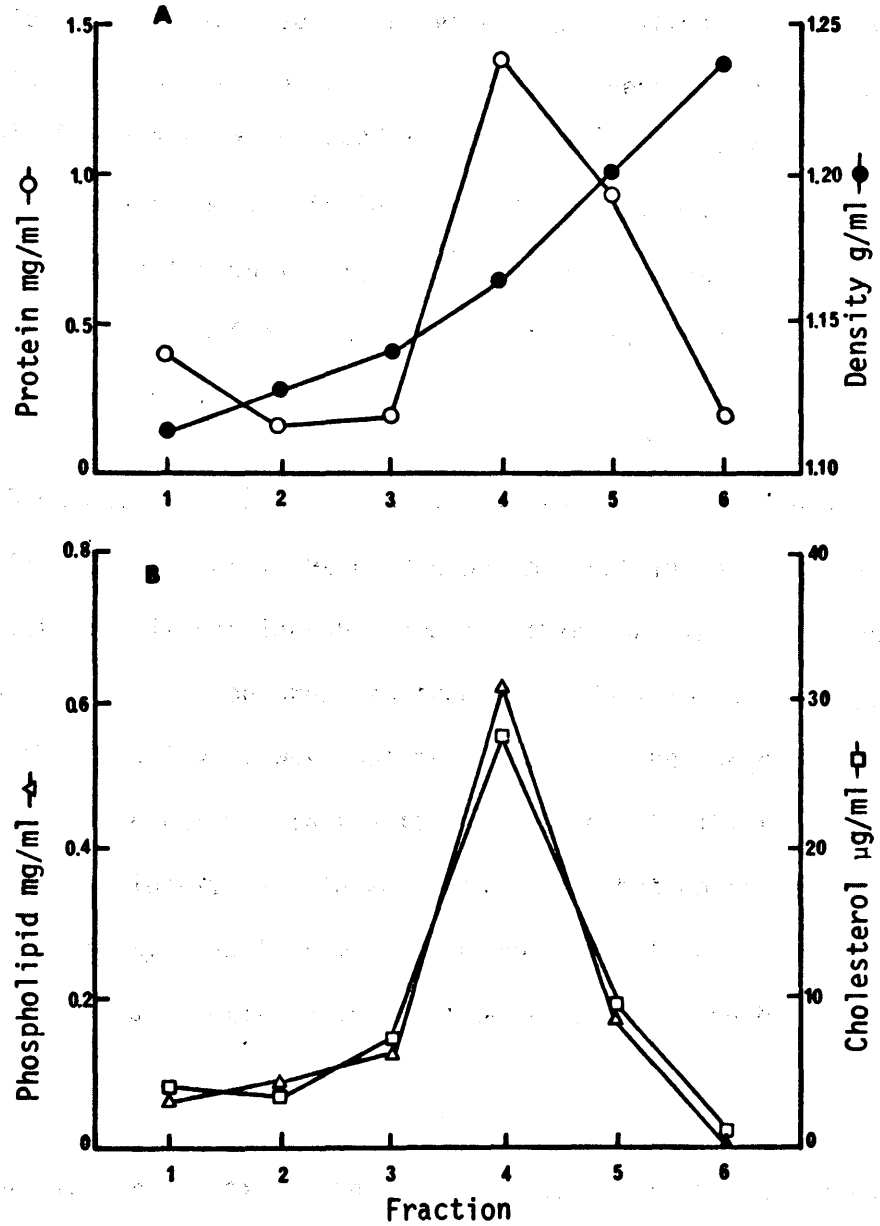


Fig. 3.54. The distribution of protein, sucrose density, phospholipid and cholesterol in the concentrated fractions prepared from the washes of Pl.

lipid and cholesterol peak sharply in fraction 4 at a modal density of 1·165. When expressed as a percentage of the total in the six fractions, 42·5% of the protein, 57·4% of the phospholipid and 53·8% of the cholesterol were found in fraction 4. The enzyme activities assayed for also peaked in this fraction. While the absolute activity of Ca^{2+}-uptake (shown in Fig. 3.55) peaked in fraction 4, the specific activity peaked in fraction 2 with an activity of 1·95 µmole per mg protein per min.

Succinate-INT reductase activity peaked in fraction 4 with a specific activity of 14·92 nmoles per mg protein per min (Fig. 3.55). This activity is considerably greater than that in the concentrated microsomal suspension prepared from the $5·76 \times 10^5$ g-min supernatant, in which the activity was routinely found in the range 2-4 nmoles per mg protein per min in fraction 4. Succinate-INT reductase activity is closely paralleled by the distribution of azide-sensitive ATPase activity - another marker enzyme for mitochondria. 56·6% of the total ATPase activity in fraction 4 is inhibited by 50 mM sodium azide (Fig. 3.55).

Assay for K^+-stimulated ATPase showed that the Mg^{2+}-ATPase activity of fractions 1, 2, 3 and 4 was stimulated by K^+, while that of fractions 5 and 6 was inhibited (Fig. 3.56). The K^+-stimulated ATPase is associated with Ca^{2+}-transport and its distribution would be expected to be similar to that for Ca^{2+}-uptake. While K^+ ions stimulate the Ca^{2+}-dependent ATPase they inhibit the myofibrillar ATPase (Martonosi, 1968a). Electrophoretic analysis of the concentrated fractions, which will be described later, has shown that myofibrillar proteins are

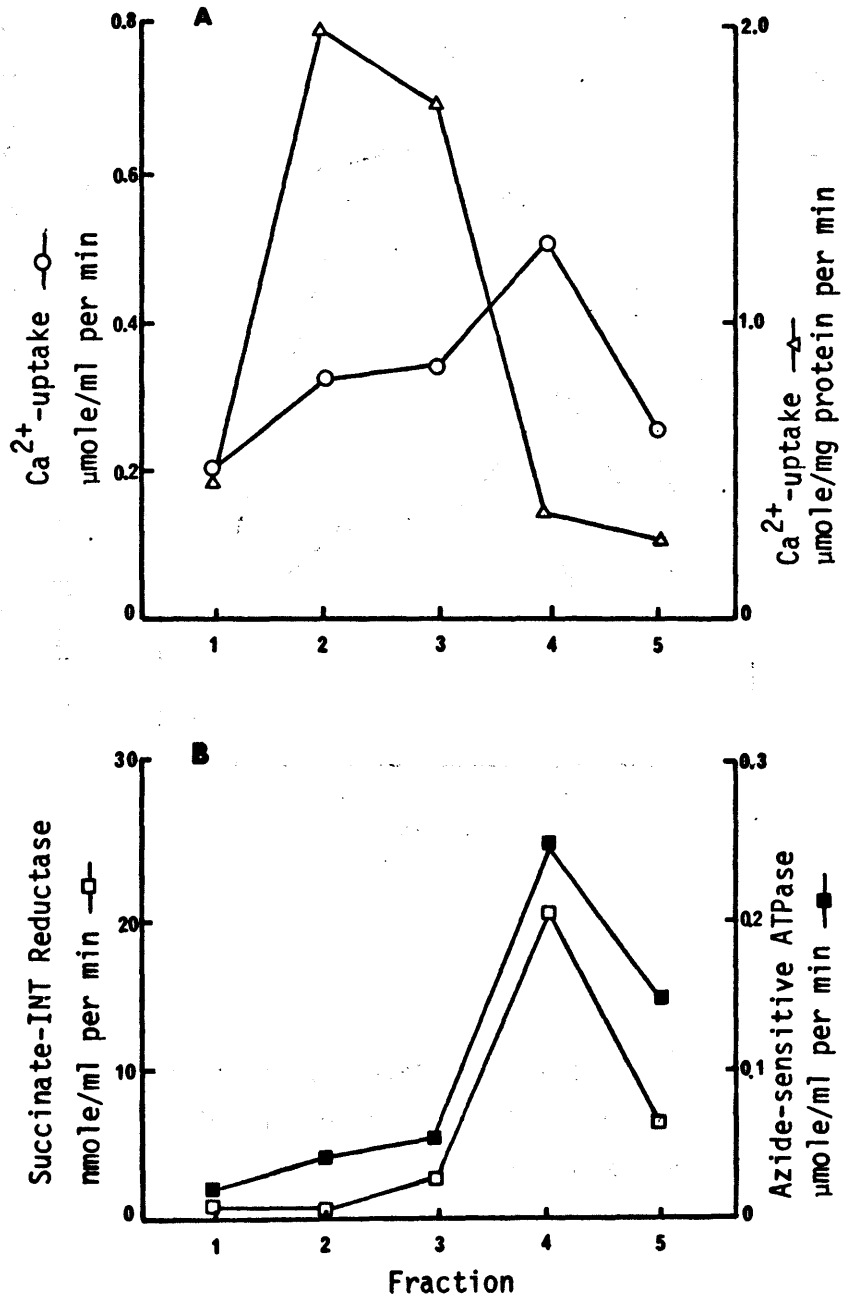


Fig. 3.55. The distribution of marker enzymes in the concentrated fractions prepared from the washes of Pl.

(A) The absolute and specific activities of Ca^{2+}-uptake.

(B) The absolute activities of succinate-INT reductase and azide-sensitive ATPase.

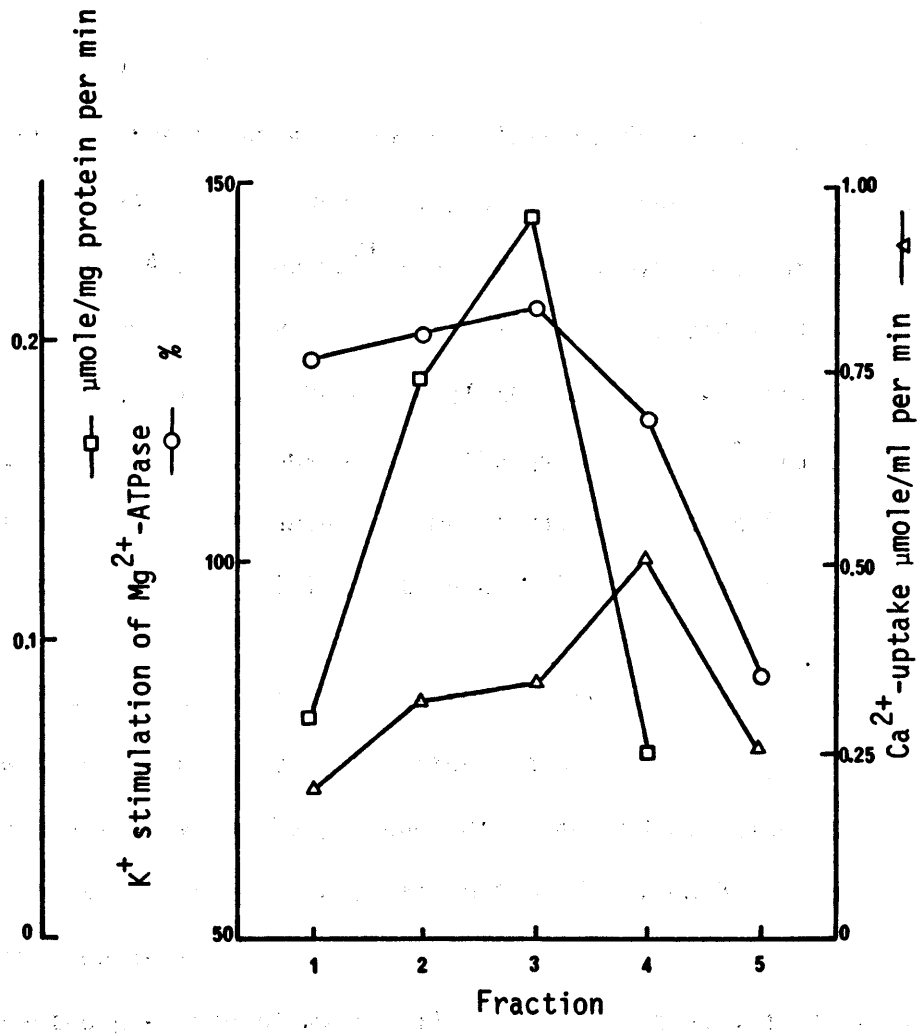


Fig. 3.56. A comparison of the distribution of Ca^{2+}-uptake and the K^{+} stimulation of Mg^{2+}-ATPase activity in the concentrated fractions prepared from the washes of P1.

present in fractions 4, 5 and 6. This evidence is supported by the sharp decrease in the phospholipid/protein ratio in fractions 4-6. In fraction 4 both the Ca^{2+}-transport and myofibrillar ATPases are present, consequently the K^{+}-stimulation observed is lower than would be expected on the basis of the Ca^{2+}-uptake activity of the fraction. In fractions 5 and 6 the amount of Ca^{2+}-transport protein decreases while the amount of myofibrillar protein increases. This results in an overall inhibition of Mg^{2+}-ATPase activity by K^{+}.

Total ATPase activity, in the presence of Mg^{2+}, peaked in fraction 4, with high activity in fraction 5 also (Fig. 3.57). Ca^{2+}-independent ATPase activity in the presence of 50 mM sodium azide decreases from fractions 1-2 and increases again from fraction 3-4. There is little difference in the activity of this enzyme in fractions 4 and 5. The suspected presence of myofibrillar ATPase activity in fractions 5 and 6 makes an interpretation of Ca^{2+}-independent ATPase data difficult.

The phospholipid/protein ratio in the six fractions peaks in fraction 3 with a ratio of 0·65 (Fig. 3.58). The ratio falls through fraction 4 to low values in fractions 5 and 6. The low values in fractions 1, 5 and 6 indicate the presence of a large amount of non-membrane protein.

The cholesterol/phospholipid ratio over fractions 1-5 is in the range 40-60 µg/mg phospholipid. The ratio peaks in fractions 1, 3 and 5 with lower values in fractions 2 and 4 (Fig. 3.58 and Table 3.13).

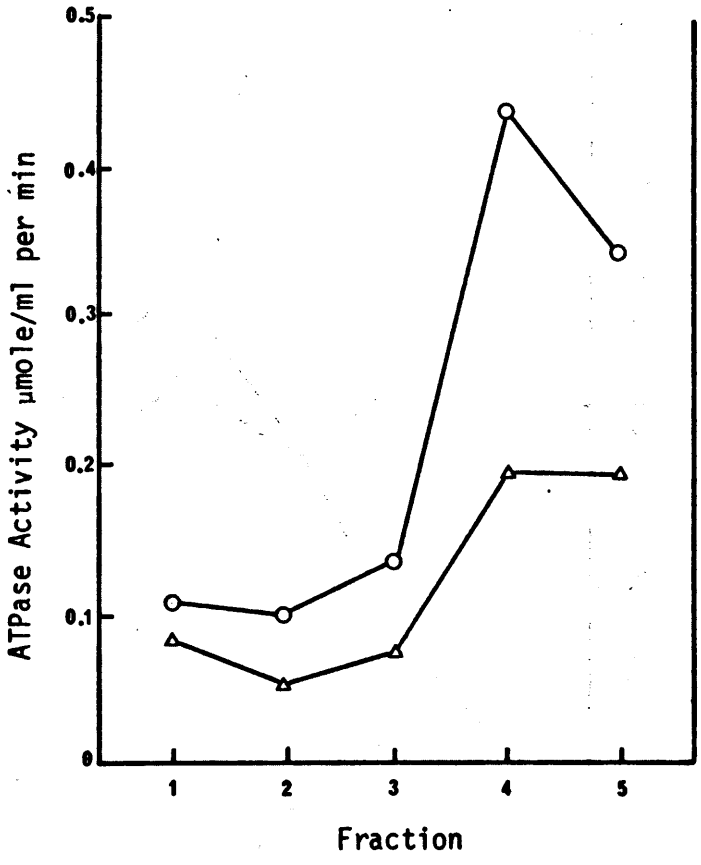


Fig. 3.57. The distribution of Mg^{2+}-ATPase activity (—o—) and azide-insensitive Ca^{2+}-independent ATPase activity (—Δ—) in the concentrated fractions prepared from the washes of P1.

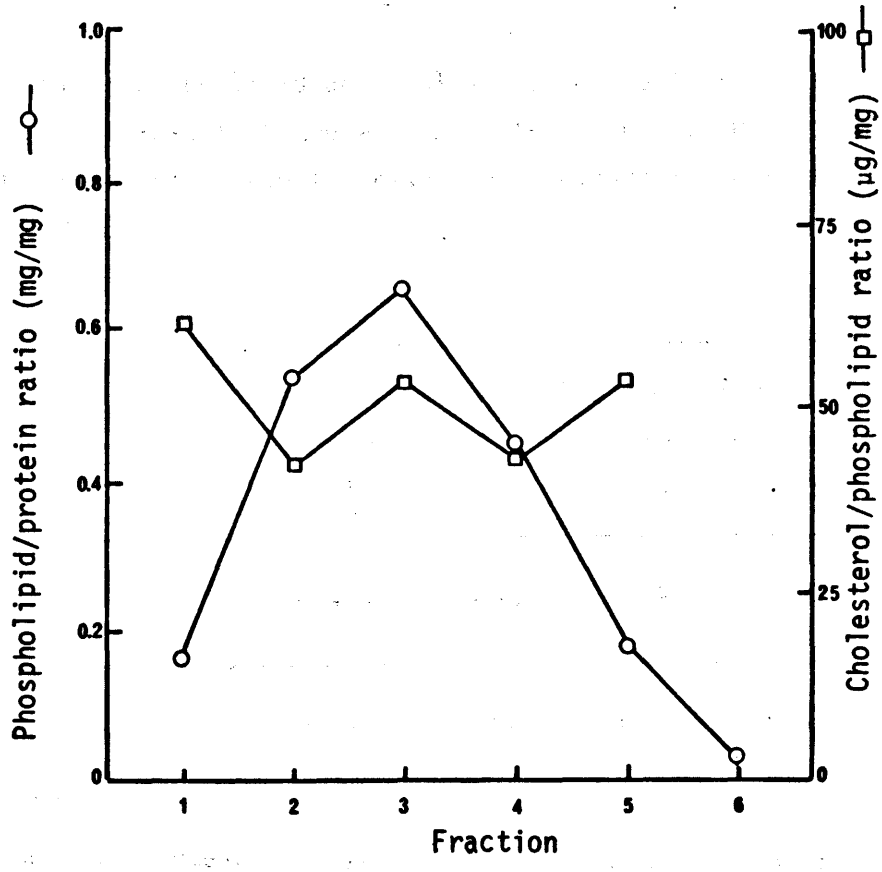


Fig. 3.58. The distribution of the phospholipid/protein (mg/mg) and cholesterol/phospholipid (µg/mg) ratios in the concentrated fractions prepared from the washes of Pl.

TABLE 3.13

The protein, phospholipid and cholesterol content of the five concentrated fractions obtained from the washes of fraction P1. Portion of the data given in this table is presented in diagrammatic form in Fig. 3.54 and 3.58

	FRACTION				
	1	2	3	4	5
Protein mg/ml	0·404	0·162	0·197	1·377	0·930
Phospholipid mg/ml	0·067	0·087	0·129	0·618	0·168
Phospholipid/Protein ratio mg/mg	0·167	0·537	0·655	0·449	0·181
Cholesterol µg/ml	4·11	3·65	6·84	27.51	8·98
Cholesterol/Protein ratio µg/mg	10·19	22·50	34·70	19·98	9·65
Cholesterol/Phospholipid ratio µg/mg	60·99	41·93	52·95	44·53	53.30

In order to obtain information on the relative differences in particle size in the six fractions, the technique of ultrafiltration was used. The experimental procedure followed was similar to that described in Section 3.2. Each fraction was diluted to a known protein concentration in the region of 100 μg/ml. 1 ml aliquots were filtered through a range of Millipore filters of average pore size ranging from 0·22 to 1·2 μm. The protein content of the unfiltered sample and that of each of the filtrates was measured. The results, which are presented in Fig. 3.59, are expressed as the percentage protein passing the filter for the range of pore sizes.

In fractions 1 and 2 the percentage protein passing the various filters is similar to that for fractions 1 and 2 of the concentrated suspension (Fig. 3.9) prepared from the $5{\cdot}76 \times 10^5$ g-min supernatant (Fig. 2.2). In fractions 3-5 the amount of protein passing the filter decreases indicating increasing particle size or the presence of aggregrates. Only 49·5%, 21% and 5% of the protein in fractions 3, 4 and 5 respectively, passes the 1·2 μm filter as opposed to 71·5% and 64·5% in fractions 1 and 2 respectively. It was not possible to filter fraction 5 through filters of pore size less than 1·2 μm. This supports the evidence already given that fraction 5 contains myofibrillar material.

The protein components of material in each fraction sedimenting after centrifugation at 100 000 g_{av} for 60 min were separated using SDS-polyacrylamide gel electrophoresis. This was done in conjunction with an electrophoretic balance sheet experiment.

Fig. 3.59. (on the following two pages)

The percentage protein in each of the concentrated fractions prepared from the washes of P1 passing through Millipore filters ranging in pore size from 0·22 μm to 1·2 μm. The unfiltered sample is indicated by nf. The experimental details are given in the text.

Fig. 3.59 (i)

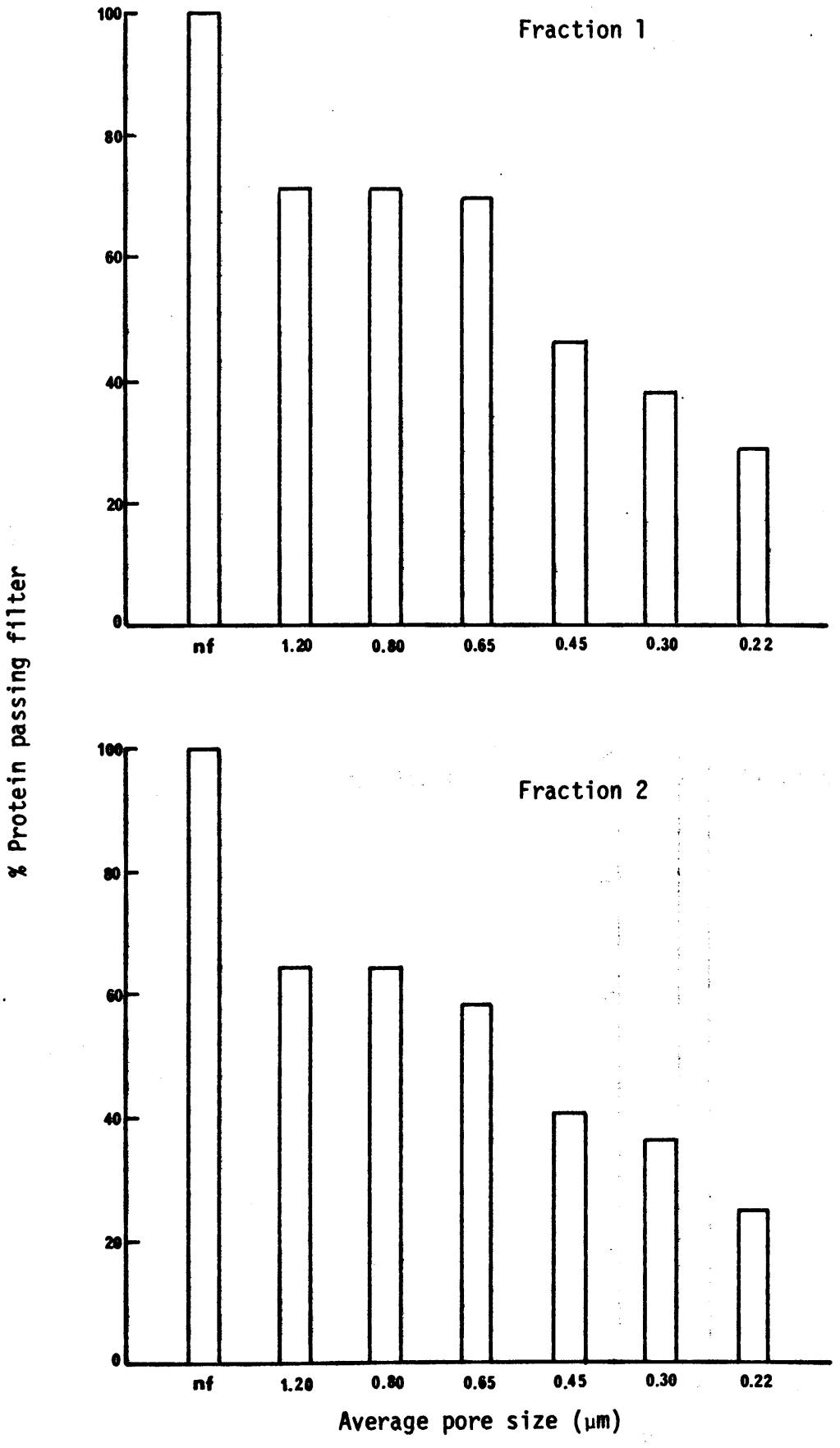
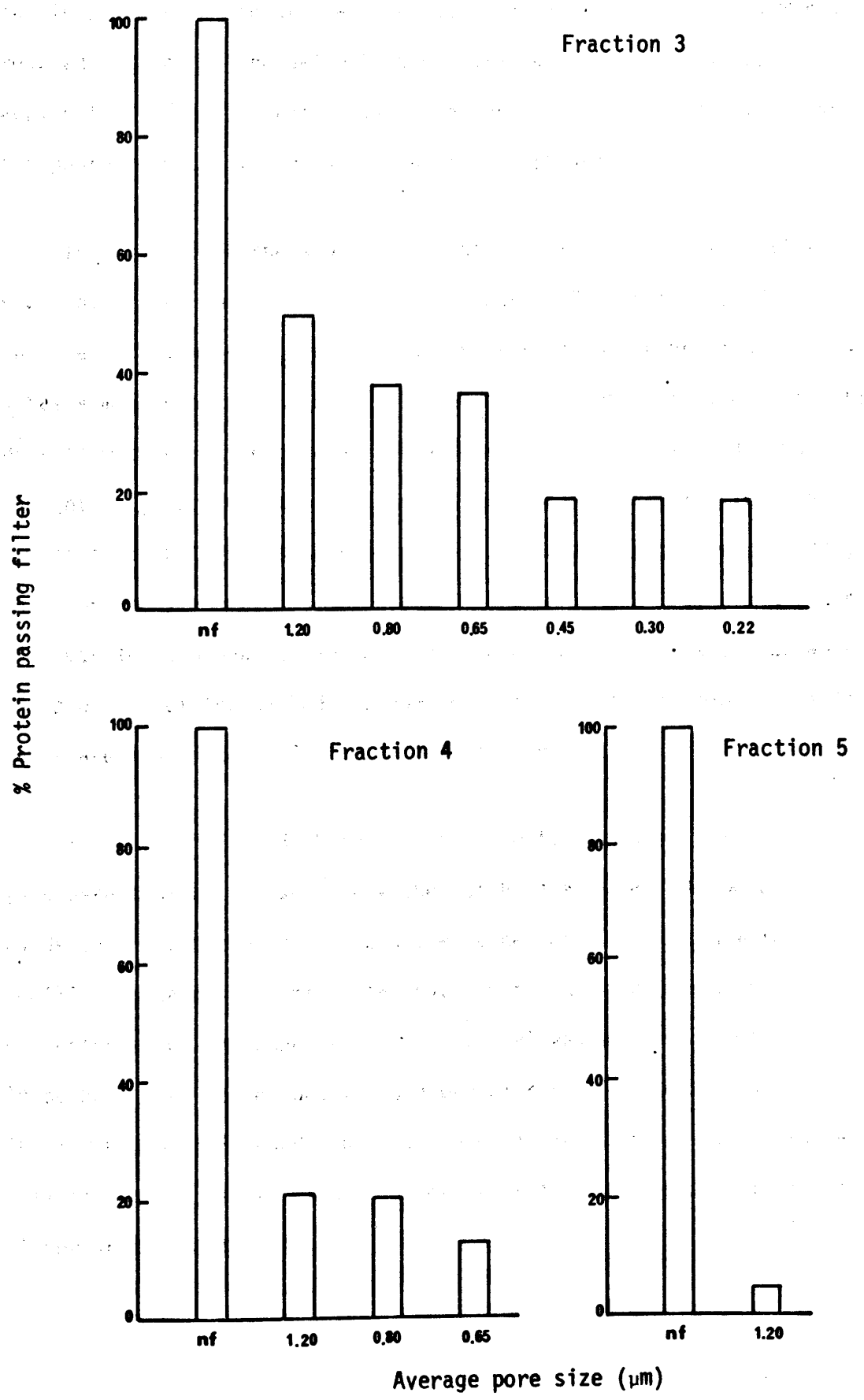

Fraction 1
Fraction 2
% Protein passing filter
100
80
60
40
20
0
nf
1.20
0.80
0.65
0.45
0.30
0.22
Average pore size (µm)

Fig. 3.59 (ii)


Fraction 3
Fraction 4
Fraction 5
% Protein passing filter
100
80
60
40
20
0
nf
1.20
0.80
0.65
0.45
0.30
0.22
Average pore size (µm)

One-dimensional polyacrylamide gel electrophoresis was carried out in polyacrylamide gradient gel slabs at 75 V until the tracker dye reached the end of the gel. Samples were applied to a fourteen-place applicator. The gels were stained for protein using amido black and are photographically reproduced in Figs. 3.60 and 3.61.

In Fig. 3.60 A the separations obtained for the SDS-solubilised proteins of the homogenate, P1 and the washed P1 are shown. Two intense bands, a and b, are present in each of the three samples. Band a has a molecular weight of 200 000 and is thought to be the contractile protein myosin, while band b, having a molecular weight of 45 000, is thought to be actin - the other principal contractile protein. The intensity of both bands correlates well with the report (Perry, 1974) that contractile proteins constitute approximately 60% of the protein of a muscle homogenate. While there are no major differences between the proteins of the washed and unwashed P1, many of the minor protein bands are less intense in the pattern for the washed pellet.

In gel B of Fig. 3.60 the electrophoretic patterns for P2, S2 (= supernatant of P2) and the washes of P1 are shown. A number of bands in the patterns for S2 and the washes of P1 have a similar mobility to bands present in the pattern for the zonal supernatant collected after the concentration of the P1 washes (Fig. 3.61 A). These bands may correspond to some proteins of the soluble phase. The 100 000 protein band, which is found in fractions with Ca^{2+}-uptake activity and is marked with an asterisk in the photographs, is relatively strong in the pattern for P2.

Fig. 3.60. (overleaf) Electrophoresis in SDS-polyacrylamide gel gradients. The protein patterns shown are those for the following:

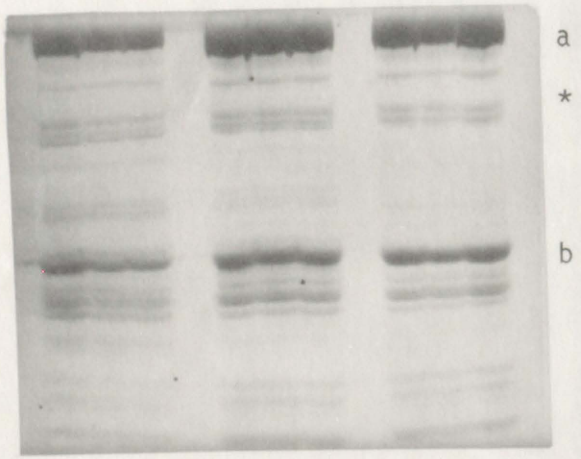
gel A (from left to right) homogenate, P1, washed P1;

gel B (from left to right) P2, S2, and the washes of P1.

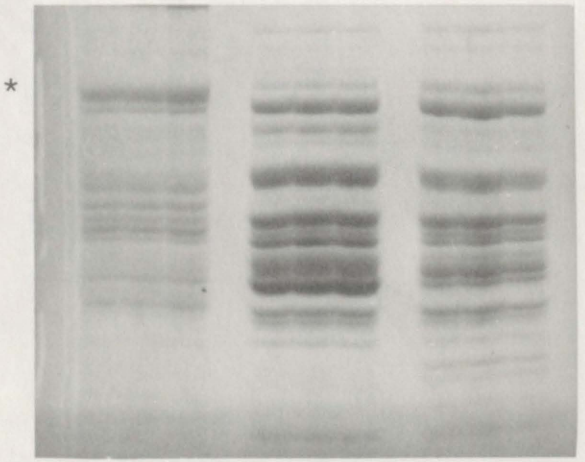
The gel gradient ranged from 6% at the top to 27% at the bottom. Migration was towards the anode, i.e. from top to bottom.

Bands a and b are thought to represent myosin and actin respectively.

The 100 000 molecular weight component is marked with an asterisk.



Gel A



Gel B

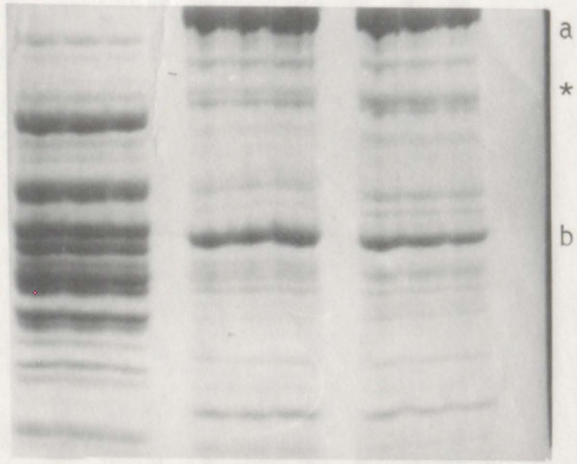
Fig. 3.60. Legend on previous page.

Fig. 3.61. (overleaf) Electrophoresis in SDS-polyacrylamide gel gradients. The protein patterns shown are those for the six concentrated fractions prepared from the washes of P1 and for the zonal supernatant (=ZS) obtained in the preparation of these fractions.

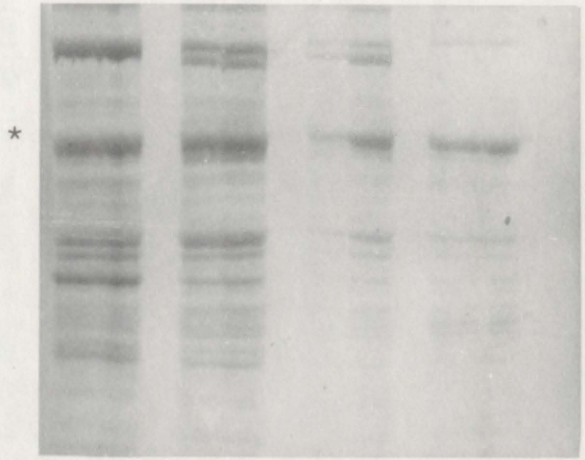
Gel A (from left to right) ZS (=zonal supernatent), fraction 6, fraction 5.

Gel B (from left to right) fractions 4, 3, 2 and 1.

The gel gradient ranged from 6% at the top to 27% at the bottom. Migration was towards the anode, i.e. from top to bottom. Bands a and b are thought to represent myosin and actin, respectively. The 100 000 molecular weight component is marked with an asterisk.



Gel A



Gel B

Fig. 3.61. Legend on previous page.

The patterns for the six concentrated fractions from the washes of P1 are shown in Fig. 3.61. Myosin (band a) and actin (band b) are the principal bands in fractions 5 and 6. The 100 000 band is strong in fractions 1-4.

The proteins of the six fractions were further analysed by two-dimensional SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out in a first dimension in 5·2% gels and in a second dimension in 6-27% polyacrylamide gradient gels. Densitometer traces of the first-dimension separation are shown in Fig. 3.62 and photographs of the second-dimension gels in Fig. 3.63.

Myosin (molecular weight = 200 000) and actin (molecular weight = 45 000) are the principal proteins in fractions 5 and 6, and are also present in fraction 4. In comparison to the other five fractions, fraction 5 has the greatest degree of heterogeneity.

A number of conclusions can be drawn from the range of analysis carried out on these fractions:

(i) The washes of P1 contain high succinate-INT reductase activity, which was largely recovered at a density of 1·165 in preparation of the concentrated fractions. Under the conditions of centrifugation, which were not isopycnic, this activity would be mainly associated with intact mitochondria.

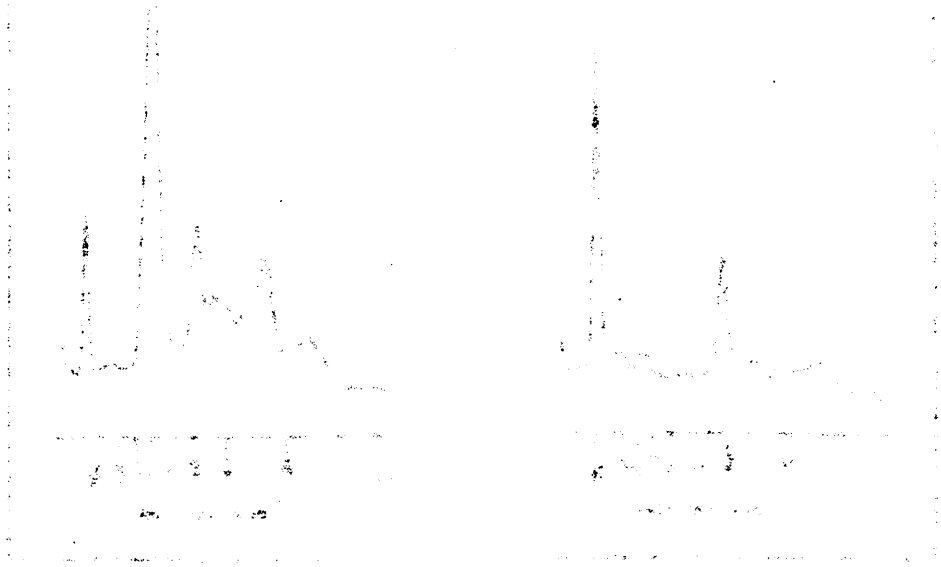
(ii) When compared to the distribution in the concentrated microsomal suspension from the $5{\cdot}76 \times 10^5$ g-min supernatant, the microsomal marker enzymes are found at a higher density than would be expected. This is probably the result of extensive aggregration of microsomal components with mitochondria, which originates from the 'packing' of particulate material in the original P1 pellet.



Fig. 3.62. (overleaf) Densitometer traces of the first-dimension separation of sedimentable protein, solubilised with SDS, in the concentrated fractions prepared from the washes of P1.

Electrophoresis in this dimension was carried out in 5·2% polyacrylamide gels. Migration was towards the anode, i.e. from left to right on the traces.

Photographs of the second-dimension separation are shown in Fig. 3.63.



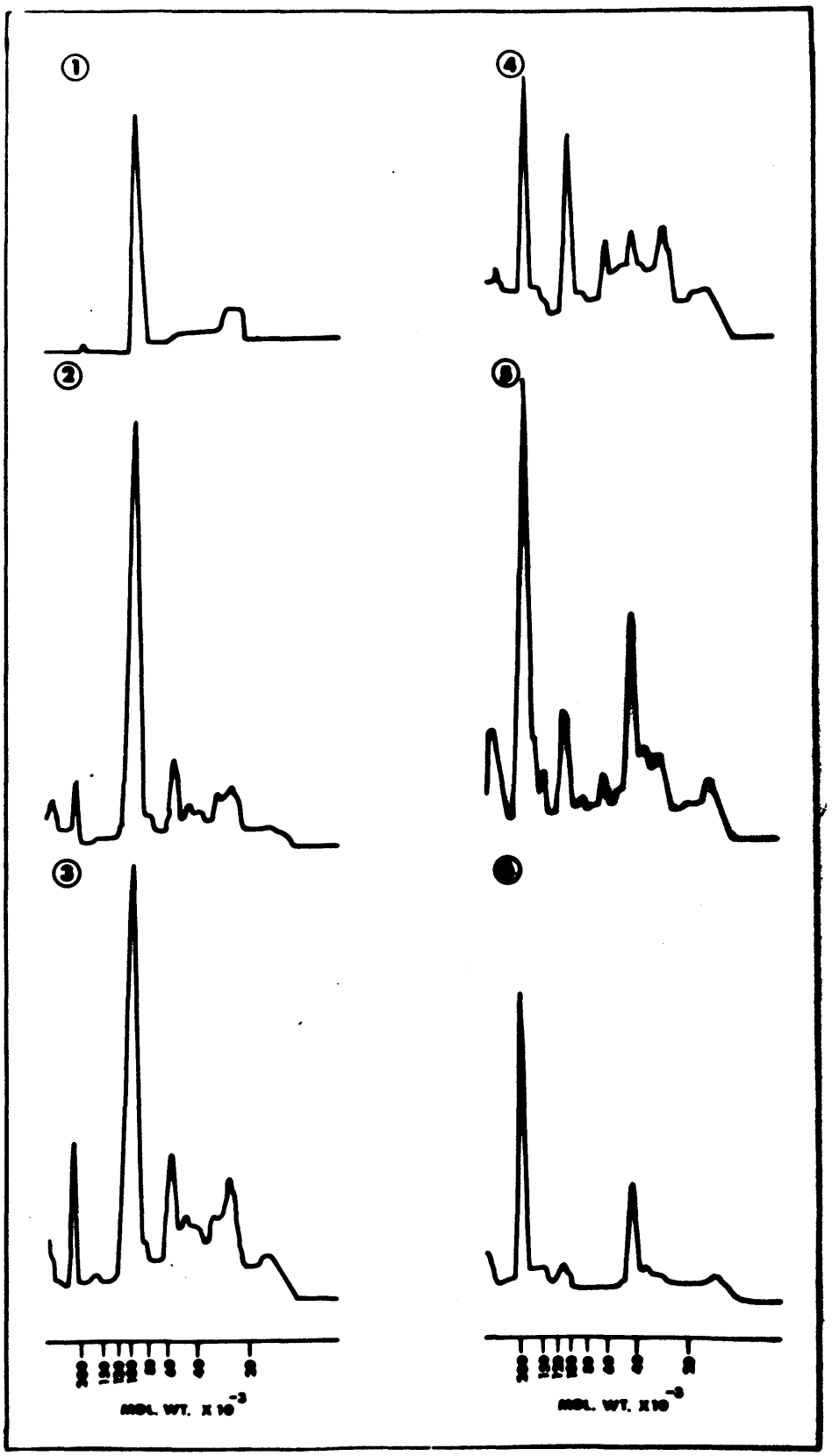

①
②
③
④
⑤
MOL. WT. X 10⁻³
MOL. WT. X10⁻³

Fig. 3.63. (overleaf) Two-dimensional SDS-polyacrylamide gel electrophoresis of sedimentable protein in the concentrated fractions prepared from the washes of P1.

Photographs of the second-dimension gradient gels after staining for protein are shown as follows:

part A fractions 1 and 2;
part B fractions 3 and 4;
part C fractions 5 and 6.

Migration in both dimensions was towards the anode. In the illustrations this corresponds to electrophoresis from left to right in the first-dimension and from top to bottom in the second dimension. In the second dimension gel the gel gradient ranged from 6% at the top to 27% at the bottom.

Densitometer traces of the first dimension separations are shown in Fig. 3.62.



Fraction 1



Fraction 2

Fig. 3.63 (A)



Fraction 3



Fraction 4

Fig. 3.63 (B)



Fraction 5



Fraction 6

Fig. 3.63 (C)

(iii) The preparation is contaminated by contractile proteins, which sediment to the interface with the cushion of 2·0 M sucrose, pH 7·4.

Difficulties were experienced in achieving the same degree of dispersion of components in the washes of P1 as existed prior to sedimentation in the pellet. In an effort to reduce this problem, particulate material in the washes was sedimented by centrifugation at 100 000 g_{av} for 90 min and carefully resuspended in 0·25 M sucrose, pH 7·4. The suspension was then subjected to isopycnic density gradient centrifugation.

15 ml sucrose density gradients were formed in 23 ml centrifuge tubes (MSE, No. 59209). Each gradient was linear with volume and ranged in density (4^{o}) from 1·064 to 1·255. A cushion consisting of 3 ml of 2·5 M sucrose, pH 7·4 (density 4^{o} = 1·316) was used. 1 ml of sample was applied to the gradient and density equilibration was carried at 4^{o} by centrifugation at 30 000 rev/min for 10 hours in an MSE 3 x 25 ml swing-out rotor. After centrifugation 20 approximately 1 ml fractions were collected.

The fractions were assayed for protein, succinate-INT reductase, Ca^{2+}-dependent ATPase and basal ATPase. The equilibrium distribution of each constituent was determined and these are plotted in the form of frequency histograms in Fig. 3.64 and 3.65 and the median densities for the distributions are summarised in Table 3.14.

The equilibrium distribution of protein is a bimodal one. One protein peak consisting of soluble or nonsedimenting protein is found in the region of the sample zone and the other at a density (4^{o}) of

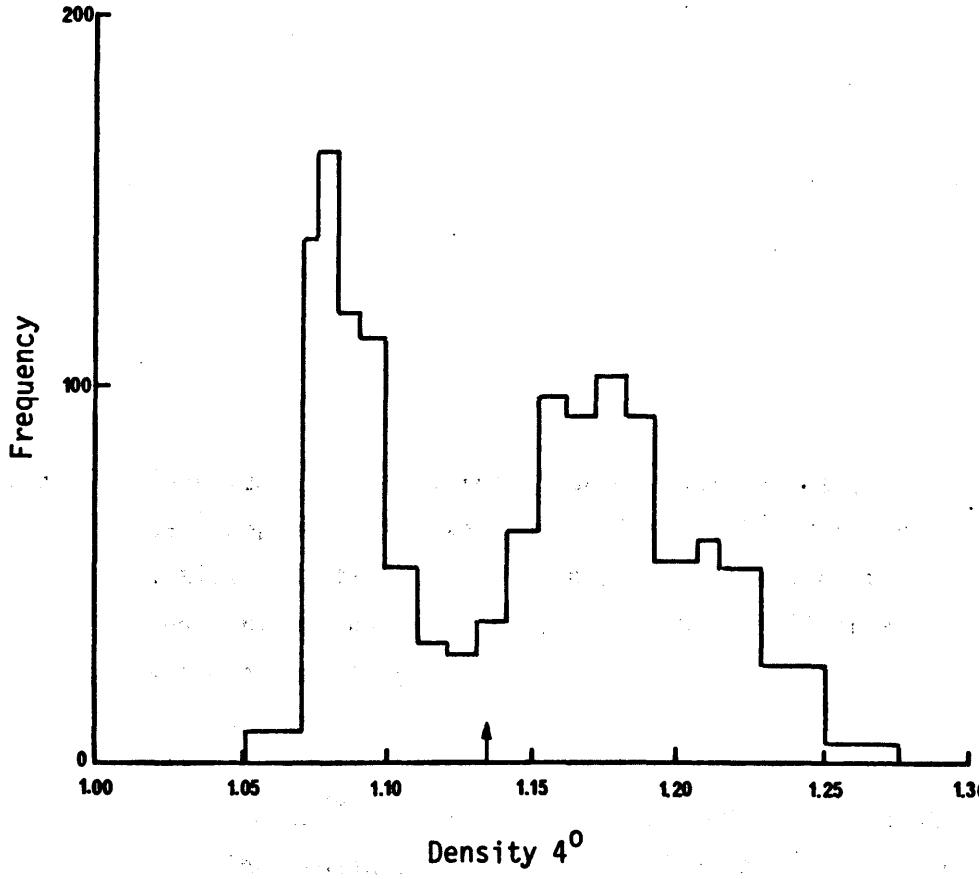


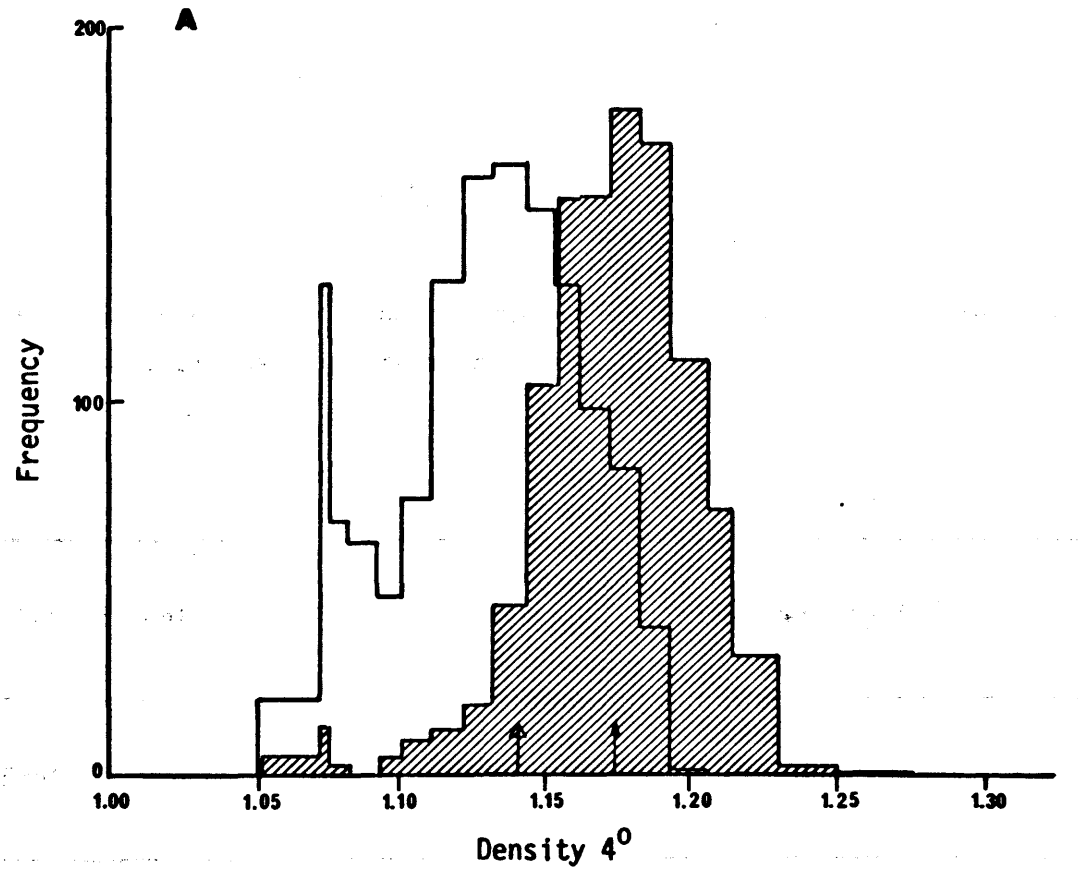
Fig. 3.64. The distribution of protein after the isopycnic equilibration of sedimentable material from the washes of Pl in a sucrose density gradient.

The median density of the distribution is indicated with an arrow.

Fig. 3.65. (overleaf) The distribution of basal ATPase, Ca^{2+}-dependent ATPase and succinate-INT reductase activities after the isopycnic equilibration of sedimentable material from the washes of P1 in a sucrose density gradient.

(A) The distributions of basal ATPase (unshaded area) and Ca^{2+}-dependent ATPase (shaded area) are shown. The median density of basal ATPase is indicated by a white arrow and that for Ca^{2+}-dependent ATPase by a black arrow.

(B) The distribution of succinate-INT reductase activity. The arrow indicates the median density of the distribution.


A
200
100
0
Frequency
1.00
1.05
1.10
1.15
1.20
1.25
1.30
Density 4^O

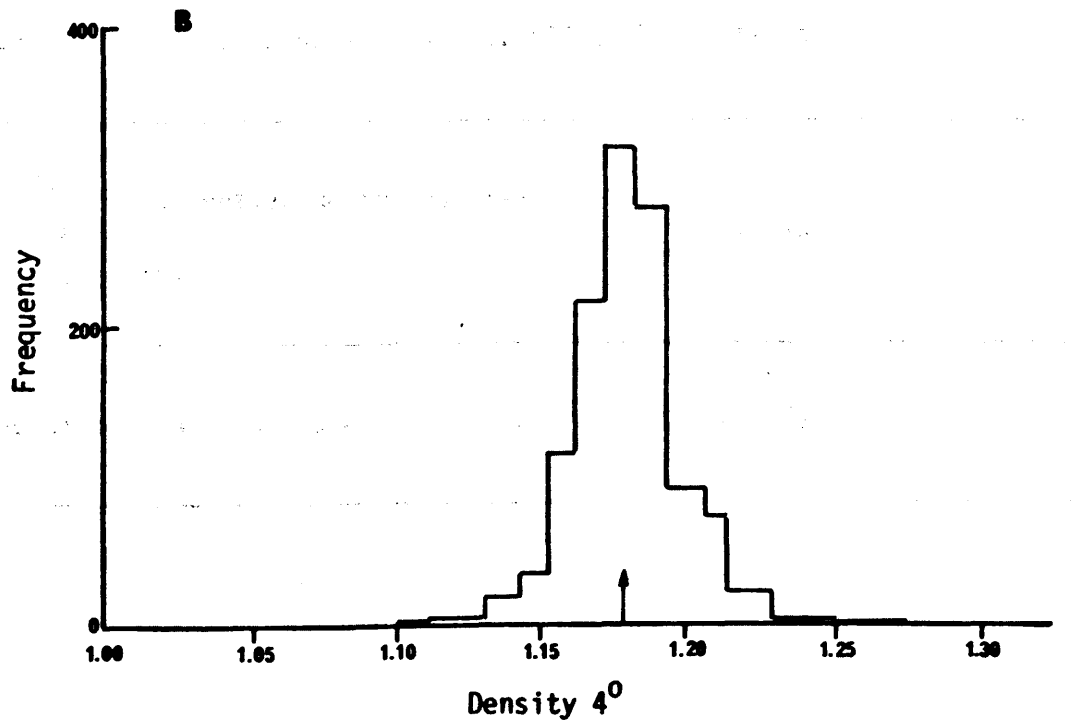

B
400
200
0
Frequency
1.00
1.05
1.10
1.15
1.20
1.25
1.30
Density 4^O

TABLE 3.14

The median densities of constituents after the isopycnic equilibration of sedimentable material from the washes of P1.

Constituent	Median Density (4^{o})
Protein	1·137
Ca^{2+}-dependent, azide-insensitive ATPase	1·174
Ca^{2+}-independent,azide insensitive ATPase = Basal ATPase	1·143
Succinate-INT reductase	1·179

1·179 g/ml. The median density of the overall distribution is 1·137 g/ml. Because of the presence of soluble protein this value is less than that obtained for a microsomal pellet (Section 3.5, Table 3.10).

The equilibrium distributions of basal ATPase and Ca^{2+}-dependent ATPase, assayed in the presence of 50 mM sodium azide, are plotted in Fig. 3.65 A. The two activities are separated. Ca^{2+}-dependent ATPase has a median density of 1·174, while that for basal ATPase is 1·143. In the calculation of the median density for basal ATPase, the peak of activity in the sample zone was disregarded as this was considered to be non-specific phosphatase activity associated with soluble protein. The median densities for both enzymes are in good agreement with the values obtained for a classical microsomal fraction (Section 3.5, Table 3.10).

The equilibrium distribution of succinate-INT reductase is a symmetrical one having a median density of 1·179 (Fig. 3.65 B).

The results given provide no evidence that microsomes found in P1 as a result of positional sedimentation are different from those in the $5·76 \times 10^5$ g-min supernatant.

Consideration will now be given to the material present in the washed P1 and constituting 74-77% of the total protein, phospholipid and cholesterol of the homogenate (Table 3.12). Fractionation of this material was undertaken by density gradient centrifugation. The separations were carried out in sucrose density gradients and in gradients supplemented by disaggregrating agents. The disaggregrating agents chosen were dioctyl sodium sulphosuccinate (=DOSSS) and dextran

sulphate. Both have been reported to dissociate clumped or aggregrated membrane material (Cline & Ryel, 1971; Wheeldon & Gan, 1971).

The washed pellet from 125 ml of homogenate was resuspended in 0·25 M sucrose, pH 7·4 to a final volume of 500 ml. The suspension was stirred at 4^{o} for 15 min and filtered through a stainless steel cloth (mesh size 30 x 30) to remove unfragmented muscle and connective tissue.

Three aliquots of the filtrate were taken and labelled A, B and C. A and B were treated with 0·000075% W/V DOSSS (= 75 µg DOSSS/100 ml) and 0·5% W/V dextran sulphate respectively, while C acted as a control. Treatment with these reagents consisted of incubation at 4^{o} for 30 min with stirring.

1 ml of treated samples were layered on 17 ml buffered sucrose density gradients supplemented with 0·000075% W/V DOSSS in the case of sample A and 0·5% W/V dextran sulphate in the case of sample B.

The gradients, which were formed in 23 ml centrifuge tubes (MSE No. 59209), were linear with volume, and ranged in density (4^{o}) from 1·044 to 1·255. A cushion consisting of 3 ml of 2·5 M sucrose (density 4^{o} = 1·316) was used.

Centrifugation was carried out at 30 000 rev/min for 3 hours at 4^{o}. After centrifugation the gradients were collected in approximately 1 ml fractions and the distribution of protein and density determined.

The results are plotted in the form of frequency histograms in Fig. 3.66, 3.67 and 3.68.

In the control 83·6% of the protein was recovered in the density (4^{o}) range 1·197 - 1·295. The remainder of the protein was found in the lighter regions of the gradient in three peaks: soluble protein peaking at a density (4^{o}) of 1·057 and two smaller peaks at densities of 1·103 and 1·171. The median density (4^{o}) of the overall protein distribution is 1·215 g/ml.

A similar distribution pattern was obtained for separations carried out in the presence of DOSSS. 85·1% of the protein was found in the density (4^{o}) range 1·197 to 1·289. The remainder of the protein consisted of nonsedimentable protein peaking at a density of 1·042 and slowly sedimenting protein in the density range 1·083 - 1·128.

Treatment with dextran sulphate results in a considerable change in the distribution pattern. In this case, 54·4% of the protein sediments to a high density region and the remainder was recovered in the sample region.

When the untreated and treated samples were allowed to stand overnight at 1^{o}, the material in the control or untreated sample and in the DOSSS-treated sample sedimented to the bottom of the test tube leaving behind a clear supernatant, which is termed the '1g for 10 hours supernatant'. In the dextran sulphate - treated sample an opaque viscous supernatant was found. These observations were confirmed by light scattering and protein measurements on the 1g supernatant from the three

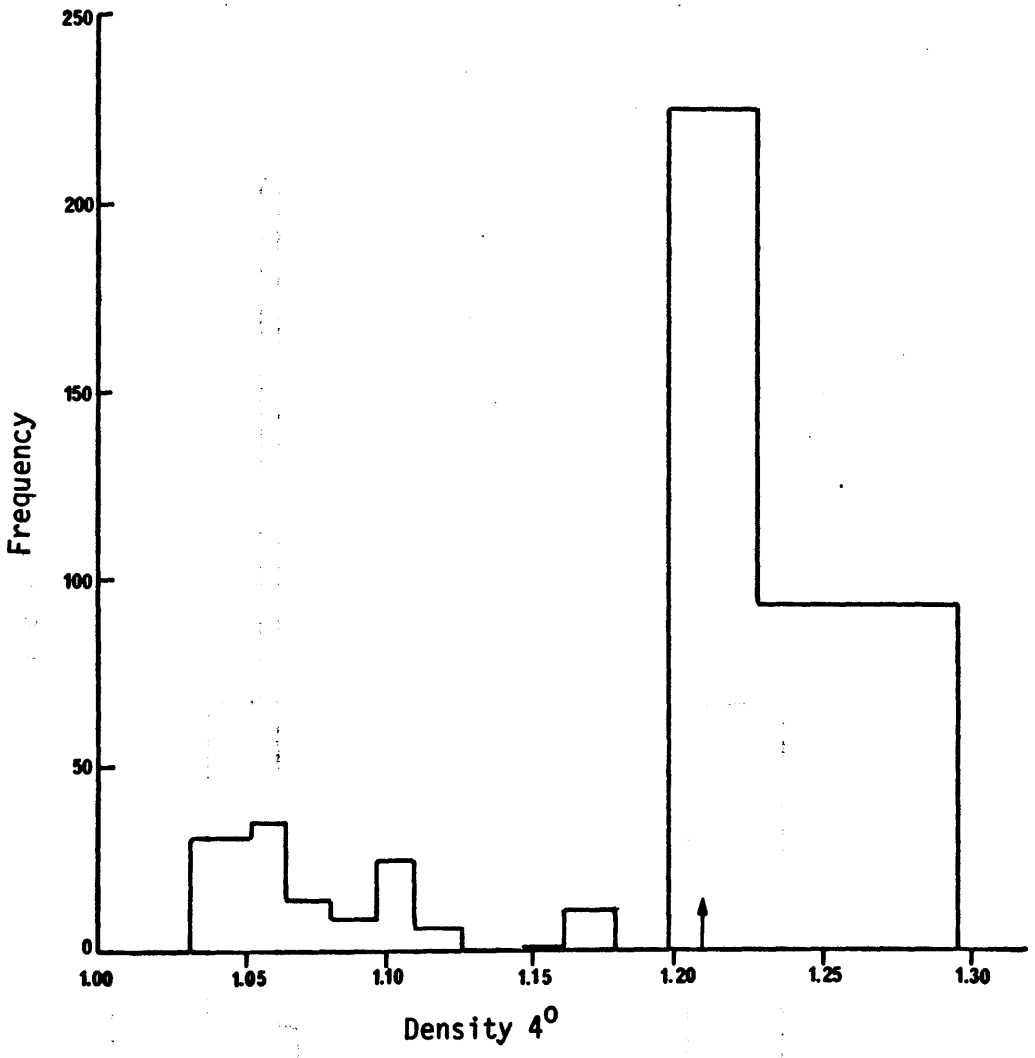


Fig. 3.66. The distribution of protein after a rate separation of the untreated washed P1 in a sucrose density gradient.

The median density of the distribution is indicated by an arrow.

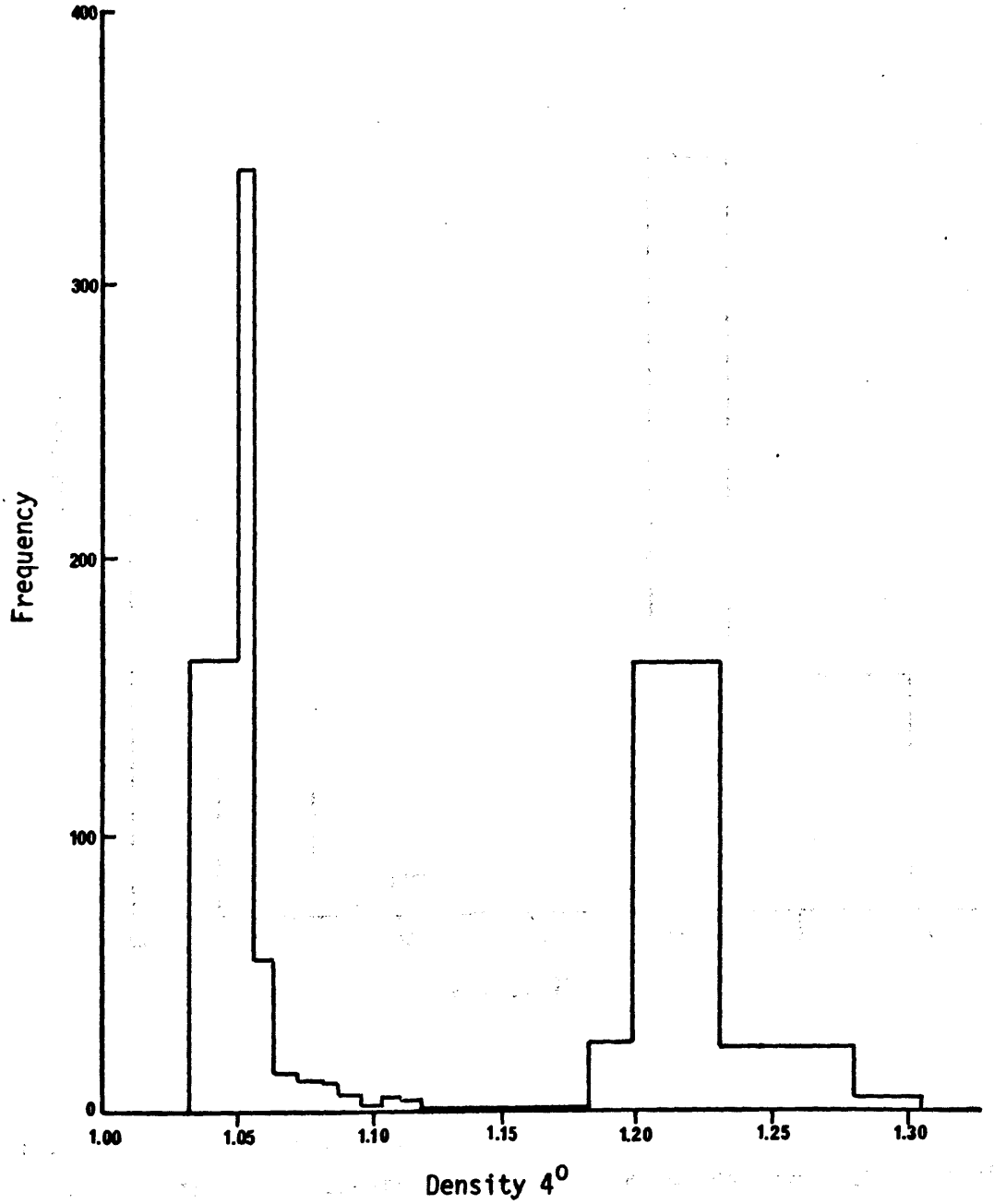


Fig. 3.67. The distribution of protein after a rate separation of the dextran sulphate-treated washed P1 in a sucrose density gradient supplemented with 0·5% W/V dextran sulphate.

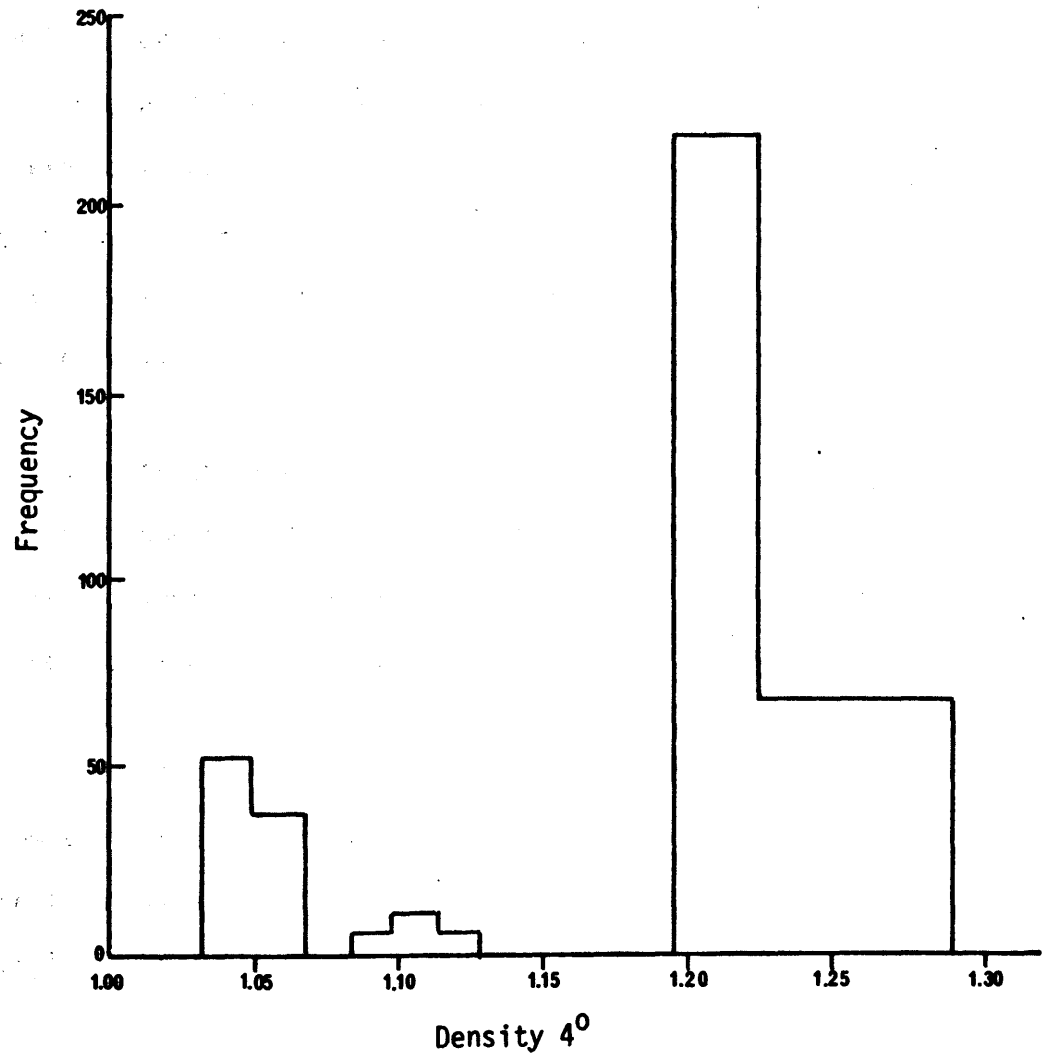


Fig. 3.68. The distribution of protein after a rate separation of the DOSSS-treated washed P1 in a sucrose density gradient supplemented with 0·000075% W/V DOSSS.

samples (Table 3.15). 79% of the protein in the dextran sulphate-treated sample was recovered in this supernatant as opposed to 8·3% and 9·3% for the control and DOSSS-treated samples. To determine whether the protein in the supernatant was soluble or particulate, the three supernatants were centrifuged at 100 000 g_{av} for 90 min. 77·1%, 78·5% and 68·1% of the protein was non-sedimentable in the 1g supernatant of the control, dextran sulphate-treated, and DOSSS treated material, respectively.

Density gradient centrifugation(approximately 75 000 g for 3 hours at the volumetric centre of the sample) of the 1g supernatant from the dextran sulphate-treated sample (Fig. 3.69) showed that the protein largely remained in the sample zone.

The pellets obtained after centrifugation of the 1g supernatants from the three samples at 100 000 g_{av} for 90 min were solubilised by treatment with SDS and the protein components separated by SDS-polyacrylamide gel electrophoresis.

A typical microsomal pattern was obtained for the DOSSS-treated and control pellets. The protein pattern for dextran sulphate-treated pellet contained only one band - the 100 000 component.

Electrophoretic analysis of the nonsedimentable protein in the 1g supernatant of the dextran sulphate-treated sample showed the presence of considerable amounts of myosin and actin.

The effect of dextran sulphate is seen as a solubilisation of contractile proteins and extrinsic membrane proteins. The solubilisation

TABLE 3.15

The effects of dextran sulphate and DOSSS (=dioctyl sodium sulphosuccinate) on the amount of protein sedimenting at 1g in the washed P1 fraction.

Treatment	Total protein mg/ml	1g for 10 hours supernatant		
		Protein mg/ml	Light scattering E660	Non-sedimentable* protein in supernatant mg/ml
Control	1·952 (100%)	0·161 (8·25%)	0·018	0·124 (6·34%)
Dextran sulphate-treated	1·952 (100%)	1·542 (79.01%)	0·050	1·211 (62·02%)
DOSSS-treated	1·952 (100%)	0·182 (9·33%)	0.018	0·124 (6·34%)

*not sedimenting after centrifugation at 100 000 g_{av} for 90 min.

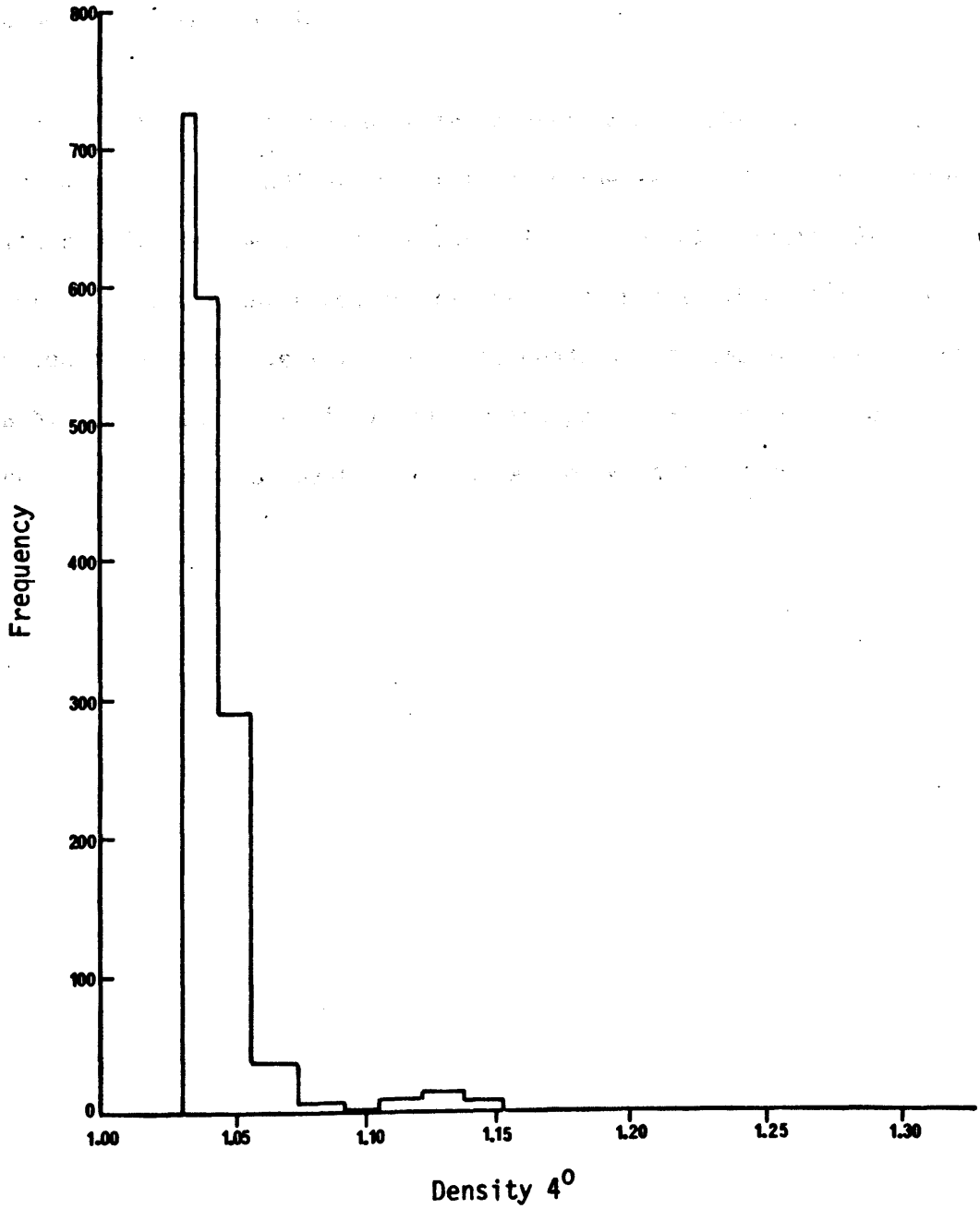


Fig. 3.69. The distribution of protein after centrifugation of the lg for 10 hours supernatant of the dextran sulphate-treated washed P1 in a sucrose density gradient, supplemented with 0·5% W/V dextran sulphate, for 3 hours at 30 000 rev/min.

of myosin and actin results in the increased viscosity of the dextran sulphate-treated sample.

The results obtained with the control and DOSSS-treated samples indicate that over 80% of the protein in the washed P1 is associated with rapidly sedimenting dense particles. The low quantity of protein in the lighter regions of the gradients is thought to represent residual entrapped vesicles and mitochondria not removed in the three washings. When the rapidly sedimenting particles were examined in the light microscope myofibrils and whole cells could be seen.

SECTION 4.

DISCUSSION

The initial step in tissue fractionation is homogenisation or rupturing of the tissue. With few exceptions (e.g. de Duve, 1967, 1971; Reid, 1967, 1971), little attention has been given to this important process.

The quality of a homogenate is best evaluated after fractionation, by a study of the distribution of marker constituents between the fractions. This has led de Duve (1967) to define the best homogenate as the one which lends itself most successfully to fractionation.

In the present study, homogenisation conditions were chosen to obtain a good yield of microsomal material with minimal loss of activity and minimum contamination by mitochondrial fragments and contractile proteins.

The choice of a mechanical device for homogenisation depends to an extent upon the nature of the tissue and, in particular, its connective framework. The Potter-Elvejhem homogeniser, which has been successfully used to homogenise liver, would be ineffective on a tough tissue such as muscle. Due to the toughness of muscle, the yield of membrane material in the microsomal fraction is low, even after a preliminary mincing of the tissue and homogenisation in a Waring blender for a relatively long time. The finding that 80-90% of the membrane material of the homogenate sediments in the P1 fraction, even after homogenisation for 120 sec, may reflect a difficulty in dislodging fragmented membranes from between the myofibrillar elements.

Palade & Siekevitz (1956) have found that the membrane systems of liver form rounded-off intact structures, which are termed vesicles or microsomes, by an active pinching-off process during homogenisation. Muscle membranes are thought to form vesicles by a similar process. Duggan & Martonosi (1970) have reported that muscle microsomes are impermeable to dextran (molecular weight = 15 000 - 90 000) and inulin (molecular weight = 5000) over the pH range 7·0 - 9·0, thus illustrating an effective resealing process.

During vesicle formation, membranes may fold in either of two directions: right-side-out (= RO) folding or inside-out (= IO) folding. Little information is available on whether this folding is random or entirely in one direction. Factors which may influence the folding in one or other direction are also unexplored, although in the case of the erythrocyte membrane, Steck et al. (1971) have developed procedures for the preparation of IO- or RO-vesicles. As regards muscle, RO-vesicles from the Ca^{2+}-transporting regions of the sarcoplasmic reticulum can be isolated after calcium oxalate loading (Section 3.4). Hannig & Heidrich (1974) have suggested that free-flow electrophoresis may be useful in the separation of RO- and IO-vesicles, provided that both sides of the membrane differ in surface charge.

The finding that muscle membrane systems are differentially fragmented during homogenisation is understandable in view of their cellular arrangement. With increasing homogenisation time, membranes containing basal ATPase and Ca^{2+}-transport ATPase sequentially appear, with high specific activity, in the microsomal fraction. With longer periods of homogenisation, a further group of membranes, not associated

with any of the activities assayed for, appear in the microsomal fraction.

While it was not investigated, homogenisation for longer than 120 sec would be expected to lead to a substantial increase in the mitochondrial contamination of the microsomal fraction, thereby complicating the analysis of the fraction.

The composition of media for homogenisation and density gradient centrifugation critically influences the aggregration of microsomal material. Aggregration of microsomes in unbuffered sucrose is attributed to a pH effect. Raising the pH to 7·0 with 1 mM imidazole reduced the aggregration, while readjusting the pH to 5·5 caused reaggregration. Dallner & Ernester (1968) have found that the pH-dependent aggregration of microsomes is considerably reduced in a concentrated suspension. This is probably due to the buffering action of the high protein concentration.

The extensive aggregration of muscle microsomes in the presence of monovalent and divalent cations is in agreement with the findings of Dallner & Nilsson (1966) in respect of liver microsomes. Wheeldon & Gan (1971), who have also observed these effects, found that clumping in microsomal preparations from muscle is greater than in comparable preparations from other tissues.

Baskin (1972) has shown that lobster muscle microsomes bear an overall negative surface charge, which he estimated to be in the order of 2×10^3 negative charges per vesicle. Cation-induced aggregration may result, therefore, from the interaction between cations and the negative charges on the vesicles.

In addition to causing aggregration, ionic media also extract myosin and actomyosin, which subsequently contaminate the microsomal fraction (Uchida et al., 1965). The use of ionic media, such as KCl (Sarzala et al., 1974) and LiBr (Meissner & Fleischer, 1971), and Ca^{2+}-chelators (Duggan & Martonosi, 1970; Sarzala et al., 1974) lead to the release of extrinsic membrane proteins from muscle microsomes.

In addition to causing microsomal aggregration, unbuffered media also effect the protein of the soluble phase. Anderson & Green (1967) have found that 24% of rat liver soluble phase nitrogen was precipitated between pH 5·0 and 5·5. Precipitation of this kind could seriously contaminate the microsomal fraction. Although unbuffered sucrose is unsuitable for density gradient centrifugation, Meissner (1975) has found that higher steady state concentrations of the ^{32}P-labelled intermediate of the Ca^{2+}-dependent ATPase can be obtained with microsomes prepared in unbuffered sucrose as opposed to sucrose buffered at pH 7·4 with 5 mM HEPES.

The effects observed after the incorporation of heparin into sucrose media support the earlier findings of Headon & Duggan (1973). These workers successfully used sucrose solutions supplemented with 50 IU heparin/ml to overcome the agglutination of muscle subcellular components. However, the usefulness of heparin is limited by its inhibitory effect on a number of microsomal enzymes, e.g. Ca^{2+}-uptake activity (Headon & Duggan, 1973).

Other results in this thesis have shown that separations in the presence of the detergent dioctyl sodium sulphosuccinate (=DOSSS) were no different

from those in the presence of buffered sucrose. Dextran sulphate (a polyanion), on the other hand, extracted considerable amounts of contractile proteins and removed all but the intrinsic protein from membranes.

Bearing all of these factors in mind, sucrose-5 mM imidazole-HCl, pH 7·4, is considered to be the medium of choice for homogenisation and density gradient centrifugation.

The preparation of muscle microsomes in the form of a concentrated suspension, according to the method of Headon & Duggan (1973), results in a partial subfractionation. Microsomal material sediments from the large volume of post $5{\cdot}76 \times 10^5$ g-min supernatant into the 100-120 ml gradient, which is formed from the original band of 1.0 M sucrose during centrifugation. Mixing during pumping and diffusion of soluble protein from the zonal supernatant during centrifugation result in the presence of up to 80% nonsedimentable protein in fraction 1, although, this can be removed by centrifugation of the diluted fraction at 100 000 g_{av} for 90 min.

In this method of preparation, neither basal ATPase or Ca^{2+}-dependent ATPase have attained an isopycnic density distribution. The median densities of Ca^{2+}-dependent ATPase (ρ= 1·127) and basal ATPase (ρ=1·135) are less than those determined in an isopycnic separation (basal ATPase, ρ=1·149; Ca^{2+}-dependent ATPase, ρ=1·172). The bimodal distribution of basal ATPase and cholesterol in this preparation, as opposed to a unimodal distribution in the isopycnic separation, would suggest that membranes containing these constituents are present in two sizes (i.e.

a group of larger vesicles which sediment more rapidly than a second group of smaller vesicles having the same density).

In the concentrated microsomal fractions, membranes containing Ca^{2+}-dependent ATPase and basal ATPase are partially separated. Basal ATPase activity (absolute and specific) peaks in fractions 1 and 4 while Ca^{2+}-dependent ATPase activity (absolute and specific) is greatest in fractions 2 and 3. The distributions of Ca^{2+}-uptake, Ca^{2+}-dependent ATPase, K^{+}-stimulated ATPase and the ^{32}P-labelled phosphoprotein intermediate parallel one another very closely. This is to be expected since Duggan (1968a, 1974) has presented evidence that the Ca^{2+}-transport enzyme requires K^{+} ions for maximum activity. The close correlation between the distribution of Ca^{2+}-dependent ATPase and the ^{32}P-labelled phosphoprotein is in agreement with the findings of Martonosi (1969b) and Makinose (1969) that the hydrolysis of ATP by the Ca^{2+}-dependent ATPase occurs through a phosphoprotein intermediate.

A peak of specific activity for Ca^{2+}-uptake of 2·0 - 2·5 μmole/mg protein per min is high compared to that obtained by other workers. Reported values for the specific activity of microsomes prepared by differential pelleting range from 0·5 - 2·0 μmole/mg protein per min.

The high specific activity in fraction 3 of the concentrated microsomal suspensions is thought to be the result of two factors: (a) the use of white skeletal muscle in the preparation, as opposed to both red and white muscle routinely used by most other workers in their preparations. The inclusion of red muscle in a preparation will lead to lower Ca^{2+}-uptake activity since Sreter (1969) has found that the specific activity

of Ca^{2+}-uptake in microsomes from red muscle is only 0·09 of that in microsomes from white muscle. (b) Vesicles in fraction 3 accumulating Ca^{2+} are partially separated from basal ATPase-containing membranes (peaking in fractions 1 and 4), mitochondrial fragments (peaking in fraction 4) and possibly other membranes.

A tentative calculation of the amount of mitochondrial protein in the concentrated microsomal fractions may be carried out. If the specific activity of succinate-INT reductase in a pure preparation of mitochondria was known, the amount of mitochondrial protein in each of the concentrated microsomal fractions could be calculated. The highest specific activity of succinate-INT reductase, measured in the studies described in this thesis, was one of 14·9 nmoles/mg protein per min in fraction 4 of a concentrated suspension prepared from the washes of P1. Since this fraction contained other components besides mitochondria (Section 3.6), a conservative estimate of 20 nmoles/mg protein per min may be taken as the succinate-INT reductase activity of mitochondria. Using this value, estimates of the content of mitochondrial protein in each fraction can be made. Of the total protein in fractions 1 to 5, 0·67%, 2·87%, 4·79%, 18·53% and 4·86% respectively, would be mitochondrial protein. These estimates should be regarded as maximum values since the true values are almost certainly lower.

The phospholipid/protein ratios of the concentrated microsomal fractions are within the range usually reported for mammalian microsomal preparations (Bretscher, 1973). The highest value which is one of 0·762, occuring in fraction 3, coincides with the highest specific

activity of all the activities associated with Ca^{2+}-transport. Phosphatidylcholine is the principal phospholipid class which is in agreement with the findings of other workers (Meissner & Fleischer, 1971; Fiehn et al., 1971; Takagi et al., 1971; Boland et al., 1974; Sarzala et al., 1974).

The cholesterol/phospholipid ratios for the concentrated microsomal fractions are lower than those reported for preparations obtained by differential pelleting (Martonosi, 1968b; Drabikowski et al., 1972; Marai & Kuksis, 1973). This is thought to be due to the failure by most workers to differentiate between red and white skeletal muscle. Red skeletal muscle microsomes have a higher cholesterol content than microsomes from white muscle (Headon, 1975). Also, the sarcoplasmic reticulum is less well developed in red muscle (Baskin & Deamer, 1969).

The distributions of cholesterol and Ca^{2+}-uptake, expressed in a variety of ways, do not parallel one another in the concentrated microsomal fractions. The coincidence of the highest specific activity of Ca^{2+}-uptake with the lowest cholesterol/phospholipid ratio suggested that cholesterol might not be associated with membranes transporting Ca^{2+}. A better correlation was found between the distributions of basal ATPase and cholesterol. Both constituents, expressed in either absolute or specific terms, peaked in the same fraction. Furthermore, the cholesterol/basal ATPase ratio did not change very much between the fractions. The ratio varied over a 1·28 fold range, as against a 3·72 fold range for the ratio of cholesterol/^{32}P-labelled intermediate.

A survey of the literature on cholesterol in muscle microsomal preparations has shown that, while all workers have found cholesterol in muscle microsomal preparations, some have proposed that it is not only a constituent of Ca^{2+}-transporting membranes but also plays an essential role in the transport process.

Martonosi (1968b) has reported on the effect of digitonin on the Ca^{2+}-uptake and ATPase activities of a microsomal preparation. With increasing concentration of digitonin (from 0 to 0·25% W/V), Ca^{2+}-uptake was inhibited and Ca^{2+}-dependent ATPase stimulated. From these results, Martonosi concluded that cholesterol was in some way involved in Ca^{2+}-transport.

The results presented in this thesis are in direct conflict with the conclusion of Martonosi. The action of digitonin, in inhibiting Ca^{2+}-transport and stimulating the transport ATPase, can be attributed to its detergent properties (Amar-Costesec et al., 1974b; Helenius & Simons, 1975) rather than to a reaction with cholesterol near the active site of the transport enzyme.

Fiehn & Hasselbach (1969) reported the increased Ca^{2+} permeability of a muscle microsomal preparation after extraction with ether. In a solution containing 7 to 8% V/V ether, Ca^{2+}-accumulation was totally abolished. Under these conditions the only lipids removed from the membranes were cholesterol esters. At this concentration of ether, the Ca^{2+}-dependent ATPase activity was stimulated, but at higher concentrations the enzyme was rapidly inactivated.

Basal ATPase, on the other hand, was insensitive to increasing ether concentration and even at an ether concentration of 20% V/V it retained full activity. On the basis of these results, Fiehn & Hasselbach postulated a role for cholesterol ester in the permeability of Ca^{2+}-accumulating vesicles.

Other workers, who have studied the effect of diethyl ether on muscle microsomes, have attributed the loss of Ca^{2+}-accumulation to the increased permeability of the vesicles (Inesi et al., 1967; Graeser et al., 1969b).

The effect of cholesterol and cholesterol ester extraction, on the enzyme activities of muscle microsomes, has been thoroughly investigated by Drabikowski et al., (1972a). In this case, lyophylised vesicles were extracted with dry diethyl ether or dry heptane in such a way that all of the cholesterol and most of the neutral lipid was removed. When this was done, there was no loss of Ca^{2+}-uptake or no effect on the Ca^{2+}-dependent ATPase. This work clearly demonstrated that cholesterol and cholesterol esters do not play a role in Ca^{2+}-transport.

The same authors, while believing that cholesterol is a constituent of Ca^{2+}-transporting vesicles, provided further evidence of its non-involvement in the transport process (Drabikowski et al., 1972b) by investigating the effect of filipin on Ca^{2+}-uptake and Ca^{2+}-dependent ATPase activities. Filipin is a polyene antibiotic which binds to cholesterol in membranes. After treatment with filipin, at concentrations which allow interaction with cholesterol only, Ca^{2+}-uptake and Ca^{2+}-dependent ATPase activities were not affected.

Further evidence that cholesterol is not essential for Ca^{2+}-transport has been provided by Fiehn & Hasselbach (1970). These workers found that muscle microsomes isolated from rats with 20, 25-diazocholesterol-induced myotonia contained a dehydrogenated and esterified product of the drug but had an essentially normal Ca^{2+}-uptake activity.

In Sections 3.4 and 3.5 two approaches were used to determine (a) if cholesterol was a constituent of Ca^{2+}-accumulating vesicles, and if not, (b) to determine which of the microsomal particles it was associated with. In these approaches, properties which are specific to Ca^{2+}-transport or cholesterol-containing vesicles were exploited.

1. The problems encountered in the purification of calcium oxalate loaded vesicles by density gradient centrifugation may originate from the very high density of the loaded vesicles. Electron micrographs (Mussini et al., 1972) show that calcium oxalate deposits occupy most of the lumen of loaded vesicles. These vesicles would therefore have a considerably greater dry weight than non-loaded vesicles. As an analogy, loaded vesicles may be compared to solid spheres as opposed to a balloon-type structure for non-loaded vesicles. When viewed in this way, the hydrodynamic instability of the sample used in the density gradient separation of loaded from non-loaded vesicles may be the reason for the contamination of the calcium oxalate loaded pellet with increasing sample concentration (Meuwissen, 1974). Only under very carefully controlled conditions can uncontaminated loaded vesicles be separated. These conditions were approximated to at the lower load factors in the experiments described in Section 3.4.

Under the ideal separatory conditions of an infinitely dilute sample, 18% of the phospholipid and 6% of the cholesterol of the sample would be found in the calcium oxalate loaded pellet. An interpretation of this data is made difficult by the ability of plasmamembrane vesicles to accumulate Ca^{2+} (Sulakhe et al., 1973). If a very small percentage of the phospholipid in the pellet originated from the plasmamembrane, it would account for all of the cholesterol in the pellet.

While plasmamembrane fragments are present in the microsomal fraction, as evidenced by the presence of ouabain-sensitive (Na^+ and K^+)-ATPase (Section 3.1), it is difficult to determine whether these fragments are in the form of vesicles capable of retaining transported Ca^{2+}.

The outer membrane of the muscle cell has a complex structure (Mauro & Adams, 1961; Franzini-Armstrong, 1973; Schapira et al., 1974) consisting of a number of layers: the innermost layer or plasmamembrane proper, which is in contact with the sarcoplasm; a basement membrane analogous to that covering epithelial cells, and a outer layer of collagen fibrils. This type of structure offers a high resistance to disruption. It is unlikely that plasmamembrane fragments would vesiculate while remaining attached to the basement membrane and collagen layer. Whether or not the homogenisation procedure used would release the plasmamembrane from the other layers and allow vesicle formation is not known. Without this crucial information, it is not possible to decide if intact plasmamembrane vesicles with the proper orientation for Ca^{2+} storage (i.e. RO-vesicles) would be found in the sample.

Overall, the preparation of calcium oxalate loaded vesicles demonstrates that the greater the purity of the Ca^{2+}-transporting regions of the sarcoplasmic reticulum the lower the cholesterol content.

The finding that 18% of the phospholipid is associated with vesicles accumulating Ca^{2+} is in good agreement with the results obtained from electron microscope studies by other workers (Hasselbach, 1963, 1964, 1966; Graeser et al., 1969a; Baskin & Deamer, 1969). After the incubation of a microsomal preparation in a calcium oxalate loading medium and examination in the electron microscope, these workers reported that only 15-20% of the vesicles were seen to have calcium oxalate deposits.

Attempts by other workers to purify calcium oxalate loaded vesicles by density gradient centrifugation have been considerably less successful than those described in this thesis. Meissner & Fleischer (1971) found that 50-75% of the protein was recovered in the loaded pellet, however, only 30% of the vesicles in the pellet contained calcium oxalate deposits.

Boland et al.,(1974) have prepared calcium oxalate loaded vesicles from 35 day old chickens by density gradient centrifugation. They found that vesicles not loading calcium oxalate and banding in the gradient were enriched 1·28 fold in cholesterol over vesicles in the pellet. In the separation described here in Section 3·4 the non-loaded vesicles were enriched 3·44 fold in cholesterol over the loaded vesicles.

The most successful density gradient separation reported by the other workers is that of Graeser et al.(1969a),who obtained a loaded

pellet in which 80% of the vesicles were loaded, the remainder being free of deposits.

The finding that only 18% of the phospholipid is associated with vesicles accumulating calcium oxalate raised the question of the origin of the remaining 82% of the phospholipid. At least some vesicles which do not load calcium oxalate contain the Ca^{2+}-transport enzyme, as evidenced by K^{+}-stimulated ATPase activity, and may represent vesicles which are unable to retain transported Ca^{2+} because of leakiness or inside-out folding. Vesicles containing an inactive Ca^{2+}-transport enzyme may also be present in the gradient. It is unlikely that vesicles not containing calcium oxalate for these reasons would account for all of the 82% of the phospholipid banding in the gradient. A proportion of the phospholipid is probably associated with membranes originating from regions of the sarcoplasmic reticulum (terminal cisternae and fenestrated collar) not transporting Ca^{2+} and from the T- system.

Baskin & Deamer (1969) have extensively used the electron microscope to study the structure of vesicles in microsomal preparations, which had been incubated in a calcium oxalate loading medium. The preparations were found to contain large and small vesicles. The small vesicles, which were the most abundant, were thought to be Ca^{2+}-transporting vesicles since some, but not all, contained calcium oxalate deposits. In contrast, none of the large vesicles were seen to contain calcium oxalate deposits. On the basis of comparative ultrastructural studies between the microsomes of heart and skeletal muscle, Baskin & Deamer postulated that the larger vesicles were derived from the T- system.

It is interesting to note that recent work by Joyce & Headon (1975) has shown that vesicles containing basal ATPase are larger than those containing the Ca^{2+}-dependent ATPase.

In the case of liver microsomes,other workers (Dallner & Ernester, 1968; Glaumann & Dallner, 1970) have shown that plasmamembrane vesicles are larger than those derived from the endoplasmic reticulum.

2. The second approach to the cholesterol problem involved a study of the effect of digitonin on the equilibrium density distribution of a number of microsomal constituents. Both basal ATPase and cholesterol were affected in an almost identical manner. This provided unambiguous evidence for the association of basal ATPase with a cholesterol-containing membrane, however,it does not mean that all cholesterol-containing membranes also contain basal ATPase. In fact, the evidence from the homogenisation studies (Sections 3.1 and 3.5) shows that there are at least two cholesterol-containing membranes. The increases in the median density of basal ATPase and cholesterol by 0·028 and 0·030 density units respectively, are similar to the values determined by Amar-Costesec et al. (1974b) for cholesterol (0·029), alkaline phosphatase (0·030), 5'-nucleotidase (0·035) and alkaline phosphodiesterase (0·028) in liver microsomes.

The median density of the Ca^{2+}-dependent ATPase distribution was not increased on digitonin treatment, in fact a slight negative shift was evident.

In their experiments, Amar-Costesec et al. (1974b) have concluded that membranes whose median density is not increased by digitonin treatment contain little or no cholesterol.

If the Ca^{2+}-dependent ATPase membranes contained 10 μg cholesterol/mg phospholipid, which is the cholesterol content of purified calcium oxalate loaded vesicles, the expected increase in density after digitonin treatment can be calculated. Assuming that cholesterol binds to digitonin on a mole to mole basis and that protein, phospholipid, cholesterol and digitonin have densities of 1·31, 0·97, 1·07 and 1·35 g/cm^3 respectively, an increase in particle density of 0·0025 density units would be expected. When compared to the negative shift of 0·0020 observed, this would indicate that cholesterol is more likely to be absent from the Ca^{2+}-transporting regions of the sarcoplasmic reticulum rather than present in low quantities.

Based on the findings presented in this thesis, skeletal muscle microsomes may be divided into two groups: group 1 are cholesterol-containing membranes; group 2 consists of membranes which do not contain cholesterol. Group 1 membranes may be sub-divided into those containing basal ATPase and those which are not associated with this enzyme.

The principal characteristics of membranes in each of the groups may be summarised as follows:

Group 1 (a): cholesterol-containing membranes associated with basal ATPase activity and having an isopycnic density of 1·15. These membranes are fragmented to microsomes early in homogenisation.

Group 1 (b): cholesterol-containing membranes not associated with either basal ATPase or Ca^{2+}-dependent ATPase. These membranes have an isopycnic density of 1·15 and are ruptured more slowly during homogenisation. They contain

a protein component with a molecular weight slightly greater than that of the 100 000 molecular weight Ca^{2+}-transport protein. Since the concentrated microsomal fractions were prepared from a 60 sec homogenate, the amount of this second cholesterol-containing membrane in the fractions would be small;

Group 2: membranes which do not contain cholesterol. These include vesicles derived from the Ca^{2+}-transporting regions of the sarcoplasmic reticulum and having an isopycnic density of 1·17.

Recent data from this laboratory (Joyce & Headon, 1975) has shown that group 1(a) vesicles and the Ca^{2+}-transporting vesicles in group 2 differ in size, in addition to particle density. Group 1(a) vesicles were found to sediment more rapidly in a sucrose density gradient than the Ca^{2+}-dependent ATPase vesicles in group 2. In other words, basal ATPase-containing vesicles are larger and have a lower isopycnic density than Ca^{2+}-transporting vesicles.

Mac Lennan (1970) has purified the Ca^{2+}-dependent ATPase from a differential pellet by a combination of deoxycholate and salt fractionation. The enzyme preparation obtained in this way consists largely of one protein component having a molecular weight of 102 000. It also contains phospholipid in amounts comparable to that in the microsomal pellet (i.e. 450 μg/mg protein).

Marai & Kuksis (1973) have examined the lipid composition of the parent membranes and the isolated ATPase and found that both had an essentially identical lipid composition. The ATPase contained 59·9 μg cholesterol/ mg protein compared to 60·3 μg cholesterol/mg protein

for the microsomal preparation. This data conflicts with the findings in this thesis. A consideration of the methodology involved in the preparation of the Ca^{2+}-dependent ATPase by the procedure of Mac Lennan (1970) may explain the differences. The isolation procedure consists of three steps. The microsomal proteins are first separated into two fractions by treatment with deoxycholate at a concentration of 0·1 mg/mg protein in 1M KCl. A large amount of protein is released leaving the ATPase in the insoluble fraction. In the second step, the enzyme is solubilised using higher concentrations of deoxycholate (0·5 mg deoxycholate/mg protein). Finally, the solubilised enzyme is purified by ammonium acetate fractionation.

Kirkpatrick et al. (1974) have shown that deoxycholate solubilises the protein, phospholipid and cholesterol of membranes. It is likely that a similar solubilisation would occur during the purification of the ATPase. If this is the case, it would result in a pooling of the phospholipid and cholesterol from the different types of membranes in the preparation. Afterwards, during ammonium acetate fractionation the enzyme would associate with a random sample of the solubilised lipid (assuming that interaction between the enzyme and lipid is non-specific). If this were to occur, the lipid composition of the isolated ATPase would be identical with that of the microsomal preparation.

Knowles & Racker (1975) have been able to reconstitute Ca^{2+}-accumulating vesicles from Mac Lennan's ATPase and a wide range of soya bean phospholipid. Warren et al. (1974a), have replaced the endogenous lipid of the ATPase with dioleoyl lecithin without loss of ATPase

activity. These investigators were also able to reconstitute Ca^{2+}-accumulating vesicles from the dioleoyl lecithin-substituted ATPase. These reports show that the phospholipid composition of the ATPase quantitatively reflects the phospholipid available in the medium during reconstitution and explain why the lipid composition of Mac Lennan's ATPase is almost identical with that of the microsomal preparation. Additional support for this conclusion comes from further work of Warren et al. (1974b, 1974c), in which they found a complete equilibration between exogenous lipid and the endogenous lipid of the ATPase in the presence of cholate. More recent work (Warren et al., 1975) has shown that when cholesterol is forced to interact directly with a number of membrane proteins (e.g. Ca^{2+}-dependent ATPase, mitochondrial ATPase, succinate dehydrogenase) it strongly inhibits their function. Furthermore, the structure of the membrane in the neighbourhood of the enzyme is such that cholesterol is excluded from the first shell of phospholipid surrounding the protein molecule (Warren et al., 1975).

Boland et al. (1974), and Sarzala et al. (1975), have studied the compositional and structural changes in microsomes prepared from the developing skeletal muscle of chicken and rabbit respectively. In the early stages of development, the skeletal muscle cell contains little or no internal membrane system. With development, considerably more internal organisation was observed. As the internal membrane system developed, the cholesterol/phospholipid ratio of the microsomal preparation decreased. Both groups of workers failed to correlate the decreasing cholesterol/phospholipid ratio with the development of the internal membrane system. The absence of cholesterol from group 2

membranes neatly explains the results obtained by both groups of investigators.

The involvement of cholesterol in the structure and function of membranes is currently under intensive investigation in several laboratories. Papahadjopoulos et al. (1973), regard the role of cholesterol as a stabilising one, by inhibiting structural changes in the membrane due to thermal or mechanical stresses. They suggest that, while cholesterol provides a stable membrane framework, it is excluded from highly functional areas. The absence of cholesterol from the Ca^{2+}-transporting regions of the sarcoplasmic reticulum would lend support to this postulate.

At a particular temperature the fatty alkyl chains of lipids in many membranes undergo a phase transition from an ordered gel state to a more disordered liquid crystalline state. The phase transition is dependent on the lipid composition of the membrane and affects its functional activities. At the transition temperature, a break in the Arrhenius plot of the activity of an enzyme located in the membrane occurs.

Cholesterol has been found to decrease the amount of energy taken up during the phase transition in both model (Ladbrooke et al., 1968; Hinz & Sturtevant, 1972) and biologically-occurring membranes (De Kruyff et al., 1972; Rottem et al., 1973). The phase transition was completely abolished in phosphatidylcholine liposomes containing 30-40 moles % cholesterol (De Kruyff et al., 1974). This effect has

also been observed in native membranes (De Kruyff, 1975). On the basis of these reports, a break in the Arrhenius plot of enzyme activities located in cholesterol-rich membranes would not be expected. Dermot Bradley of this laboratory has found (Bradley & Headon, 1975) that basal ATPase activity (which is located in a cholesterol-containing membrane) does not undergo a temperature-dependent phase transition. Ca^{2+}-transport activity, on the other hand, undergoes a sharp phase transition.

In addition to the lipid and enzyme differences described, the concentrated microsomal fractions have a heterogenous protein composition. A simple protein pattern was obtained for each fraction after one-dimensional SDS- electrophoresis in homogenous polyacrylamide gels. The separations obtained by other workers (Martonosi & Halpin, 1971; Mac Lennan et al., 1972; Meissner & Fleischer, 1971; Louis & Shooter, 1972; Sarzala et al., 1974) for unfractionated microsomes correspond to the summation of the bands obtained for the concentrated fractions. Mac Lennan et al. (1972, 1974),have extensively studied the protein composition of microsomes prepared by differential pelleting. In these studies, they have characterised seven protein components: a 102 000 molecular weight protein which is labelled with ^{32}P after preincubation of the microsomes with γ-^{32}P ATP; a 55 000 molecular weight or high affinity calcium binding protein; calsequestrin, a 46 500 molecular weight protein which also binds Ca^{2+}; low molecular weight acidic proteins having molecular weights of 20 000, 33 000 and 38 000; and finally, a proteolipid with an approximate molecular weight of 12 000.

The use of the more highly resolving two-dimensional separation has shown that many of the bands seen after the first-dimension separation are resolved into 2-5 components on the second-dimension separation in gradient gels. It is unlikely that this heterogeneity results from contaminating soluble protein or proteolysis. During the preparation of sedimentable material from each of the concentrated fractions, the pellets were washed three times with 0·25 M sucrose, pH 7·4. The solubilisation conditions were such (i.e. heating at 100^{o} in the presence of EDTA) that proteolysis would not occur (Juliano, 1973). Furthermore, quantitatively identical separations were obtained whether samples were solubilised by SDS-treatment at 30^{o} for 8 hours or by heating at 100^{o} for 5 min. This finding conflicts with that of Pucell & Martonosi (1972) who reported that prolonged incubation in the presence of SDS and β-mercaptoethanol lead to the breakdown of the 100 000 component to subunits. Others, Louis & Irving (1974) and Stewart & Mac Lennan (1974), have been unable to repeat the findings of Pucell & Martonosi. Sarzala et al. (1974), have found that after incubation for several days there was a partial dissociation of the 100 000 molecular weight component into subunits.

Mac Lennan (1974) has detected two proteins in the 46 500 molecular weight band after one-dimensional electrophoresis. This second protein found in the microsomes from some rabbits was found to be a mutant form of calsequestrin. In the separations shown in this thesis, there would appear to be even more than two proteins in this molecular weight range.

While several groups of workers (Migala et al., 1973); Thorley-Lawson & Green 1973; Stewart & Mac Lennan, 1974; Inesi & Scales, 1974; Louis et al., 1974) have investigated the effect of trypsin on the Ca^{2+}-dependent ATPase or 100 000 molecular weight component, Louis et al. observed that KCl influences the susceptibility of the ATPase to trypsin hydrolysis. At KCl concentrations greater than 50 mM, the ATPase polypeptide is hydrolysed at only one site. In the absence of KCl, many proteolytic fragments were formed. This observation is interesting in that it suggests that K^+ ions may interact with the Ca^{2+}-dependent ATPase producing a conformational change. This may be a possible mechanism for the K^+ stimulation of the Ca^{2+}-dependent ATPase (Duggan, 1967, 1968a,1974).

Margreth et al. (1974) have found that all the protein components of a microsomal preparation except the 100 000 component can be removed by osmotic shock and sonication. This is in agreement with Mac Lennan's classicication of muscle microsomal proteins. In this classification the proteins were divided into two groups:

(i) those which are water soluble after detachment from the membrane (=extrinsic proteins). All of the proteins except the 100 000 component were assigned to this group;

(ii) proteins which bind phospholipid and are water insoluble (=intrinsic proteins).

Margreth et al. (1974) also suggest that the 80 000 molecular weight component detected after SDS-electrophoresis represents the glycolytic enzyme, phosphofructokinase. This suggestion is based on two observations: (a) Coelectrophoresis of crystalline

phosphofructokinase with a microsomal preparation. The purified enzyme moved as a single band coincident with the 80 000 band. (b) 15% of the phosphofructokinase activity of the homogenate was recovered in the microsomal fraction. The specific activity of the enzyme in the fraction was three times greater than in the soluble phase.

In order to fully evaluate the suggestion of Margreth et al., two further items of information are required. An estimate of the relative contribution of phosphofructokinase protein to the total microsomal protein is needed to determine the amount of phosphofructokinase protein in the 80 000 molecular weight band. Secondly, a knowledge of the distribution of phosphofructokinase after the density gradient centrifugation of the microsomal fraction would be required to determine whether or not this enzyme is associated with a certain type of membrane. Since Margreth et al. have carried out neither of these investigations, further work is required to characterise the nature of the association, if any, between phosphofructokinase and the microsomal fraction.

While glycolytic enzymes are generally regarded as being located in the sarcoplasm (de Duve, 1971), Clarke & Masters (1973) have provided new evidence for the existence of a glycolytic complex. These workers have studied the boundry sedimentation of rat muscle aldolase, lactate dehydrogenase and pyruvate kinase under physiological conditions of pH and ionic strength. The sedimentation profiles for the three enzymes occurred in parallel. Evidence of this kind was used by the authors to postulate the existence of a multienzyme aggregrate.

The presence of periodic acid-Schiff staining material with an electrophoretic mobility close to that of the tracker dye was first reported by Martonosi & Halpin (1971). Material with these characteristics is also present in the concentrated microsomal fractions. It has been characterised in considerable detail by Mac Lennan et al. (1972). Their analyses have shown that it is a proteolipid with high affinity for phospholipid and accounts for 2-5% of the total protein in the microsomal pellet. Its actual molecular weight is approximately 12 500, although, the apparent molecular weight by SDS-electrophoresis is only 50% of this.

The advantages of using calcium oxalate loaded vesicles to study the disposition of protein in membranes transporting Ca^{2+} are weakened by the interference of calcium oxalate in the solubilisation process. Despite these difficulties, some useful information was obtained from these studies. The 100 000 molecular weight component was found to constitute 75-76% of the protein of the vesicles. Components in the 45 000-65 000 molecular weight range were partially released from the vesicles during the loading procedure. The extraction of these proteins by EDTA or EGTA was first reported by Duggan & Martonosi (1970) and confirmed by Mac Lennan et al. (1972) and Sarzala et al. (1974). The partial loss of these proteins during calcium oxalate loading did not result in vesicles losing their ability to retain transported Ca^{2+}. That these proteins can be released from the membrane into the medium may be an indication that they are located on the outside of the vesicle. However, the possibility that they are released from the inside of the membrane cannot be excluded since the vesicles are permeable to EGTA under the conditions

of calcium oxalate loading (Duggan & Martonosi, 1970).

Proteins in the molecular weight range 15 000 - 45 000 were more heavily iodinated by lactoperoxidase, relative to the amount of protein they contained, than were proteins in the 45 000 - 110 000 molecular weight range. The lower molecular weight proteins had a bound iodine/protein ratio of 2·13, as compared to one of 0·76 for the 100 000 molecular weight protein, indicating that the latter had less tyrosine and histidine residues available for labelling. The 100 000 molecular weight protein either has a lower content of these residues, or it is embedded in the membrane. In order to carry out its function, the protein should have access to both sides of the membrane and would, therefore, be a transmembrane protein.

There is increasing evidence that integral or intrinsic membrane proteins have an amphipathic structure in the native membrane, with one, or two in the case of a transmembrane protein, exposed hydrophilic segments and a hydrophobic segment embedded in the membrane (Singer, 1974).

Mac Lennan & co-workers (1972) have studied the location of proteins in membranes of the microsomal fraction prepared by differential pelleting. The use of a microsomal preparation which not only has a heterogenous membrane composition but, also, contains right-side-out, inside-out and leaky vesicles,considerably limits the value of the study. However, the results obtained are interesting. The 100 000 molecular weight component accounted for 45% of the protein and 44% of the protein-bound iodine. On the other hand, the lower molecular weight bands, of molecular weight 20 000 - 40 000, constituted 15% of the protein but had 41% of the bound iodine.

The location of membrane proteins in a microsomal preparation, similar to that of Mac Lennan et al., has been studied by Thorley-Lawson & Green (1973) using the lactoperoxidase system. As a control, these workers used a membrane preparation solubilised with 1% deoxycholate in which all of the protein was fully labelled. They compared the amount of labelled protein in the untreated vesicles with that in the control. All protein bands separated by electrophoresis were labelled, however, proteins in the 15000 - 30000 molecular weight range were labelled maximally relative to the control.. The iodination of the 100 000 molecular weight component was 30-40% of that in the control. The authors also investigated the accessibility of the ATPase to trypsin and lactoperoxidase. From the results, they believe that about half of the molecule is exposed on the outer surface of the membrane, while the remainder is embedded in the lipid bilayer. Hasselbach & Elfvin (1967) have specifically labelled the active site of the ATPase using Hg-phenylazoferritin and found, in the electron microscope, that all of the label was clustered at the outer surface of the membrane.

The 100 000 molecular weight protein is also present in vesicles not loading calcium oxalate, although the membranes in this case are heterogenous as evidenced by differences in the composition of light and heavy fractions prepared by zonal centrifugation. An 80 000 molecular weight protein component is present in high concentration in the light fraction, while the protein composition of the heavy fraction resembles that of the calcium oxalate loaded vesicles. The 80 000 molecular weight component has also been found in microsomes

prepared by differential pelleting for four other species besides the rabbit (Louis & Irving, 1974).

The widespread distribution of the 100 000 molecular weight component in muscle membranes suggests that a protein of this size and electrophoretic mobility may not be unique to the regions of the sarcoplasmic reticulum transporting Ca^{2+}.

Most intrinsic membrane proteins have transport functions and, for this reason, are thought to extend through the membrane. Bretscher (1971b) has calculated that if a 105 000 molecular weight membrane protein were an average globular protein it would have dimensions of 50Å X 50Å X 100Å. In any one dimension, it would be large enough to extend through a lipid bilayer of about 40Å. On the basis of these calculations, one may make a plausible suggestion that most transmembrane proteins would have a molecular weight in the region of 100 000. Indeed, the limited number of studies carried out on transport enzymes to date indicate that this may be the case.

The(Na^+ and K^+)-ATPase isolated from cardiac muscle by Pitts & Schwartz (1975) has a molecular weight of 94 000 as determined by SDS-electrophoresis and was found in association with a 58 000 molecular weight glycoprotein. The 94 000 molecular weight polypeptide was labelled with ^{32}P after incubation of the membrane with γ-^{32}P-labelled ATP in the presence of Na^+ and K^+. The enzyme isolated from other tissues was found to have similar molecular weight and other properties, e.g. the electric organ of Electrophorus electricus (Dixon & Hokin, 1974); beef brain (Uesugi et al., 1971) and dog kidney (Kyte, 1971).

Knauf et al. (1974) have determined the molecular weights of the phosphoprotein intermediates of the (Na^+ and K^+)-ATPase and Ca^{2+}-ATPase of human red cell ghosts to be 103 000 and 150 000 respectively. Somewhat similar work on the ATPases of the red cell membrane has been carried out by Drickamer (1975). Three phosphorylated intermediates of ATPases were found and their molecular weights determined as follows: Mg^{2+}-ATPase, 160 000; (Na^+ and K^+)-ATPase, 110 000; and Ca^{2+}-ATPase, 135 000.

Bastide et al. (1973) undertook a comparative study of the (Na^+ and K^+)-ATPase from guinea pig kidney and the Ca^{2+}-dependent ATPase from rabbit skeletal muscle. There was a considerable similarity at the site of covalent attachment of the phosphate group in both enzymes. The ^{32}P-labelled phosphoprotein intermediate in both cases had a molecular weight of approximately 100 000, although the Ca^{2+}-dependent ATPase had a marginally greater mobility.

It seems possible that the 100 000 molecular weight component, while it represents the Ca^{2+}-dependent ATPase in fractions with high Ca^{2+}-uptake activity, is not unique to the Ca^{2+}-transport system and may represent other transport enzymes in other fractions.

A good deal of information can be obtained on the protein composition of group 2 membranes transporting Ca^{2+} from a study of the protein composition of calcium oxalate loaded vesicles. However, the lack of an isolation procedure for group 1a and 1b membranes has hampered an investigation into their protein composition. There is no

information on the protein composition of basal ATPase-containing membranes in the literature and in fact this area has received little or no attention from other workers in the field.

A consideration of the protein composition of preparations enriched in basal ATPase can give tentative information on the possible protein composition of group 1a membranes. Examples of these preparations are: fractions 1 and 4 of the concentrated microsomal fractions (this thesis); vesicles not loading calcium oxalate (this thesis); the microsomal preparation from newly-born rabbits (Sarzala et al., 1975); and microsomes from red skeletal muscle (Sarzala et al., 1974).

The 80 000 molecular weight protein, present in high concentration in fraction 1 and in vesicles not loading calcium oxalate, is unlikely to be located in basal ATPase-containing membranes, since it is not present in fraction 4. Proteins in the 25 000 - 30 000 molecular weight range are present in high concentration both in the light fraction (gradient fraction 1) from the calcium oxalate loading experiment and in fractions 1 and 4, but only present in trace quantities in the heavy fraction from the loading experiment and in the loaded vesicles.

The 15-20% contamination of fraction 4 by protein of the inner mitochondrial membrane complicates the protein composition of the fraction. The protein composition of the mitochondrial inner membrane is a complex one. Capaldi (1974), using SDS-electrophoresis, has separated twenty-one protein bands ranging in molecular weight from 10 000 - 90 000.

The possibility of basal ATPase being associated with a 100 000 molecular weight protein is unlikely in view of the recent results of Sarzala et al. (1975). These authors found that the amount of 100 000 molecular weight protein in the microsomes of a 3 day-old rabbit was 0.24 of that in the adult microsomes, while the basal ATPase activity was seven times greater. Also, the Ca^{2+}-dependent ATPase activity in the microsomes of the 3 day-old rabbit was only 0·24 of that in the adult.

The microsomal fraction prepared from red muscle contains a greater proportion of T-system and sarcolemma fragments than an equivalent preparation from white muscle (Baskin & Deamer, 1969). Sreter (1969) found this was reflected in an 11·25 fold greater rate of Ca^{2+}-uptake in white muscle. Of more importance to this discussion is the 9 fold greater activity of basal ATPase in red muscle microsomes (Sreter, 1969). Despite the higher mitochondrial content (Baskin & Deamer, 1969), microsomes from red muscle would contain a higher proportion of basal ATPase membrane proteins. Sarzala et al. (1974) have found that the amount of 100 000 molecular weight protein is greatly reduced. Proteins of lower molecular weight were present in high quantities, in particular, a 32 000 molecular weight hydrophobic protein.

Any discussion on the possible protein composition of basal ATPase membranes would not be complete without a reference to the possible involvement of proteins with a molecular weight greater than 100 000. These proteins have been found by several workers (Louis & Shooter,

1972; Mac Lennan et al., 1972; Martonosi & Halpin, 1971; Thorley-Lawson & Green, 1973; Meissner & Fleischer, 1971) and in a number of species (Louis & Irving, 1974). Louis & Shooter (1972) have provided evidence from molecular weight and cross-linking studies suggesting that these proteins are aggregrates of the 100 000 molecular weight component. This evidence is strongly supported by the finding that these bands were labelled with ^{32}P after preincubation of the membranes with γ-^{32}P-ATP under Ca^{2+}-transport conditions (Louis & Shooter, 1972; Thorley-Lawson & Green, 1973).

SUMMARY

(1) The structure of muscle, the intracellular movements of Ca^{2+} ions, subcellular methodology and the structure and composition of membranes were reviewed.

(2) The effect of varying homogenisation time on the distribution of a number of constituents in the four classical subcellular fractions was investigated. Membranes containing basal ATPase and the Ca^{2+}-transport ATPase were differentially ruptured during homogenisation. A third class of membranes, not associated with either of these two enzyme activities, appeared in the microsomal fraction with homogenisation for longer times (90 and 120 sec).

(3) Factors to be considered in the choice of media for homogenisation and density gradient centrifugation were discussed.

Muscle microsomes aggregrated not only in an unbuffered sucrose medium, but also, in buffered sucrose media supplemented with monovalent and divalent cations. In the light of the results obtained, sucrose buffered with 5 mM imidazole-HCl, pH 7·4, appeared to be the medium of choice for muscle subcellular fractionation.

(4) Microsomes were prepared in the form of a concentrated suspension by zonal centrifugation. This method of preparation resulted in the collection of five fractions which differed in protein, enzyme and lipid content, and was equivalent to a partial subfractionation. Basal ATPase and cholesterol had similar distributions which were different from those of activities associated with Ca^{2+}-transport. Sedimentable material in the fraction with the highest Ca^{2+}-uptake

activity contained 762 µg phospholipid per mg protein, of which 65% was phosphatidylcholine.

The protein components in the sedimentable material of each fraction were solubilised by treatment with sodium dodecyl sulphate and separated by a highly resolving two-dimensional electrophoretic procedure,consisting of electrophoresis in one dimension in 5·2% polyacrylamide gels, followed by electrophoresis in a second dimension in 6-27% polyacrylamide gel gradients. The separations obtained confirmed the membrane heterogeneity indicated by the enzyme and cholesterol data.

(5) The purity of calcium oxalate loaded vesicles prepared by density gradient centrifugation was found to be critically dependent on the load applied to the gradient. Under ideal separatory conditions, only 18% of the phospholipid in fractions 2 and 3 of the concentrated microsomal suspension was associated with vesicles storing calcium oxalate. The cholesterol/phospholipid ratio in the loaded vesicles was approximately one third of that in the sample applied to the gradient. The possibility of vesicles from the cholesterol-rich sarcolemma also loading calcium oxalate,and thereby accounting for the cholesterol found in the calcium oxalate loaded pellet,was considered.

Since calcium oxalate loaded vesicles have defined permeability properties and right-side-out folding, they were considered suitable for a study of the distribution of the protein components in the membrane. Proteins on the external surface were labelled by a

lactoperoxidase-catalysed iodination. The 100 000 molecular weight Ca^{2+}-transport protein was found to be less extensively labelled, relative to the amount of protein it contained, than any of the other components.

(6) After equilibration of microsomes in a sucrose density gradient, Ca^{2+}-dependent ATPase, basal ATPase and cholesterol had median densities of 1·172, 1·149 and 1·151 respectively. Treatment of the microsomal preparation with digitonin altered the density distribution of basal ATPase and cholesterol in an identical fashion. The protein distribution showed a less marked change in median density, while the distribution of Ca^{2+}-dependent ATPase was largely unaffected. It was concluded that:

(i) basal ATPase is associated with a group of cholesterol-containing membranes;

(ii) the Ca^{2+}-transporting regions of the sarcoplasmic reticulum do not contain cholesterol.

(7) An investigation into the effect of varying homogenisation time on the enzyme and cholesterol content of the microsomal fraction provided evidence for the presence of an additional class of cholesterol-containing membranes which are not associated with basal ATPase.

(8) A fractionation of the particulate material sedimenting in the low speed pellet (= P1 fraction) was undertaken. This pellet contained 80-90% of the protein, phospholipid, and cholesterol of the homogenate.

Extensive washing of the pellet removed entrapped material, which accounted for 13% of the phospholipid of the homogenate. The washes contained soluble protein, mitochondria, contractile protein, and membranes containing the Ca^{2+}-dependent ATPase and basal ATPase which were similar in isopycnic density and protein composition to membranes in the classical microsomal fraction.

Material not removed from the Pl fraction by washing accounted for 74-77% of the protein, phospholipid and cholesterol of the homogenate and was found to consist of rapidly sedimenting particles,which banded at a density of 1·215 in a sucrose density gradient.

REFERENCES

Amar-Costesèc, A., Beaufay, H., Wibo, M., Thines-Sempoux, D., Feytmans, E., Robbi, M. & Berthet, J. (1974a) J. Cell Biol. 61, 201-212.

Amar-Costesec, A., Wibo, M., Thines-Sempoux, D., Beaufay, H. & Berthet, J. (1974b) J. Cell Biol. 62, 717-745.

Anderson, N.G. (1966) editor: National Cancer Institute Monograph 21, U.S., Department of Health, Education and Welfare.

Anderson, N.G. (1967) in:Methods of Biochemical Analysis (Glick, D., ed.) Vol. 15, pp.271-310, Interscience Publishers, New York, London and Sydney.

Anderson, N.G. & Green, J.G. (1967) in: Enzyme Cytology (Roodyn, D.B., ed.), pp.475-509, Academic Press, New York and London.

Anderson, N.G., Waters, D.A., Fisher, W.D., Cline, G.B., Nuley, C.E., Elrod, L.H. & Rankin Jnr., C.T. (1967) Anal. Biochem. 21, 235-252.

Andersson-Cedergren, E. (1959) J. Ultrastruct, Res. Suppl. 1, 5-191.

Bach, C. & Lewis, C.P. (1973) J. Physiol. (Lond.) 235, 477-492.

Baker, P.F. (1972) in: Metabolic Pathways (Hokin, L.E., ed.), Vol. 6, 3rd edn, pp.243-268, Academic Press, New York and London.

Barber, E.J. (1966) in: National Cancer Institute Monograph 21 (Anderson, N.G., ed.), pp.219-239, U.S. Dept. of Health, Education and Welfare.

Bartlett, G.R. (1959) J. Biol, Chem. 234, 466-468.

Baskin, R.J. (1972) Bioenergetics 3, 249-269.

Baskin, R.J. & Deamer, D.W. (1969) J. Cell Biol. 43, 610-617.

Bastide, F., Meissner, G., Fleischer, S. & Post, R.L, (1973) J. Biol, Chem. 248, 8385-8391.

Baudhuin, P., Evrard, P. & Berthet, J. (1967) J. Cell Biol. 32, 181-191.

Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M. & Berthet J. (1974a) J. Cell Biol. 61,188-200.

Beaufay, H., Amar-Costesec, A., Thines-Sempoux, D., Wibo, M., Robbi, M. & Berthet, J. (1974b) J. Cell Biol. 61, 213-231.

Bendall, J.R. (1952) Nature 170, 1058-1060.

Bendall, J.R. (1953) J. Physiol. 121, 232-254.

Bendall, J.R. (1958) Nature 181, 1188-1190.

Bendall, J.R. (1969) Muscles, Molecules and Movement, 1st edn., Heinemann Education Books Ltd., London.

Berg, H.C. (1969) Biochim. Biophys. Acta 183, 65-78.

Birbeck, M.S.C. & Reid, E. (1956) J. Biophys. Biochem. Cytol. 2, 609.

Birnie, G.D. & Harvey, D.R. (1968) Anal. Biochem. 22, 171-174.

Bishop, B.S. (1966) in: National Cancer Institute Monograph 21 (Anderson, N.G., ed.) pp. 175-188, U.S. Dept. of Health, Education and Welfare.

Boland, R., Martonosi, A & Tillack, T.W. (1974) J. Biol. Chem. 249, 612-623.

Bradley, D.P. & Headon D.R. (1975), in the press.

Bretscher, M.S. (1971a) J. Mol. Biol. 58, 775-781.

Bretscher, M.S. (1971b) J. Mol. Biol. 59, 351-357.

Bretscher, M.S. (1973) Science 181, 622-629.

Bruns, R.R. & Palade, G.E. (1968a) J. Cell. Biol. 37, 244-276.

Bruns, R.R. & Palade, G.E. (1968b) J. Cell Biol. 37, 277-299.

Bullock, G.R., Carter, E.E. & White, A.M. (1973) Biochim. Biophys. Acta 292, 350-359.

Burton, K. (1969) in: Data for Biochemical Research (Dawson, R.M.C., Elliot, D.C. & Jones, K.M. eds.), 2nd edn, 169-179, Oxford University Press.

Cabantchik, Z.I. & Rothstein, A. (1974) J. Membrane Biol. 15, 207-226.

Canonico, P.G. & Bird, J.W.C. (1970) J. Cell Biol. 45, 321-333.

Capalaldi, R.A. (1974) Arch. Biochem. Biophys. 163, 99-105.

Carroll, K.K. (1961) Nature 191, 377-378.

Clarke F.M. & Masters, C.J. (1973) Biochem. Biophys. Acta 327, 223-226.

Cline, G.B. & Ryel, R.B. (1971) in: Methods in Enzmyology (Jackoby, W.B., ed.) vol. 22, 168-204, Academic Press, New York and London.

Cline, G.B., Dagg, M.K. & Ryel, R.B. (1974) in: Methodological Developments in Biochemistry (Reid, E., ed.), 4, 39-46. Longman Group Ltd., London.

Dahl, J.L. & Hokin, L.E. (1974) Ann. Rev. Biochem. 43, 327-356.

Dallner, G. & Ernester, L. (1968) J. Histochem. Cytochem. 16, 611-632.

Dallner, G. & Nilsson, R. (1966) J. Cell Biol, 31, 181-193.

Davis, G.A. & Bloom, F.E. (1973) Anal. Biochem. 51, 429-435.

Deamer, D.W. & Baskin, R.J. (1969) J. Cell Biol. 42, 296-307.

de Duve, C.(1964) J. Theoret. Biol. 6, 33-59.

de Duve, C. (1967) in: Enzyme Cytology (Roodyn, D.B., ed.), 1-26, Academic Press Inc., New York and London.

de Duve, C. (1971) J. Cell Biol. 50, 20D-55D.

de Haan, E.J., Groot, G.S.P., Scholte, H.R., Tager, J.M. & Wit-Peeters, E.M. (1973) in: The Structure and Function of Muscle (Bourne, G.H., ed.), 3, 2nd edn., 417-469, Academic Press, New York and London.

de Kruyff, B., Demel, R.A. & van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 331-347.

de Kruyff, B., van Dijck, P.W.M., Demel, R.A., Schuijff, A., Brants, F. & van Deenan, L.L.M. (1974) Biochem. Biophys. Acta 356, 1-7.

Dixon, J.F. & Hokin, L.E. (1974) Arch. Biochem. Biophys. 163, 749-758.

Dobrota, M. & Reid, E. (1971) in: Separations with Zonal Rotors (Reid, E., ed.), Z-6·1 - Z-6.2, Wolfson Bioanalytical Centre, University of Surrey, Guildford, Surrey.

Drabikowski, W., Dominas, H. & Dabrowska, M. (1966) Acta Biochem. Pol. 13, 11-24.

Drabikowski, W., Sarzala, M.G., Wroniszewska, A., Lagwinska, E. & Drzewiecka, B. (1972a) Biochem, Biophys. Acta 274, 158-170.

Drabikowski, W., Lagwinska, E. & Sarzala, M.G. (1972b) Biochem. Biophys. Acta 291, 61-70.

Drickamer, L.K. (1975) J. Biol. Chem. 250, 1952-1954.

Duggan, P.F. (1967) Life Sci. 6, 561-567.

Duggan, P.F. (1968a) Life Sci. 7, 913-919.

Duggan, P.F. (1968b) Life Sci. 7, 1265-1271.

Duggan, P.F. (1971) Irish J. Med. Sci. 140, 71-78.

Duggan, P.F. (1974) Biochem. Soc. Trans. 2, 1380-1383.

Duggan, P.F. & Martonosi, A (1970) J. Gen. Physiol. 56, 147-167.

Ebashi, S., (1958) Arch. Biochem. Biophys. 76, 410-423.

Ebashi, S. (1960) J. Biochem. (Tokyo) 48, 150.

Ebashi, S (1961) J. Biochem. (Tokyo) 50, 236-244.

Ebashi, S & Lipmann, F. (1962) J. Cell Biol. 14, 389-400.

Eisenberg, B.R., Kuda, A.M. & Peter, J.B. (1974) J. Cell Biol. 60, 732-754.

El-Aaser, A.A. (1971) in: Separations with Zonal Rotors (Reid, E.,ed.), B-5.1 - B-5.2, Wolfson Bioanalytical Centre, University of Surrey, Guildford, U.K.

El-Aaser, A.A. (1973), Spectra 2000, 4, 185-189.

Emmelot, P. & Bos, C.J. (1962) Biochim. Biophys. Acta 59, 495-497.

Emmelot, P., Bos, C.J. Bendetti, E.L. & Rumke, P. (1964) Biochim. Biophys. Acta 90, 126-145.

Endo, M. (1964) Nature 202, 1115-1116.

Evans, W.H. (1974) Nature 250, 391-394.

Fairbanks, G., Steck, T. & Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.

Fanburg, B. & Gergely, J. (1965) J. Biol. Chem. 240, 2721-2728.

Fiehn, W. & Hasselbach, W. (1969) Eur. J. Biochem. 9, 574-578.

Fiehn, W. & Hasselbach, W. (1970) Eur. J. Biochem. 12, 375-379.

Fiehn, W., Peter, J.B., Mead, J.F. & Gan-Elepano, M. (1971) J. Biol. Chem. 246, 5617-5620.

Field, M.E., Leigh, O.C., Heim, J.W. & Drinker, C.K. (1934) Am. J. Physiol. 110, 174-181.

Fischman, D.A. (1973) in: The Structure and Function of Muscle (Bourne, G.H., ed.), 1, 2nd edn., 75-148, Academic Press, New York and London.

Folch, J., Lees, M. & Sloane-Stanley, G.H. (1957) J. Biol. Chem. 266, 497-509.

Franzini-Armstrong, C. (1973) in: The Structure and Function of Muscle (Bourne, G.H., ed.), 2, 2nd ed., 531-619, Academic Press, New York and London.

Glaumann, H. & Dallner, G. (1970) J. Cell Biol. 47, 34-48.

Gornall, A.G., Bardawill, C.J. & David, M.M. (1949) J. Biol. Chem. 177, 751-756.

Gould, R.P. (1973) in: The Structure and Function of Muscle (Bourne, G.H., ed.), 2, 2nd ed., 185-241, Academic Press, New York and London.

Graeser, M.L., Cassens, R.G., Hoekstra, W.G. and Briskey E.J. (1969a) J. Cell. Physiol. 74, 37-50.

Graeser, M.L., Cassens, R.G., Hoekstra, W.G. & Briskey, E.J. (1969b) Biochem. Biophys. Acta 193, 73-81.

Grotte, G. (1956) Acta Chir, Scand, Suppl. 211, 1-83.

Hannig, K. & Heidrich, H.G. (1974) in: Methods in Enzymology (Fleischer, S. & Packer, L., eds.), 31, 746-761, Academic Press, New York and London.

Hardwicke, P.M.D. & Green N.M. (1974) Eur. J. Biochem. 42, 183-193.

Hartman, G.C., Black, N., Sinclair, R. & Hinton, R.H. (1974) in: Methodological Developments in Biochemistry (Reid, E., ed.), 4, 93-102, Longman Group Ltd., London.

Hasselbach, W. (1963) Fortschr, Zool. 15, 1-91.

Hasselbach, W. (1964) Progr. Biophys. Mol. Biol. 14, 167-222.

Hasselbach, W. (1966) Ann. New York Acad. Sci. 137, 1041-1048.

Hasselbach, W. & Elfvin, L.G. (1967) J. Ultrastruct. Res. 17, 598-622.

Hasselbach, W. & Makinose, M. (1961) Biochem. Z. 333, 518-528.

Hasselbach, W. & Makinose M. (1963) Biochem. Z. 339, 94-111.

Hasselbach, W. & Weber, H.H. (1953) Biochim. Biophys. Acta 11, 160.

Headon, D.R. (1972) Ph.D. Thesis, The National University of Ireland.

Headon, D.R. (1975), personal communication of unpublished results.

Headon, D.R. & Duggan, P.F. (1972) Biochem. Soc. Trans, 1, 294-297.

Headon, D.R. & Duggan, P.F. (1973) Spectra 2000, 4, 175-183.

Heilbrunn, L.V. & Wiercinski, F.J. (1947) J. Cell. Comp. Physiol. 29, 15-32.

Helenius, A. & Simons, K. (1975) Biochim. Biophys. Acta 415, 29-79.

Henderson, J.F. & Paterson, A.R.P. (1973) Nucleotide Metabolism, Academic Press, New York and London.

Hinton, R.H. & Dobrota, M. (1969) Anal. Biochem. 30, 99-110.

Hinton, R.H. & Norris, K. A. (1972) Anal. Biochem. 48, 247-258.

Hinton, R.H. Burge, M.L.E. & Hartman, G.C. (1969) Anal. Biochem. 29, 248-256.

Hinz, H.J. & Sturtevant, J.M. (1972) J. Biol. Chem. 247, 3697-3699.

Hokin, L.E. & Dahl, J.L. (1972) in: Metabolic Pathways (Hokin, L.E., ed.), 6, 3rd edn., 269-315, Academic Press,New York and London.

Hooke, R. (1665) Micrographia, London.

Huxley, H.E. (1964) Nature 202, 1067-1071.

Inesi, G. (1972) Ann. Rev. Biophys. Bioeng. 1, 191-210.

Inesi, G. & Scales, D. (1974) Biochemistry 13, 3298-3306.

Inesi, G., Goodman, J.J. & Watanabe, S. (1967) J. Biol. Chem. 242, 4637-4643.

Jacques, P.J. (1974) in: Methodological Developments in Biochemistry (Reid, E., ed.), 4, 145-156, Longman Group Ltd., London.

Jennings, M.A., Marchesi, V.T. & Florey, H. (1962) Proc. R. Soc. Lond. B. Biol. Sci. 156, 14-19.

Joyce, N. & Headon, D.R. (1975) Biochem. Soc. Trans. 3, in the press.

Juliano, R.L. (1974) Biochim. Biophys. Acta 300, 341-378.

Juliano, R.L. & Behar-Bannelier, M. (1975) Biochim. Biophys. Acta 375, 249-267.

Kamath, S.A. & Narayan, K.A. (1972) Anal. Biochem. 48, 53-61.

Karnovsky, M.J. (1968) J. Gen. Physiol. 52, 64S-95S.

Karnovsky, M.J. (1970) in: Capillary Permeability (Crone, C. & Lassen, N.A., eds.), Alfred Benzon Symposium 2, 341-350, Academic Press, New York.

Kirkpatrick, F.H., Gordesky, S.E. & Marinetti, G.V. (1974) Biochim. Biophys. Acta 345, 154-161.

Kitchin, F.D. (1965) Proc. Soc. Exp. Biol. 119, 1153.

Knauf, P.A., Proverbio, F. & Hoffman J.F. (1974) J. Gen. Physiol. 63, 324-336.

Knowles, A.F. & Racker, E. (1975) J. Biol. Chem. 250, 3538-3544.

Krebs, H.A. & Henseleit, K..(1932) Hoppe-Seyler's Z.T. Physiol. Chem. 210, 33-36.

Kreibich, G. & Sabatini, D.D. (1974) J. Cell Biol. 61, 789-807.

Kumagai, H., Ebashi, S. & Takeda, F. (1955) Nature 176, 166.

Kyte, J. (1971) J. Biol. Chem. 246, 4157-4165.

Ladbrooke, B.D., Williams, R.M. & Chapman, D. (1968) Biochim. Biophys. Acta 150, 333-340.

Landis, E.M. & Pappenheimer, J.R. (1963) in: Handbook of Physiology (Hamilton, W.F. & Dow, P., eds.), 2(2) 961-1034, Amer. Physiol. Soc. Washington, D.C.

Leighton, F., Poole B., Beaufay, H., Baudhuin, P., Coffey, J.W., Fowler, S. & de Duve, C. (1968) J. Cell Biol. 37, 482-513.

Leskes, A., Siekevitz, P. & Palade, G.E. (1971a) J. Cell Biol. 49, 264-287.

Leskes, A., Siekevitz, P.& Palade, G.E. (1971b) J. Cell Biol. 49, 288-302.

Loftfield, R.B. & Eigner, E.A. (1960) Biochem. Biophys. Res. Commun. 2, 72-76.

Louis, C.F. & Irving, I, (1974) Biochem. Biophys. Acta 365, 193-202.

Louis, C. & Shooter, E.M. (1972) Arch. Biochem. Biophys. 153, 645-655.

Louis, C.F., Buonaffina, R. & Binks, B. (1974) Arch. Biochem. Biophys. 161, 83-92.

Lowry, R.R. (1968) J. Lipid Res. 9, 397.

Lowry, O.H. & Lopez, J.A. (1946) J. Biol. Chem. 162, 421.

Lowry, O.H. Rosenbrough, N.J. Farr, A.L. & Randall R.J. (1951) J. Biol. Chem. 193, 265-275.

Luzio, J.P., Newby, A.C. & Hales, C.N. (1974) Biochem. Soc. Trans. 2, 1385-1386.

Mac Lennan, D.H. (1970) J. Biol. Chem. 245, 4508-4518.

Mac Lennan, D.H. (1974) J. Biol. Chem. 249, 980-984.

Mac Lennan, D.H. & Wong, P.T.S. (1971) Proc.Natl. Acad. Sci. USA 68, 1231-1235.

Mac Lennan, D.H., Ostwald, T.J. & Stewart P.S. (1974) Ann. New York Acad. Sci. 227, 527-536.

Mac Lennan, D.H., Yip, C.C., Iles, G.H. & Seeman, P. (1972) Cold Spring Harbor Symp. Quant, Biol. 37, 469-477.

Maddy, A.H. (1974) in: Methodological Developments in Biochemistry (Reid, E., ed.), 4, 383-391, Longman Group Ltd.,London.

Makinose, M. (1969) Eur. J. Biochem. 10, 74-82.

Marai, L. & Kuksis, A. (1973) Can. J. Biochem. 51, 1248-1261.

Margolis, J. (1969) Anal. Biochem. 27, 319-322.

Margolis, J. (1973) Lab. Practice 22, 107-109.

Margolis, J. & Kenrick, K.G. (1969) Nature 221, 1056-1057.

Margreth, A., Carraro, U. & Salviati, G. (1974) Biochem. J. 139, 509-513.

Marsh, B.B.(1951) Nature 167, 1065-1066.

Marsh, B.B. (1952) Biochim. Biophys. Acta 9, 247-260.

Martonosi, A. (1968a) J. Biol. Chem. 243, 71-81.

Martonosi, A. (1968b) Biochim. Biophys. Acta 150, 694-704.

Martonosi, A. (1969a) Biochem. Biophys. Res. Commun. 36, 1039-1044.

Martonosi, A. (1969b) J. Biol. Chem. 244, 613-620.

Martonosi, A (1972a) in: Metabolic Pathways (Hokin, L.E., ed.), 3rd edn., 6, 317-349, Academic Press,London and New York.

Martonosi, A. (1972b) in: Current Topics in Membranes and Transport (Bronner, F. & Kleinzeller, A., eds.), 3, 83-197, Academic Press, London and New York.

Martonosi, A. & Halpin, R.A. (1971) Arch. Biochem. Biophys. 144, 66-77.

Marzban, M.T. & Hinton, R.H. (1970) Eur. J. Biochem. 12, 360-361.

Mauro, A. & Adams, W.R. (1961) J. Biophys. Biochem. Cytol. 10, 177-185.

Meissner, G. (1975) Biochim. Biophys. Acta 389, 51-68.

Meissner, G. & Fleischer S. (1971) Biochim. Biophys. Acta 241, 356-378.

Meissner, G., Conner, G.E. & Fleischer, S. (1973) Biochim. Biophys. Acta 298, 246-269.

Merz, D.C., Good, R.A. & Litman, G.W. (1972) Biochem. Biophys. Res. Commun. 49, 84-91.

Meuwissen, J.A.T.P. (1973) Spectra 2000, 4, 21-31.

Meuwissen, J.A.T.P. (1974) personal communication.

Migala, A., Agostini, B. & Hasselbach, W. (1973) Z. Naturforsch 28, 178-182.

Morre, D.J., Yunghans, W.N., Vigil, E.L. & Keenan, T.W. (1974) in: Methodological Developments in Biochemistry (Reid, E., ed.), 4, 195-236, Longman Group Ltd., London.

MSE Technical Publications No. 49 & 57, Measuring & Scientific Equipment Ltd., Manor Royal, Crawley, Sussex, U.K.

Muscatello, U., Andersson-Cedergren, E., Azzone, G.F., & von der Decken, A. (1961) J. Biophys. Biochem. Cytol. 10, 201-218.

Mussini, I., Margreth, A. & Salviati, G. (1972) J. Ultrastruct. Res. 38, 459-465.

Nagai, T., Uchida, K. & Yasuda, M. (1962) Biochim. Biophys. Acta 56, 205-215.

National Cancer Institute Monograph 21 (Anderson, N.G., ed.), 1966. U.S. Department of Health, Education and Welfare.

Needham, D.M. (1973) in: The Structure and Function of Muscle (Bourne, G.H., ed.), 3, 2nd edn., 363-415, Academic Press, New York and London.

Oldfield, E. & Chapman, D. (1972) FEBS Lett. 23, 285-297.

Ostwald, T.J. & Mac Lennan, D.H. (1974) J. Biol. Chem. 249, 974-979.

Page, S.G. (1964) J. Physiol. (London) 175, 10p-11p.

Palade, G.E. (1961) Circulation 24, 368-384.

Palade, G.E. & Siekevitz, P. (1956) J. Biophys. Biochem. Cytol. 2, 171-191.

Pang, D.C. & Briggs, F.N. (1973) Biochemistry 12, 4905-4911.

Papahadjopoulos D., Cowden, M. & Kimelberg, H. (1973) 330, 8-26.

Pappenheimer, J.R. (1953) Physiol. Rev. 33, 387-423.

Patterson, B.D., Atkins, C.A., Graham, D. & Wills, R.B.H. (1971) Anal. Biochem. 44, 388-391.

Peachey, L.D. (1965) J. Cell Biol. 25, 209-232.

Peachey, L.D. & Schild, R.F. (1968) J. Physiol. (London) 194, 249-258.

Pennington, R.J. (1961) Biochem. J. 80, 649-654.

Perry, S.V. (1974) in: Calcium and Cell Regulation (Smellie, R.M.S. ed.), 115-132, The Biochemical Society, London.

Phillips, M.C. (1972) in: Progress in Surface and Membrane Science (Danielli, J.F., Rosenberg, M.D. & Cadenhead, D.A., eds.), 5, 139-221, Academic Press, New York.

Phillips, D.R. & Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284-289.

Pitts, B.J.R. & Schwartz, A. (1975) Biochim. Biophys. Acta 401, 184-195.

Porteous, J.W. & Clark, B.J. (1965) Biochem. J. 96, 159-171.

Porter, K.R. & Palade, G.E. (1957) J. Biophys. Biochem. Cytol. 3, 269-300.

Portzehl, H. (1957) Biochim. Biophys. Acta 26, 373-377.

Pucell, A.G. & Martonosi, A. (1973) Arch. Biochem. Biophys. 151, 558-564.

Reid, E. (1967) in: Enzyme Cytology (Roodyn, D.B., ed.), 321-406, Academic Press, London and New York.

Reid, E. (1971) editor: Separations with Zonal Rotors, published by Wolfson Bioanalytical Centre, University of Surrey, Guildford, U.K.

Reid, E. (1973a) editor: Methodological Developments in Biochemistry, 2, Longman Group Ltd., London.

Reid, E. (1973b) editor: Methodological Developments in Biochemistry, 3, Longman Group Ltd., London.

Reid, E. (1974) editor: Methodological Developments in Biochemistry, 4, Longman Group Ltd., London.

Reid, E. & Williamson, R. (1974) in: Methods in Enzymology (Fleischer, S. & Packer, L., eds.), 31, 713-733, Academic Press, New York and London.

Retzius, G. (1881) Biol. Untersuch, 1, 1-26.

Ringer, S. (1883) J. Physiol. (London) 4, 29-42.

Rottem, S., Cirillo, V.P., de Kruyff, B., Shinitsky, M. & Razin, S. (1973) Biochim. Biophys. Acta 323, 509-519.

Sarzala, M.G. Zubrzycka, E. and Drabikowski, W. (1974) in: Calcium Binding Proteins (Drabikowski, W., Strzelecka-Golaszewska, H. & Carafoli, Ed., eds.), 315-346, Elsevier Scientific Publishers and Polish Scientific Publishers.

Sarzala, M.G.,Pilarska, M., Zubrzycka, E. & Michalak, M. (1975) Eur. J. Biochem. 57, 25-34.

Schapira, G., Dobocz, I., Piau, J.P. & Delain, E. (1974) Biochim. Biophys. Acta 345, 348-358.

Schleiden, M.J. & Schwann, T. (1839) "Beitrage zur Phytogenesis".

Sheridan, J.W., Kenrick, K.G. & Margolis, J. (1969) Biochem. Biophys. Res. Commun, 35, 474-477.

Simionescu, N., Simionescu, M. & Palade, G.E.(1973) J. Cell Biol. 57, 424-452.

Simionescu, N., Simionescu, M. & Palade, G.E. (1975) J. Cell. Biol. 64, 586-607.

Singer, S.J. (1974) Ann. Rev. Biochem. 43, 805-833.

Sreter, F.A.(1969) Arch. Biochem. Biophys. 134, 25-33.

Stagni, N. & de Bernard, B. (1968) Biochim. Biophys. Acta 170, 129-139.

Stauber, W.T. & Bird, J.W.C.(1974) Biochim. Biophys. Acta 338, 234-245.

Steck, T.L. (1974) J. Cell Biol. 62, 1-19.

Steck, T.L. Fairbanks, G. & Wallach, D.F.H. (1971) Biochemistry 10, 2617-2624.

Stewart, P.S. & Mac Lennan, D.H. (1974) J. Biol. Chem. 249, 985-993.

Sreter, F.A. (1969) Arch. Biochem. Biophys. 134, 25-33.

Sulakhe, P., Drummond, G.I. & Ng, D.C. (1973) J. Biol. Chem. 248, 4150-4157, 4158-4162.

Takagi, A. (1971) Biochim. Biophys. Acta 248, 12-20.

Thines-Sempoux, D. (1974) in: Methodological Developments in Biochemistry (Reid, E., ed.), 4, 157-166, Longman Group Ltd., London.

Thines-Sempoux, D., Amar-Costesec, A., Beaufay, H. & Berthet, J. (1969) J. Cell Biol, 43, 189-192.

Thorley-Lawson, D.A. & Green, N.M. (1973) Eur. J. Biochem. 40, 403-413.

Tolbert, N.E. (1974) in: Methods in Enzymology (Fleischer, S. & Packer, L., eds.), 31, 734-746, Academic Press, New York and London.

Uchida, K., Mommaerts, W.F.H.M. & Meretsky, D. (1965) Biochim. Biophys. Acta 104, 287-289.

Uesugi, S., Dulak, N.C., Dixon, J.F., Hexum, T.D., Dahl, J.L., Perdue, J.F. & Hokin,L. E. (1971) J. Biol. Chem. 246, 531-543.

van Deenen, L.L.M. (1965) in: Progress in the Chemistry of Fats and Related Lipids (Holman, R., ed.) 8, Pergamon Press, Oxford.

van Deenen, L.L.M. & Gier, J. (1964) in: The Red Blood Cell (Bishop, C. & Surgenor, D.M., eds.), 234-307, Academic Press, New York and London.

Veratti, E. (1902) Mem. Ist. Lombardo Sci. Lettere 19, 87-133.

Virchow, R. (1885) Arch. Pathol. Anat. Physiol. 8, 23.

Wallach, D.F.H. & Winzler, R.J. (1974) Evolving Strategies and Tactics in Membrane Research, Springer-Verlag, New York.

Wallach, D.F.H., Kranz, B., Ferber, E. and Fischer, H. (1972) FEBS Lett. 21, 29-33.

Warren, G.B., Houslay, M.D., Metcalfe, J.C. & Birdsall, N.J.M. (1975) Nature 255, 684-687.

Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. & Metcalfe, J.C. (1974a) Proc. Natl. Acad. Sci. USA, 71, 622-626.

Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. & Metcalfe, J.C. (1974b) FEBS Lett. 41, 122-124.

Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. & Metcalfe, J.C. (1974c) Biochemistry 13, 5501-5506.

Weber, A. (1959) J. Biol. Chem. 234, 2764-2769.

Weber, A. & Murray, J.M. (1973) Physiol. Rev. 53, 612-672.

Weber, A., Herz, R. & Reiss, I. (1966) Biochem. Z. 345, 329-369.

Weber, K. & Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.

Wheeldon, L.W. & Gan, K. (1971) Biochim. Biophys. Acta 233, 37-48.

Wiederhielm, C.A. (1968) J. Gen. Physiol. 52, 29s-63s.

Wilkie, D.R. (1968) Muscle, 1st eds., Edward Arnold Ltd., London.

Winegrad, S. (1968) J. Gen. Physiol. 51, 65-83.

Winegrad, S. (1970) J. Gen. Physiol. 55, 77-88.

Wissig, S.L. (1958) Anat. Rec. 130, 467-468.

APPENDIX A

A computer program used for calculation of gradient profiles.

This program is written in FORTRAN IV.

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C                              BIOCHEMISTRY                                    NJ900010
      DIMENSION D(25)                                                          NJ900020
    2 WRITE(6,700)                                                             NJ900030
  700 FORMAT( 'GIVE MOLARITY OF SOLUTION IN MIXING VESSEL')                    NJ900040
      READ(5,100)DM                                                            NJ900050
      WRITE(6,701)                                                             NJ900060
  701 FORMAT( ' GIVE MOLARITY OF SOLUTION IN RESERVOIR ')                      NJ900070
      READ(5,100)DR                                                            NJ900080
      WRITE(6,702)                                                             NJ900090
  702 FORMAT( 'GIVE VOLUME OF FRACTION TO BE COLLECTED ')                      NJ900100
      READ(5,100)X                                                             NJ900110
      Y=-(X/2.0)                                                               NJ900120
      WRITE(6,703)                                                             NJ900130
  100 FORMAT(F5.3)                                                             NJ900140
  703 FORMAT( 'GIVE VOLUME OF SOLUTION IN MIXING VESSEL')                      NJ900150
      READ(5,50)VX                                                             NJ900160
      WRITE(6,704)                                                             NJ900170
  704 FORMAT( ' GIVE VOLUME OF SOLUTION IN RESERVOIR')                         NJ900180
      READ(5,50)VY                                                             NJ900190
      WRITE(6,705)                                                             NJ900200
  705 FORMAT ('GIVE NUMBER OF FRACTIONS REQUIRED')                             NJ900210
      READ(5,706)N                                                             NJ900220
  706 FORMAT(I3)                                                               NJ900230
   50 FORMAT(F5.1)                                                             NJ900240
      WRITE(6,210)                                                             NJ900250
  210 FORMAT(' MOLARITIES CALCULATED TO THE CENTRE OF EACH FRACTION')          NJ900260
      WRITE(6,51)VX                                                            NJ900270
   51 FORMAT(' VOLUME OF SUCROSE IN MIXING VESSEL=',F5.1)                      NJ900280
      WRITE(6,52)VY                                                            NJ900290
   52 FORMAT(' VOLUME OF SUCROSE IN RESEVOIR=',F5.1)                           NJ900300
      WRITE(6,103)DM                                                           NJ900310
  103 FORMAT(' MOLARITY OF SUCROSE IN MIXING VESSEL=',F5.3)                    NJ900320
      WRITE(6,104)DR                                                           NJ900330
  104 FORMAT(' MOLARITY OF SUCROSE IN RESERVOIR=',F5.3)                        NJ900340
      V=Y                                                                      NJ900350
      WRITE(6,707)                                                             NJ900360
  707 FORMAT( ' WANT LINEAR (5.0) OR EXPONENTIAL (1.0) GRADIENT ? ')           NJ900370
      READ(5,708)ABC                                                           NJ900380
       IF(ABC.GT.2.0)GO TO 709                                                 NJ900390
  708 FORMAT(F3.1)                                                             NJ900400
      DO 1 I=1,N                                                               NJ900410
       V=V+X                                                                   NJ900420
      D(I)=DM+(DR-DM)*(1.0-EXP(-V/VX))                                         NJ900430
      WRITE(6,200)I,D(I)                                                       NJ900440
    1 CONTINUE                                                                 NJ900450
      GO TO 710                                                                NJ900460
  709 DO 714 I=1,N                                                             NJ900470
      V=V+X                                                                    NJ900480
      D(I)=DM+(DR-DM)*(V/(2.0*VX))                                             NJ900490
      WRITE(6,200)I,D(I)                                                       NJ900500
  714 CONTINUE                                                                 NJ900510
      GO TO 710                                                                NJ900520
  200 FORMAT(' ',I4,3X,F5.3)                                                   NJ900530
  710 WRITE(6,711)                                                             NJ900540
  711 FORMAT( ' DO YOU REQUIRE ANOTHER GRADIENT ? YES(1.0)  NO(5.0) ')         NJ900550
```

```
      READ(5,712)BCA                                                    NJ900560
  712 FORMAT(F3.1)                                                      NJ900570
      IF(BCA.GT.2.0) GO TO 713                                          NJ900580
       GO TO 2                                                          NJ900590
  713  CONTINUE                                                         NJ900600
      END                                                               NJ900610
```

MOLARITIES CALCULATED TO THE CENTRE OF EACH FRACTION
VOLUME OF SUCROSE IN MIXING VESSEL=200.0
VOLUME OF SUCROSE IN RESEVOIR=700.0
MOLARITY OF SUCROSE IN MIXING VESSEL=0.550
MOLARITY OF SUCROSE IN RESERVOIR=1.500

1	0.596
2	0.682
3	0.760
4	0.830
5	0.894
6	0.951
7	1.004
8	1.051
9	1.093
10	1.132
11	1.167
12	1.199
13	1.227
14	1.253
15	1.277
16	1.298
17	1.317
18	1.334
19	1.350
20	1.364
21	1.377
22	1.389
23	1.399
24	1.409
25	1.418

EXPONENTIAL GRADIENT

MOLARITIES CALCULATED TO THE CENTRE OF EACH FRACTION
VOLUME OF SUCROSE IN MIXING VESSEL=200.0
VOLUME OF SUCROSE IN RESEVOIR=400.0
MOLARITY OF SUCROSE IN MIXING VESSEL=0.500
MOLARITY OF SUCROSE IN RESERVOIR=2.000

1	0.537
2	0.612
3	0.688
4	0.762
5	0.837
6	0.912
7	0.987
8	1.063
9	1.137
10	1.212
11	1.287
12	1.362
13	1.438
14	1.512
15	1.587
16	1.662
17	1.737
18	1.813
19	1.887
20	1.962
21	2.037
22	2.112
23	2.188
24	2.262
25	2.337

LINEAR GRADIENT

A P P E N D I X B

A computer program, entitled SEDIMENTATION JOB, used for the calculation of sedimentation coefficients. This program is written in FORTRAN IV.

```
C     SEDIMENTATION JOB; C HEADON,E BARRETT,BIOCHEMISTRY DEPT UCC        NB3CCC10
      DIMENSION R(30),POLAR(30),PPOLF(3C),VIS(3C),FAC2(30),DEN(30),SUP(3NB3CCC20
     1C), S20W(30)                                                      NB3CCC30
      DIMENSION C(8)                                                    NB3CC040
      B1=1.0C03698E0                                                    NB3CCC50
        B2=3.9680504E-05                                                NB3CCC60
        B3=-5.8513271E-06                                               NB3CCC70
        B4=3.898237E-01                                                 NB3C0080
        B5=-1.057891?E-03                                               NB3CCC90
        B6=1.2392833E-05                                                NB3CC1CC
        B7=1.709759E-01                                                 NB3CC110
        B8=4.753C081E-04                                                NB3C0120
        B9=8.9239737E-06                                                NB3CC13C
      C(1)=-0.15C1832E+01                                               NB3CC14C
      C(2)=+0.9411213E+01                                               NB3CC15C
      C(3)=-0.1143574E+04                                               NB3C0160
      C(4)=+0.1050413E+06                                               NB3CC170
      C(5)=-0.4692710E+07                                               NB3C0180
      C(6)=+0.1032335E+09                                               NB3CC190
      C(7)=-0.1102898E+10                                               NB3C02C0
      C(8)=+0.4592191E+10                                               NB3CC210
    1 WRITE(6,965)                                                      NB3CC22C
  965 FORMAT(1H1,'SEDIMENTATION  COEFFICIENTS')                         NB3C0230
      READ(5,5C0)RPM                                                    NB3C0240
  500 FORMAT(F7.1)                                                      NB3CC250
      RPS=RPM/6C.0                                                      NB3CC260
      READ(5,3C0)TIME                                                   NB3CC270
      T=TIME*60.0                                                       NB3C0280
      READ(5,3C0)VOOL                                                   NB3CC290
      READ(5,3C0)VOSAM                                                  NB3CC3CC
  300 FORMAT(F5.1)                                                      NB3CC310
      READ(5,3C0)VOCF                                                   NB3CC320
  502 FORMAT(F5.1)                                                      NB3CC330
      READ(5,501)N                                                      NB3CC34C
  501 FORMAT(I3)                                                        NB3C0350
      READ(5,971)(POLAR(I),I=1,N)                                       NB3C036C
  971 FORMAT(16F5.3)                                                    NB3C0370
      READ(5,505)TT                                                     NB3CC380
  505 FORMAT(F4.1)                                                      NB3CC390
      FORCE=((12.0*3.14*RPS)**2)*T)                                     NB3C04C0
      READ(5,2)DP                                                       NB3CC410
      READ(5,2)DP1                                                      NB3CC420
    2 FORMAT(F4.2)                                                      NB3C0430
  670 DP=DP+0.01                                                        NB3C0440
      IF(DP-DP1)602,602,1                                               NB3CC450
  602 CONTINUE                                                          NB3CC46C
      V=(VOOL+(VOSAM/2.0)-VOCF)                                         NB3CC470
      WRITE(6,911)DP                                                    NB3CC480
  911 FORMAT(1HO,'PARTICLE DENSITY =',F6.4)                             NB3C0490
      WRITE(6,350)VOOL                                                  NB3C05C0
  350 FORMAT(' VOLUME OF OVERLAY IN MILLILITRES =',F5.1)                NB3C0510
      WRITE(6,351)VOSAM                                                 NB3CC520
  351 FORMAT(' VOLUME OF SAMPLE IN MILLILITRES =',F5.1)                 NB3CC53C
      WRITE(6,352)RPM                                                   NB3C0540
  352 FORMAT(' SPEED OF CENTRIFUGATION IN REVOLUTIONS PER MINUTE =',F7.1NB3C0550
```

```
     1)                                                                 NB3C056C
      WRITE(6,353)TIME                                                  NB3CC570
  353 FORMAT(' TIME OF CENTRIFUGATION IN MINUTES =',F5.1)               NB3CC580
      WRITE(6,354)TT                                                    NB3C0590
  354 FORMAT(' TEMPERATURE OF CENTRIFUGATION IN DEGREES CENTIGRADE =',F4NB3CC6C0
     1.1)                                                               NB3CC610
      WRITE(6,966)                                                      NB3CC620
  966 FORMAT(' ',15X,'S20W',8X,'DEN',8X,'VISCOSITY',6X,'MOLARITY',3X,'RANB3CC630
     1DIUS',4X,'MOLE FR')                                               NB3C0640
      FAC1=(DP-1.0)/(FORCE*0.010C19EC)                                  NB3C0650
      DO 590 I=1,N                                                      NB3CC660
  8C0 V=V+VOCF                                                          NB3CC670
      R(I)=2.262+0.0100*V+(-0.CCCCC049)*V**2                            NB3C0680
  990 CONTINUE                                                          NB3C0690
      DO 920 I=1,N                                                      NB3CC7C0
  921 IF(POLAR(I)-0.40)922,922,9C1                                      NB3CC710
  922 Y=PCLAR(I)*0.130/0.4                                              NB3CC720
      GO TO 910                                                         NB3CC730
  9C1 IF(POLAR(I)-0.90)923,923,902                                      NB3CC740
  923 Y=PCLAR(I)*0.3+0.01                                               NB3C0750
      GO TO 910                                                         NB3CC760
  9C2 IF(POLAR(I)-1.80)924,924,9C3                                      NB3CC770
  924 Y=PCLAR(I)*0.244+0.C6                                             NB3CC780
      GO TO 910                                                         NB3CC790
  903 Y=PCLAR(I)*0.225+0.C9                                             NB3CC8C0
  910 CONTINUE                                                          NB3C0810
      PMOLF(I)=Y*18.016/(Y*18.016+(1-Y)*342.3)                          NB3C0820
      AA=C.0                                                            NB3C0830
      DO 480 KK=1,8                                                     NB3C0840
      AA=AA+D(KK)*(PMOLF(I)**(KK-1))                                    NB3C0850
  480 CONTINUE                                                          NB3C0860
      GO TO 982                                                         NB3C0870
  982 IF(Y-0.48)925,925,952                                             NB3CC880
  925 BB=2.11699070E02+PMCLF(I)*1.6C77G73E03+PMOLF(I)**2*1.6911611E05   NB3CC890
     1+PMCLF(I)**3*(-1.4184371EC7)+PMOLF(I)**4*6.0654775E08+PMCLF(I)**5*NB3C0900
     2(-1.2985834E10)+PMCLF(I)**6*1.35329C7E11+PMOLF(I)**7*(-5.4970416E1NB3CC910
     31)                                                                NB3CC920
      GO TO 983                                                         NB3CC930
  952 BB=1.3975568E02+PMOLF(I)*6.674732SEC3+PMCLF(I)**2*(-7.8716105E04)+NB3C0940
     1PMCLF(I)**3+9.096757E05+PMCLF(I)**4*(-5.5380830EC6)+PMOLF(I)**5*1.NB3CC950
     22451219E07                                                        NB3CC960
  983 CC=1.4606635E02-25.251728*SQRT(1.C+(PMOLF(I)/7.06748E-02)**2)     NB3CC970
      DEN(I)=(B1+B2*TT+B3*TT**2)+Y*(B4+B5*TT+B6*TT**2)+Y**2*(B7+B8*TT+B9NB3C0980
     1*TT**2)                                                           NB3C059C
  920 VIS(I)=10.0**(AA+BB/(TT+CC))/1CC.C                                NB301CC0
      K=N-1                                                             NB3C1C10
      DO 960 I=2,K                                                      NB3C1C20
  960 FAC2(I)=(VIS(I)*(R(I)-R(I-1)))/((DP-DEN(I))*(R(I)+R(I-1)/2))      NB3C1C30
      SUM(2)=FAC2(2)                                                    NB3C1C40
      DO 961 I=3,K                                                      NB3C1C50
  961 SUM(I)=SUM(I-1)+FAC2(I)                                           NB3C1C60
      DO 962 I=2,K                                                      NB301070
      S20W(I)=(FAC1*SUM(I))/1.0E-13                                     NB3C1C80
      IF(S20W(I))601,601,962                                            NB301C90
  601 S20W(I)=0.0                                                       NB3011C0
```

```
  962 WRITE(6,964)I,S2OW(I),CEN(I),VIS(I),POLAR(I),R(I),PMCLF(I)        NB3C1110
  964 FORMAT(' ',I4,4X,F14.4,4X,F6.4,4X,E14.8,4X,F6.4,4X,F7.4,4X,F6.4) NB3C1120
      GO TO 670                                                         NB3C1130
      END                                                               NB3C1140
```

PARTICLE DENSITY =1.1900
VOLUME OF OVERLAY IN MILLILITRES =100.0
VOLUME OF SAMPLE IN MILLILITRES = 40.0
SPEED OF CENTRIFUGATION IN REVOLUTIONS PER MINUTE =30000.0
TIME OF CENTRIFUGATION IN MINUTES = 60.0
TEMPERATURE OF CENTRIFUGATION IN DEGREES CENTIGRADE = 5.0

	S20W	DEN	VISCOSITY	MOLARITY	RADIUS	MOLE FR
2	48.0919	1.0798	0.29904298E-01	0.6000	3.5660	0.0122
3	107.1825	1.0935	0.34534443E-01	0.7000	3.7366	0.0146
4	182.7033	1.1075	0.40388256E-01	0.8000	3.9032	0.0172
5	284.1807	1.1218	0.47905788E-01	0.9000	4.0660	0.0201
6	417.4087	1.1335	0.55545937E-01	1.0000	4.2248	0.0225
7	604.6670	1.1455	0.65325797E-01	1.1000	4.3798	0.0251
8	894.9351	1.1578	0.77859104E-01	1.2000	4.5308	0.0279
9	1435.6650	1.1703	0.94208658E-01	1.3000	4.6778	0.0309

PARTICLE DENSITY =1.2000
VOLUME OF OVERLAY IN MILLILITRES =100.0
VOLUME OF SAMPLE IN MILLILITRES = 40.0
SPEED OF CENTRIFUGATION IN REVOLUTIONS PER MINUTE =30000.0
TIME OF CENTRIFUGATION IN MINUTES = 60.0
TEMPERATURE OF CENTRIFUGATION IN DEGREES CENTIGRADE = 5.0

	S20W	DEN	VISCOSITY	MOLARITY	RADIUS	MOLE FR
2	46.4113	1.0798	0.29904298E-01	0.6000	3.5660	0.0122
3	102.7723	1.0935	0.34534443E-01	0.7000	3.7366	0.0146
4	173.6757	1.1075	0.40388256E-01	0.8000	3.9032	0.0172
5	266.8455	1.1218	0.47905788E-01	0.9000	4.0660	0.0201
6	386.0034	1.1335	0.55545937E-01	1.0000	4.2248	0.0225
7	546.9353	1.1455	0.65325797E-01	1.1000	4.3798	0.0251
8	780.0781	1.1578	0.77859104E-01	1.2000	4.5308	0.0279
9	1157.7463	1.1703	0.94208658E-01	1.3000	4.6778	0.0309

A P P E N D I C C

A computer program used to fit a curve to given data. This program is written in FORTRAN IV.

```
PAGE  1
// JOB
LOG DRIVE   CART SPEC   CART AVAIL  PHY DRIVE
 0000         0C02         0C02        0000
                           0C26        0002

V2 M07   ACTUAL 32K  CONFIG 32K

// FOR
*LIST ALL
*ONE WORD INTEGERS
*EXTENDED PRECISION
*IOCS(2501 READER,1442 PUNCH,1403 PRINTER,DISK,PLOTTER)
C    ---------    PROGRAM TO FIT IN A LEAST-SQUARES SENSE A CURVE TO GIVEN DATA.
C------- IT IS UNDERSTOOD THAT EITHER A STRAIGHT LINE OR A SECOND DEGREE CURVE
C---------------------- IS BEING SOUGHT.
      DIMENSION   X(100),Y(100)
      DATA  LPL/1/,PI/3.14159265/
      WRITE(5,408)
  408 FORMAT(1H1///,45X'REGRESSION OF RADIUS(R) ON THE VOLUME(V)'///)
      WRITE(5,413)
  413 FORMAT(1H ,1X'CURVE/COUNT',3X'COEFFS. OF EQ.',20X'SUMS OF SQUARES'
     1,11X'MS',9X'S.E. B AND C',6X'MOD. OF',/19X'RADIUS  = A+B*V+C*V**2'
     2,64X'COR. COEFF.'//,7X'NUMBER',6X'A',10X'B',10X'C',8X'TOTAL',6X'RE
     3SIDUAL',39X'R')
  304 READ(8,401)  N,K,IPLOT,NCURV
  401 FORMAT(I2,I1,I1,I3)
      READ(8,400)  (X(I),I=1,N),(Y(I),I=1,N)
  400 FORMAT( 13F6.2)
      IF(N)  900,900,402
  402 IF(K-1) 900,403,404
  403 XBAR=0.
      YBAR=0.
      DO 405  I=1,N
      YBAR=YBAR+Y(I)
  405 XBAR = XBAR + X(I)
      YBAR = YBAR/N
      XBAR = XBAR/N
      COV=0.
      VARX = 0.
      VARY = 0.
      DO 406  I=1,N
      COV=COV+(X(I)-XBAR)*(Y(I)-YBAR)
      VARX=VARX+(X(I)-XBAR)**2
  406 VARY=VARY+(Y(I)-YBAR)**2
      BETA=COV/VARX
      ALPHA=YBAR-XBAR*BETA
      TSS=0.
      DO407 I=1,N
      YEST   =ALPHA+BETA*X(I)
  407 TSS=TSS+(YEST   -YBAR)**2
      RES=VARY-TSS
      RMS=RES/(N-K-1)
      COR=(TSS/VARY)**0.5+0.0005
      SEB=(RMS/VARX)**0.5+0.00005
      AR=ALPHA+0.0005
      BR=BETA+0.00005
      VARY=VARY+0.000005
      RES=RES+0.000005
      RMS=RMS+0.00000005
      WRITE(5,409)NCURV,AR,BR,VARY,RES,RMS,SEB,COR
  409 FORMAT(1HO/,8X13,4XF7.3,4XF7.4,15XF9.5,4XF8.5,4XF9.7,4XF6.4,14XF5.
```

```
     A3)
      GO TO 301
  404 XBAR=0.
      X2BAR=0.
      X3BAR=0.
      X4BAR=0.
      YBAR=0.
      COV=0.
      COV2=0 .
      DO 410 I=1,N
      XBAR=XBAR+X(I)
      X2BAR = X2BAR+(X(I))**2
      X3BAR = X3BAR+(X(I))**3
      X4BAR = X4BAR+(X(I))**4
      YBAR = YBAR +Y(I)
      COV=COV+X(I)*Y(I)
  410 COV2=COV2+X(I)**2*Y(I)
      XBAR=XBAR/N
      X2BAR = X2BAR/N
      X3BAR = X3BAR/N
      X4BAR = X4BAR/N
      YBAR = YBAR/N
      COV=COV/N
      COV2=COV2/N
      GG=XBAR*X2BAR-X3BAR
      UK=X4BAR-X2BAR**2
      HM=XBAR*X4BAR-X2BAR*X3BAR
      GM=X2BAR*X4BAR-X3BAR**2
      UM=XBAR*X3BAR-X2BAR**2
      HH=X3BAR-XBAR*X2BAR
      HZ=X2BAR-XBAR**2
      AMATR=GM-XBAR*HM+X2BAR*UM
      ALPHA=(YBAR*GM-COV*HM+COV2*UM)/AMATR
      BETA=(-YBAR*HM+COV*UK+COV2*GG)/AMATR
      CETA=(YBAR*UM-COV*HH+COV2*HZ)/AMATR
      VARY=0.
      TSS=0.
      DO 411 I=1,N
      YEST=ALPHA+BETA*X(I)+CETA*X(I)**2
      TSS=TSS+(YEST-YBAR)**2
  411 VARY=VARY+(Y(I)-YBAR)**2
      RES=VARY-TSS
      RMS=RES/(N-K-1)
      COR=(TSS/VARY)**0.5+0.0005
      SEB=(RMS*(X2BAR*UK**2+HM**2-2*XBAR*UK*HM+X4BAR*GG**2+2*X3BAR*UK*GG
     A-2*X2BAR*GG*HM)/N)**0.5/AMATR+0.00005
      SEC=(RMS*(UM**2+X2BAR*HH**2-2*XBAR*UM*HM+X4BAR*HZ**2+2*X2BAR*HZ*UM
     A-2*X3BAR*HZ*HM)/N)**0.5/AMATR +0.00005
      AR=ALPHA+0.0005
      BR=BETA+0.00005
      CR=CETA+0.00000005
      VARY=VARY+0.000005
      RES=RES+0.000005
      RMS = RMS+0.00000005
      WRITE(5,412)NCURV,AR,BR,CR,VARY,RES,RMS,SEB,SEC,COR
  412 FORMAT(1HO,/8X13,4XF7.3,4XF7.4,4XF10.7,4XF9.5,4XF8.5,4XF9.7,4XF6.4
     A,4XF6.4,4XF5.3)
  301 IF(IPLOT-1)304,309,900
  309 IF(LPL-1)900,310,302
  310 READ (8,2) XMX,XMN,YMX,YMN,UX,UY,IX,IY
    2 FORMAT(6F6.2,2I2)
```

```
PAGE  3

      CALL SCALE(10./(XMX-XMN),9./(YMX-YMN),XMN,(YMX+YMN)/2.)
      NYTK = (YMX-YMN) /UY
      NXTK = (XMX-XMN) /UX
      CALL EGRID (0,XMN,YMN,UX,NXTK)
      CALL EGRID (1,XMN,YMN,UY,NYTK)
      NXTK=NXTK+IX
      DO 311 I=IX,NXTK,IX
      CALL ECHAR ((I-IX)*UX+XMN-3*(XMX-XMN)/100.,YMN-2*(YMX-YMN)/90.,0.1
     1,0.1,0.)
      F = (I-IX)*UX+XMN+0.005
  311 WRITE (7,313) F
  313 FORMAT (F6.2)
      NYTK = NYTK+IY
      DO 312 I=IY,NYTK,IY
      CALL ECHAR (XMN-7*(XMX-XMN)/100.,(I-IY)*UY+YMN,0.1,0.1,0.)
      F = (I-IY)*UY+YMN+0.005
  312 WRITE(7,313)F
      CALL ECHAR((XMX+XMN)/2.,YMN-4*(YMX-YMN)/90.,0.1,0.1,0.)
      WRITE(7,314)
  314 FORMAT('VOLUME')
      CALL ECHAR(XMN-6*(XMX-XMN)/100.,(YMX+YMN)/2.,0.1,0.1,PI/2.)
      WRITE(7,315)
  315 FORMAT('RADIUS')
      LPL = 2
  302 DO 303  I=1,N
      CALL EPLOT(-2,X(I),Y(I))
      CALL POINT(1)
  303 CALL EPLOT(1,X(I),Y(I))
       UR=XMN
      DX=PI/225
      IF(K-1)  900,316,317
  316 CALL EPLOT(-2,UR,ALPHA+BETA*UR)
  318 UR =UR+DX
      CALL EPLOT(0,UR,ALPHA+BETA*UR)
      IF(XMX-UR)319,319,318
  319 CALL ECHAR(XMN,YMN-8*(YMX-YMN)/90.,0.2,0.2,0.)
      WRITE(7,320)  AR,BR
  320 FORMAT('RADIUS = ',F7.3,'+',F7.4,'*V')
      GO TO 304
  317 CALL EPLOT(-2,UR,ALPHA+BETA*UR+CETA*UR**2)
  321 UR =UR+DX
      CALL EPLOT(0,UR,ALPHA+BETA*UR+CETA*UR**2)
      IF(XMX-UR) 322,322,321
  322 CALL ECHAR(XMN,YMN-8*(YMX-YMN)/90.,0.2,0.2,0.)
      WRITE(7,323) AR,BR,CR
  323 FORMAT('RADIUS = ',F7.3,'+',F7.4,'*V+',F7.4,'*V**2')
      GO TO 304
  900 CALL EXIT
          END
 VARIABLE ALLOCATIONS
      X(R )=0129-0000     Y(R )=0255-012C   XBAR(R )=0258     YBAR(R )=025B      COV(R )=025E      VARX(R )=0261
   VARY(R )=0264       BETA(R )=0267      ALPHA(R )=026A      TSS(R )=026D      YEST(R )=0270       RES(R )=0273
    RMS(R )=0276        COR(R )=0279       SEB(R )=027C       AR(R )=027F       BR(R )=0282     X2BAR(R )=0285
  X3BAR(R )=0288      X4BAR(R )=028B     COV2(R )=028E       GG(R )=0291       UK(R )=0294       HM(R )=0297
     GM(R )=029A         UM(R )=029D        HH(R )=02A0       HZ(R )=02A3     AMATK(R )=02A6     CETA(R )=02A9
     SEC(R )=02AC        CR(R )=02AF       XMX(R )=02B2      XMN(R )=02B5      YMX(R )=02B8       YMN(R )=02BB
     UX(R )=02BE        UY(R )=02C1         F(R )=02C4       PI(R )=02C7       UR(R )=02CA       DX(R )=02CD
      N(I )=02E2          K(I )=02E3     IPLOT(I )=02E4     NCURV(I )=02E5       I(I )=02E6      LPL(I )=02E7
     IX(I )=02E8        IY(I )=02E9       NYTK(I )=02EA      NXTK(I )=02EB

$STATEMENT ALLOCATIONS
   408  =0322  413  =0340  401  =03A3  400  =03A8  409  =03AB  412  =03BF  2    =03D7  313  =03DC  314  =03DE  315  =03E3
```

REGRESSION OF RADIUS(R) ON THE VOLUME(V)

CURVE/COUNT	COEFFS. OF EQ. RADIUS = A+B*V+C*V**2			SUMS OF SQUARES		MS	S.E. B AND C	MOD. OF COR. COEFF.
NUMBER	A	B	C	TOTAL	RESIDUAL			R
1	3.466	0.0175	-0.0000087	89.28211	0.18341	0.0131005	0.0005 0.0000	0.999

A P P E N D I X D

A computer program, entitled OPTIMISING JOB, used for optimising the separatory conditions. This program is written in FORTRAN IV.

```
C     OPTIMISING JOB ,D HEADON,E BARRETT   BIOCHEMISTRY DEPT UCC          NB7CCC10
      DIMENSION R(30),POLAR(30),PMOLF(30),VIS(30),FAC2(30),DEN(30),SUM(3NB700020
     1C), S20W(30)                                                       NB7C0C30
      DIMENSION C(8)                                                     NB70C040
      C(1)=-0.1501832E+01                                                NB7CCC50
      C(2)=+0.9411213E+01                                                NB7CCC6C
      C(3)=-0.1143574E+04                                                NB7CCC70
      C(4)=0.1050413E+06                                                 NB7CC080
      C(5)=-0.4692710E+07                                                NB700C90
      C(6)=+0.1032335E+09                                                NB7CC1C0
      C(7)=-0.1102898E+10                                                NB7C0110
      C(8)=+0.4592191E+10                                                NB7CC120
      B1=1.000369BE0                                                     NB7C0130
        B2=3.9680504E-05                                                 NB7C0140
        B3=-5.8513271E-06                                                NB7CC150
        B4=3.898237E-01                                                  NB7CC160
        B5=-1.0578919E-03                                                NB700170
        B6=1.2392833E-05                                                 NB7C0180
        B7=1.709759E-01                                                  NB7C0190
        B8=4.7530081E-04                                                 NB7C02C0
        B9=8.9239737E-06                                                 NB700210
      READ(5,500)RPM                                                     NB7CC220
  500 FORMAT(F7.1)                                                       NB7C023C
      RPS=RPM/60.0                                                       NB7CC240
      READ(5,300)VCOL                                                    NB700250
      READ(5,300)VOSAM                                                   NB7CC260
      READ(5,300)VOCF                                                    NB7CC270
  300 FORMAT(F5.1)                                                       NB700280
      READ(5,501)N                                                       NB700290
  501 FORMAT(I3)                                                         NB7CC3C0
      READ(5,971)(POLAR(I),I=1,N)                                        NB7C0310
  971 FORMAT(16F5.2)                                                     NB7CC320
      READ(5,505)TT                                                      NB7C0330
  505 FORMAT(F4.1)                                                       NB700340
  481 WRITE(6,965)                                                       NB7C035C
  965 FORMAT(1H1,'SEDIMENTATION  COEFFICIENTS')                          NB7C0360
      WRITE(6,350)VOOL                                                   NB700370
  350 FORMAT(' VOLUME OF OVERLAY IN MILLILITRES =',F5.1)                 NB700380
      WRITE(6,351)VOSAM                                                  NB700390
  351 FORMAT(' VOLUME OF SAMPLE IN MILLILITRES =',F5.1)                  NB7CC4CC
      WRITE(6,928)VOSAM                                                  NB700410
  928 FORMAT(' VOLUME OF APPLIED SAMPLE = ',F5.1)                        NB7CC420
      WRITE(6,352)RPM                                                    NB700430
  352 FORMAT(' SPEED OF CENTRIFUGATION IN REVOLUTIONS PER MINUTE =',F7.1NB7CC440
     1)                                                                  NB7C0450
      WRITE(6,354)TT                                                     NB7CC460
  354 FORMAT(' TEMPERATURE OF CENTRIFUGATION IN DEGREES CENTIGRADE =',F4NB7CC470
     1.1)                                                                NB7CC480
      READ(5,3C1)DP                                                      NB7C0490
      READ(5,3C0)TIME                                                    NB7CC5C0
  301 FORMAT(F4.2)                                                       NB7CC51C
      T=TIME*60.0                                                        NB7C0520
      TTIME=T/60.0                                                       NB7CC530
      WRITE(6,353)TTIME                                                  NB7C054C
  353 FORMAT(' TIME OF CENTRIFUGATION IN MINUTES =',F5.1)                NB7CC550
```

```
      FORCE=(((2.0*3.14*RPS)**2)*T)                                     NB7C0560
      V=(VOOL+(VOSAM/2.0)-VOCF)                                         NB7C0570
      WRITE(6,911)DP                                                    NB7C0580
  911 FORMAT(1HO,'PARTICLE DENSITY =',F6.4)                             NB7C0590
      WRITE(6,966)                                                      NB7C0600
  966 FORMAT(' ',11X,'S2Ow',8X,'DEN',8X,'VISCOSITY',6X,'MCLARITY',3X,'RANB7C0610
     1DIUS',4X,'MOLE FR')                                               NB7C0620
      FAC1=(DP-1.0)/(FORCE*0.C1CC19EC)                                  NB7C0630
      DO 990 I=1,N                                                      NB7C0640
  800 V=V+VOCF                                                          NB7C0650
      R(I)=5.88*V/(3.1416*1.08**2)                                      NB7C0660
  990 CONTINUE                                                          NB7C0670
      DO 920 I=1,N                                                      NB7C0680
  921 IF(POLAR(I)-0.40)922,922,9C1                                      NB7C0690
  922 Y=PCLAR(I)*0.130/0.4                                              NB7C0700
      GO TO 910                                                         NB7C0710
  901 IF(POLAR(I)-0.90)923,923,9C2                                      NB7C0720
  923 Y=PCLAR(I)*0.3+0.01                                               NB7C0730
      GO TO 910                                                         NB7C0740
  902 IF(POLAR(I)-1.80)924,924,903                                      NB7C0750
  924 Y=PCLAR(I)*0.244+0.C6                                             NB7C0760
      GO TO 910                                                         NB7C0770
  903 Y=PCLAR(I)*0.225+0.C9                                             NB7C0780
  910 CONTINUE                                                          NB7C0790
      PMOLF(I)=Y*18.016/(Y*18.016+(1-Y)*342.3)                          NB7C0800
      AA=C.0                                                            NB7C0810
      DO 480 KK=1,8                                                     NB7C0820
      AA=AA+D(KK)*(PMOLF(I)**(KK-1))                                    NB7C0830
  480 CONTINUE                                                          NB7C0840
      GO TO 982                                                         NB7C0850
  982 IF(Y-0.48)925,925,952                                             NB7C0860
  925 BB=2.11699070E02+PMCLF(I)*1.6C77073E03+PMOLF(I)**2*1.6911611E05   NB7C0870
     1+PMCLF(I)**3*(-1.4184371E07)+PMOLF(I)**4*6.0654775E08+PMCLF(I)**5*NB7C0880
     2(-1.2985834E10)+PMCLF(I)**6*1.3532907E11+PMOLF(I)**7*(-5.4970416E1NB7C0890
     31)                                                                NB7C0900
      GO TO 983                                                         NB7C0910
  952 BB=1.3975568E02+PMOLF(I)*6.6747329EC3+PMCLF(I)**2*(-7.8716105E04)+NB7C0920
     1PMOLF(I)**3*9.096757E05+PMCLF(I)**4*(-5.5380830EC6)+PMOLF(I)**5*1.NB7C0930
     22451219E07                                                        NB7C0940
  983 CC=1.4606635E02-25.251728*SQRT(1.C+(PMOLF(I)/7.06748E-02)**2)     NB7C0950
      DEN(I)=(B1+B2*TT+B3*TT**2)+Y*(B4+B5*TT+B6*TT**2)+Y**2*(B7+B8*TT+B9NB7C0960
     1*TT**2)                                                           NB7C0970
  920 VIS(I)=10.0**(AA+BB/(TT+CC))/1CC.C                                NB7C0980
      K=N-1                                                             NB7C0990
      DO 960 I=2,K                                                      NB7C1000
  960 FAC2(I)=(VIS(I)*(R(I)-R(I-1)))/((DP-DEN(I))*(R(I)+R(I-1)/2))      NB7C1010
      SUM(2)=FAC2(2)                                                    NB7C1020
      DO 961 I=3,K                                                      NB7C1030
  961 SUM(I)=SUM(I-1)+FAC2(I)                                           NB7C1040
      DO 962 I=2,K                                                      NB7C1050
      S2Ow(I)=(FAC1*SUM(I))/1.0E-13                                     NB7C1060
      IF (S2Ow(I))601,601,962                                           NB7C1070
  601 S2Ow(I)=0.0                                                       NB7C1080
  962 WRITE(6,964)I,S2Ow(I),DEN(I),VIS(I),POLAR(I),R(I),PMCLF(I)        NB7C1090
  964 FORMAT(' ',I4,4X,F14.4,4X,F6.4,4X,E14.8,4X,F6.4,4X,F7.4,4X,F6.4)  NB7C1100
```

```
  927 CONTINUE                                                    NB7C1110
      GO TO 481                                                   NB7C1120
      END                                                         NB7C1130
```

SEDIMENTATION COEFFICIENTS
VOLUME OF OVERLAY IN MILLILITRES = 6.0
VOLUME OF SAMPLE IN MILLILITRES = 2.0
VOLUME OF APPLIED SAMPLE = 2.0
SPEED OF CENTRIFUGATION IN REVOLUTIONS PER MINUTE =30000.0
TEMPERATURE OF CENTRIFUGATION IN DEGREES CENTIGRADE = 5.0
1.20
120.0
TIME OF CENTRIFUGATION IN MINUTES =120.0

PARTICLE DENSITY =1.2000

	S20W	DEN	VISCOSITY	MOLARITY	RADIUS	MOLE FR
2	27.9251	1.1073	0.40322531E-01	0.7990	8.0632	0.0172
3	64.7148	1.1205	0.47146175E-01	0.8910	8.3361	0.0198
4	111.1772	1.1307	0.53604230E-01	0.9770	8.6090	0.0219
5	170.0537	1.1401	0.60634181E-01	1.0550	8.8819	0.0239
6	245.7965	1.1489	0.68519473E-01	1.1280	9.1548	0.0259
7	344.5203	1.1572	0.77152431E-01	1.1950	9.4277	0.0277
8	475.8501	1.1649	0.86623549E-01	1.2570	9.7006	0.0296
9	655.2664	1.1720	0.96882343E-01	1.3140	9.9735	0.0313
10	911.5330	1.1788	0.10805511E+00	1.3670	10.2464	0.0330
11	1300.5334	1.1849	0.11981308E+00	1.4150	10.5193	0.0346
12	1980.4197	1.1907	0.13253462E+00	1.4600	10.7922	0.0362
13	3738.5281	1.1961	0.14616519E+00	1.5020	11.0651	0.0377
14	0.0	1.2011	0.16021448E+00	1.5400	11.3380	0.0391
15	0.0	1.2058	0.17527872E+00	1.5760	11.6109	0.0404

SEDIMENTATION COEFFICIENTS
VOLUME OF OVERLAY IN MILLILITRES = 6.0
VOLUME OF SAMPLE IN MILLILITRES = 2.0
VOLUME OF APPLIED SAMPLE = 2.0
SPEED OF CENTRIFUGATION IN REVOLUTIONS PER MINUTE =30000.0
TEMPERATURE OF CENTRIFUGATION IN DEGREES CENTIGRADE = 5.0
1.20
135.0
TIME OF CENTRIFUGATION IN MINUTES =135.0

PARTICLE DENSITY =1.2000

	S20W	DEN	VISCOSITY	MOLARITY	RADIUS	MOLE FR
2	24.8223	1.1073	0.40322531E-01	0.7990	8.0632	0.0172
3	57.5243	1.1205	0.47146175E-01	0.8910	8.3361	0.0198
4	98.8242	1.1307	0.53604230E-01	0.9770	8.6090	0.0219
5	151.1588	1.1401	0.60634181E-01	1.0550	8.8819	0.0239
6	218.4857	1.1489	0.68519473E-01	1.1280	9.1548	0.0259
7	306.2402	1.1572	0.77152431E-01	1.1950	9.4277	0.0277
8	422.9858	1.1649	0.86623549E-01	1.2570	9.7006	0.0296
9	582.4500	1.1720	0.96882343E-01	1.3140	9.9735	0.0313
10	810.2515	1.1788	0.10805511E+00	1.3670	10.2464	0.0330
11	1156.0295	1.1849	0.11981308E+00	1.4150	10.5193	0.0346
12	1760.3726	1.1907	0.13253462E+00	1.4600	10.7922	0.0362
13	3323.1357	1.1961	0.14616519E+00	1.5020	11.0651	0.0377
14	0.0	1.2011	0.16021448E+00	1.5400	11.3380	0.0391
15	0.0	1.2058	0.17527872E+00	1.5760	11.6109	0.0404

APPENDIX E

A computer program, entitled ANYL JOB, used in processing results of protein, succinate-INT reductase and Ca^{2+}-uptake analyses. This program is written in FORTRAN IV.

```
C     ANYL JOB NED BARRETT BIOCHEMISTRY DEPARTMENT,UCC                    NB1CCC10
      DIMENSION ODPR(40),POLAR(4C),Y(40),BODPR(4C),CCCPR(40)              NB1CC020
      DIMENSION DIL(40),PROT(40)                                          NB1CC030
      DIMENSION PPER(37)                                                  NB100040
      DIMENSION ODSDH(40),YY(40),SDH(40),CPER(37),SASDH(40)               NB1CCC50
      DIMENSION PURIF(37)                                                 NB1CCC60
      DIMENSION CALUA(40), CALUP(4C), SCAL(4C), DPER(37)                  NB1CCC70
      DIMENSION CALUB(40)                                                 NB1CCC80
      DIMENSION AMP(37),ACUP(37),UNAP(37)                                 NB1CCC90
  1CC FORMAT(' FR NO',6X,'PRCTEIN CCNC MGS/ML')                           NB1CC1C0
  101 FORMAT(1H1,' FR NO',6X,'PERCENT PROTEIN')                           NB1CC110
    1 READ (5,302)STDOH                                                   NB1CC120
      READ(5,303)DIL                                                      NB1CC13C
      READ(5,302)STDSU                                                    NB1CC140
      READ(5,106)ODPR                                                     NB1CC150
      READ(5,106)POLAR                                                    NB1CC160
      READ(5,304)VOAS                                                     NB100170
      READ(5,304)VOCF                                                     NB1CC180
  302 FORMAT(F5.3)                                                        NB1CC190
  303 FORMAT(16F5.1)                                                      NB1CC2C0
  106 FORMAT(16F5.3)                                                      NB1CC210
  304 FORMAT(F4.1)                                                        NB1CC220
  115 FORMAT(1H1,'  PROTEIN RECOVERY =',F5.3)                             NB1CC230
  116 FORMAT(' ',I4,6X,F14.3)                                             NB100240
  117 FORMAT(' ',I4,3X,F14.6)                                             NB1CC250
  112 FORMAT(' ',I4,8X,F5.3,10X,F5.3,9X,F5.1)                             NB1CC260
  119 FORMAT(' ',I4,10X,F5.3)                                             NB1CC270
  8C4 FORMAT (10F8.1)                                                     NB1CC280
  805 FORMAT (F8.1)                                                       NB1CC290
  806 FORMAT (1HO, 'FR NC', 6X, 'CPM CF ANALYTICAL', 6X, 'CPM OF BLANK')  NB1CC3C0
  808 FORMAT (' ', I4, 1CX, F8.1, 12X, F8.1)                              NB1CC310
  809 FORMAT (1HO, 'CPM RESULTING FRCM THE UPTAKE OF 1.0 MICROMOLE CF     NB1CC320
     1CALCIUM ION PER ML CF FRACTION = ', F8.1)                           NB1CC330
      WRITE(6,999)                                                        NB1CC340
  999 FORMAT(' ANALYSIS CF COLLECTED FRACTICNS FCR PRCTEIN AND SDH')      NB1CC35C
      WRITE(6,1C8)STDOH                                                   NB1CC360
  108 FORMAT(1HO,' ABSORBANCE OF 1CC MICROG. BSA/ML STANDARD = ',F5.3)    NB1CC370
      WRITE(6,109)VOAS                                                    NB1CC380
  109 FORMAT(1HO,' VOLUME(ML) OF APPLIED SAMPLE =',F4.1)                  NB1CC390
      WRITE(6,110)VOCF                                                    NB1CC4C0
  110 FORMAT(1HO,' VOLUME(ML) OF COLLECTED FRACTICN =',F4.1)              NB1CC410
      WRITE(6,111)                                                        NB1CC420
  111 FORMAT(1HO,' FR NO ',6X,'POLARITY',6X,'ABSORBANCE',6X,'DILUTICN')   NB1CC430
      CO 120 I=1,40                                                       NB1CC440
      WRITE(6,112)I,POLAR(I),ODPR(I),DIL(I)                               NB1CC450
  120 CONTINUE                                                            NB1CC460
      DO 5 I=1,40                                                         NB1CC470
      IF(POLAR(I)/DIL(I)-C.3CC)2C,2C,3                                    NB1CC480
   20 Y(I)=100.C-60.0*POLAR(I)/DIL(I)                                     NB1CC49C
      GO TO 5                                                             NB1CC5CC
    3 IF(POLAR(I)/DIL(I)-C.700)21,21,8                                    NB1CC510
   21 Y(I)=90.25-27.5*POLAR(I)/DIL(I)                                     NB1CC52C
      GO TO 5                                                             NB1CC53C
    8 IF(POLAR(I)/DIL(I)-1.500)22,22,9                                    NB1CC540
   22 Y(I)=63.69-1.125*POLAR(I)/DIL(I)                                    NB1CC550
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```
      GO TO 5                                                          NB1CC560
    9 Y(I)=61.0                                                        NB1CC570
      GO TO 5                                                          NB1CC580
    5 CONTINUE                                                         NB1CC590
      DO 395 I=1,40                                                    NB1CC600
      BCDPR(I)=POLAR(I)/OIL(I)*STDSU                                   NB1CC610
  395 CONTINUE                                                         NB1CC620
      DO 300 I=1,40                                                    NB1CC630
      CODPR(I)=(ODPR(I)-BCDPR(I))*(100.0/Y(I))                         NB1CC640
  300 CONTINUE                                                         NB1CC650
      DO 301 I=1,40                                                    NB1CC660
      PROT(I)=(CODPR(I)*OIL(I)*0.1)/STDCM                              NB1CC670
      IF(PROT(I))16,16,301                                             NB1CC680
   16 PROT(I)=0.0001                                                   NB1CC690
  301 CONTINUE                                                         NB1CC700
      SUM=0.0                                                          NB1CC710
      DO 18 I=1,37                                                     NB1CC720
      SUM=SUM+VOCF*PROT(I)                                             NB1CC730
   18 CONTINUE                                                         NB1CC740
      RECV=SUM/(VOAS*PROT(38))                                         NB1CC750
      WRITE(6,115)RECV                                                 NB1CC760
      WRITE (6,100)                                                    NB1CC770
      TSUM=0.0                                                         NB1CC780
      DO 624 I=1,37                                                    NB1CC790
      TSUM=TSUM+VOCF*PROT(I)                                           NB1CC800
  624 CONTINUE                                                         NB1CC810
      DO 625 I=1,37                                                    NB1CC820
      PPER(I)=VOCF*PROT(I)/TSUM*100.0                                  NB1CC830
  625 CONTINUE                                                         NB1CC840
      DO 626 I=1,40                                                    NB1CC850
      WRITE(6,117)I,PROT(I)                                            NB1CC860
  626 CONTINUE                                                         NB1CC870
      WRITE(6,101)                                                     NB1CC880
      DO 627 I=1,37                                                    NB1CC890
      WRITE(6,117)I,PPER(I)                                            NB1CC900
  627 CONTINUE                                                         NB1CC910
  103 FORMAT(1HO,'FR NO',8X,'ABSCLUTE ACTIVITY',8X,'SPECIFIC ACTIVITY') NB1CC920
  104 FORMAT(14X,'MICROMOLES/ML/MIN',6X,'MICROMOLES/MG PRCTEIN/MIN')   NB1CC930
  113 FORMAT(16F5.3)                                                   NB1CC940
  434 FORMAT(1H1,' SDH RECOVERY=',F5.1)                                NB1CC950
      WRITE(6,432)                                                     NB1CC960
  432 FORMAT(1H1,'SDH')                                                NB1CC970
      DO 700 I=1,40                                                    NB1CC980
      YY(I)=100.0/(100.0-62.0*(PCLAR(I)/2.0))                          NB1CC990
  700 CONTINUE                                                         NB1C1CC0
      READ(5,113)CDSDH                                                 NB1C1C10
      READ(5,304)STIME                                                 NB1C1020
      WRITE(6,118)                                                     NB1C1C30
  118 FORMAT(1HO,' FR NO',6X,'ABSCRBANCE')                             NB1C1C40
      DO 121 I=1,40                                                    NB1C1050
      WRITE(6,119)I,ODSDH(I)                                           NB1C1C60
  121 CONTINUE                                                         NB1C1C70
      WRITE(6,555)STIME                                                NB1C1C80
  555 FORMAT(1HO,' REACTION TIME IN MIN.=',F4.1)                       NB1C1090
      DO 430 I=1,40                                                    NB1C11C0
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```
      VOLPR=0.5                                                          NB1C1110
      SDH(I)=(OOSDH(I)*0.C5*YY(I))/(0.370*VOLPR*STIPE)                   NB1C1120
  430 CONTINUE                                                           NB1C1130
      SUM=0.0                                                            NB1C1140
      DO 433 I=1,37                                                      NB1C1150
  433 SUM=SUM+VOCF*SDH(I)                                                NB1C1160
      RECV=SUM/(VOAS*SDH(38))                                            NB1C1170
      WRITE(6,434)RECV                                                   NB1C1180
      WRITE(6,103)                                                       NB1C1190
      WRITE(6,104)                                                       NB1C1200
      DO 431 I=1,40                                                      NB1C1210
  435 SASDH(I)=SDH(I)/PRCT(I)                                            NB1C1220
      IF(PROT(I)-0.00010C)801,801,431                                    NB1C1230
  801 SASDH(I)=0.0C0000                                                  NB1C1240
  431 CONTINUE                                                           NB1C1250
      DO 605 I=1,40                                                      NB1C1260
      WRITE(6,170)I,SDH(I),SASDH(I)                                      NB1C1270
  605 CONTINUE                                                           NB1C1280
      WRITE(6,800)                                                       NB1C1290
  800 FORMAT(1H1,'FR NO',6X,'PERCENT ABSOLUTE ACTIVITY')                 NB1C1300
      TSUM=0.0                                                           NB1C1310
      DO 504 I=1,37                                                      NB1C1320
      TSUM=TSUM+VOCF*SDH(I)                                              NB1C1330
  504 CONTINUE                                                           NB1C1340
      DO 604 I=1,37                                                      NB1C1350
      CPER(I)=VOCF*SDH(I)/TSUM*1C0.C                                     NB1C1360
      WRITE(6,176)I,CPER(I)                                              NB1C1370
  604 CONTINUE                                                           NB1C1380
  170 FORMAT(' ',I4,3X,F14.6,3X,F14.6)                                   NB1C1390
  176 FORMAT(' ',I4,3X,F14.6)                                            NB1C1400
  107 FORMAT(1H1,' FR NO',6X,'PURIFICATION FACTOR')                      NB1C1410
      WRITE(6,107)                                                       NB1C1420
      DO 505 I=1,37                                                      NB1C1430
      PURIF(I)=SASDH(I)/SASDH(38)                                        NB1C1440
      WRITE(6,116)I,PURIF(I)                                             NB1C1450
  505 CONTINUE                                                           NB1C1460
      WRITE (5, 803)                                                     NB1C1470
  803 FORMAT (1H1, 'CALCIUM UPTAKE ACTIVITIES OF COLLECTED FRACTIONS ')  NB1C1480
      READ (5, 804) CALUA                                                NB1C1490
      READ (5, 804) CALUB                                                NB1C1500
      READ (5, 805) CALST                                                NB1C1510
      WRITE (6, 806)                                                     NB1C1520
      DO 807 I = 1, 40                                                   NB1C1530
      WRITE (5, 808) I, CALUA(I), CALUB(I)                               NB1C1540
  807 CONTINUE                                                           NB1C1550
      WRITE (6, 809) CALST                                               NB1C1560
      WRITE (6, 803)                                                     NB1C1570
      WRITE (6, 103)                                                     NB1C1580
      WRITE (6, 104)                                                     NB1C1590
      DO 810 I = 1, 40                                                   NB1C1600
      CALLP(I) = (CALUA(I) - CALUB(I))/CALST                             NB1C1610
      IF (CALUP(I)) 811,811,810                                          NB1C1620
  811 CALLP(I) = 0.0C0C0C                                                NB1C1630
  810 CONTINUE                                                           NB1C1640
      DO 812 I = 1, 40                                                   NB1C1650
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      SCAL(I) = CALUP(I)/PROT(I)                                        NB101660
      IF(PROT(I) - 0.000100) 813, 813, 812                              NB101670
  813 SCAL(I) = 0.000000                                                NB101680
  812 CONTINUE                                                          NB101690
      TSUM = 0.0                                                        NB101700
      DO 814 I = 1, 37                                                  NB101710
      TSUM = TSUM + VOCF * CALUP(I)                                     NB101720
  814 CONTINUE                                                          NB101730
      DO 815 I = 1, 37                                                  NB101740
      CPER(I) = VOCF * CALUP(I)/TSUM * 100                              NB101750
  815 CONTINUE                                                          NB101760
      DO 816 I = 1, 40                                                  NB101770
      WRITE (5, 170) I, CALUP(I), SCAL(I)                               NB101780
  816 CONTINUE                                                          NB101790
      WRITE (6, 800)                                                    NB101800
      DO 817 I = 1, 37                                                  NB101810
      WRITE (6, 176) I, CPER(I)                                         NB101820
  817 CONTINUE                                                          NB101830
  951 FORMAT( ' ',I4,15X,F7.3,15X,F7.3,19X,F7.3,4X,F14.6)               NB101840
      DO 944 I=1,37                                                     NB101850
      AMP(I) = SDH(I)/0.015                                             NB101860
  944 CONTINUE                                                          NB101870
      DO 945 I = 1,37                                                   NB101880
      ACUP(I) = CALUP(I)/2.0                                            NB101890
  945 CONTINUE                                                          NB101900
      DO 946 I=1,37                                                     NB101910
      UNAP(I) = PROT(I)-AMP(I)-ACUP(I)                                  NB101920
  946 CONTINUE                                                          NB101930
      WRITE (6,947)                                                     NB101940
  947 FORMAT(1H1, 'TENTATIVE ASSIGNMENT OF PROTEIN IN FRACTIONS ')      NB101950
      WRITE (6,948)                                                     NB101960
  948 FORMAT(1HO, 'FR NO',6X,'SDH PRCTEIN',6X,'CALCIUM UPTAKE PRCTEIN',6NB101970
     1X,'UNASSIGNED PROTEIN',10X,'PRCTEIN')                             NB101980
      WRITE(6,949)                                                      NB101990
  949 FORMAT(1H ,14X,'MILLIGRAMS/ML',9X,'MILLIGRAMS/ML',13X,'MILLIGRAMS/NB102000
     1ML',7X,'MILLIGRAMS/ML')                                           NB102010
      DO 950 I = 1,37                                                   NB102020
      WRITE(6,951)I,AMP(I),ACUP(I),UNAP(I),PRCT(I)                      NB102030
  950 CONTINUE                                                          NB102040
       GO TO 1                                                          NB102050
      END                                                               NB102060
*
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