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A METHOD FOR RAPID FRACTIONATION OF PARTICULATE SYSTEMS BY GRADIENT DIFFERENTIAL CENTRIFUGATION

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In initial experiments on the fractionation of mitochondria into subparticulate constituents, it became apparent that for isolation of the particles, a method more satisfactory than the customary methods of differential centrifugation was needed. The subject of this report is a simple and rapid method for obtaining relatively uncontaminated preparations of particulate cell components by gradient differential centrifugation, in quantities sufficient for further subfractionation. The method depends on the use of a centrifuge distribution head that allows rapid construction of liquid density gradients. Although we have been primarily concerned with the isolation of mitochondria free of nuclei and small particles, the method is applicable, with suitable modification, to a variety of particulate systems.

The resolution of suspensions of particles, such as tissue breis, into fractions of relatively uniform size and density is greatly improved if the centrifugation is accomplished in continuous density gradients [1, 2, 4, 10]. This is because centrifugation anomalies that lead to mixing and cross contamination are largely eliminated [1, 2]. The literature describing the experiences of several workers in adapting gradient differential centrifugation to various systems is reviewed by Anderson [2] and Svensson *et al.* [19]. Papers not mentioned by these authors are by Weber [22], who describes the separation of the particulate components of *Xenopus* eggs in density gradients, and by Thomson and Klipfel [20], who relate enzymatic activity to the size of particles isolated by gradient differential centrifugation from liver breis. Cardinal difficulties with previous methods of gradient differential centrifugation have been (*a*) the time required to produce the gradients and (*b*) the small volume of brei fractionated per tube. In the method previously described by Anderson [2], for example, 2–3 ml of material could be fractionated per tube, and the filling of each required 90 minutes.

[1] Operated by Union Carbide Nuclear Comapny for the U.S. Atomic Energy Commission.

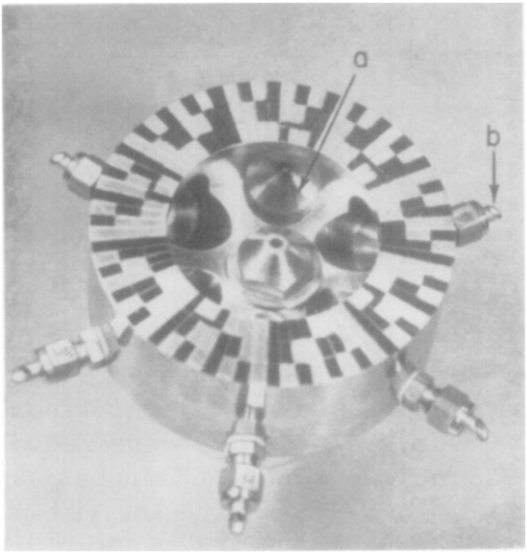


Fig. 1 *A*.

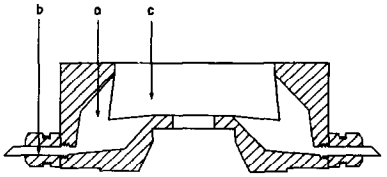


Fig. 1 *B*.

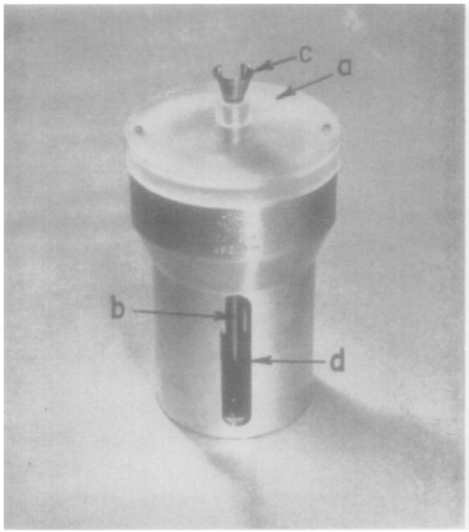
Fig. 1*A*.—Centrifuge distribution head. *a*, Radially oriented concavity; *b*, outlet tube. Scored and painted for stroboscopic speed control.

Fig. 1*B*.—Centrifuge distribution head. *a*, Radially oriented concavity; *b*, outlet tube; *c*, receiving bowl.

Increase in capacity can be achieved only through an increase in the number and in the cross-sectional area of the tubes. However, tubes cannot be filled rapidly with a continuous density gradient without swirling and mixing unless the gradient is stabilized, as in a centrifugal field. We, therefore, explored methods for filling centrifuge tubes during centrifugation and designed a gradient distribution head that apportions one continuously produced density gradient among six 250 ml centrifuge tubes. At 1400 rpm, the distribution head divides the density gradient stream into 140 segments per second, thus ensuring even distribution among the tubes.

MATERIALS AND METHODS

Construction and operation of the distribution head.—The circular distribution head (Fig. 1) is 5 inches in diameter and 2 inches high and was fabricated from type 347 stainless steel. In operation, it is mounted on the shaft of the International Equipment Company's model PR-2 centrifuge resting on the conventional yoke-type centrifuge head No. 259. The distribution head is divided into six equally spaced, radially oriented concavities shown in Fig. 1*A* that are tapered at the bottom and connect with outlet tubes (Fig. 1*B*) projecting from the periphery of the head.



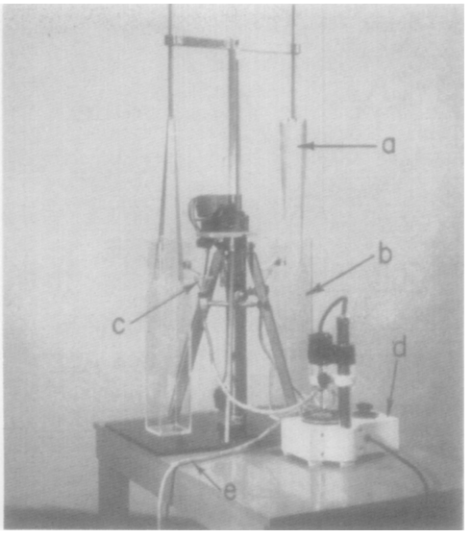


Fig. 2. Fig. 3.

Fig. 2.—Duralumin cup. *a*, Lucite cap; *b*, conduit tube; *c*, slotted funnel; *d*, observation slit.
Fig. 3.—Gradient engine. *a*, Lucite blocks; *b*, lucite vessels; *c*, funnels; *d*, high-speed mixer; *e*, conduit to distribution head.

The six duralumin cups that accompany head No. 259 are equipped with lucite caps held in place by brass pins (Fig. 2). The latter support stainless steel tubes (Fig. 2) flared and slotted at the centripetal end. The flared ends of the tubes are designed to enclose the centrifugal ends of the outlet tubes on the distribution head. The centrifugal ends of the conduit tubes extend almost to the bottom of 250 ml Pyrex bottles that are placed inside the duralumin cups (the necks of the bottles have been cut off to permit the use of the lucite caps).

During acceleration, the cups swing to the horizontal position, and the flared ends of the conduit tubes enclose the tips of the outlet tubes. Liquid is introduced into the bowl of the distribution head through a funnel and pipe that extend through the hinged top of the centrifuge. Liquid entering the rotating bowl is distributed equally and continuously into the concavities and is forced outward into the glass bottles.

In operation, the centrifuge is accelerated to 1400–2200 rpm; and the tissue brei, or other suspension, is introduced. This is followed at once by the gradient, beginning with the least dense liquid and progressing to the denser mixture. The brei is thus displaced centripetally, remaining on the surface of the gradient. Mixing is prevented by the centrifugal force. In this manner,

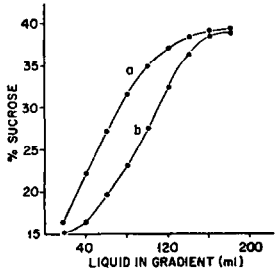


Fig. 4.

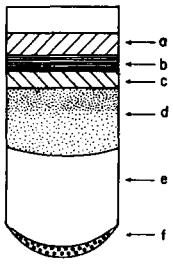


Fig. 6.

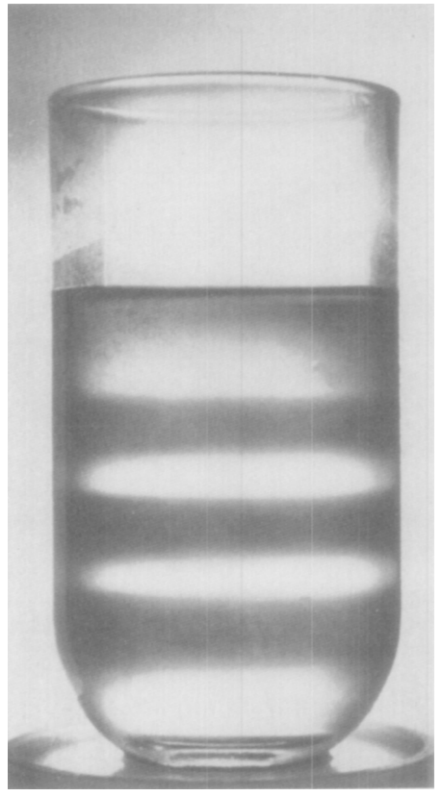


Fig. 5.

Fig. 4.—Curves describing gradient produced by (*a*) pyramid, inverted-wedge system, (*b*) double pyramid system.

Fig. 5.—Stratification of sucrose solutions of eight different densities; alternate solutions were colored with toluidine blue dye.

Fig. 6.—Stratification of rat liver brei in a sucrose gradient. Average particle diameter increases from near the limit of microscopic resolution (*a*) to 1.8 μ (*d*); nuclei found only in (*f*).

the time required for the construction of the sucrose gradient in all six 200-ml glass bottles is less than 10 minutes. For evaluation of the performance of the apparatus, the centrifuge was modified to allow the production of the gradient and the subsequent separation to be observed directly during centrifugation. We did this by cutting slits in the duralumin cups (Fig. 2) and providing a glass port in the bottom of the guard bowl and a hinged top of 1-inch lucite on an International Model PR-2 centrifuge. Illumination was provided by a high-intensity stroboscopic lamp[1] mounted below the guard bowl port. For accurate stroboscopic determination of speed of revolution, the top of the distribution head was scored and painted (Fig. 1*A*) as previously described [1].

[1] Strobolux, Type 648-A and Strobotac, Type 631-B, manufactured by General Radio Company.

Construction and operation of the gradient engine.—The gradients were prepared with a gradient engine developed by Brown [7] (Fig. 3), slightly modified to deliver a gradient of the desired character. The device lowers two lucite blocks of suitable shape (Fig. 3) into two vessels containing liquids of different density. The liquid displaced by the lucite blocks is caught in two funnels and conducted to a high-speed mixer (Fig. 3) to which a flask with three side arms was adapted. It is essential that the two liquids be rapidly and thoroughly mixed. From the mixer, the gradient liquid is conveyed to the bowl of the distribution head.

The shapes of the lucite blocks depend on the nature of the gradient desired. Gradients of two different types were tested for the fractionation of rat liver breis. Fig. 4 (curve 6) shows the sigmoidal curve that characterizes the gradient produced when a lucite pyramid (base: 5.71 × 5.71 cm, height 43.8 cm), suspended upright, descends into 15 per cent sucrose while an identical pyramid, suspended in the inverted position, descends into 40 per cent sucrose. Curve *a* in Fig. 4 was obtained by replacement of the inverted pyramid, which descends into 40 per cent sucrose, with a lucite wedge having the same over-all dimensions.

The composition of the gradient at any instant is given by the ratios of the horizontal cross sections of the lucite shapes at the level of the surface of the liquid. If the upright pyramid and inverted wedge have two sides parallel, the ratios of the two cross sections may be calculated as follows: both shapes have square bases of equal size (b^2) and have the same total height L. The cross section (S^2) at height l for the pyramid is

$$S_p^2 = \frac{b^2 (L-l)^2}{L^2}, \tag{1}$$

and for the inverted wedge

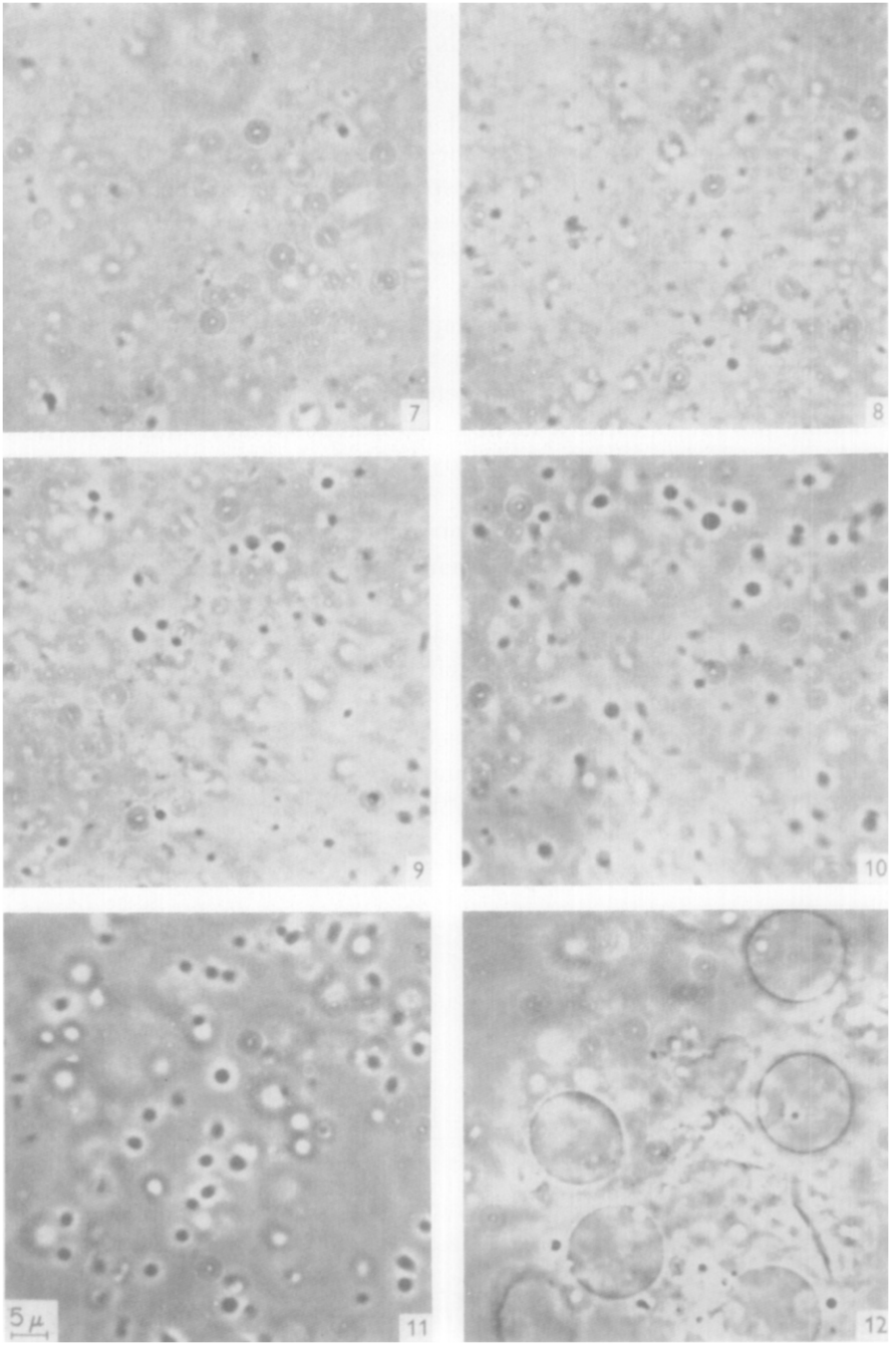
$$S_w^2 = \frac{b^2 l}{L}. \tag{2}$$

Combined, the ratio is found to be

$$\frac{S_p^2}{S_w^2} = \frac{(L-l)^2}{Ll}. \tag{3}$$

The shape of the gradient is easily calculated from expression (3) and the formula for the volume (V) delivered as a function of l:

$$V = \frac{b^2 L}{3} - \frac{b^2 (L-l)^3}{3L^2} + \frac{b^2 l^2}{2L}. \tag{4}$$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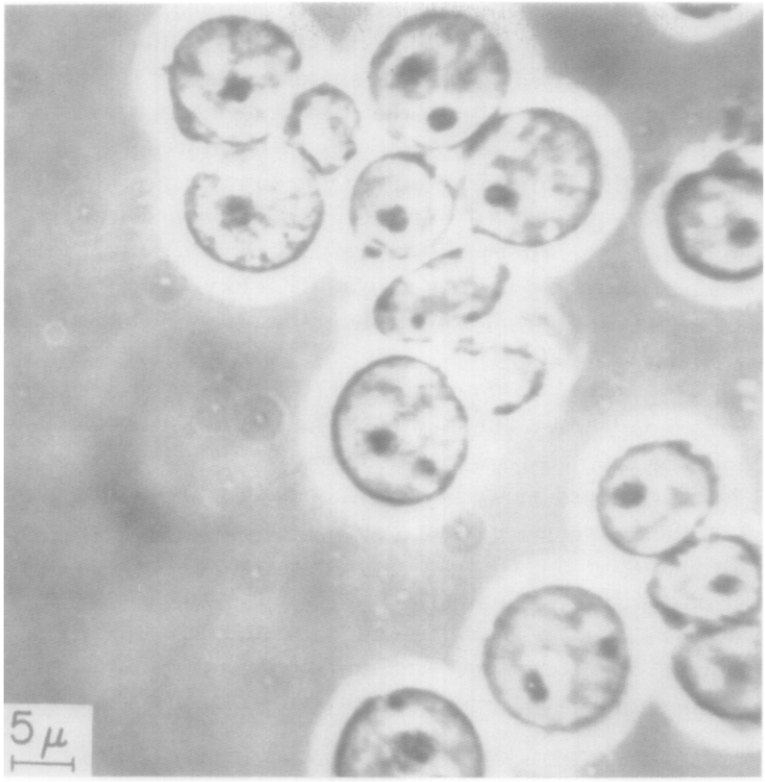

Fig. 13.—Phase photomicrograph of nuclei obtained after recentrifugation of layer *f* in sucrose gradient at 450 × *g* for 10 minutes. Note absence of contamination. Oil immersion, dark-M objective.

Fig. 7.—Phase photomicrograph of layer *a* of stratified rat liver brei. Oil immersion, dark-M objective.

Fig. 8.—Phase photomicrograph of layer *b* of stratified rat liver brei. Average particle diameter, 1.0 μ. Oil immersion, dark-M objective.

Fig. 9.—Phase photomicrograph of layer *c* of stratified rat liver brei. Average particle diameter 1.4 μ. Oil immersion, dark-M objective.

Fig. 10.—Phase photomicrograph of layer *d* of stratified rat liver brei. Average particle diameter 1.8 μ. Oil immersion, dark-M objective.

Fig. 11.—Phase photomicrograph of layer *d* of stratified rat liver brei after centrifugation through gradient a second time. Oil immersion, dark-M objective.

Fig. 12.—Phase photomicrograph of layer *f* of stratified rat liver brei after centrifugation at 800 × *g* for 90 minutes. Note contamination of nuclei by mitochondria. Oil immersion, dark-M objective.

Rat liver breis.—Sprague-Dawley rats were stunned, exsanguinated, and the livers rapidly perfused with Lock's solution. The livers were removed, weighed, and homogenized in 0.25 *M* sucrose plus 1×10^{-4} *M* $CaCl_2$. The total brei volume was three times the liver weight. The brei was strained through four layers of cheesecloth. It was then subjected to gradient fractionation either without further dilution (concentrated brei) or after dilution with 0.25 *M* sucrose plus 1×10^{-4} *M* $CaCl_2$ to a final concentration of one part liver in ten parts of brei (*w/v*) (dilute brei). The temperature of the brei and of the sucrose gradient did not rise above 6°C at any time.

The sucrose solutions from which the gradients were constructed usually contained 1×10^{-4} *M* $CaCl_2$.

Chemical estimations.—Ribonucleic acid (RNA) was extracted from freshly isolated mitochondria by the Schmidt-Thannhauser-Schneider procedure [15, 21] and was estimated spectrophotometrically by the orcinol reaction for pentose [6]. A standard curve based on a commercial sample of yeast RNA was used as the reference. Protein was estimated both in fresh, unextracted mitochondria and in the residue remaining after extraction. The analysis was based on the biuret reaction as modified by Weichselbaum [23]. Total nitrogen was estimated by the micro-Kjeldahl method [14].

The chemical data are expressed in amount per million mitochondria. Counts of mitochondria were made as described by Shelton *et al.* [17].

RESULTS

To establish that the operation of the distribution head did not lead to appreciable mixing of the liquid traversing it, we made preliminary tests by introducing into the head equal volumes of solutions of 8 different concentrations of sucrose of specific gravity from 1.03 (10 per cent) to 1.17 (40 per cent). Alternate concentrations were colored with a few drops of toluidine blue dye. Fig. 5 shows the stratification resulting in the receiving bottles. Very little mixing was observed.

The success of fractionation of the breis depended in part on the character of the gradient. Although a gradient characterized by a sigmoidal curve (Fig. 4, plot of concentration vs. volume of gradient) afforded slightly greater stratification of the brei, the various strata were noticeably contaminated by particles that, from microscopic examination, should have been localized elsewhere. A gradient described by curve *a* (Fig. 4) allowed the best resolution of a brei into layers of uniform particle size. The stratification achieved in a typical experiment after 90 minutes' centrifugation at $960 \times g$ (center

of bottle) is shown diagramatically in Fig. 6. The dimensions of the particles and the uniformity of size in samples from the various strata are shown in Figs. 7–13. The centrifugal force developed under the conditions described is not sufficient for satisfactory resolution of the very small particles according to their size and density. Phase microscopic examination of the top layer, *a* (Figs. 6, 7), reveals a heterogeneous population of small particles, with a few large particles and many lipid droplets. The size of the majority of the visible particles is near the limit of optical resolution. Layer *b* (Figs. 6, 8) is rich in particles 0.3–1.6 μ in diameter (average diameter, 1.0 μ). The diameter of the majority of the particles in layer *c* ranges from 1.0–2.1 μ (Figs. 6, 9). The average diameter of these particles, 1.4 μ, is greater than that of the particles in layer *b* but less than that in layer *d*. The particles in layer *d* (Figs. 6, 10) appear to be typical large mitochondria. The size of the particles in this layer is quite uniform (1.6–2.6 μ; average diameter, 1.8 μ). We can achieve even greater uniformity of particle size by removing this layer after the first gradient centrifugation, concentrating it by high-speed centrifugation and centrifuging in a gradient a second time (Fig. 11). An additional indication of the uniformity of layer *d* results from centrifugation at high speed (21,000 $\times$ *g*) after recovery from the gradient. The resulting pellet is devoid of a "fluffy" layer that is generally attributed to concomitant sedimentation of large microsomes [see 16]. Layer *e* (Fig. 6) is almost devoid of particulate material. The cell nuclei are concentrated in layer *f* (Fig. 6) at the bottom of the bottle. Microscopic examination (Fig. 12) reveals that they are highly contaminated with aggregates of large mitochondria. However, recentrifugation of layer *f* in a gradient one or two times (less than 12 minutes' centrifugation time is required to sediment the nuclei at 450 $\times$ *g*) permits the preparation of a relatively uncontaminated batch of nuclei (Fig. 13). Two or more times through the gradient seems to result in some shrinkage and granulation of the nuclei (see Figs. 12, 13), although other media might prevent nuclear damage. All the nuclei seem to be sedimented by this procedure; *no* nuclei were discovered in other layers.

It should be understood that the centrifugal fractionation of a particulate system depends on both size and density of the various particles. Thus the separation, according to size, of a suspension into layers in which the particle size is perfectly uniform cannot be achieved if the particles also vary in density and vice versa. This largely accounts for the range in particle size found in the separate layers.

By this method, the time required for the establishment of a total volume of 1200 ml (six bottles, 200 ml per bottle) of gradient of continuously varying

TABLE I. *Some chemical analyses of mitochondria isolated by differential gradient centrifugation.*

Number of determinations	Substance	Amount per million mitochondria ($\mu g \times 10^{-2}$)
4	RNA	1.1
4	Nitrogen	3.8
4	Protein	21.0

density is less than 10 minutes. A volume of 120 ml of brei (20 ml per bottle), corresponding to 1–2 rat livers, is satisfactorily fractionated in about 90 minutes' centrifugation time. The entire operation may be easily performed in 2 hours.

Since our interest is in the procurement of uncontaminated mitochondria, we have adjusted conditions to permit maximum development of layer *d*. The particulate matter in this layer has been partially characterized chemically as shown in Table I.

DISCUSSION

The method described should be applicable in the fractionation of particulate systems in general. Thus, in addition to the separation of liver subcellular fractions, it may be useful in the resolution of mixtures of cells of different types into groups of cells, each of which is relatively uniform with respect to size and density. This should permit more meaningful analyses of changes in tissues under various conditions by permitting the analysis of changes in several cell types. It should also facilitate studies of the interactions of different cell types under various circumstances. For example, Anderson *et al.* [3] used differential gradient centrifugation for the resolution of suspensions of bone marrow cells into several distinct groups.

Gradient differential centrifugation for fractionation of cell particles for biochemical studies has not been widely used. This may be attributed, in part, to the relatively low yield and complex apparatus required. It should be pointed out that the use of discontinuous gradients for achievement of cell fractionation, such as those used by Kuff and Schneider [12], suffers from the same sources of error as does differential centrifugation in which a gradient is not used.

Work to date [8, 9, 11, 13, 18] indicates that a significant portion of cyto-

plasmic protein may be held in common by microsomes, mitochondria, and the soluble phase. Yet the proteins that are restricted to these sites are likely to be the most interesting for further biological experimentation. Although a number of enzymes are known to be largely localized at certain intracellular sites, we believe, temporarily ignoring enzymatic activity, that a fresh approach to the characterization of cytoplasmic proteins may be rewarding. The availability of an analyzable quantity of reasonably homogeneous starting material should make future studies on the protein composition of mitochondria more meaningful.

SUMMARY

A method is designed for the rapid fractionation of large volumes of suspensions of particles varying in size and density by gradient differential centrifugation. A centrifuge distribution head is used that permits the rapid construction of continuous liquid density gradients since the gradients are stabilized by centrifugal force as they are formed. The application of the method to the fractionation of rat liver breis is described.

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