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Analytical Differential Centrifugation: An Analysis of the Sedimentation Properties of Synaptosomes, Mitochondria and Lysosomes from Rat Brain Homogenates

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A newly developed analytical differential centrifugation procedure for use in angle rotors was used to analyze the sedimentation of certain brain subcellular particles. Particle sedimentation was measured over a wide range of applied forces, and a curve descriptive of this sedimentation was computed. Precise conditions for complete and fractional sedimentation of mitochondria, choline acetyltransferase-containing nerve-end particles, and lysosomal activities from rat brain cerebral cortex homogenates were established. Mitochondria, nerve-end particles, and lysosomal activities began to sediment together, but all mitochondrial cytochrome oxidase sedimented before nerve-end particles and lysosomes. Even though populations overlapped, a discrete sizing of mitochondria and nerve-end particles was accomplished. Mitochondria sedimentation was similar to an ideal particle even though brain mitochondria are composed of at least two populations. These data are compared with those used by other investigators to provide a basis for making a decision on significant differences among diversified separation conditions.

Differential centrifugation is widely used as a preliminary enrichment and purification procedure for the separation of subcellular particles. The selection of optimum separation conditions for these procedures is therefore essential. Brain tissue, because of its heterogeneity and variety of components, presents a particularly difficult problem in subcellular separations. Existing methods for the separation of mitochondria, nerve-end particles (NEP) or synaptosomes,[4] and lysosomes employ different differential centrifugation conditions. Data are not available to enable the investigator to decide the differential centrifugation conditions optimal for his particular purposes. The objective of these studies is to quantify the precise sedimentation of these subcellular particles and to show the applicability of analytical differential centrifugation to other such separation problems.

The method of analytical differential centrifugation involves sedimentation in an angle rotor in defined solution volumes while monitoring the total sedimentation forces

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[4] Abbreviations used: NEP, nerve-end particles or synaptosomes; ChA, choline acetyltransferase; S_{obs}, sedimentation coefficient of a particle under conditions stated; η, viscosity in centipoises; ρ, density in g/cc; ω, angular acceleration in radians per sec.

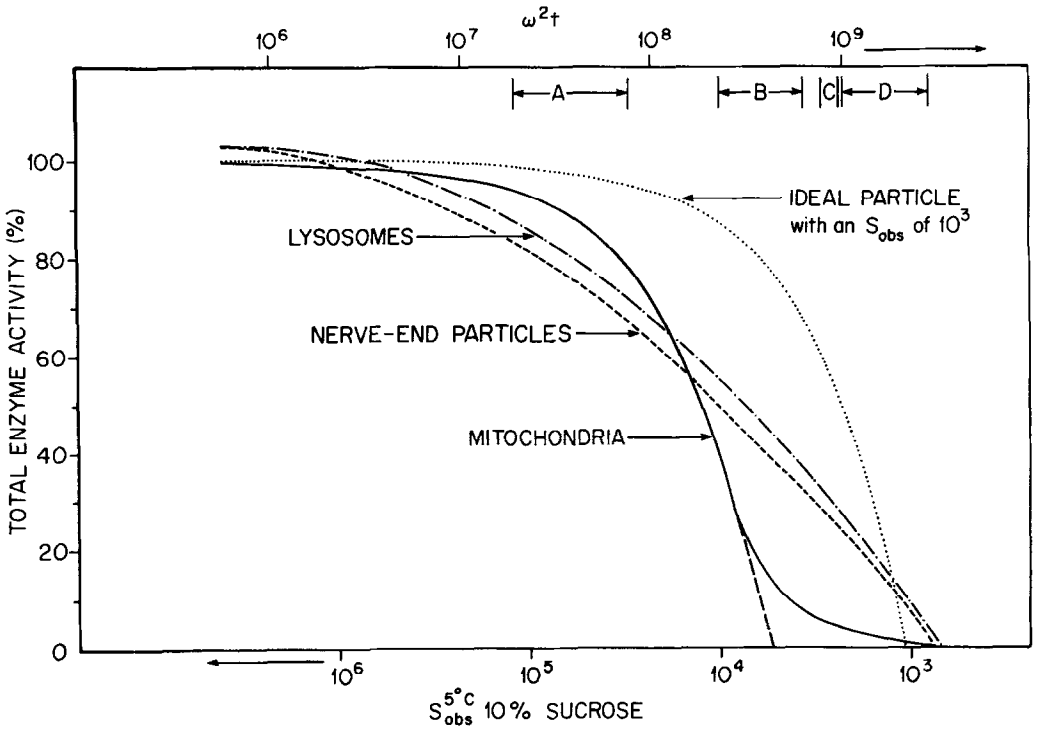

FIG. 1. A composite of the mitochondria, NEP, and lysosomal sedimentation curves shown in Figs. 2–4. The ordinate represents the particle enzyme activity remaining in the supernatant after centrifugation of a 2% brain homogenate in 10% sucrose for the integrals ($\omega^2 t$) shown on the abscissa. The sedimentation scale on the abscissa was aligned with the $\omega^2 t$ scale by solving equation in Methods section for a series of values of $\omega^2 t$, with $C_f/C_i = 0$. The ideal particle curve was obtained by solving the same equation for a series of values of C_f/C_i for a 10^3S particle.

with a digital integrator (1). This method, developed by Anderson (2), is precise enough to provide accurate measurements of the sedimentation coefficients of bovine serum albumin, red blood cells, and latex beads. We find it to be quite applicable to subcellular particles as well. We studied the sedimentation profile of three brain particles in isotonic sucrose over a variety of forces by following them with enzyme markers and morphology.

All steps of the method are quantitative, repeatable, and reproducible from one investigator to another regardless of the type of centrifuge employed. The need to approach sedimentation problems empirically is eliminated even for particles with unknown sedimentation characteristics. The method also allows the investigator to estimate the sedimentation coefficients of particles, permitting him to select the gradient centrifugation program best suited for subsequent purification of the particles. The theoretical aspects and preliminary studies of the method have been discussed in a previous paper (2).

We studied the sedimentation of three well-defined subcellular components from brain: mitochondria, NEP, and lysosomes. The conditions used for the separation of brain subcellular particles are particularly critical and in need of precise definition because of the wide variety of structures contained in brain homogenates and the overlapping sedimentation properties of these structures.

MATERIALS AND METHODS

Mitochondria were assayed by cytochrome oxidase. The sedimentation of NEP was followed

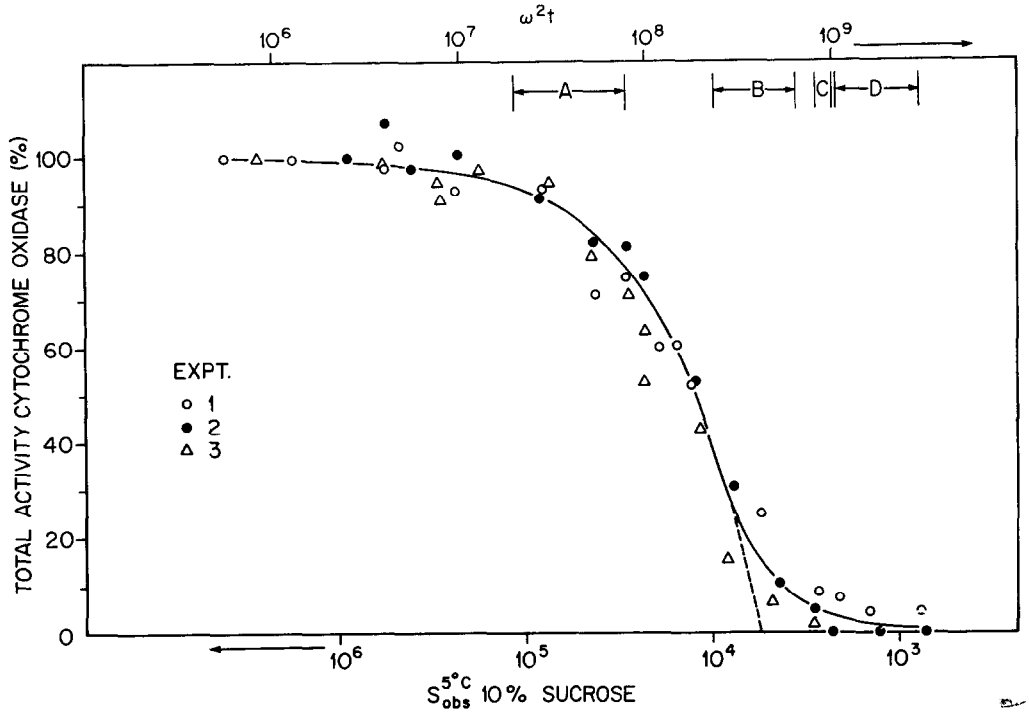

FIG. 2. Sedimentation curve for brain mitochondria. The points (from three separate experiments) represent the percent cytochrome oxidase activity remaining in the supernatant after centrifugation of a 2% brain homogenate (in 10% sucrose) in a No. 30 Spinco rotor for measured integrals (ω^2). The curve that fits the data best was obtained by computer computations utilizing equation described in Methods section. (Points were deleted where C_f/C_i was less than 10.2% of the enzyme activity.) The dotted projection intercepts the X axis at 5.4×10^3 S, which represents an average sedimentation coefficient for approximately 85% of the mitochondrial population.

TABLE I

SUMMARY OF EXPERIMENTS OF BRAIN HOMOGENATE SEDIMENTATION IN A NO. 30 SPINCO ROTOR

Particle enzymes	$\omega^2 t$	$g \cdot \min$[a]	$S_{50,10\%}$
Cytochrome oxidase	4.2×10^8[b]	57×10^3	5.4×10^3
Choline acetylase	3.1×10^9[c]	420×10^3	7.2×10^2[c]
Acid phosphatase	3.5×10^9[d]	475×10^3	6.5×10^2[c]

[a] $g \cdot \min$ are related to $\omega^2 t$ by the formula: $\omega^2 t = \dfrac{g \cdot \min \cdot 60 \cdot 980}{R_{av}}$ where g = force times gravity at R_{av}; min = minutes of total centrifugation time; R_{av} = average radius in cm.

[b] Value obtained for "large" particle mitochondrial population representing approximately 85% of the cytochrome oxidase activity removed from the supernatant after an $\omega^2 t$ integral of 4.2×10^8 $rad^2\ sec^{-1}$. Sedimentation coefficient given is for a hypothetical particle that would have the sedimentation curve nearly identical to that observed.

[c] Value obtained for complete sedimentation of particulate enzyme activity.

[d] Values shown represent the smallest class of hypothetical particles that would sediment in that integral ($\omega^2 dt$). The values are not to be construed as the sedimentation coefficients of the respective particles, but are an indication of the range of particle sizes observed.

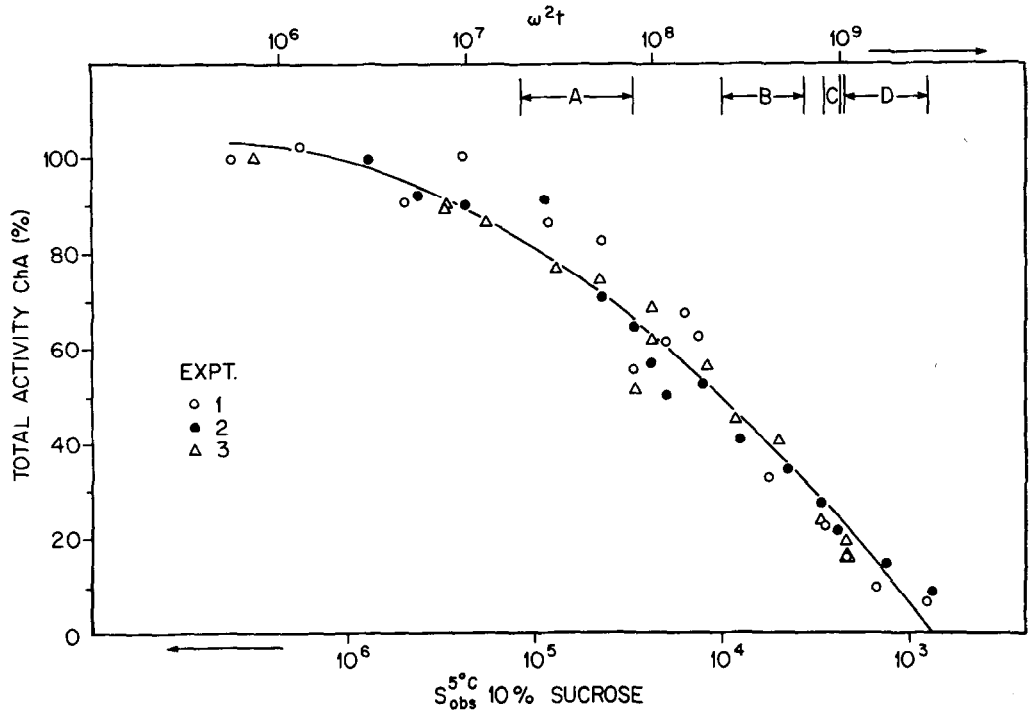

FIG. 3. Sedimentation curve for brain NEP. The data represent pelletable ChA (C_f/C_i) activity from supernatant samples obtained as described in Fig. 2. The fitted curve follows the equation

$$Y = -102.9 + 74.4 \ln X - 6.7 (\ln X)^2.$$

TABLE II

BRAIN PARTICLES S_{obs} CONVERTED TO $S_{20,w}$[a]

Particle	Assumed[b] $\rho_{5°}$	$S_{5°,10\%}$[c]	$S_{20,w}$[d]
Mitochondria	1.176	5.4×10^3	1.5×10^4
NEP	1.156	7.2×10^2	2.0×10^3
Lysosomes	1.156	6.5×10^2	1.8×10^3

[a] $S_{20,w} = S_{obs} \times \frac{\eta_{T,M}}{\eta_{20,w}} \times \frac{(\rho_p - \rho_{20,w})}{(\rho_p - \rho_{T,M})}$ $\eta_{5°,10\% \text{ sucrose}}$ = 2.073; $\eta_{20,w}$ = 1.004; ρ_p = as indicated in Table; $\rho_{5°,10\% \text{ sucrose}}$ = 1.0406; $\rho_{20,w}$ = 0.9988.

[b] Assumed from sucrose banding densities.

[c] Compare footnotes, Table I.

[d] The $S_{20,w}$ values were used for comparative purposes since the conditions (temperature and sucrose concentration) may vary from one investigator to another.

by ChA activity, which is localized largely in NEP (3–6), although there is evidence that the enzyme is also contained in other neuronal structures (7, 8). Lysosomes were assayed by measuring acid phosphatase activity. Lysosomes and/or lysosomal activities, as well as being distinct subcellular particles in brain (9), are contained in NEP (10). From the data obtained, sedimentation curves were plotted for each particle.

Preparative procedures. The cerebral cortices from 13 to 15 male Sprague-Dawley rats, weighing approximately 250 g, were used for each experiment. Removal of the cortex was achieved within 40 sec after decapitation. The tissue was placed in tared vessels containing ice-cold 10% sucrose for a final weight/volume ratio of 20%. Approximately 16 g of brain tissue were homogenized in two lots with Potter-Elvehjem type homogenizers, manually operated. Initial breakage of the tissue was performed in a smooth-walled glass tube fitted with a Plexiglas pestle (0.026 cm clearance). Three passes were made with this homogenizer prior to transferring the tissue to a second homogenizer with a pestle clearance of 0.051 cm. Ten passes were made with this homogenizer. The pestles were not rotated and the speed of each complete pass was approximately 20 sec. The homogenate was diluted with 10% sucrose to a 4% (w/v) con-

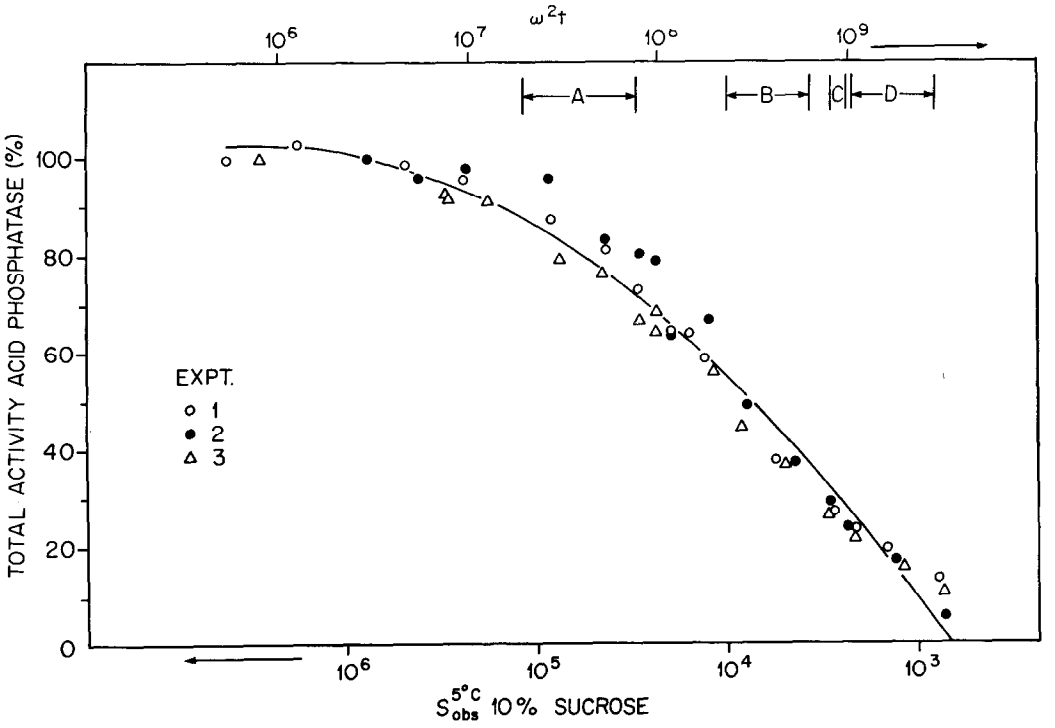

FIG. 4. Sedimentation curve for brain lysosomes. The data were obtained as described in Fig. 3. Acid phosphatase activity was measured to determine lysosome content of the samples. The fitted curve follows the equation

$$Y = -167.3 + 91.6 \ln X - 7.8 (\ln X)^2.$$

TABLE III

COMPARISON OF DIFFERENTIAL CENTRIFUGATION CONDITIONS USED TO PREPARE CRUDE NUCLEAR AND MITOCHONDRIAL FRACTIONS FROM BRAIN HOMOGENATES
(g·min have been converted to their approximate $\omega^2 t$)

Investigator	Nuclear		Mitochondrial	
	g·min	$\omega^2 t$	g·min	$\omega^2 t$
Autilio *et al.*, 1968 (20)	10×10^3	7.4×10^7	210×10^3	154×10^7
de Robertis *et al.*, 1962 (21)	9×10^3	6.6×10^7	230×10^3	169×10^7
Kurkowa *et al.*, 1965 (6)	10×10^3	7.4×10^7	150×10^3	110×10^7
Gray *et al.*, 1962 (22)	11×10^3	8.1×10^7	1050×10^3	768×10^7
Whittaker *et al.*, 1964 (23)	11×10^3	8.1×10^7	200×10^3	147×10^7

centration and passed through eight double layers of cheesecloth. The filtrate was further diluted to 2% (w/v) with 10% sucrose. All operations were performed in the cold room (4°), and all materials were maintained cold in ice baths. Precise 20-ml samples of the homogenate were measured with a graduate and decanted into No. 30 Spinco centrifuge tubes.[5] No pipeting of the homogenate was performed up to this point of the procedure.

[5] Oak Ridge polycarbonate type, obtained from International Equipment Co., Needham, Mass.

Centrifugation procedures. Centrifugation of the brain homogenate samples was performed in No. 30 Spinco angle-head rotors. The centrifuges were equipped with Beckman digital integrators that permit the operator to determine accurately the integral of $\omega^2 dt$ during the period of centrifugation. An International Equipment Co. digital integrator was adapted to one centrifuge and was rewired to permit the accurate determination of $\pm 4 \times 10^5$ $\omega^2 dt$. This centrifuge was used for all short runs that were routinely operated at a plateau speed of 3000 rpm. All other integrals were determined by

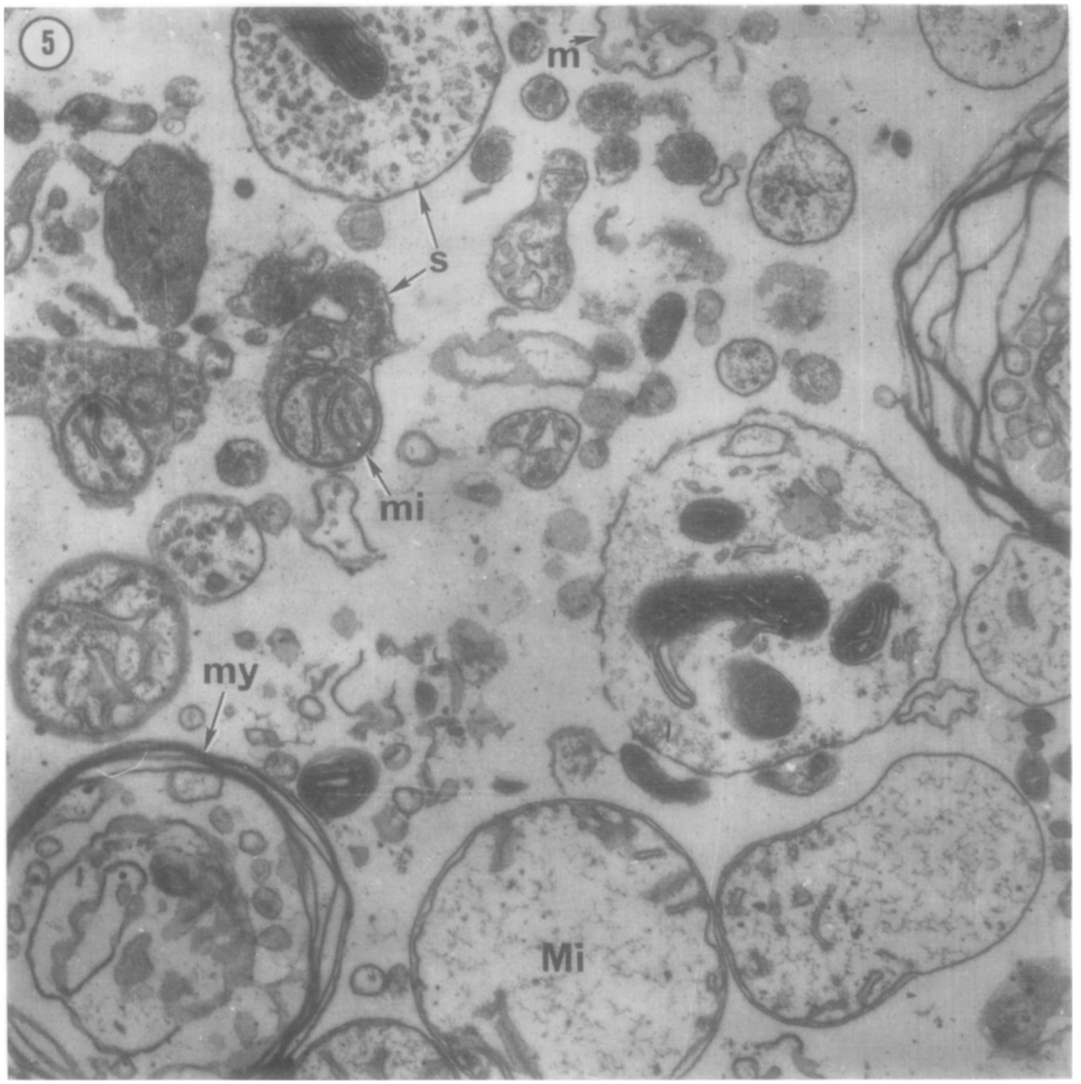

FIG. 5. Fraction A, Plate 1, 2–8 × 10^7 $\omega^2 t$. Large NEP (s) (1 to 1.5 μ); huge mitochondria (Mi), many of which are swollen; large membrane fragments (m); and myelin (my) are present. The mitochondria associated with NEP (mi) are large and more numerous than those in the other fractions. There are a number of vesicular structures containing mitochondria that are not NEP because synaptic vesicles are absent. Some of these can be identified as arising from neuronal plasma membrane due to the presence of attached nerve endings. A substantial portion of the NEP population characteristically has a large postsynaptic sector in the form of a vesicle. ×30,000.

operating the centrifuges at a plateau speed of 10,000 rpm. The remaining part of the centrifugation procedure was performed according to the method described by Anderson (2). In these experiments, however, the top 15 ml of the centrifugate were removed for analysis; the remaining 5 ml were defined as the pellet. These volumes result in the radius of the meniscus (R_0) at 7.07 and the radius of partition or plane surface of the "pellet volume" (R_p) at 8.88 cm. By utilizing the above values and the equation:

$$S = -\frac{1}{2\omega^2 t}\left(\ln\left[\frac{R_0^2}{R_p^2} + \frac{C_f}{C_i}\left(1 - \frac{R_0^2}{R_p^2}\right)\right]\right)$$

where (C_i) is the concentration of particles at the

start of the run and (C_f) is the concentration of particles in the supernatant at the end of the run (2), we obtained the range of sedimentation coefficients that were fitted graphically to the measured $\omega^2 t$ values. The $\omega^2 t$ values of interest ranged from 10^6 to 5×10^9 (equivalent to 136 $g \cdot$min to 680,000 $g \cdot$min). The activity of the enzymes representing specific particles remaining in the supernatant after centrifugation is expressed as percent of the starting material. Two milliliters of the 15-ml supernatant withdrawn were used for the cytochrome oxidase assay; the remainder was centrifuged at 30,000 rpm for 1 hr in a Spinco No. 30 rotor, and the pellet was resuspended for analysis of acid phosphatase, choline acetylase activity, and protein.

All centrifugations were completed within 2 hr after the homogenate was prepared, and all samples were measured in duplicate.

Analytical procedures. Cytochrome oxidase (cytochrome *c*: O_2 oxidoreductase EC 1.9.3.1) was measured with minor modifications by the method described by Duncan and Mackler (11).

Enzyme activity is assayed by measuring the initial rate of aerobic oxidation of reduced ferrocytochrome *c* by following the decrease in absorbance at 550 mμ. The limit of detectability, based on the millimolar extinction coefficient of 19.6 for the beef heart cytochrome *c*, is 0.1 μM/min. The absolute activities of the 2% homogenates were approximately 40 IUB units cytochrome *c* reduced per milligram protein.

Acid phosphatase (orthophosphoric monoester phosphohydrolase EC 3.1.3.2) activity was determined by the automated procedure described in the Technicon Laboratory Manual (12), except that Levor IV (Technicon, an anionic detergent) was introduced in the wash cycle (0.5 ml per liter of water). We found that enzyme activity was not further increased (released) by adding detergent (Triton X100 or Levor IV) directly to the sample. The limit of detectability of this method is 4 μg of phenol per milliliter. The absolute activity of the starting material was approximately 266 IUB units of phenol produced per milligram of protein.

Choline acetyltransferase (acetyl-CoA: choline-*O*-acetyl transferase EC 2.3.1.6) activity was measured according to the method of Shrier and Shuster (13). Enzyme activity is determined by measuring the amount of ^{14}C-acyl groups transferred from ^{14}C-acetyl-CoA to choline. The reaction mixture contains 0.23 M phosphate buffer (Na^+ salt, pH 7.6), 0.14 M NaCl, 0.045 M choline chloride (Sigma), 0.034 mM neostigmine (Sigma), 302 μM ^{12}C-acetyl-CoA (Sigma) containing 1.42 μmoles ^{14}C-acetyl-CoA (New England Nuclear, S.A. 45 μCi/μM) and 0.4% Triton X-100 (Rohm and Haas).

Fifty- to 100-μl samples to be determined for ChA content are mixed with 100 μl of the reaction buffer (total volume 200 μl) and incubated at 37° for 30 min. The entire sample is then pipeted on a small anion exchange column (Pasteur pipets containing Dowex 1-X8 resin, 200–400 mesh in the Cl^- form; Baker Chemical Co.). The ^{14}C-acetyl choline formed in the reaction is eluted from the column with two washes (0.4 and 0.8 ml) of distilled water. The eluates are collected directly in scintillation vials that are then filled with 10 ml of scintillation fluid (dioxane:naphthalene mix) (14). The unreacted acetyl-CoA remains behind bound to the resin. The specific activity of the starting material varied from 5 to 7 $\times$ 10^{-3} IUB units of acyl groups transferred per milligram of protein. The enzyme activity was linear over the range studied. Triton X100 activated the enzyme to the same extent as treatment with ether (15).

Protein was determined by an automated Lowry procedure (16).

Electron microscopy. Pellets of the differential centrifugation cuts, noted as A, B, C, and D in the figures, were fixed with Caufield's Veronal osmium buffer fixative (17) for 30 min and then rinsed with distilled water. The samples were then dehydrated stepwise with 70–100% ethanol, followed by two 15-min changes of propylene oxide. Infiltration of the pellets was done with a 1:1 mixture of propylene oxide and epoxy resin for 1 hr, followed by a 1:2 mixture overnight, and finally with the epon alone.

The embedded material was sectioned with a LKB microtome, and thin sections were examined with an RCA electron microscope.

RESULTS

With the development of the digital integrator used for accurate determination of the integral, $\omega^2 t$, during the centrifugation cycle, it is possible to obtain precise reproducible centrifugal forces. The integral, $\omega^2 t$, measures the acceleration, running, and deceleration forces for any rotor in any centrifuge. Thus any investigator can rereproduce a specific set of conditions accurately.

Brain homogenates centrifuged in an angle-head rotor showed sedimentation profiles, as illustrated in Figs. 1–4. A particle of uniform size and density would behave as shown by the dotted line in Fig. 1. If the mitochondrial sedimentation curve is compared with the ideal particle, it is apparent

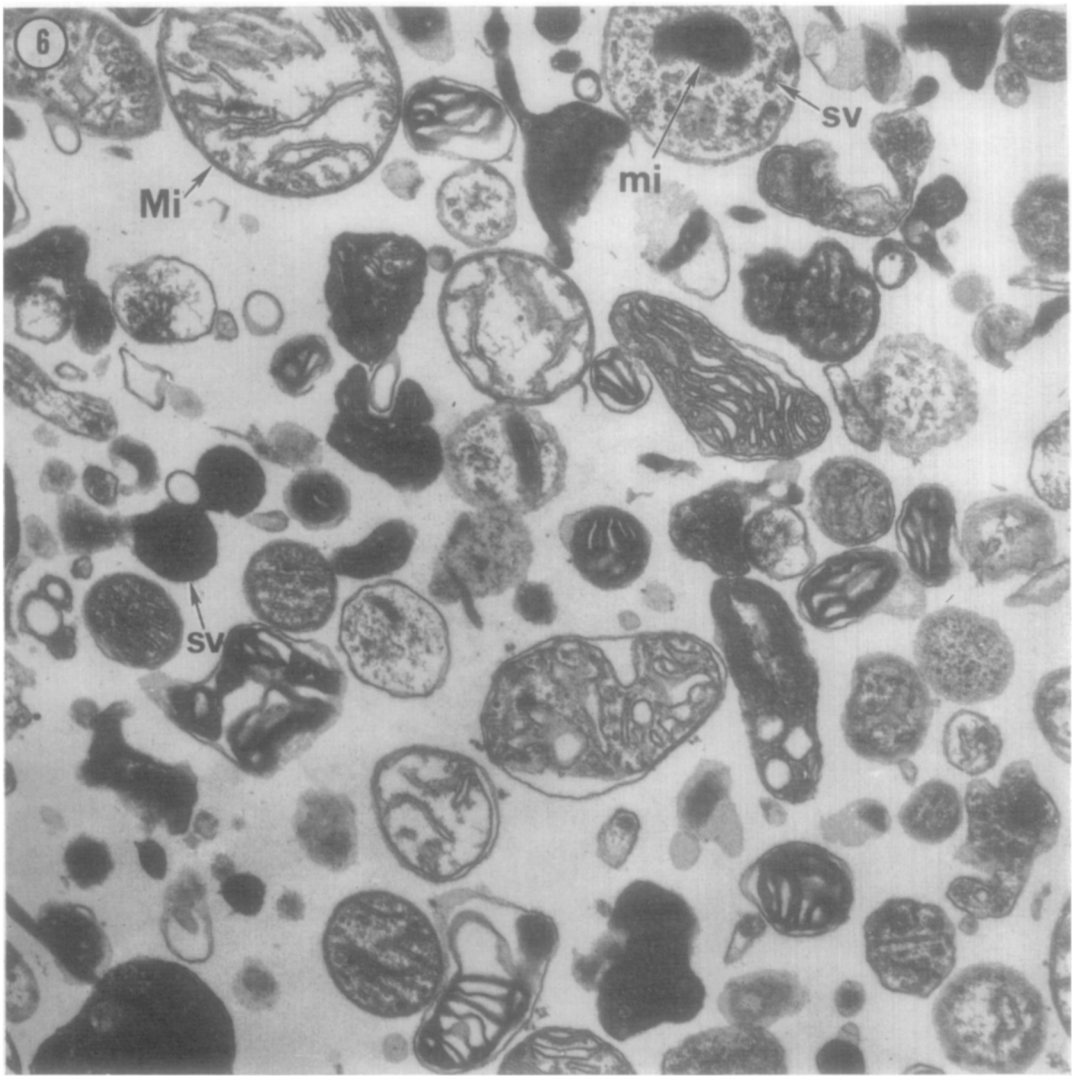

FIG. 6. Fraction B, Plate 2, 24–67 $\times$ 10^7 $\omega^2 t$. The free mitochondria (Mi) are large and nearly all NEP contain at least one mitochondrion (mi). Some membrane vesicles, the same size as NEP but devoid of synaptic vesicles (sv), are present. Typically these structures contain an electron dense matrix similar to cell cytoplasm. These vesicles probably originate as fragments of dendrites or cell soma. $\times$30,000.

that the large majority of mitochondria were homogeneous, at least with respect to their sedimentation characteristics. These particles were completely removed from the supernatant after 4 $\times$ 10^8 $\omega^2 t$ or 5.7 $\times$ 10^4 $g \cdot$min. The remaining smaller mitochondria (approx. 15%) represented another size class of particles that were not completely sedimented until the integral of 1 $\times$ 10^9 $\omega^2 t$. In contrast to the mitochondria, NEP and lysosomes exhibited considerable heterogeneity, as is suggested by their enzyme activity curves. The curves plotted were fitted by computer computation. The best fit for the mitochondria data was obtained from the equation describing the sedimenta- of an "ideal" particle (see Methods). A second-order polynomial was the simplest equation for describing the NEP and lysosomal data.

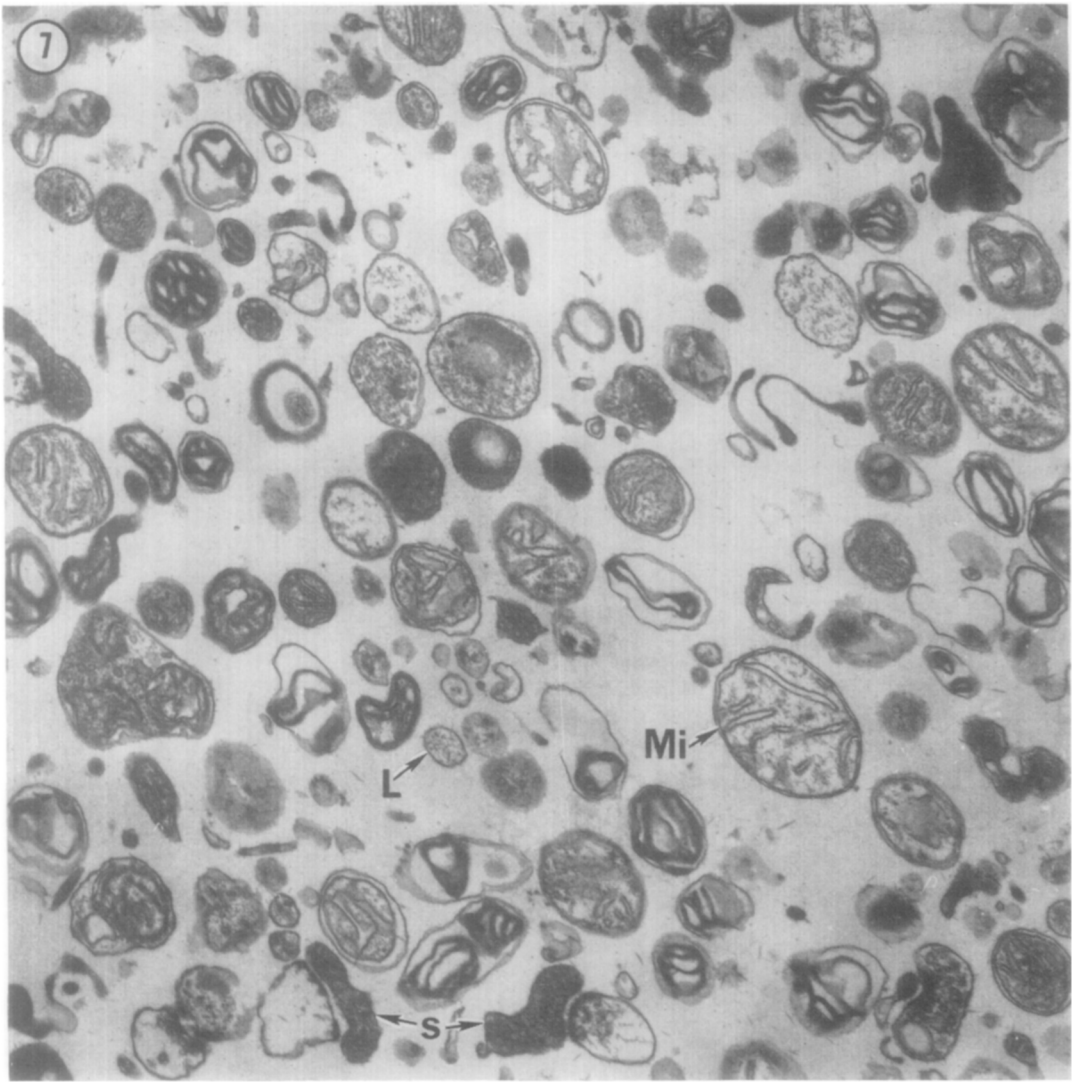

FIG. 7. Fraction C, Plate 3, 85–102 × 10^7 $\omega^2 t$. The free mitochondria (Mi) are smaller and there is a greater preponderance of NEP (s) without any mitochondria. Very few NEP contain more than one mitochondrion. The average size NEP is 0.5 μ compared to 0.75 to 1.0 μ in fractions A and B. Lysosomes (L) are more distinguishable than in other fractions and are in larger number. Very few free membrane fragments are present. Rough endoplasmic reticulum is present but not in large quantity. ×30,000.

Tables I and II summarize the sedimentation data obtained from the experiments. The morphological contents of four different cuts, A, B, C, and D, noted at the top of Figs. 1–4, are shown in Figs. 5–8. Analysis of these specific integrals shows that a sizing and grouping of the various populations was achieved. NEP were separated with respect to mitochondrial content, and mitochondria were grouped with respect to size.

DISCUSSION

The differential centrifugation procedure developed by Anderson (2) showed that the sedimentation of monodispersed particle populations behaved in a predictable and

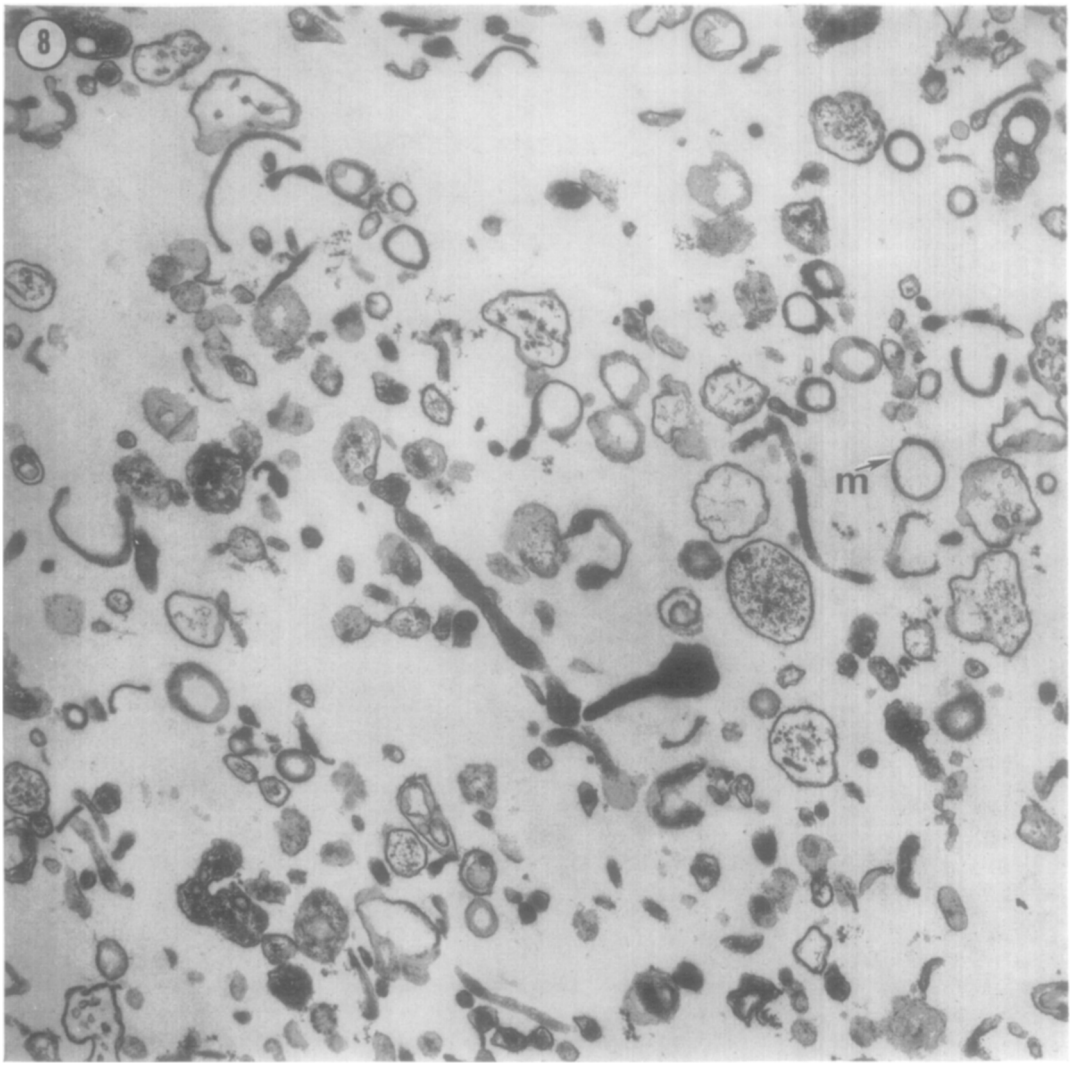

FIG. 8. Fraction D, Plate 4, 106–301 × 10^7 $\omega^2 l$. This fraction contains mostly free membrane (m). Some of the membrane structures have a clear matrix, while an equal quantity of others have a characteristic granularity. Some of the membrane arise from broken NEP. There are very few intact NEP. More rough endoplasmic reticulum is present than in other fractions. ×30,000.

reproducible manner in angle-head rotors. The question arose as to the suitability of the technique for studies on polydispersed suspensions. Although the question is only partly answered, we have shown that the method is applicable for characterizing the sedimentation of several particles from brain homogenates.

In the cases studied, the particle populations overlap because their properties are graded rather than sharply defined. Synaptosomes and lysosomes show characteristics of heterogeneously dispersed particles, while mitochondria quite surprisingly give a diagram very close to that expected theoretically for a homogeneous particle population. On the other hand, the two mitochondrial populations, those in NEP and those free, cannot be distinguished. Neither a break in the curves nor a prominent shoulder is

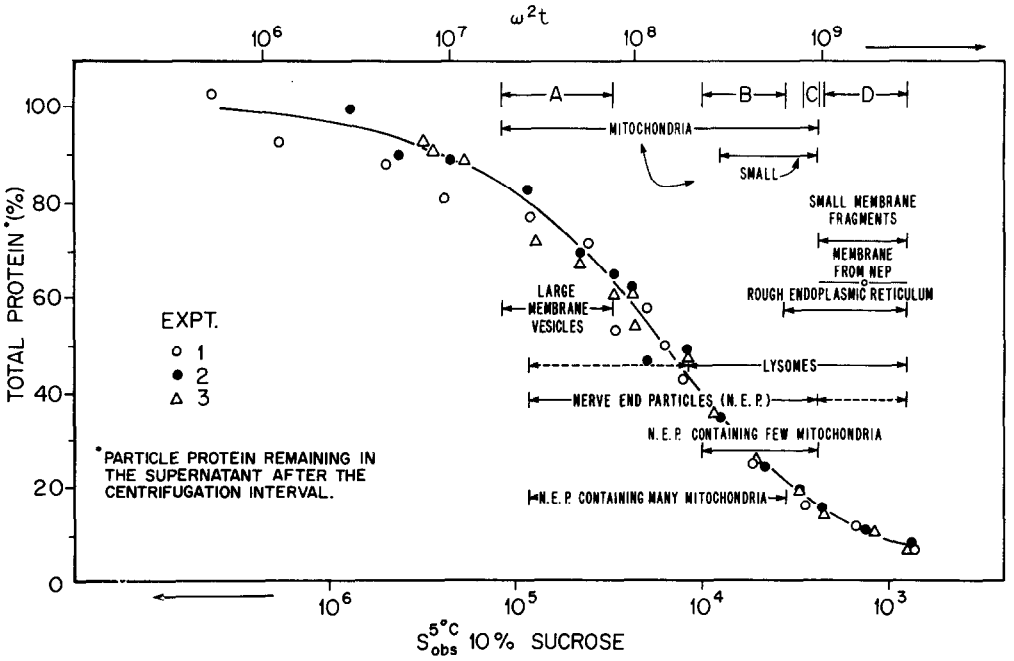

FIG. 9. Summary of morphological and enzymatic analysis on the sedimentation of particles from brain homogenates. The size data on the sedimentation range of particular particles (small mitochondria, membrane fragments, rough endoplasmic reticulum, lysosomes, and mitochondria content of NEP) were obtained from morphological analysis.

evident (Fig. 2). Acid phosphatase and ChA activities begin to sediment in close proximity to mitochondria, but significantly greater forces are required to sediment 90% of the population. For the smallest particles or complete sedimentation of brain lysosomes a maximum of 4.8×10^5 $g \cdot$min is required. The subcellular organization of brain acid phosphatase-containing particles is quite different from liver (18). The presence of lysosomes in NEP is at least one definite difference (9).

Sedimentation plots, such as Fig. 1, can be used to investigate problems involving subcellular particles in a quantitative and reproducible manner. Figure 9 summarizes our results on the sedimentation of characteristic particle sizes and features. Information of this type permits the selection of those preliminary centrifugation conditions preliminary centrifugation conditions that would allow the greatest percentage collection and the greatest percentage elimination of the particles desired.

In Table III we summarized the differential centrifugation conditions selected by a number of investigators. The differences among procedures represent selected incremental portions of particle populations. The particles separated and grouped under these different conditions can be ascertained from Figs. 1 and 9 for the particles assayed. As seen, mitochondria sediment over the range of 2 to 100×10^7 $\omega^2 t$. In order to sediment almost all of the mitochondria and 80% of the NEP, forces greater than 100×10^7 $\omega^2 t$ are *not* required; in fact, greater forces only reduce the purity of the fraction. Eighty-five percent of the mitochondrial population sediments by 42×10^7 $\omega^2 t$. Approximately 55% particulate ChA would sediment with the mitochondrial population. Procedures to collect ChA-containing structures at $\omega^2 t$ values higher than approximately 100×10^7 $\omega^2 t$ will include membrane from broken NEP and other sources as well as quantities of rough endoplasmic reticulum.

Knowledge of the sedimentation data

obtained from curves of this type may provide a guide to the development of simplified procedures for the use of density gradients in zonal rotors. Preliminary differential centrifugation can be accomplished directly in zonal rotors. The sample is run for a specified period of time depending on the sedimentation coefficient of the particles entering the gradient; the particles of lower sedimentation coefficient still in the starting zone are backed out of the rotor. Sample pelleting and resuspension are eliminated. As was demonstrated with liver homogenates (19), particles with larger sedimentation coefficients (e.g., mitochondria) can be recovered at the edge of the rotor utilizing the most recent zonal rotor, B-XXIX. The part of the gradient that was removed is replaced and centrifugation is continued for separation of the particles with smaller sedimentation coefficients. All of these procedures are easily reproducible when the $\omega^2 t$ integral is counted. The sedimentation of mitochondria, NEP, and lysosomes in sucrose density gradients over a wide range of $\omega^2 t$ values will be described in a subsequent paper.

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