INTRODUCTION

The centrifuge is now firmly established as an indispensable tool in virtually all phases of modern biology. Centrifugation may be used either: (1) to separate a mixture of different substances in suspension or solution and to isolate these substances into suspensions or solutions containing only one kind of particle; or (2) to characterize the size, shape and density of the particles after they have been separated into purified solutions or suspensions. Consequently, centrifugation can be used in both purification and characterization of a wide range of biological substances. This universality of application makes the centrifuge invaluable and very widely used in cellular and molecular biology. Because of its practically universal application, an exorbitantly lengthy discussion would be required to review all biological uses of the centrifuge. Consequently, this account will be limited to a discussion of only the first of the two general applications of the centrifuge; namely, use of the centrifuge to separate mixtures of different particles into suspensions or solutions of purified particles. The second general application of the centrifuge, use of centrifugation to characterize size and shape of particles, will not be discussed explicitly in this review. Whenever possible, separations involving muscle tissue or subcellular organelles from muscle tissue will be used to illustrate the usefulness of the centrifuge in muscle biology and meat science.

The mixtures of different substances that are commonly fractionated by centrifugation may contain either whole intact cells or subcellular components of cells released by rupture of the outer cell membrane. For example, different kinds of cells may be resolved into homogeneous cell populations by centrifugation (Boone et al., 1968). More frequently, centrifugation is used to purify a given subcellular component from the entire mixture of subcellular components released upon rupture of the cell membrane. In muscle tissue, centrifugation has not yet been widely used on the first of these two kinds of samples, i.e., on suspensions of

* Journal Paper No. J-7956 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Projects 1795 and 1796. The original studies on muscle polysomes reported in this review were supported in part by grants from the National Institutes of Health (AM 12654), the Muscular Dystrophy Associations of America, the American Heart Association (Grant No. 71-679), and the Iowa Heart Association (Grants No. 72-G-15 and 73-G-20). Presented at the 27th Annual Reciprocal Meat Conference of the American Meat Science Association, 1974.
intact muscle cells. It seems likely, however, that centrifugation could be used advantageously to purify whole muscle cells from muscle cell cultures grown in vitro. Fibroblasts normally contaminate cultures of muscle cells, sometimes constituting up to 30% of total cells in such cultures, and fibroblasts are very difficult to separate completely from cultured muscle cells by ordinary procedures. Because fibroblasts differ from muscle cells in several ways that may cause hydrodynamic differences, it seems probable that centrifugation under the proper conditions would separate muscle cells and fibroblasts, although to the best of our knowledge, such centrifugal resolution has not yet been seriously attempted. Even though centrifugation has not been widely used for separation and purification of intact muscle cells, it has been used to purify a number of other cell types (Boone et al., 1968). For example, centrifugation in density gradients has been used to separate malignant from normal or nonmalignant cells (Pretlow, 1971; Pretlow and Boone, 1968; 1970), to separate most cells in different stages of maturation differentiation (Pretlow and Cassady, 1970), to purify human leukemia cells (Abeloff et al., 1970; Boone et al., 1969; Pretlow and Boone, 1969), and for numerous similar separations (Miller and Cudkowicz, 1971). Rotors and procedures for purification of cells by centrifugation have been developed only recently, and it is likely that use of the centrifuge to prepare suspensions of homogeneous cells will increase in the future.

FRACTIONATION OF SUBCELLULAR ORGANELLES
BY DIFFERENTIAL CENTRIFUGATION

Centrifugation is probably most frequently used in biology to fractionate the mixture of subcellular components produced by rupture of the outer cell membrane into purified preparations containing only one kind of subcellular particle, and the remainder of this discussion will be concerned specifically with this use of centrifugation. It should be clearly specified, however, that the principles used to separate subcellular organelles by centrifugation are also directly applicable to centrifugal separation of intact cells, and that the only differences between centrifugal separation of cells and subcellular organelles are the procedural protocols, such as centrifuge speed and suspending solution used, associated with the two different kinds of sample material. As shown in Figures 1 and 2, skeletal muscle cells are composed of at least nine different kinds of subcellular components that can be identified and structurally characterized with the electron microscope. These nine components include the nucleus, mitochondria, the sarcolemma or outer cell membrane, myofibrils, which are the contractile elements of muscle, T- or transverse-tubules, the sarcoplasmic reticular membranes, glycogen granules, ribosomes, and the sarcoplasm, which is not structural in nature but can be identified as the protoplasmic fluid in which the structural elements are embedded. In addition to the nine components that can be identified in Figures 1 and 2, muscle cells may also contain membranes of the Golgi complex, lysosomes, and lipid inclusions. With the possible exception of lysosomes, all these subcellular components can be identified in electron micrographs of intact muscle cells, but it is evident that determining the biochemical functions of these subcellular components depends on isolation of each
Fig. 1. This electron micrograph of an intermediate type rat diaphragm muscle fiber shows four of the nine or ten different components that constitute muscle cells. Part of the nucleus seen in the lower left-hand corner of the micrograph illustrates the very large size of this subcellular organelle. Numerous mitochondria can be seen just above the nucleus (M) and scattered elsewhere in the cell. Myofibrils, which are a subcellular organelle unique to muscle fibers, course almost vertically in this micrograph (Z-disk of one myofibril indicated by Z). The outer cell membrane, or sarcolemma, can just be distinguished in the upper left-hand corner of the micrograph. X13,500. Reproduced from Gauthier (1970) by permission of the author and The University of Wisconsin Press, Madison; © 1970 by the Regents of the University of Wisconsin, p. 111.
Fig. 2. The membranous subcellular components of a muscle cell are evident in this electron micrograph of frog (Rana pipiens) sartorius muscle. Areas indicated by T are triads and show various aspects of the T-tubule system that passes transversely to the long axis of the muscle cell. The presence of large membrane-enclosed sacs called lateral cisternae on either side of the T-tubule is responsible for the name, triad. Note that triads in this muscle occur at the level of every Z-disk. L. designates the sarcoplasmic reticular membranes that run parallel with the long axis of the cell. These membranes are analogous to the endoplasmic reticulum of nonmuscle cells. Myofibrils also course almost vertically in this micrograph. Small, darkly staining spherical granules are glycogen. F = fenestrated collar. Line is 1 μm. Reproduced from Peachey (1970) by permission of the author and the University of Wisconsin Press, Madison; © 1970 by the Regents of the University of Wisconsin, p. 283.
of these components in a purified form. Such isolation requires that the outer cell membrane be broken, and the individual components be separated from the resulting mixture. Although it is not difficult to rupture cell membranes, it was virtually impossible before development of modern high-speed refrigerated centrifuges to separate the complex mixture of substances produced by such rupture. Even before development of high-speed centrifuges, however, it was clear that simply letting cell homogenates (i.e., suspensions of subcellular components after the outer cell membrane had been ruptured) stand under the influence of gravity caused some separation of the subcellular components because the largest components settled out of the homogenate rapidly and could be partly separated from the other components by decanting the supernatant solution. If the supernatant solution from this first decanting was allowed to set for a slightly longer period under the influence of gravity, the next largest component settled out and could also be obtained in a partly purified form by again decanting the supernatant. This procedure could be repeated with successively longer settling periods to obtain partly purified preparations of successively smaller particles (Figure 3). Although this procedure enabled early investigators to obtain partly purified preparations of the largest subcellular components, the time required for the smallest subcellular particles to settle out of the homogenate was prohibitively long (indeed, several centuries would be required for some of the smallest subcellular particles to settle out of a homogenate), and it was impossible to obtain preparations of the smallest subcellular preparations by using this settling procedure.

Sedimentation of Subcellular Components in the Centrifuge

It soon became evident that separation of subcellular components produced by settling in a gravitational field could be accelerated tremendously by increasing the force causing settling or sedimentation of the particles, and that this force could conveniently be increased by spinning cell homogenates in a centrifuge. Spinning at low speeds for brief periods of time generates low sedimentation forces, and partly purified preparations of the heaviest subcellular components can be obtained with only slight contamination by the lighter subcellular components. By increasing the speed, greater sedimentational forces are developed, and even the lightest subcellular components can be sedimented in an acceptable period of time. Theoretical calculations showed that sedimentation of particles due to centrifugal forces obeyed the same physical laws as sedimentation of particles due to gravitational forces, and that differences in rates of sedimentation of two particles in the same solvent or suspending medium depended only on differences in the size, shape, and density of the two particles (table 1). Because of the direct relationship described earlier between sedimentation of subcellular components in a centrifugal field and the differential settling of components in a gravitational field, separation of subcellular components by a series of centrifugation cycles each done at different speeds is called differential centrifugation, and the force developed during centrifugation is commonly given in terms of how many times greater it is than the force of gravity; e.g., 2000 times gravity or, for short, 2000 x g.
Fig. 3. Diagrammatic representation of how particles of different sizes are separated by differential centrifugation. Initially, all particles are distributed uniformly through the centrifuge (A). As centrifugation proceeds (B), all particles sediment at their respective sedimentation rates, resulting first in complete sedimentation of the largest particles present (C), and then in sedimentation of progressively smaller particles (D and E). The degree of contamination of larger particles in the pellet by smaller particles at the exact moment when the larger particles have just been completely sedimented (e.g., see C) is approximately proportional to the ratio of the sedimentation coefficients of the two particles. Distribution of particles in tube E is shown in the bar graph at the right. Reproduced from Anderson (1966) by permission of the author and the United States Department of Health, Education and Welfare.
TABLE 1

Differences in rate of sedimentation of two particles

depend on
1. Size
2. Shape
3. Density
of the two particles

\[ v = \frac{dr}{dt} = \frac{2a^2(p_p - p_s)w^2r}{g_s(f/f_0)_p} \]

where \( v \) = velocity
\( a \) = particle radius
\( p \) = density
\( w \) = angular velocity
\( r \) = radial distance from center of rotation
\( \eta \) = viscosity
\( f/f_0 \) = frictional coefficient

As shown in table 1, velocity of sedimentation increases with the square of the rotational speed of the centrifuge rotor. Consequently, much emphasis has been placed on obtaining the greatest possible centrifugal speeds to permit sedimentation of the smallest particles, and centrifuges capable of speeds up to 75,000 rpm are now commercially available. Indeed, it is not difficult to construct centrifuges capable of speeds in excess of 100,000 rpm, but it has been impossible to find metals or metal alloys of sufficient strength to build rotors capable of withstanding the tremendous forces developed at these very high speeds. At 75,000 rpm, the outer radius of the commercially available rotor is traveling faster than the muzzle velocity developed by most high-velocity rifles, and a force slightly over 500,000 \( x \) \( g \) is developed. At this force, one gram of metal in the rotor "weighs" 500,000 grams (i.e., a centripetal binding force of at least 500,000 grams is required to keep the rotor together), and a 170-pound man would weigh 85,000,000 pounds or 42,500 tons--imagine your legs trying to support that! Rotors capable of withstanding these very high forces are usually made of titanium alloys, and cost in excess of $2000.

The equation in table 1 also shows that sedimentation velocity of a particle is proportional to the difference between density of the particle and density of the solvent in which the particle is suspended. Solvents used in differential centrifugation generally are dilute aqueous solutions with densities only slightly greater than 1.0. Because most subcellular particles have densities between 1.15 and 1.75, the difference in density between subcellular particles and the common
solvents varies only fivefold, whereas the difference in radius of the same subcellular particles ranges from 20 to 5000 nm or 250-fold. Therefore, at the solvent densities commonly used in differential centrifugation, rate of sedimentation of particles depends principally on particle size and shape, and less on particle density. This dependence on particle size and shape is characteristic of the differential centrifugation procedure that we have been describing. A second centrifugal procedure, called density gradient centrifugation, has also been developed and will be described in more detail later in this review. One of the two different kinds of density gradient centrifugation separates particles solely on the basis of their density. Consequently, a combination of differential centrifugation, which separates particles on the basis of their size and shape, with the particular kind of density gradient centrifugation that separates particles solely on the basis of their density, provides a high-resolution technique for purification of subcellular particles. Use of this technique to isolate homogeneous fractions of muscle membranes will be discussed later in this review.

Factors Affecting Sedimentation Coefficients of Subcellular Components

The availability of refrigerated ultracentrifuges capable of speeds up to 75,000 rpm has made it possible to sediment even relatively small protein molecules from cell homogenates. Consequently, sedimentation velocities, expressed as velocity per unit of centrifugal acceleration so that they are independent of the speed used to sediment the particle, have been determined for many subcellular components. Some of these sedimentation coefficients, expressed in Svedberg units (one Svedberg unit = $10^{-13}$ sec), are listed in table 2. It is evident from the data in table 2 that size is the predominant factor determining sedimentation coefficients of subcellular components in differential centrifugation. Almost without exception, larger components have greater sedimentation coefficients than smaller components, and there is no obvious ordering of sedimentation coefficients according to density of the components (cf. table 9). For example, RNA, DNA, and ribosomes are all much denser than microsomes or plasma membranes (cf. table 9), but RNA, DNA, and ribosomes all have smaller sedimentation coefficients than microsomes or plasma membranes because RNA, DNA, and ribosomes are smaller than microsomes or plasma membranes. A range of sedimentation coefficients is given for each of the components listed in table 2. For nuclei, mitochondria, lysosomes, RNA, DNA, and proteins, this range of sedimentation coefficients probably indicates that these components exist in a range of sizes in living cells. For example, polysomes range considerably in size and when only muscle cells are considered, the range of sedimentation coefficients listed for polysomes in table 2 is too small because the polysomes that code for myosin synthesis are among the largest polysomes known and have a sedimentation coefficient in the range of 1000-5000 S. On the other hand, the wide variety of different sizes of myofibrils, plasma membranes, and microsomes that exists in a muscle cell homogenate is produced during homogenization of the cell,
### TABLE 2. APPROXIMATE SEDIMENTATION COEFFICIENTS OF MAJOR COMPONENTS OF MAMMALIAN CELLS$^a$

<table>
<thead>
<tr>
<th>Cell Component</th>
<th>Sedimentation Coefficient (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble proteins</td>
<td>1 - 25</td>
</tr>
<tr>
<td>RNA</td>
<td>4 - 50</td>
</tr>
<tr>
<td>DNA</td>
<td>up to 100</td>
</tr>
<tr>
<td>Ribosomal subunits</td>
<td>30 - 60</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>70 - 80</td>
</tr>
<tr>
<td>Polysomes</td>
<td>100 - 400</td>
</tr>
<tr>
<td>Glycogen</td>
<td>100 - 10,000</td>
</tr>
<tr>
<td>Microsomes</td>
<td>100 - 15,000</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>100 - 100,000</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>10,000 - 20,000</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>20,000 - 70,000</td>
</tr>
<tr>
<td>Myofibrils</td>
<td>100,000 - 4,000,000 (?)</td>
</tr>
<tr>
<td>Nuclei</td>
<td>4,000,000 - 10,000,000</td>
</tr>
</tbody>
</table>

$^a$ Sedimentation coefficients are expressed in Svedberg units where one Svedberg = $10^{-13}$ sec.

and sizes of these components in muscle cell homogenates are not indicative of their size in the living cell. Indeed, it is obvious from electron micrographs of intact muscle cells that myofibrils and the sarcoplasmic reticular and T-tubule membranes that constitute the microsomal fraction in homogenates are very extensive in situ and actually extend unbroken throughout the intact cell. Homogenization, and consequent rupture, of the outer cell membrane produces fragments of the plasma membrane and also ruptures the myofibrils, T-tubules, and sarcoplasmic reticular membranes inside the cell. The round, membrane-enclosed vesicles that constitute the microsomal fraction in muscle homogenates (see figures 11 and 12) are formed by sealing of the broken ends of ruptured sarcoplasmic reticular or T-system membranes due to surface tension forces generated by exposure of hydrophobic membrane.
lipoproteins to an hydrophilic homogenizing solvent. It is clear, therefore, that size of the particles in myofibril, plasma membrane, and microsomal fractions is related directly to the kind and duration of homogenization used to rupture the outer cell membrane; a mild or short homogenization will result in large particles with large sedimentation coefficients in these fractions, but severe or prolonged homogenization will cause very small particles with small sedimentation coefficients in the same fractions. If homogenization is severe or prolonged, other subcellular components such as nuclei, mitochondria, or lysosomes may also be damaged, and these damaged components will not sediment normally. Consequently, the homogenization procedure used to rupture cells has important effects on sedimentation properties of subcellular components during differential centrifugation, and homogenization conditions should always be specified when describing fractionation of subcellular components by differential centrifugation.

In addition to homogenization procedure, the type of centrifuge rotor or head can also have pronounced effects on separation achieved by differential centrifugation. Two general kinds of centrifuge rotors may be used for differential centrifugation: (1) an angle-head or conical rotor in which tubes are inclined at a fixed angle to the horizontal plane (figure 4); and (2) a swinging bucket or swing-out rotor in which tubes are suspended from a rotatable pin and are free to swing out and assume a perfectly horizontal position during rotation (figure 4). Thus far, it has not been possible to construct swinging bucket rotors capable of swinging large volumes at very high speeds, and most differential centrifugation has been done with angle-head rotors. Fortunately, it has been shown (Anderson, 1968; Pickels, 1943; Schumaker and Rees, 1969) that the theory for sedimentation of particles in a centrifugal field holds for sedimentation in angle-head rotors as well as it does for sedimentation in swinging bucket rotors. Regardless of the type of rotor, sedimentation always occurs radically in a direction perpendicular to the axis of rotation (second and fourth parts of figure 4). When sedimenting particles strike the outer inclined wall in angle-head rotors, they pellet there, and the pellet gradually slides toward the bottom of the tube depending on the interplay of frictional and gravitational forces (Anderson, 1968; Schumaker and Rees, 1969). Although earlier investigators (Pickels, 1943) suggested that sliding of pellet material along the outer inclined tube wall in angle-head rotors would produce convective disturbances serious enough to affect the rates at which particles sediment radially, more recent results (Charlwood, 1963) have shown that remarkably little convection occurs during sedimentation in angle-head rotors, and that angle-head rotors may even be used very effectively for density gradient centrifugation (Brentani et al., 1967; Fisher et al., 1964; Martin and Ames, 1961) where convective disturbances have serious effects. Because sedimentation in angle-head rotors does not seem to be accompanied by large convective movements, the principal difference between sedimentation in angle-head rotors and swinging bucket rotors is the much shorter sedimentation distances required to pellet materials against the tube wall in angle-head rotors (arrows in second and fourth parts of figure 4). Consequently,
Fig. 4. Schematic representation of sedimentation in angle-head and swinging bucket rotors. A cut-away side view of the angle-head rotor is shown in the tube held at an angle of 23.5° to the vertical (or 66.5° to the horizontal). At far right, a top view is shown of the swinging bucket rotor as it would appear when spinning; tubes all project directly outward radially (0° to the horizontal). In both kinds of rotors, sedimentation proceeds radially as indicated by the arrows.
the time required to sediment a given subcellular component out of a cell homogenate will likely be less when centrifugation is done in an angle-head rotor than when a swinging bucket rotor is used. Because of this, the kind of rotor used can also have important effects on the conditions necessary to obtain optimal separation by differential centrifugation.

Because velocity of sedimentation, or what is equivalent, centrifugal force developed by the centrifuge rotor, depends on radial distance from the center of rotation (see table 1), it therefore follows that centrifugal force varies depending on position of the particle in the centrifuge tube. For example, a particle near the bottom of the tube in a swinging bucket rotor is being subjected to a larger centrifugal force than the same particle would be if it were near the top of the same tube because its radial distance from the center of rotation is greater. These differences can become very large at high centrifuge speeds (e.g., between 180,000 x g and 420,000 x g for a commercial rotor at 65,000 rpm), and it is therefore important that they be considered when attempting to do careful differential centrifugal separation of subcellular components. Most centrifuge manufacturers publicize only the maximum forces obtainable with their instruments (i.e., the force at the bottom of the tube) to place themselves in the best competitive position. When listing centrifugal conditions in a scientific publication, however, the average centrifugal force (i.e., the centrifugal force developed at a position halfway between the maximum and minimum radial distances) should be given because this is the average force to which particles in that centrifuge tube would be subjected. At the very least, any departure from the procedure of listing the average centrifugal force should be explicitly indicated. The notation customarily used for listing centrifugal forces at different positions in the centrifuge tube is $\varepsilon_{\max}$, $\varepsilon_{\min}$ or $\varepsilon_{\text{ave}}$; hence 220,000 x $\varepsilon_{\text{ave}}$.

Separation of Subcellular Components of Muscle Cells by Differential Centrifugation.

With this brief introduction to some of the important factors affecting separation of subcellular components by differential centrifugation, the use of differential centrifugation to separate subcellular components from muscle cells will be discussed. Muscle tissue presents several unique problems to the application of differential centrifugation. First, muscle cells are surrounded by a tough basement membrane, 40-70 nm in thickness, and this basement membrane is in turn connected to the endomysial connective tissue layer. The presence of these two layers makes it very difficult to rupture the outer cell membrane, or sarcolemma, of muscle cells. Consequently, to obtain reasonably complete disintegration of muscle cells, particularly in mature muscle, it is necessary to mince the muscle very finely in an instrument like a silent chopper or to grind the muscle in a meat grinder. Such mincing or grinding generally fragments the sarcolemma into small pieces that sediment with the microsomal fraction, and may also damage the other large subcellular components such as nuclei.
or mitochondria. If large, relatively intact sarcolemmal sheaths are desired, the muscle tissue must be cut into small pieces with a scissors, and these pieces homogenized in a Waring Blender or similar device without subjecting the cells to severe mincing or grinding. Dr. Cedric Matsushima will probably discuss the procedure necessary to obtain intact sarcolemmal sheaths from muscle tissue in his paper later today. Mincing with a scissors followed by homogenization is not very efficient at rupturing cells from mature muscle cells, but it is possible to obtain reasonably complete rupture of embryonic muscle cells simply by using a Potter-Elvehjem or similar homogenizer (table 3). Occasionally, muscle cells from very young animals can also be ruptured without grinding or fine mincing. After the cells have been ruptured by grinding or fine mincing, a muscle cell homogenate is prepared by suspending the ruptured cells in a suitable medium with a Waring Blender or other similar device (table 3). Cell rupture, suspension of the ruptured cells, and all subsequent steps in separation of subcellular components by differential centrifugation should be done at cold temperatures (1-3°C) with pre-cooled apparatus and solutions to prevent undesired degradation of subcellular components by endogenous enzymes and to stop other metabolic and cellular reactions as completely as possible.

Some recommendations on ingredients that should be included in the homogenizing medium used to suspend ruptured muscle cells and the functions of these ingredients are given in table 4. Generally, KCl, which is the most abundant soluble substance in living mammalian muscle cells, or sucrose is used to maintain proper osmotic conditions surrounding the liberated subcellular components. Inclusion of KCl or sucrose is very important, for without these substances, mitochondria, nuclei, lysosomes, and microsomal vesicles will swell and burst. Myofibrils will also swell and lose all structure if they are suspended in a solution of zero osmolarity. In some instances, the homogenizing medium is deliberately made hypertonic to cause membranous subcellular components to shrink slightly in anticipation that the shrunken components will be more resistant to breakage during homogenization than their normal or slightly swollen counterparts. A small amount of buffer is included in the homogenizing medium to maintain pH of the homogenate near in vivo levels (table 4). Because phosphate has a much larger buffering capacity at pH 6.8-7.0, it is generally to be preferred over Tris (abbreviation for tris-(hydroxymethyl)-aminomethane). Phosphate buffer, however, must later be removed if the subcellular component is to be assayed for any enzymic activity involving release of inorganic phosphate (such as assaying myofibrils for ATPase activity). Inclusion of a buffer is particularly important in studies comparing postmortem muscle, which has a low pH, with muscle obtained immediately after death when it has a pH near 7.0. Omission of buffer from the homogenizing medium in such studies will result in data that are hopelessly confounded by the difference in pH under which the subcellular fractionations are done. A Ca²⁺-chelator and Mg²⁺ are included to limit the amount of myofibrillar contraction caused by grinding and homogenization. Even in the presence of a Ca²⁺-chelator and Mg²⁺, it is very difficult to
### TABLE 3. PREPARATION OF MUSCLE TISSUE FOR DIFFERENTIAL CENTRIFUGAL SEPARATION OF SUBCELLULAR COMPONENTS

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rupture cells by passing through a meat grinder or other cutter</td>
<td>mature muscle cells cannot be ruptured by Potter-Elvejhem or similar homogenizers and must be minced or ground. Meat grinders fragment the sarcolemma into small pieces.</td>
</tr>
<tr>
<td>2. Suspend ruptured cells in isotonic or hypertonic medium by use of Waring Blender or similar device</td>
<td>Potter-Elvejhem or similar homogenizer can be used for young muscle. Type of medium used and severity of homogenization can have important effects on the way subcellular components sediment during differential centrifugation.</td>
</tr>
</tbody>
</table>

### TABLE 4. INGREDIENTS IN MEDIUM USED TO SUSPEND BROKEN MUSCLE CELLS PRIOR TO DIFFERENTIAL CENTRIFUGATION

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 0.25 to 0.8 M sucrose or 50 to 150 mM KCl</td>
<td>maintain osmotic relationships to prevent disruption of organelles.</td>
</tr>
<tr>
<td>2. 10 to 50 mM Tris or K-phosphate buffer, pH 6.8-7.4</td>
<td>maintain pH at near in vivo levels to insure reproducible centrifugal separations.</td>
</tr>
<tr>
<td>3. 1 to 5 mM EGTA or EDTA</td>
<td>chelate Ca$^{2+}$ and deleterious heavy metals such as Fe$^{3+}$, Cu$^{2+}$, etc. Ca$^{2+}$ chelation helps to limit extent of contraction during homogenization.</td>
</tr>
<tr>
<td>4. 1 to 5 MgCl$_2$</td>
<td>help prevent actin-myosin interaction and contraction during homogenization. Use only with EGTA.</td>
</tr>
</tbody>
</table>

*a These ingredients are recommendations for general purpose differential centrifugal of muscle cells and different ingredients may be necessary for specific purposes.
prepare myofibrils completely in the relaxed state from muscle cells obtained immediately after death of the animal. Some reports (de Duve and Berthet, 1954) have indicated that, in liver cell homogenates, chelation of Ca\textsuperscript{2+} enhances mitochondrial stability, but that a trace of Ca\textsuperscript{2+} is necessary for preparation of intact nuclei by using differential centrifugation. To our knowledge, these assertions have not been tested in muscle cell homogenates. Obviously, because it chelates both Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, EDTA (abbreviation for ethylenediamine tetraacetic acid) should not be included in a homogenizing medium if Mg\textsuperscript{2+} is also included. As noted in table 4, the presence of EDTA or EGTA (abbreviation for 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane) in the homogenizing medium has the additional beneficial effect of chelating trace amounts of heavy metal contaminants that may be in the homogenizing medium or be released from the grinder or homogenizer, and that generally have deleterious effects on subcellular components.

A summary of the centrifugal conditions that may be used to fractionate muscle cell homogenates into myofibrillar plus nuclear, mitochondrial, lysosomal, microsomal and sarcoplasmic fractions is given in table 5. This table illustrates the second unique problem encountered when attempting to fractionate muscle cell homogenates by differential centrifugation; muscle cells contain myofibrils, a subcellular component not possessed by other cells, and myofibrils sediment with nuclei. Therefore, it is very difficult to obtain muscle nuclei not heavily contaminated with myofibrils by using differential centrifugation alone. On the other hand, myofibrils relatively free from nuclei can be obtained simply by subjecting the myofibrillar fraction to repeated homogenization in the Waring Blender. Such homogenization ruptures the nuclear membrane, and the fragmented membrane and nuclear contents are not sedimented at low centrifugal forces. The conditions of centrifugal speed and time listed in table 5 are for homogenizing times of 10-20 seconds and a homogenizing medium similar to that given in table 4. If a more viscous homogenizing medium or if longer homogenizing times are used, centrifugal speeds or times or both will need to be increased to obtain adequate separation. Of course, the converse would apply if shorter homogenizing times are used. Also, if only one of the five fractions listed in table 5 is desired, the centrifugal boundary conditions given as necessary to sediment that fraction might be widened to increase the yield of that particular fraction at the expense of purity of the fraction and yields of the bordering fractions. Although the 100,000 x g supernatant is listed as the final fraction in table 5, it is possible, with modern preparative ultracentrifuges, to sediment proteins and high molecular weight substances out of this supernatant. For example, any protein or other particle having a sedimentation coefficient of 5S or greater can be sedimented from the 100,000 x g supernatant by centrifuging for 5-8 hours at 75,000 rpm (500,000 x g).
TABLE 5. SEPARATION OF SUBCELLULAR COMPONENTS OF THE MUSCLE CELL BY DIFFERENTIAL CENTRIFUGATION

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Components Sedimented</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 2000 x g for 10-15 min</td>
<td>- Sediments nuclei, myofibrils, unbroken cells, connective tissue, and sarcolemmal sheaths.</td>
</tr>
<tr>
<td>2000 to 10,000 x g for 10-20 min</td>
<td>- Sediments mitochondria</td>
</tr>
<tr>
<td>10,000 to 25,000 x g for 20-30 min</td>
<td>- Sediments lysosomes and &quot;heavy&quot; microsomes</td>
</tr>
<tr>
<td>25,000 to 100,000 x g for 120-180 min</td>
<td>- Sediments microsomes, polysomes, ribosomes and fragments of sarcolemma</td>
</tr>
<tr>
<td>100,000 x g supernatant</td>
<td>- Sarcoplasmic proteins, t-RNA, and other soluble substances.</td>
</tr>
</tbody>
</table>

*a These conditions are useful for complete fractionation of a muscle cell homogenate. Different conditions may be more appropriate for separation of a specific component.

Because myofibrils are the contractile elements of muscle and because preparation of myofibrils is often the first step in purification of one or more of the myofibrillar proteins, myofibrils are probably the subcellular component most frequently prepared from muscle cell homogenates by differential centrifugation. Although a large number of different procedures have been described in the literature for preparation of myofibrils, several important improvements in these procedures have been made recently. Table 6 shows a procedure that our laboratory has developed for preparation of highly purified myofibrils. Removal of connective tissue by passing through a nylon strainer in Steps III and IV and solubilization of membranes adhering to the myofibrillar surface in Steps V and VI are particularly important features of this procedure. We have found that a common household nylon strainer is the most efficient device available for removing connective tissue from myofibrils. Treatment with 1% Triton X-100 (steps V and VI) has no detectable effect on the biological or structural properties of the myofibrillar proteins but almost completely removes the membranes that normally adhere to the surface of myofibrils. Because these membranes contain a Mg$^{2+}$-modified ATPase activity, it is important that they be absent from myofibrillar preparations that will be assayed for ATPase activity.
### TABLE 6. PREPARATION OF PURIFIED MYOFIBRILS BY USING DIFFERENTIAL CENTRIFUGATION

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Sediment</td>
<td>Ground or minced muscle</td>
</tr>
<tr>
<td>1</td>
<td>Suspend in 10 volumes (v/w) 100 mM KCl, 20 mM K phosphate, pH 6.8, 2 mM MgCl₂, 2 mM EGTA, 1 mM NaN₃ (standard salt solution) by homogenizing for 10 sec in Waring Blender.</td>
</tr>
<tr>
<td>2</td>
<td>Centrifuge at 1000 x g for 10 min.</td>
</tr>
<tr>
<td>II. Sediment</td>
<td>Supernatant (contains CAF and other sarcoplasmic proteins)</td>
</tr>
<tr>
<td>1</td>
<td>Suspend in 6 volumes (v/w) of standard salt solution by homogenizing for 10 sec in Waring blender.</td>
</tr>
<tr>
<td>2</td>
<td>Centrifuge at 1000 x g for 10 min.</td>
</tr>
<tr>
<td>III. Sediment</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>1</td>
<td>Suspend in 8 volumes (v/w) of standard salt solution by homogenizing for 10 sec in Waring blender.</td>
</tr>
<tr>
<td>2</td>
<td>Pass suspension through household nylon net strainer.</td>
</tr>
<tr>
<td>3</td>
<td>Centrifuge at 1000 x g for 10 min.</td>
</tr>
<tr>
<td>IV. Sediment</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>1</td>
<td>Suspend in 8 volumes (v/w) of standard salt solution by homogenizing for 10 sec in Waring blender.</td>
</tr>
<tr>
<td>2</td>
<td>Pass suspension through household nylon net strainer.</td>
</tr>
<tr>
<td>3</td>
<td>Centrifuge at 1000 x g for 10 min.</td>
</tr>
<tr>
<td>V. Sediment</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>1</td>
<td>Suspend in 6 volumes (v/w) of standard salt solution plus 1% (v/w) Triton X-100 by homogenizing for 10 sec in Waring Blender.</td>
</tr>
<tr>
<td>2</td>
<td>Centrifuge at 1500 x g for 10 min.</td>
</tr>
<tr>
<td>VI. Sediment</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>1</td>
<td>Suspend in 6 volumes (v/w) of standard salt solution plus 1% (v/w) Triton X-100 by homogenizing for 10 sec in Waring blender.</td>
</tr>
<tr>
<td>2</td>
<td>Centrifuge at 1500 x g for 10 min.</td>
</tr>
<tr>
<td>VII. Sediment</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>1</td>
<td>Suspend in 8 volumes (v/w) of standard salt solution by stirring vigorously with polyethylene stirring rod.</td>
</tr>
<tr>
<td>2</td>
<td>Centrifuge at 1500 x g for 10 min.</td>
</tr>
</tbody>
</table>
Supernatant (discard) 1) Repeat Step VII four times, but suspending in 8 volumes (v/w) of 100 mM KCl instead of standard salt solutions; centrifuge at 1500 x g after each suspension.

XIII. Sediment

Supernatant (discard) 1) Suspend in 8 volumes (v/w) of 100 mM KCl by homogenizing for 3 sec in Waring blender. 2) Centrifuge at 1500 x g for 10 min.

XIV. Sediment

Supernatant (discard) 1) Suspend in 2 volumes (v/w) of 100 mM KCl by homogenizing for 3 sec in Waring blender. 2) Do protein analysis of suspension.

Purified Myofibrils free of membranes
FRACTIONATION OF SUBCELLULAR ORGANELLES BY DENSITY GRADIENT CENTRIFUGATION

Although differential centrifugation is an invaluable method for separation of crude whole cell homogenates into fractions containing particles of the same size, it is almost impossible by using differential centrifugation alone to obtain these particles in highly purified form. Closer examination of figure 3 shows why this is so. In differential centrifugation, all subcellular components are distributed uniformly throughout the centrifuge tube at the beginning of the fractionation procedure (left tube, figure 3). During the time required to sediment the largest particles initially at the top of the tube all the way to the bottom of the tube (tube C, figure 3), the small particles that initially were near the bottom of the tube have also been pelleted because, even though the sedimentation coefficients of these small particles are far less than that of the large particles, they have a much shorter distance to travel before they are pelleted. It can be calculated from the equation in Table 1 that if the sedimentation coefficient of the heavier particles is three times larger than that of the smaller particles, 40% of the smaller particles will be sedimented in the time required to sediment 100% of the heavier particles. Even if the sedimentation coefficient of the heavier particles is ten times greater than that of the smaller particles, 13% of the smaller particles will be sedimented in the time required to pellet 100% of the heavier particles. Although the amount of contaminating, slowly sedimenting particles present in a fraction can be reduced by resuspending the pellet and resedimenting the suspension, such a procedure is very time consuming and subjects the subcellular component to undesirable additional homogenization and handling. Moreover, complete purity of the desired subcellular component is approached asymptotically when using repeated cycles of differential centrifugation, and many cycles of centrifugation and suspension are required to obtain 90% purity or greater for fractions that contain contaminants with sedimentation coefficients very similar to that of the main component. This situation is exacerbated when the same subcellular components exist in different sizes and have a range of sedimentation coefficients (see table 2 and earlier discussion in this review). Even though the optimal combination of conditions necessary to obtain purified fractions by differential centrifugation has been investigated theoretically (Schumaker and Rees, 1969), differential centrifugation clearly is not very useful for purifying subcellular particles after their initial fractionation from a crude cell homogenate.

Because preparations of subcellular particles must be as pure as possible for their biochemical functions in a cell to be assayed accurately, intensive efforts were made to develop satisfactory procedures for additional purification of the partly purified fractions obtained from crude cell homogenates by differential centrifugation. In 1951, Brakke, (Brakke, 1951, 1953) at the University of Nebraska introduced the idea of centrifugation in a density gradient, and this technique has now been expanded and developed until density gradient centrifugation is the commonly accepted procedure for purification of subcellular organelle
fractions obtained by differential centrifugation of crude cell homogenates. It should be clearly understood that in spite of the development of zonal rotors and zonal ultracentrifuges designed to accommodate the largest possible samples (Anderson, 1966), high-resolution, density gradient centrifugation is inherently limited to relatively small samples (for example, even zonal rotors cannot accommodate samples larger than 100 ml without loss of resolution). Because differential centrifugation, particularly at low centrifugal speeds, can easily be applied to samples of two liters or more, density gradient centrifugation is most appropriately used to purify the partly purified fractions obtained by differential centrifugation of crude cell homogenates. Hence, the "lysosomal fraction" obtained from as much as two liters of muscle cell homogenate by differential centrifugation (table 5) can easily be resuspended in 10-20 ml, and this latter volume can be readily accommodated by density gradient centrifugation.

Categories of Density Gradient Centrifugation

As a result of the intense development of density gradient centrifugation since its inception in 1951 (de Duve et al., 1959), at least two general categories of density gradient centrifugation can now be distinguished. Although both these categories employ a density gradient in the centrifuge tube, the purpose of the gradient and the physical basis underlying separation of the subcellular components in the two categories are completely different. The first category is the rate-zonal method, frequently referred to simply as the zonal method. The essential features of this method are illustrated in figure 5. The density gradient in zonal density gradient centrifugation is preformed and is represented by the gradual increase in shading between the top and bottom of the tubes in figure 5. Density gradients in zonal density gradient centrifugation are commonly made of sucrose because sucrose is inexpensive and readily available, but other gradient materials may also be used (see table 8 and subsequent discussion). The partly purified sample of subcellular components obtained after sedimentation by differential centrifugation and resuspension in a small amount of the appropriate solvent is carefully layered in a thin zone on top of the density gradient (hence, the name zonal centrifugation, figure 5). The density gradient in zonal centrifugation is used principally to support and stabilize the zone of layered material. Therefore, the solvent used to suspend samples destined for zonal centrifugation should not be denser than the top of the density gradient or the sample will sink in the gradient before centrifugation is started with resulting mixing and spreading of the zone. During centrifugation, the different subcellular components present in the starting zone sediment through the density gradient at rates determined primarily by their size and shape; these are the same factors that affect rate of sedimentation in differential centrifugation. In zonal centrifugation, however, all subcellular components are in a thin zone at the top of the tube when centrifugation begins rather than being distributed uniformly throughout the tube as
Fig. 5. A schematic diagram showing separation of particles by rate-zonal density gradient centrifugation. In rate-zonal centrifugation, samples are initially layered in a thin zone over the density gradient (Tube A). After centrifugation (Tube B), the different particles have sedimented different distances into the gradient; the distance the particles are sedimented is a function principally of the size and shape of the particles, and density of the particles is of lesser importance (note that larger particles have sedimented farther than medium-sized particles which have sedimented farther than small particles in the illustration in Table B). The density gradient in rate-zonal centrifugation is used to stabilize the initial and the sedimenting zones rather than cause separation on the basis of density. From Anderson (1965) and reproduced by permission of the author and Beckman Instruments, Inc.; © 1965 by Beckman Instruments, Inc.
TABLE 7. CATEGORIES OF DENSITY GRADIENT CENTRIFUGATION

<table>
<thead>
<tr>
<th>Type of density gradient centrifugation</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rate--zonal or zonal centrifugation</td>
<td>- gradient is preformed and components to be separated are layered on top of the gradient. Purpose of the gradient is to prevent convection during centrifugation. Different components sediment as bands or zones moving at different rates through the density gradient. Centrifugation may be at low or high forces but generally is not done for long (&gt;4-5 hr) periods of time. Separation depends on size, shape, and density of components.</td>
</tr>
<tr>
<td>2. Isopycnic--zonal or isopycnic centrifugation</td>
<td>- gradient may be preformed and the sample layered on it or sample and gradient material may be dispersed uniformly through the tube. Centrifugation is done at very high speeds (up to 75,000 rpm) and for long periods of time (24 hr or longer). Components are banded in the gradient at a point equal to their density and separation depends only on density.</td>
</tr>
</tbody>
</table>

they are at the start of differential centrifugation. Consequently, all particles must travel the same distance to be sedimented to the bottom of the tube. This allows faster sedimenting particles to be obtained completely free from more slowly sedimenting particles (figure 5). This procedure may be contrasted with differential centrifugation where some slowly sedimenting particles initially near the bottom of the tube must travel only a small distance to be pelleted and are therefore unavoidably sedimented with larger particles (cf. Figures 3 and 5). Zonal centrifugation separations are normally run until the heaviest components have sedimented nearly to the bottom of the tube. At this point, maximum resolution of the different sedimenting components is obtained. Most zonal centrifugation are done in swinging bucket rotors because it was thought that sedimentation in angle-head rotors was accompanied by serious convective distances that would disrupt the gradient. As indicated earlier in this review, however, the most recent information shows that sedimentation in angle-head rotors occurs relatively free from convective movements, and several studies have shown that high resolution zonal centrifugation separations can be done in angle-head rotors (Fisher et al., 1964; Martin and Ames, 1961). Swinging bucket rotors have continued to be popular for zonal centrifugation because they generally provide a longer path length for sedimentation than
angle-head rotors (figure 4), and greater path lengths produce greater resolution between similarly sedimenting components. It might be noted that although zonal centrifugation is considered a form of density gradient centrifugation, density of the components is not a major factor affecting their separation.

The preformed gradients illustrated in figure 5 can be prepared in a variety of ways. Figure 6 shows a simple, homemade apparatus that can be used to prepare linear density gradients. In this device, two vessels of identical inside diameter are placed at identical heights on two magnetic stirrers and connected at their base with a piece of tubing. The tubing connecting the two vessels and the outlet tubing from the front vessel (i.e., the vessel to the right in figure 6) are both clamped shut, and the material that will constitute the densest part of the gradient (e.g., 50% sucrose) is placed in the front vessel; in figure 6, the denser material is symbolized by greater density of dots. An exactly equal volume of the material that will constitute the light end of the gradient (e.g., 10% sucrose) is placed in the rear vessel. The combined volume of the two solutions is chosen to just equal the volume of one centrifuge tube. The clamp on the tubing connecting the two vessels is then removed, and the magnetic stirrers are started and adjusted to produce thorough mixing of the solution in the front vessel. Then the clamp on the outlet tubing from the front vessel is loosened, and the gradient solution is allowed to flow slowly down the side of the centrifuge tube. Every ml of solution removed from the front vessel into the centrifuge tube results in one-half ml of solution flowing from the rear vessel into the front vessel. The solution in the rear vessel is of low density, and this low-density solution is mixed with the high-density solution in the front vessel, so that density of the solution in the front vessel gradually decreases as solution flows from it into the centrifuge tube. The result is a solution of gradually decreasing density from the bottom of the centrifuge tube to the top (as symbolized in figures 5 and 6). If not disturbed or exposed to drafts or large temperature fluctuations, such gradients are stable for many hours. In general, however, it is wise not to prepare density gradients until shortly before they are to be used.

The second general category of density gradient centrifugation is isopycnic--zonal or simply isopycnic centrifugation (Meselson et al., 1957). This category of density gradient centrifugation is also sometimes referred to as "sedimentation equilibrium in a buoyant density gradient" or as "band centrifugation in CsCl" (Schumaker, 1967). The general features of this method are described in table 7 and are illustrated in figure 7. The gradient may either be preformed and the sample layered on it (right tube in figure 7) as in zonal centrifugation, or sample and gradient material may be uniformly dispersed throughout the tube (left side of figure 7) before starting the run. In either instance, centrifugation continues for a very long period of time, and the density gradient formed in the tube represents an equilibrium between sedimentation of the solute forming the gradient and diffusion of this solute against the centrifugal field. Hence, the final density gradient in isopycnic centrifugation is a function of speed of the centrifuge and size of the
Fig. 6. Schematic diagram of an apparatus for preparing linear density gradients. Two vessels of equal diameters are filled to the same level with the solutions chosen to make the gradient. The solution chosen for the dense end of the gradient is placed in the front vessel (greater density of dots), and the solution chosen for the light end of the gradient is placed in the rear vessel (fewer dots). When the tube connecting the vessels is opened and the outlet tube is opened, a linear density gradient such as that shown in the tubes is produced.
Fig. 7. A diagrammatic representation showing separation of particles by isopycnic-zonal density gradient centrifugation. At the beginning of centrifugation, the particles to be separated may be distributed uniformly in a homogeneous suspending medium (A) or the particles may be layered in a thin zone on a preformed gradient (B) as in rate-zonal density gradient centrifugation. In either situation, the final density gradient is formed by the centrifugal field itself (of course, the change involved in formation of the gradient is much greater for A than for B) and separation of the particles depends solely on the differences in their densities (note that the larger particles are banded higher in the gradient (C) than the smaller particles). From Anderson (1966) and reproduced by permission of the author and the U.S. Department of Health, Education and Welfare.
gradient-forming solute and is formed during the run itself. Even if a preformed gradient is used, the shape of this gradient may change during the run depending on speed of the centrifuge, etc. Isopycnic centrifugation runs are continued until each subcellular component has sedimented and banded in the gradient at a point exactly equal to its density (center tube, figure 7). Consequently, in contrast to differential centrifugation and zonal centrifugation, isopycnic centrifugal separations are based solely on density of the components, and size and shape have no effect on isopycnic centrifugal separations (note the larger particles banded higher in the schematic diagram in center tube, figure 7). Because width of sample bands decreases with increasing rotor speed, and because high rotor speeds move components to their densities more quickly, isopycnic centrifugation runs are normally done at the highest possible speeds. This combination of high speed and long runs places severe demands on centrifugal equipment, but very high resolutions are possible at the very great centrifugal forces used. For example, one of the classical separations done by isopycnic centrifugation involved resolution of single strands of DNA that differed only in that one of the strands contained a heavy isotope of nitrogen, $^{15}$N, whereas the other strand contained only $^{14}$N, the naturally occurring isotope of nitrogen. Density of these two DNA strands differed by only 0.0014 g/cm$^3$ (approximately 1.710 and 1.724 g/cm$^3$), and yet they were completely separated (Meselson and Stahl, 1958). Under favorable conditions, density differences of only 0.0005 g/cm$^3$ can be resolved by isopycnic centrifugation (Hearst and Schmid, 1973).

It is obvious, of course, that the material used to form the density gradient in isopycnic centrifugation must be dense enough to "float" all subcellular components in the sample. On the other hand, the density gradient in zonal centrifugation is used only to stabilize the sedimenting zones, and there is no requirement that the gradient be as dense or denser than the sedimenting components in zonal centrifugation. These differences in requirements obviously affect the choice of material used to form the gradient in density gradient centrifugation. As shown in table 8, sucrose and Ficoll, the two most popular gradient materials for zonal centrifugation do not form extremely dense solutions, and these materials are, therefore, not as useful for isopycnic centrifugation as they are for zonal centrifugation. Ficoll is a copolymer of sucrose and epichlorohydrin, and because it forms solutions with low osmolarity, it is frequently used in density gradient centrifugation studies involving intact cells where high concentrations of sucrose or salts would cause irreversible damage to the cell. Although cesium chloride forms very dense solutions and would, therefore, seem to be the material of choice for forming density gradients, it is extremely expensive ($100-400 of CsCl would be required to fill six, 25-ml centrifuge tubes) and irreversibly destroys many subcellular organelles at the high concentrations (up to 7M) used in density gradients. Metrizamide (2-(3 acetamido - 5-N-methylacetamido-2,4,6-triodobenzamido)-2-deoxy-D-glucose) has recently been introduced as a density gradient material. It is capable of forming fairly dense solutions at lower osmolarities than sucrose and may be useful for some applications involving density gradient centrifugation of proteins or ribosomes. At present, however, Metrizamide is also quite expensive.
TABLE 8. MATERIALS USEFUL FOR FORMING DENSITY GRADIENTS

<table>
<thead>
<tr>
<th>Material</th>
<th>Maximum density at 5°C (g/cm³)</th>
<th>Approximate maximum viscosity at 5°C (Centipoise)</th>
<th>Approximate maximum concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>1.32</td>
<td>620</td>
<td>860</td>
</tr>
<tr>
<td>Ficoll</td>
<td>1.16</td>
<td>1020</td>
<td>465</td>
</tr>
<tr>
<td>Cesium chloride</td>
<td>1.85</td>
<td>---</td>
<td>1160</td>
</tr>
<tr>
<td>Potassium bromide</td>
<td>1.32</td>
<td>---</td>
<td>510</td>
</tr>
<tr>
<td>Potassium citrate</td>
<td>1.51</td>
<td>---</td>
<td>960</td>
</tr>
<tr>
<td>Potassium tartrate</td>
<td>1.48</td>
<td>---</td>
<td>950</td>
</tr>
<tr>
<td>Metrizamide</td>
<td>1.47</td>
<td>246</td>
<td>850</td>
</tr>
</tbody>
</table>

Densities of Cells and Subcellular Components

Densities of cells and some subcellular components as determined by isopycnic centrifugation are given in table 9. Because the gradient material in which the density determination was made affects the hydration and, therefore, the density of the component, the gradient material used for the density determination in table 9 is given in parentheses after each density. It is evident from comparing component densities in table 9 with properties of the gradient materials in table 8 that CsCl would be necessary for isopycnic studies of glycogen, DNA, and RNA. Although it would be possible to use sucrose in isopycnic studies of proteins and nuclei, such studies would be extremely difficult because of the exceedingly high viscosities of the very concentrated sucrose solutions necessary for such studies. Hence, in practice, the use of sucrose in isopycnic studies is limited to components with the density of lysosomes or less. Because they are useful at opposite ends of the density range in table 9, sucrose and CsCl have been by far the most widely used materials in density gradient centrifugation, and approximately 95% of all density gradient studies done use one of these two materials to form the gradient.
TABLE 9. DENSITIES OF SOME SUBCELLULAR CONSTITUENTS⁴

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Approximate density (g/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>1.06 - 1.12 (Ficoll)</td>
</tr>
<tr>
<td>Microsomes</td>
<td>1.12 - 1.18 (sucrose)</td>
</tr>
<tr>
<td>T-tubules and sarcolemma</td>
<td>1.10 - 1.12 (sucrose)</td>
</tr>
<tr>
<td>Sarcoplasmic reticular membranes</td>
<td>1.13 - 1.17 (sucrose)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.14 - 1.19 (sucrose)</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>1.15 - 1.20 (sucrose)</td>
</tr>
<tr>
<td>Muscle lysosomes</td>
<td>1.18 (sucrose)</td>
</tr>
<tr>
<td>Protein</td>
<td>1.28 - 1.33 (sucrose)</td>
</tr>
<tr>
<td>Nuclei</td>
<td>1.29 - 1.35 (sucrose)</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>1.43 (sucrose) to 1.54 (CsCl)</td>
</tr>
<tr>
<td>Glycogen</td>
<td>1.61 - 1.65 (CsCl)</td>
</tr>
<tr>
<td>DNA</td>
<td>1.69 - 1.75 (CsCl)</td>
</tr>
<tr>
<td>RNA</td>
<td>1.85 - 1.95 (CsCl)</td>
</tr>
</tbody>
</table>

⁴ Gradient material used to determine the density of each component is given in parentheses immediately after each density. In many instances, the type of gradient material used will affect the hydration and, therefore, the density of the particle. Some densities were determined at temperatures above 25℃ to permit greater solute solubility and, therefore, greater density (cf. CsCl density for RNA and in table 8).
Application of Density Gradient Centrifugation to Crude Muscle Cell Homogenates

The remainder of this review will discuss three different examples of density gradient purification of subcellular components. The first example (figure 8) is an attempt to purify subcellular components directly from crude muscle cell homogenates by using zonal centrifugation. Because crude muscle homogenates contain all the subcellular components of muscle cells in one suspension, the quantity of any single component in the homogenate is necessarily limited. Consequently, large volumes of the homogenate are required to obtain appreciable quantities of any single purified subcellular component and such large volumes require that a zonal rotor be used. In the experiment shown in figure 8, a rotor with 1500 ml capacity was used; 60 ml of a muscle homogenate made by homogenizing frog skeletal muscle in three volumes (v/w) of 0.25 M sucrose, 1.8 mM CaCl₂, 1 mM Tris buffer at pH 8 for three minutes in a Waring Blender was applied to the gradient. Only four clearly defined fractions could be obtained by zonal centrifugation of the crude muscle homogenate under these conditions (figure 8) even though it is possible to resolve crude muscle homogenates into five partly purified fractions by using only differential centrifugation (table 5). Furthermore, biochemical and electron microscope analyses of the four fractions obtained by zonal centrifugation showed that all four fractions were mixtures of several components. The fraction labeled "1" contained soluble proteins, nucleic acids, and other soluble material as well as smaller particulate material (probably the microsomal fraction in table 5). The fraction labeled "2" contained larger particulate material (probably the heavy microsomal fraction of table 5), some mitochondrial fragments, and some small filaments. Fraction 3 contained collagen fibers and myofibrils that appeared free from sarcoplasmic reticular membranes, whereas fraction 4 contained collagen fibers and myofibrils that had an abundant amount of sarcoplasmic reticular membranes adsorbed to their surface. Mitochondria and nuclei were not clearly resolved into any single fraction. It is clear, therefore, that zonal centrifugation, when used directly on crude muscle cell homogenates, does not satisfactorily resolve the subcellular components present in such homogenates. Although there are several reasons for the ineffectiveness of zonal centrifugation when applied to whole cell homogenates, one of the principal sources of difficulty is the presence of components with such very widely differing sedimentation coefficients in the sample that it is impossible to choose a centrifuge speed and time that will not completely sediment the heaviest components but will also sediment the lighter components at least a small distance into the gradient (note that fraction 1 in figure 8 is still on the surface of the gradient).
Fig. 8. Separation obtained by zonal density gradient centrifugation of a crude homogenate of frog skeletal muscle. A total of 60 ml of crude muscle homogenate in 250 mM sucrose, 1.8 mM CaCl$_2$, 1 mM Tris·HCl, pH 8, was layered onto a sucrose gradient made by layering 200 ml of 13% sucrose over a 700 ml continuous, linear 33 to 54% sucrose gradient with an underlay of 300 ml of 55% sucrose. An overlay of 140 ml of 1.8 mM CaCl$_2$, 1 mM Tris·HCl, pH 8, was introduced over the sample. Loading was done in a zonal rotor at 1000 rpm, the rotor was accelerated to 2000 rpm for 10 min, then decelerated to 1000 rpm, and immediately unloaded by pumping 55% sucrose into the outer edge of the rotor. The effluent steam was monitored at 260 nm (solid line) and collected in 40 ml fractions. Sucrose concentration of each fraction was measured refractometrically (dotted line). Aliquots of each fraction were pelleted and resuspended for electron microscope and biochemical assays. Reproduced from Barber and Canning (1966) by permission of the authors and the U.S. Department of Health, Education and Welfare.
Fractionation of the Muscle Microsomal Fraction by Density Gradient Centrifugation

The data in figure 8 demonstrate that zonal centrifugation cannot be used effectively to separate all subcellular components from a crude muscle cell homogenate. Figures 9, 10, 11, and 12 show the results when density gradient centrifugation is used to purify fractions that have been separated and partly purified by differential centrifugation. A microsomal fraction (see table 5) was obtained by differential centrifugation between 15,000 x g for 20 minutes and 123,000 x g for 57 minutes of a crude homogenate of rabbit skeletal muscle (Heuson-Stiennon et al., 1972). This microsomal fraction was resuspended in 0.25 M sucrose, 0.05 M KC1, and the suspension was layered onto a 0.74 M to 2.05 M continuous linear sucrose gradient. The subcellular components in the microsomal suspension were separated by isopycnic density gradient centrifugation (figures 9 and 10) into three fractions: (1) a low-density fraction (density 1.10-1.12 labeled 1 in figure 9 and 10); (2) a high-density fraction (density 1.13-1.17 labeled 2 in figures 9 and 10); and (3) a contaminating fraction (labeled C in figure 9 and unlabeled peak just before letter b in figure 10). Biochemical analyses showed that the low-density fraction had no Ca2+-sequestering ability, but that the high-density fraction possessed potent Ca2+-binding ability. Neither the low-density nor the high-density fractions had any cytochrome oxidase activity, so both fractions were therefore probably free from contamination by mitochondrial membranes. The contaminating fraction contained nearly all cytochrome oxidase activity present in the original partly purified microsomal fraction, so this fraction probably represented the mitochondrial contamination present in the original microsomes.

Electron microscope observations (figures 11 and 12) showed that the low-density fraction was heterogeneous and contained many tubular or tadpole-like structures (figure 11) that resembled the T-tubules seen in sections of intact muscle (cf. figure 2 and No. 12-16 in figure 11) as well as fragments of the outer cell membrane (sarcolemmal fragments, No. 17 in figure 11) that had been ruptured when the tissue had been passed through the meat grinder (see paper by Dr. Matsushima later in this volume). Indeed, in a few instances (no. 14 in figure 11), what appeared to be a T-tubule still lying between two lateral cisternae could be seen in sections of the low-density fraction. The high-density fraction, on the other hand, was structurally homogeneous and consisted of vesicular structures 90-120 nm in diameter in either negatively stained (no. 18 in figure 12) or positively stained (numbers 19 and 20 in figure 12) sections. It is very likely that these vesicular structures are pieces of the sarcoplasmic reticular membrane (see figure 2) that have been ruptured by grinding and homogenization. Membrane thicknesses in both the low-density and high-density fractions are approximately 7.5-10.0 nm, and in some instances (no. 20 in figure 12), a triple-layered or subunit structure of the membrane can be discerned. Dr. Stromer will discuss structure of the unit membrane in detail later in this volume. Because the high-density fraction accumulates Ca2+ but the low-density fraction does not, these electron microscope observations indicate that all Ca2+ accumulating ability in skeletal muscle is in
Fig. 9. Subfractions of a muscle microsomal fraction are seen as light-colored bands in this centrifuge tube after centrifugation at 25,000 rpm for 15 hrs. Rabbit leg muscle was ground, suspended in 180 mM sucrose, 100 mM phosphate buffer, pH 7.2, by using a Potter homogenizer for 3 min, and sedimented at 1500 x g for 5 min to remove nuclei and myofibrils. KCl was added to the supernatant to give a final KCl molarity of 250 mM (to keep myofibrillar proteins soluble), and a microsomal pellet was sedimented between 15,000 x g for 20 min and 123,000 x g for 57 min. The microsomal pellet was resuspended in 250 mM sucrose, 50 mM KCl and centrifuged at low speed to remove contaminating myofibrillar protein. The resulting supernatant was layered onto a continuous linear sucrose density gradient (25 to 70% sucrose) and centrifuged at 25,000 rpm for 15 hours to produce the separation seen in this tube. Subfraction 1 (area labeled 1 in figure) is banded at a density of 1.10-1.12 and subfraction 2 is banded at a density of 1.13-1.17. C=contaminating band, arrow indicates top of the gradient. From Heuson-Stiennon et al. (1972) and reproduced by permission of the Rockefeller University Press and the authors.
Fig. 10. The distribution of proteins (solid line labeled "a") and optical densities (dashed line labeled "b") measured at 280 nm of a muscle, microsomal fraction after density gradient centrifugation at 39,000 rpm for 2 hr is shown in this figure. The muscle microsomal fraction was prepared as described in the legend to Fig. 9. A continuous, linear sucrose density gradient from 25 to 70% sucrose was used. The fractions labeled 1 and 2 correspond to those similarly labeled in Fig. 9. The dotted line labeled "c" is the molar concentration of the sucrose gradient. From Heuson-Stiennon et al. (1972) and reproduced by permission of the Rockefeller University Press and the authors.
Fig. 11. These electron micrographs of the low density fraction labeled 1 in Fig. 9 and 10 show that this fraction contains numerous tubular, tail-like structures (arrows in 12) and several triads (T in 13) that are reminiscent of the triads seen in sectioned muscle. The area labeled T in 13 is shown at higher magnification in 14 where its resemblance to the triad is quite striking. Higher magnifications of the tubular structures observed in 12 are shown in 15 and 16; these structures may be segments of T-tubules broken during grinding and homogenization. The typical, folded, sarcolemmal membranes seen in the low-density fraction are shown in 17; the granular material (arrows) may represent fragmented, fibrous proteins. The sarcolemma in this fraction has been broken during grinding and homogenization because if the sarcolemmal sheath had remained intact, it would have sedimented with the myofibrillar fraction. 12, 13 and 17; X25,900. 14, 15 and 16; X105,000. From Heuson-Stiennon et al. (1972) and reproduced by permission of the Rockefeller University Press and the authors.
Fig. 12. These electron micrographs of the high-density fraction labeled 2 in Fig. 9 and 10 show that this fraction is structurally more homogeneous than the low density fraction, and consists almost entirely of thin-walled vesicles. A negatively stained view is shown in 18 and a positively stained section is shown in 19. At the higher magnifications shown in 19, the positively stained membranes appear triple-layered (arrows), and minute spots are sometimes observed. The vesicles in this fraction are probably formed by resealing of sarcoplasmic reticular tubules that are ruptured during grinding and homogenization. 18 and 19; X25,000; 20; X137,000. From Heuson-Stiemmon et al. (1972) and reproduced by permission of the Rockefeller University Press and the authors.
the sarcoplasmic reticular membranes and that the T-tubules, lateral cisternae, and sarcolemmal membranes of skeletal muscle have no Ca\(^{2+}\) accumulating ability. It is evident from this brief summary of the evidence in figures 9, 10, 11, and 12 that careful density gradient purification of partly purified subcellular components obtained by differential centrifugation can result in precise localization of biochemical functions. The microsomal purification summarized in figures 9-12 also illustrates the usefulness of density gradient centrifugation to purify partly purified fractions obtained by differential centrifugation in contrast with the inability of density gradient centrifugation to purify subcellular components from crude muscle cell homogenates (figure 8).

**Purification of Muscle Polysomes by Density Gradient Centrifugation**

The last example of the use of density gradient centrifugation to purify subcellular components from skeletal muscle involves zonal centrifugal purification of the polysomes that translate for synthesis of myosin (figure 13). Homogenates of 76-hour cell cultures of fused chicken skeletal muscle myoblasts are prepared by aspirating the cells gently in 250 mM KCl, 10 mM MgCl\(_2\), 10 mM Tris \(\cdot\) HCl, pH 7.4, 0.5% Triton X-100. The lysed cells are centrifuged for 10 minutes at 12,000 \(x\) g, and the supernatant is then layered onto a continuous linear 15 to 40% sucrose gradient. This concentration of sucrose is obviously not sufficient to "float" polysomes (see tables 8 and 9) so the separation is rate-zonal. Centrifugation at 150,000 \(x\) g for 90 minutes separates the 12,000 \(x\) g supernatant into a range of different fractions (figure 13). The fractions on the right side in figure 13 are those that did not sediment very far and probably represent proteins and other soluble, ultraviolet-absorbing substances in the 12,000 \(x\) g supernatant that was layered onto the gradient. Because myosin contains very large polypeptide chains, the m-RNA that codes for synthesis of myosin must also be large, and polysomes \(i.e.,\) m-RNA plus ribosomes\) that translate for myosin would be expected to have sedimented a considerable distance into the gradient. Assay of fractions obtained from the density gradient indicated that only fractions 3-6 were able to catalyze synthesis of the heavy chains of myosin (figure 13). Electron microscope examination of the material in these fractions indicated (figure 14) that it contained very large polysomes similar to those described by Heywood et al. (1967) as capable of supporting myosin synthesis. Consequently, it is possible, by using a few differential centrifugal steps followed by density gradient centrifugation, to isolate from a crude muscle homogenate a class of polysomes that translate for synthesis of a single protein.

**CONCLUSIONS**

Although this review has been restricted to a discussion of the use of centrifugation to separate subcellular components and other particles from each other, the universal applicability and unique
Fig. 13. Density gradient profile of a polysome fraction prepared from a homogenate of 76-hr culture of fused chick skeletal myoblasts. Fused chick skeletal myoblasts were lysed by aspirating gently in 250 mM KCl, 10 mM MgCl₂, 10 mM Tris·HCl, pH 7.4, 0.5% Triton X-100, and this homogenate was centrifuged for 10 min at 12,000 x g to remove myofibrils, unbroken cells, nuclei, and mitochondria. The supernatant from this centrifugation is layered onto a continuous, linear 15 to 40% sucrose density gradient and centrifuged for 90 min at 150,000 x g. The tubes are unloaded by pumping dense sucrose solution into the bottom of the tube and monitoring the effluent at 260 nm (heavy continuous line). Approximately two ml fractions were collected. Polysomes in each fraction were pelleted by centrifuging at 150,000 x g for 2 hrs through 1.5 M sucrose and were then assayed for ability to incorporate ³H-leucine into the heavy chain of myosin (line connected by dots). Incorporation was measured as amount of radioactivity incorporated into the 200,000 molecular weight band isolated by SDS-polyacrylamide gel electrophoresis of the incorporation mixture.
Fig. 14. This electron micrograph of the heavy polysome fractions isolated from a 15-40% linear, continuous sucrose density-gradient centrifugation of the polysome fraction prepared from chick skeletal myoblasts (see Fig. 13) shows that these heavy polysome fractions contain polysomes large enough to be responsible for synthesis of the heavy chain of myosin. Fifty to sixty ribosomes may be counted in the polysome shown in the upper center of this micrograph (cf. Heywood et al., 1967). X36,200.
competency of the centrifuge to purify subcellular particles should be evident from the few examples given here. Muscle biologists and meat scientists have, in general, not used density gradient purification as widely as many other molecular and cellular biologists, and significant advances in our understanding of the muscle cell and of meat quality may be possible by careful application of density gradient purification to some of the problems of muscle biology and meat science. Hopefully, this review might prompt such applications.

ACKNOWLEDGEMENTS

We are grateful to Darlene Markley for drawing figures 4 and 6 and to Janet Stephenson for assistance with the tissue culture studies. The patience and assistance of Joan Andersen and Barbara Hallman in drafting this manuscript is also gratefully acknowledged.

LITERATURE CITED


M. D. Judge: We thought it would be appropriate to call the speakers back one at a time now and let them "top off" their presentations. Specifically we asked them to make a comment about a way of applying the research method discussed and then you may have questions or comments.

R. M. Robson: As I was saying before, we are interested in this proteolytic enzyme and its effect on myofibrillar proteins. Before, I just went through individually purified proteins; however, in some work done by Dennis Olson in our laboratory we actually digested the entire myofibrill with this enzyme. This is an example of putting the entire myofibrill in SDS and then electrophoresing it. You can identify the myosin heavy chains, the alpha actinin, actin and tropomyosin. Essentially we found that this component, which we presume to be troponin T in bovine muscle, is very quickly lost from the myofibril. In approximately the same time one gets a degradation product showing up. One gets a somewhat similar occurrence if one simply ages muscle after death. As post-mortem aging progresses, the band disappears.

C. S. Chung, is our laboratory, is studying myofibrillar proteins prepared from muscle that has been cooked for different times and temperatures. This is another type of problem that can be done with this technique. As you heat the muscle past about 60°C you start to pick up extra bands. Now these are not degradation products; they are, in fact, sarcoplasmic proteins which are precipitated out on the myofibrils during cooking and then are isolated on the myofibrils.

M. L. Greaser, University of Wisconsin: Could we go back to the second slide? What are the molecular weights on the left side of those second and third bands up from the bottom?

R. M. Robson: This is tropomyosin at 35,000 and this one probably alkali 1 and perhaps troponin I at about 25,000.

M. L. Greaser: In other words, there is no troponin C there?

R. M. Robson: Troponin C in this gel would probably be in this region, although it does not show up very well.

P. B. Addis, University of Minnesota: I was interested in the slide of the heat denatured protein. By use of SDS electrophoresis, you seem to get good electrophoretic separation of the heat denatured proteins; do you think that it might be possible to do a species identification on cooked meat samples or resolve a mixture of two different species?

R. M. Robson: It might be possible but it would be a little difficult. Perhaps I could have Marion Greaser comment on troponins. This is one of the myofibrillar proteins that will change slightly in the subunit molecular weights. For instance, different muscles from the same animal (i.e., cardiac and skeletal muscle) will differ. Marion, have you checked different species to see if they also vary between animals or between species?
M. L. Greaser: We have not done much checking on this. The few species that we have looked at do not appear to be much different in the subunit molecular weights. I think there is a little difference in chicken muscle, for instance, in the troponin T component, but for horse, beef and pig muscle I think all have approximately the same molecular weights for all the myofibrillar components.

R. M. Robson: One, of course, can identify mixtures of products that contain soy protein or milk protein.

C. E. Allen, University of Minnesota: Rich, I think all of your gels here have been on myofibrillar proteins. Perhaps the key to Dr. Addis' question is found in the sarcoplasmic proteins after heating. Did you find a precipitation of sarcoplasmic proteins onto your myofibrillar proteins? Would this be possible for identification of species?

R. M. Robson: If one prepares the sarcoplasmic proteins from different muscle, one does get a tremendous difference in quantity of glycolytic enzymes. I would guess that it would be a little difficult to use this but I think that it is a fruitful area that someone should look at. Mr. Chung feels that the bands that he is getting after heating are not made up of sarcoplasmic proteins because, if he makes an SDS gel of the extract and compares it, they line up perfectly with these extra bands on the myofibril.

J. J. Guenther, Oklahoma State: Did you define CAF treatment?

R. M. Robson: We are using a purified enzyme and it is normally added in most of these results with a weight to weight ratio of one to two hundred done at 25°C for one hour.

J. J. Guenther: Did you indicate that you could not electrophorese myosin?

R. M. Robson: It is extremely difficult to electrophorese it in the absence of a denaturing solvent. Florini and Brewer tried under a number of circumstances and failed every time unless they put in urea, which, of course, is another dissociating reagent.

T. R. Dutson: By heating above 60°C, there seem to be some changes that take place in the myofibrillar protein. Are some of these being heat denatured and just not coming on with the gel?

R. M. Robson: I think the problem you refer to is that the same protein load was not applied to each gel. I think it is a case of just not having equal protein loads. If one maintains the same concentration on the gels one does not see any difference even in the bands down in the troponin and the light chain region of myosin.

T. R. Dutson: Then the heating of these proteins up to these high temperatures apparently doesn't cause enough change at least in the protein structure to alter electrophoretic patterns?
R. M. Robson: It is certainly not breaking any covalent bonds. You are, in fact, getting out intact polypeptide chains.

M. D. Judge: Let's move on to the other speakers and then if we have time we can come back to Dr. Robson's paper. Dr. Dutson, may we hear from you?

T. R. Dutson: As a brief reintroduction, I would like to recall the last micrographs I showed of electronmicroprobe x-ray analysis. I think this technique is something that meat scientists or muscle biologists could be working with to a greater extent. The preparation seems to be very difficult, but I think that, with use of various techniques such as critical point drying, the tissue could be prepared with the elements remaining localized fairly well in the tissue as in the intact state. There are also problems of analyzing tissue components or tissue elements at the low levels that are sometimes present. I think one particular application here might be in determining certain additives or certain chemicals such as metals and contaminants that might be present in the muscle tissues. Also, the electron microscope and the scanning electron microscope could be used in analyzing systems such as meat emulsions using freeze fracture techniques to break the emulsion. Breakage would probably occur at the membranous unit around the fat portion of an emulsion. It seems to me that we could utilize the microscope to give us an idea of how this structural system imparts texture. If we could also utilize the microscope and various microscope techniques in combination with biochemical and histochemical techniques, we could get an idea of the way some of these components exist in a structural relationship and then possibly relate this to some of the textural properties of muscle.

P. B. Addis: You showed that the Z line of the red fiber was thicker than that of the white fiber. How does the intermediate fiber Z line thickness compare with the others?

T. R. Dutson: When you look at the ultra-structure there seems to be considerable variation between the two types. The intermediate fiber, although when stained by various enzymes seems to be rather distinct, is quite variable in the ultra-structure. However, there does seem to be a definite intermediate group, the Z lines of which appear to have characteristics in between the red and white types as far as mitochondrial content and other structures are concerned.

R. L. Henrickson, Oklahoma State University: Elaborate a little further on why the Z line is wider in one case than the other.

T. R. Dutson: There could be a relationship with the contraction speed; whether the pressure on the myofibril in the white fiber is any different than that in the red fiber I really don't know. Another possibility might be that there is more of the amorphous material in the Z line surrounding the heavier structural proteins in the red fibers.
R. G. Kauffman, University of Wisconsin: I would like to have you clarify a point regarding your ability to prepare materials in a completely relaxed form. Have you ever attempted to use the technique you described in a biopsy situation in which you did not remove the tissue until after it was fixed?

T. R. Dutson: We have taken some biopsies and, of course, as soon as we cut into the muscle we saw a tremendous amount of contraction. Our techniques could be used by biopsy but it would result in the necessity to sacrifice the animal because of the rather large sample size. I don't believe you could call the muscle as I prepare it a relaxed sample. It is a restrained sample. A relaxed sample would be something different.

F. C. Parrish, Iowa State University: Can you or have you looked at changes occurring in muscle fibers during postmortem aging?

T. R. Dutson: We have looked at the post mortem changes in isolated collagen fibers in section material but we have not looked at the differences between post mortem muscle samples. I think that the techniques involved in preparation are particularly important. We need to break this muscle tissue apart in the proper place to get the perspective of the connective tissues. As it looks now in shattering the muscle, you don't really have much control of the site of breakage. I think we need to devise some technique for tearing the muscle apart to look at these connective tissues between early and late post mortem times, after enzyme treatments, and other variations.

M. H. Stromer, Iowa State University: I would like to respond to Dr. Kauffman's comment. It is possible to do a biopsy on a living animal and I have modified various surgical clamps that are available on the market. One can maintain a relaxed sarcomere length without too much difficulty. The problem that one gets into is this: When you subject an intact piece of muscle with its excitable membranes and its load of ATP to the temperature shock that necessarily accompanies biopsy, you, of course, cause depolarization and a resultant shortening.

M. D. Judge: Thank you. Let's move on to the third paper. Dr. Goll, may we have your summary comments?

D. E. Goll: I have a slide or two to show an example of the use of isopycnic centrifugation for separation of subcellular components. This is a tube that has had a microsomal fraction from muscle layered on top and centrifuged for 15 hours at 25,000 RPM. This microsomal fraction has been separated into three bands: band labeled number 1, band labeled number 2, and then a contaminating band. These three bands were isolated and studied biochemically. The contaminating band contained all the mitochondria that were originally present as an impurity in the microsomal fraction. The next slide will show a micrograph of the material in band number 1. These micrographs are all at approximately 37,000 magnification. You will see areas where it appears that we have T tubules with the lateral cisternae on either side of them.
These, in fact, also might be fragments of T tubules that have been broken by homogenization; consequently that upper band labeled number 1 seems to contain T tubules and also lateral cisternae. It was of interest that this band did not bind any calcium, suggesting that neither lateral cisternae nor T tubules possess calcium accumulating ability. Also found in this fraction that was labeled number 1 in the preceding slide, are sarcolemma membranes that have been fragmented by passing through a meat grinder. The next slide shows an electron micrograph of that fraction labeled number 2. This is a much more homogenous looking field. This is negatively stained at the top and positively stained at the bottom. This material does accumulate calcium and is apparently sarcoplasmic reticular membranes.

R. M. Robson: I was wondering what would be a relative proportion in a muscle cell of the T tubule plus lateral cisternae compared to the SR?

D. E. Goll: Other workers found three parts by weight of the SR fraction as compared to one part by weight of the T tubules.

C. E. Allen: With the isopycnic centrifugation, do you think there is a possibility of applying this to things like fat cells?

D. E. Goll: I would guess that you would have to start with layering the gradient on top of the sample. You would start almost in reverse with the material at the bottom and then centrifuging it up. You would probably separate different populations of fat cells according to their fat content.

R. G. Cassens, University of Wisconsin: Would you comment on the use of enzymes such as collagenase as a prestep to the centrifugation that you described?

D. E. Goll: There has been one report that I know of using collagenase to "soften up" muscle cells prior to homogenization so you don't have to subject the cell to a meat grinder or something so severe to disrupt the cell. Apparently it worked. I have never used collagenases myself because I would be concerned about the presence of contaminating proteases and their effect on the cell membrane. It is difficult to purify collagenases although I understand that purified preparations are now available.

R. A. Merkle, Michigan State University: I have a question for Dr. Robson. Based on the effect that the CAF factor had on tropomyosin, are you thinking along the lines that the Z line disintegration might be an effect on the tropomyosin rather than alpha actinin?

R. M. Robson: We are thinking along several lines as a matter of fact. Since the complete ultra-structure of the Z disc is not known for certain, and since we don't know how far tropomyosin and troponin go into the structure itself, it is quite possible that neither actin
nor alpha actinin are altered but rather are released. There is another
slight possibility which we have not yet completely ruled out and that
is that there is a very small amount of another protein present which
is not widely recognized as myofibrillar protein.

R. A. Merkel: If you accept the idea of an effect on tropomyosin,
then how do you rectify the differences between red and white fiber
types, since there is much less disintegration of the Z line in red
fibers during aging as compared to white fibers?

R. M. Robson: I would explain that primarily not from a difference
in the Z line itself but from a difference in the amount of the calcium
activated factor present in the different types of cells. That might be
the most logical explanation.

W. W. Migura, Wilson and Company: If enzymic activity can be
characterized within one particular subunit of the protein and that
subunit subsequently eluted, is the enzymic activity negated by the use
of SDS?

R. M. Robson: Yes, of course, it would be. When you add SDS you
denature the protein so you would lose the activity. If you are thinking
of actually preparing the protein and still having it active you would
have to electrophorese it in the absence of SDS and then work with that
fraction.

M. L. Greaser: There is actually a method of removing SDS from
proteins and it has been shown that certain enzymes can, in fact, be
renatured after being put in SDS. It involves passing the purified
protein in a mixture with SDS through an ion exchange resin and in
many cases, one can recover 75 to 90% of the enzymatic activity, so
SDS is not necessarily a permanent denaturing agent but in the presence
of SDS, of course, it is unfolded.

R. M. Robson: I didn't mean to imply that it was totally impossible.
The reference he is talking about is Weber and Kooder in JBC, 1971.

C. E. Allen: I'd like to ask Dr. Dutson if, in the scanning scope,
you can distinguish between collagen, reticulin, and elastin fibers?

T. R. Dutson: No, I really don't think there is any way unless
you are able to label one protein with something like silver staining
or some electron dense substance and then take a scanning electron
micrograph or secondary electron image and use the electron microprobe
to pick up that specifically stained substance. In this way you might
be able to get the structural relationship of those two. In our work
thus far, all we can see is fibrous networks which might not even be
collagen, elastin or reticulin. They might just be other proteins or
mucopolysaccharide-protein complexes that are in the ground substance
connecting the other fibrillar proteins. All of the proteins in this
fixed state would appear rather fibrous so it is difficult to say at
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* * *

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fixed state would appear rather fibrous so it is difficult to say at
this point.

* * *
B. B. Marsh: In opening this session on "Muscle Membranes and Meat Properties," I would first like to express my thanks to the committee who have so ably assisted me. Their names are listed in the back of your program, and I would particularly mention the cooperation I received from Fred Parrish, the coordinator of this session.

To the initiated, membranes are an exciting area; to the uninitiated, they are a very complex and involved field full of sometimes incomprehensible jargon. For this reason we feel it is particularly appropriate that the topic should be discussed at length today. If we are ever going to control meat quality, it is clear that we must first understand meat and, of course, the muscle from which it is derived. The empirical try-it-and-see methods of improving meat quality have just about had their day. From now on we've got the difficult problems to solve—the ones which are not amenable to the direct trial-and-error approach and we hope that our topic today will lay the groundwork for a phase of renewed vigor in this area.

We've assembled four outstanding workers today, and I'm personally very grateful to them for agreeing to present papers under this general title. The first paper, "The Structure and Function of Cell Membranes," is to be given by Dr. Marv Stromer of Iowa State University.