

# FREE-FLOW ELECTROPHORETIC SEPARATION AND ELECTRICAL SURFACE PROPERTIES OF SUBCELLULAR PARTICLES FROM GUINEA PIG BRAIN

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## ABSTRACT

Continuous free-flow electrophoretic separation has been used to obtain relatively pure preparations of synaptosomes and synaptic vesicles from crude fractions of guinea pig brain homogenates. Measurements of the contents of protein, neuraminic acid, and bound acetylcholine; the activities of succinic dehydrogenase, adenosine triphosphatase, choline acetylase, and 5'-nucleotidase; and the uptake of  $^{14}\text{C}$ -labeled choline and acetylcholine in the presence and absence of hemicholinium, all confirm the electron microscope evidence that the electrophoretic preparations are at least as pure as those obtained by ultracentrifugal methods. The electrophoretic mobility measurements have been used to calculate zeta potentials and surface charge densities for these particles.

## INTRODUCTION

Previous isolations of brain subcellular particles have been achieved primarily by means of differential centrifugal techniques, which have been reviewed by Whittaker (1) and De Robertis (2). Kadota and Kanaseki (3) have combined centrifugation and diethylaminoethyl(DEAE)-Sephadex techniques to purify synaptic vesicle fractions. Vos et al. (4) and Sellinger and Borens (5) have used the zonal density gradient electrophoresis method of Svensson (6) in the further fractionation of centrifugal preparations of brain subcellular particles.

In the present work continuous free-flow electrophoresis, as described by Hannig (7), has been used to increase the purity of fractions of subcellular particles, to reduce the preparation time, and to allow electrokinetic interpretation of data obtained during the separation steps. This paper describes the optimum electrophoretic separation conditions for preparations of synaptosomes, mitochondria, and synaptic vesicles, as well as

some morphological and biochemical studies of the isolated fractions.

## METHODS

### *Centrifugal Preparations*

Four types of centrifugal preparation of guinea pig cortex were used in this work. All four fractions were isolated by the methods outlined by Whittaker (1) and are designated, using Whittaker's terminology, as: supernatant of crude homogenate, crude synaptosomal-mitochondrial fraction ( $P_2$ ), purified synaptic vesicle fraction ( $D_1$ ), and purified synaptosomal fraction ( $P_2B$ ).

### *Electrophoretic Isolation*

The apparatus used for the electrophoretic separations was originally designed by Hannig and is marketed as the Brinkmann FF-3 Continuous Free Flowing Electrophoretic Separator (Brinkmann Instruments Inc., Westbury, N.Y.). The principle of

operation may be briefly outlined as follows: the sample to be separated flows vertically downward in a rectangular chamber measuring 50 cm in length, 12 cm in width, and 0.5 mm in thickness. The vertical velocity of the sample is equal to that of the flowing chamber buffer. The individual particles of the sample move horizontally with a velocity determined by the electric field applied across the chamber and the electrostatic characteristics of the particles. The continuously injected sample divides into bands, each containing particles of equal mobility; the bands are then isolated at the bottom of the separating chamber by a manifold of 92 tubes.

#### *Distribution of Material in Output Fractions*

The output fractions of the separator were analyzed by nephelometry using a Photovolt 520M fluorimeter (Photovolt Corporation, New York) in order to determine quickly the distribution of particulate matter in the array of collector tubes.

#### *Electron Microscopy*

Samples were studied and photographed in a Zeiss EM9A electron microscope. For negative stain preparations, 1–2% (w/v) phosphotungstic acid, pH 7.0, was used according to the methods of Horne and Whittaker (8) and Whittaker (9). Positive stain fixation was done according to Farquhar and Palade (10). Sections were cut on a Porter-Blum MT2 microtome, and stained with 6% uranyl acetate and Reynold's lead citrate solutions.

#### *Mobility Measurement*

The total deflections of the individual bands of particles within the separating chamber were recorded photographically. The lateral displacement of particles was made up of one component due to the electrophoretic velocity of the particles and a second component due to the electro-osmotic velocity of the chamber buffer. To correct for the latter component a zero mobility marker (chamber buffer containing 0.4 M sucrose) was injected into the chamber. The resultant diffraction pattern, established at the interface between the marker containing 0.4 M sucrose and the normal chamber buffer containing 0.32 M sucrose, could be observed visually. A fine thread was used to mark the position of the interface for photographic recording. The angle of deflection of the bands due to the electrophoretic velocity of the particles was measured as the difference between the angles of total deflection of the individual bands of material and the angle of deflection of the marker.

The volume flow rate of the chamber buffer was measured directly by collection of the output of the separating chamber for a measured time interval.

The maximum linear flow rate of the chamber buffer was calculated from the volume flow rate and the geometry of the separating chamber. The chamber buffer was assumed to exhibit laminar flow, with a parabolic velocity profile across the narrowest dimension of the chamber.

The electric field within the chamber was determined directly by measuring the voltage drop between two test probes of measured separation. The mobility of a given band of particles was calculated using the formula:

$$u = \frac{V_{\max}}{E} [\tan \theta_b - \tan \theta_m],$$

where  $u$  = electrophoretic mobility,  $V_{\max}$  = maximum vertical velocity of the buffer,  $E$  = electric field within the chamber,  $\theta_b$  is the angle of deflection of the band of particles, and  $\theta_m$  is the angle of deflection of the zero mobility marker.

#### *<sup>14</sup>C-Label Studies*

Choline chloride (methyl-<sup>14</sup>C), SA 54 mCi/mmol, and acetyl-1-<sup>14</sup>C-choline chloride, SA 9.2 mCi/mmol, were used as markers for mitochondria and synaptosomes. For a validation of the use of these markers, see the uptake experiments of Marchbanks (11, 12) and Burton (13). P<sub>2</sub> crude preparations of mitochondria and synaptosomes were incubated in the presence of 5 μCi of choline chloride (1 μCi/ml) or 10 μCi of acetylcholine chloride (2 μCi/ml) in a medium described by Marchbanks (11). In the acetylcholine experiments, eserine was included in the medium (0.1 mg/ml), and also non-labeled acetylcholine chloride (50 mM). The P<sub>2</sub> fraction was divided into two samples of equal volume, one of which was incubated in the presence of either choline-<sup>14</sup>C or acetylcholine-<sup>14</sup>C, and the other of which was incubated in the presence of the same labeled compound and hemicholinium No. 3 (100 μM). After a preincubation of 20 min at 25°C, the labeled compound was added to each sample and the incubation was continued for 30 min. At the end of the 50-min period the samples were centrifuged at 17,500 *g* for 30 min and resuspended in chamber buffer. Electrophoretic separation was carried out for 45 min. The output fractions of the separator were then analysed by liquid scintillation counting techniques. 1 ml of each fraction was added to 10 ml of Bray's (14) solution and counted by the channels ratio method in a Nuclear-Chicago Liquid Scintillation Counter Model 720 (Nuclear-Chicago, Des Plaines, Ill.).

#### *Biochemical Assays*

Neuraminic acid concentrations were measured by the procedure of Warren (15) with the following

modification. Each electrophoretic fraction was centrifuged to a pellet (18,000 *g*), resuspended in 1.0 ml of 0.1 *N* H<sub>2</sub>SO<sub>4</sub>, and incubated for 1.0 hr at 80°C. The hydrolyzed sample was placed in a narrow dialysis sac and dialyzed against 10 ml of twice-distilled water at room temperature, with continuous shaking, for 24 hr. A blank of H<sub>2</sub>SO<sub>4</sub> without tissue was carried through the same procedure. The 10 ml of outer phase was evaporated under nitrogen to a volume of 0.3 ml. Subsequent steps followed Warren's method. Graphical techniques were used to determine the *N*-acetylneuraminic acid levels after spectrophotometric scanning from 420 to 600 *mμ*. Pure standards were used for comparison in each experiment.

Na,K-ATPase and ouabain-resistant ATPase activities were determined by the method of Post and Sen (16). Succinic dehydrogenase activity was measured by means of the Warburg respirometer technique, with the method outlined by Umbreit et al. (17).

Measurement of 5'-nucleotidase activity was done by the method of Heppel and Hilmo (18). Choline acetylase activity was measured by the method of Berry and Whittaker (19). Bound acetylcholine content in fractions of the electrophoresis output, as well as acetylcholine produced in the reaction mixtures for choline acetylase activity, were measured by the spectrophotometric method of Hestrin (20), initially validated against the frog heart bioassay method of Zapata and Eyzaguirre (21). For this purpose, samples were adjusted to pH 4-4.5 by addition of dilute (0.2 *N*) HCl, heated at 100°C for 10 min, cooled, and centrifuged for 10 min at 8000 *g*. The supernatant was used for analysis. Protein was determined by the method of Lowry et al. (22).

## RESULTS

### *Optimum Separating Conditions*

Preliminary experiments were undertaken with a crude preparation (supernatant of 1000 *g* × 11 min) of guinea pig cortex homogenized in 0.32 *M* sucrose (10% w/v) at pH 7.0. The heterogeneous mixture was fractionated in the Brinkmann FF-3, using five different buffers and the following range of operating conditions: chamber voltage 550-1200 v, chamber current 50-150 ma, pH 4.5-8.6, buffer flow 0.04-0.117 ml/sec, and chamber conductivity  $4.1 \times 10^{-4}$ - $6.64 \times 10^{-4}$  ohm<sup>-1</sup>cm<sup>-1</sup>.

Fig. 1 indicates the conditions which produced optimum separation as judged by turbidity measurements performed on the electrophoretic fractions of a crude mitochondrial-synaptosomal preparation. The nephelometric measurements

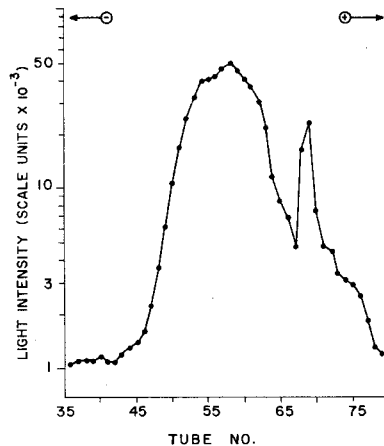


FIGURE 1 Turbidity profile of the electrophoretic output fractions of a crude mitochondrial-synaptosomal preparation. The injected material was the P<sub>2</sub> fraction from guinea pig cortex prepared according to Whittaker (1). The separation conditions were: 1200 v, 55 ma, 6°C, electrode buffer pump setting 50, and dosing pump setting 10.1. Michaelis-Veronal buffer of pH 7.15 and conductivity  $4.1 \times 10^{-4}$  ohm<sup>-1</sup>cm<sup>-1</sup> was used for the chamber buffer. The broad peak appears to consist of two incompletely resolved peaks.

indicated two distinct peaks and a wide separation of material. When a centrifugal pure preparation of synaptosomes (P<sub>2</sub>B) was run under the same conditions as in Fig. 1, the main distribution occurred around tube 68, the location of the side peak in Fig. 1. This provided preliminary evidence that synaptosomes could be isolated from a very crude preparation.

### *Electron Microscopy*

The synaptosomal-mitochondrial preparation P<sub>2</sub> and the synaptic vesicle preparation D<sub>1</sub> were independently subfractionated by electrophoresis under the conditions given in Fig. 1. The subfractions were then studied by electron microscopy.

Fig. 2 shows a negative stain preparation of fraction D<sub>1</sub> before electrophoresis. This fraction resembles closely the synaptic vesicle preparations described by Whittaker (1) and De Robertis (2). Note the white-cored and hollow-cored vesicles, broken vesicles, vesicles on edge, and contamination by stringlike figures and larger particles.

After electrophoresis of D<sub>1</sub> a fraction composed primarily of white-cored vesicles (Fig. 3) was obtained; the major contaminant materials were

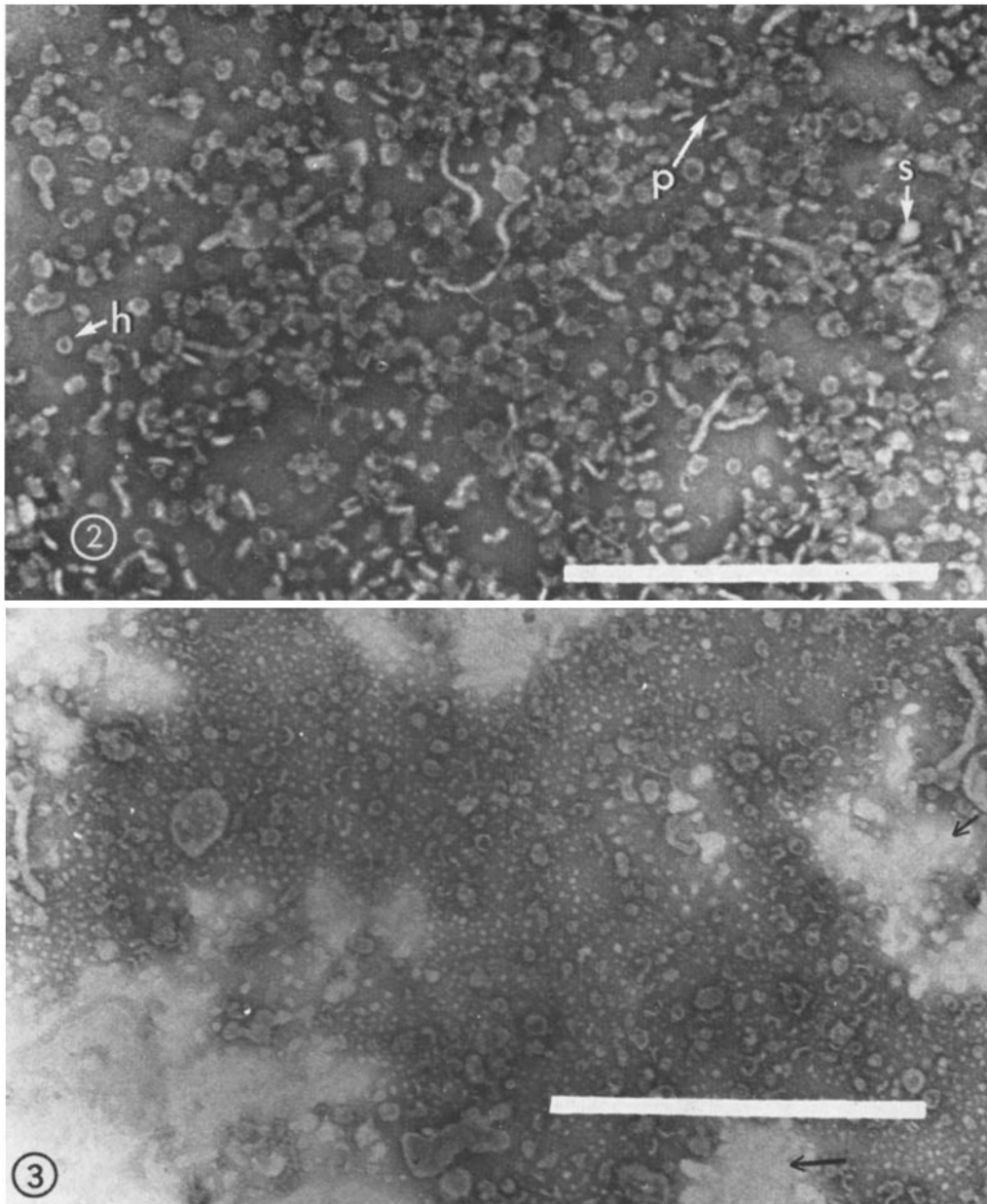


FIGURE 2 Electron micrograph of the synaptic vesicle fraction,  $D_1$ , prepared from guinea pig cortex according to Whittaker (1). The negative stain methods of Horne and Whittaker (8) were used. Arrows indicate hollow vesicles (*h*), white-cored or "solid" vesicles (*s*), and vesicles in profile (*p*). Bar =  $1\mu \times 51,000$ .

FIGURE 3 Electron micrograph of one electrophoretic subfraction of the same fraction,  $D_1$ , as is shown in Fig. 2. The conditions used for separation were the same as in Fig. 1. Note the increase in the relative number of white-cored vesicles, clumps of which are indicated by arrows. Negative stain was used. Bar =  $1\mu \times 51,000$ .

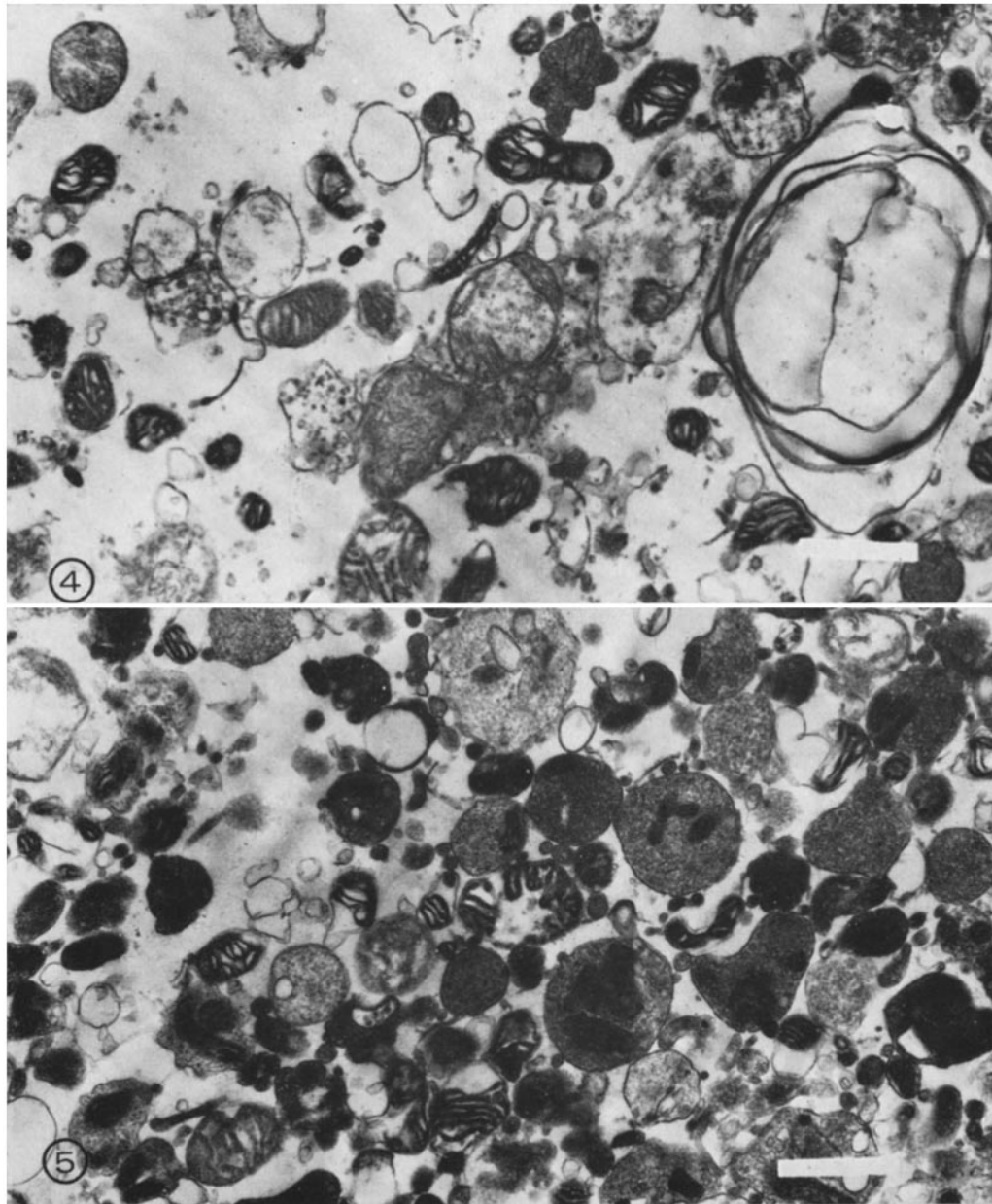


FIGURE 4 Electron micrograph of a synaptosomal-mitochondrial preparation,  $P_2$ , from guinea pig cortex prepared according to the method of Whittaker (1) and observed using the positive stain procedures of Farquhar and Palade (10). Three main types of particles were observed: synaptosomes, myelin, and mitochondria. Bar =  $1\mu$ .  $\times 16,000$ .

FIGURE 5 Electron micrograph of a synaptosomal fraction purified by electrophoresis from the control material,  $P_2$ , shown in Fig. 4. Bar =  $1\mu$ .  $\times 16,000$ .

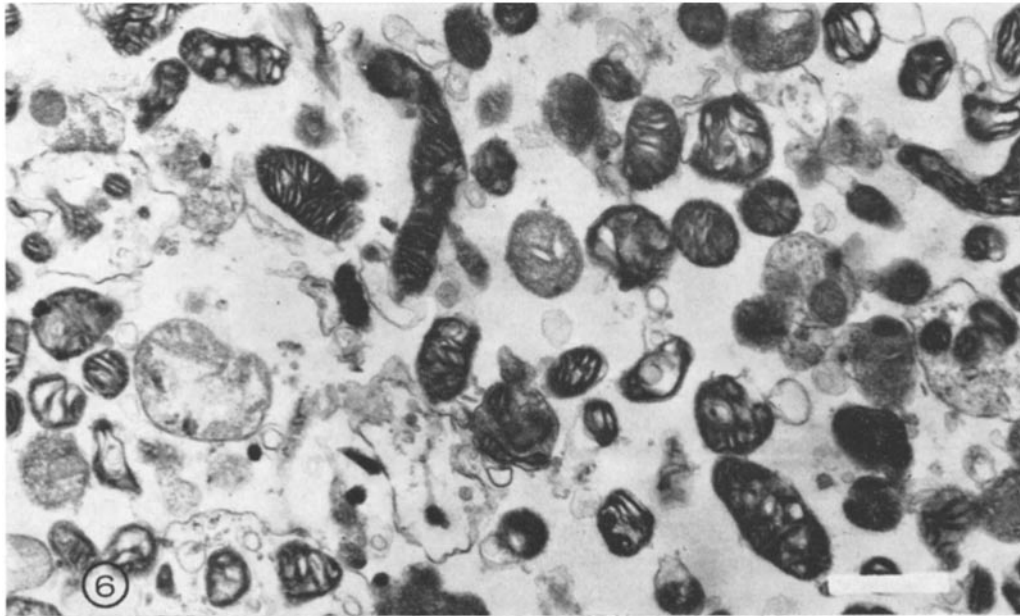


FIGURE 6 Electron micrograph of a mitochondrial fraction purified by electrophoresis from the control material,  $P_2$ , shown in Fig. 4. Bar =  $1\mu$ .  $\times 16,000$ .

found in an adjacent fraction in the output spectrum.

Fraction  $P_2$  is represented by Fig. 4 which contains three main types of particles: mitochondria, synaptosomes, and myelin. After continuous electrophoresis  $P_2$  was resolved into two distributions. The band of particles showing the highest negative charge (i.e. migrating to the positive electrode side of the main protein peak) was made up primarily of synaptosomes, as can be seen in Fig. 5. This band corresponded to the side peak at tube 68 in Fig. 1. The second band was made up of two poorly separated distributions: mitochondria (Fig. 6) which were displaced least and collected on the negative electrode side of the lowest mobility band, and unseparated material which constituted the major portion of the low mobility band. In Fig. 1, tube 53 corresponds to the center of the mitochondrial distribution.

#### *Mobility Measurements*

An example of the bands produced by the electrophoretic subfractionation of the crude mitochondrial-synaptosomal preparation  $P_2$  is given in Fig. 7. Fig. 8 represents the angle of deflection due to the electro-osmotic velocity of the buffer. The

required angles of deflection were measured in  $7 \times 10$  inch prints made from photographs taken during repeated separations of  $P_2$  and  $D_1$  fractions. The mobilities of synaptic vesicles, synaptosomes, and mitochondria were calculated from the measured deflections, the observed chamber buffer flow rates, and the determined field strengths within the separating chamber (Table I). The order of increasing magnitude of mobility for the three particles agrees with that reported by Vos et al. (4). However, the individual magnitudes appear to be about twice as large.

Table I also includes the zeta potentials of the three subcellular particles, calculated from the mobility data and buffer characteristics by application of Henry's (23) equation according to the theory reviewed by Overbeek and Lijklema (24). The method and tabulated solutions of Loeb and Wiersema (25) were used to determine the surface charge densities of the three particles. Full details of these calculations have been provided elsewhere (26).

#### *Radioactive Labeling*

Fig. 9 illustrates the distribution of acetylcholine- $^{14}C$  in the electrophoretic output fractions after

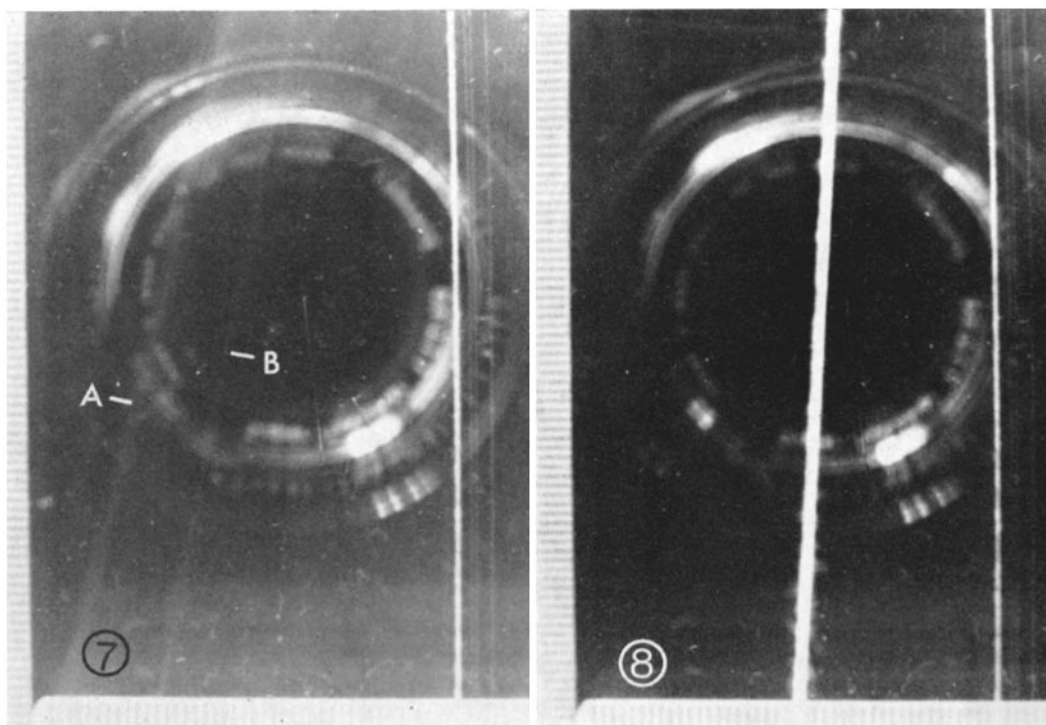


FIGURE 7 Bands produced by the electrophoretic subfractionation of the mitochondrial-synaptosomal preparation  $P_2$ . The bands were photographed through the separating chamber face plate. The vertical line on the right represents the "zero mobility" reference line. The oblique line (A) farthest from the reference line is the synaptosomal band. The fraction corresponding to this band is shown in Fig. 5. The lower mobility band (B) corresponds to the mitochondrial fraction and impurities shown in Fig. 6.

FIGURE 8 The angle of deflection due to the electro-osmotic velocity of the buffer. Chamber buffer containing 0.4 M sucrose was injected into the normal chamber buffer which contained 0.32 M sucrose. A white thread was superimposed on the resultant diffraction pattern to allow a photographic record of the angle of deflection caused by the motion of the chamber buffer under the influence of the applied electric field and other conditions used in the separation procedure.

TABLE I  
*Electrical Characteristics of Synaptic Vesicles, Synaptosomes, and Mitochondria*

Particle	No. of repetitions	Mobility	Zeta potential	Surface charge density
		$cm^2 \times 10^4/volt\text{-}sec$	$mv$	$\mu coul/cm^2$
Synaptic vesicles	4	$2.08 \pm 0.05^*$	$-69.2 \pm 2.0$	$-1.35 \pm 0.06$
Synaptosomes	2	$3.63 \pm 0.04$	$-90.3 \pm 0.6$	$-1.87 \pm 0.01$
Mitochondria	6	$2.71 \pm 0.23$	$-67.5 \pm 3.5$	$-1.09 \pm 0.09$

\* Values shown indicate mean  $\pm$ SE.

incubation of the  $P_2$  pellet in the presence of eserine (0.1 mg/ml), unlabeled acetylcholine (50 mM), and acetylcholine- $^{14}C$ , and subsequent electrophoretic separation for 45 min. The curve represents the

best separation of a series of seven which yielded essentially the same information.

The smaller peaks in the curves for disintegrations per minute indicate the position of the

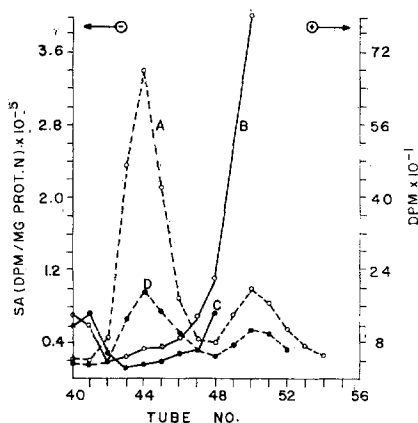


FIGURE 9 Distribution of acetylcholine- $^{14}\text{C}$  in the electrophoretic output fractions of a  $\text{P}_2$  preparation separated under the conditions shown in Fig. 1. The  $\text{P}_2$  preparation was preincubated for 20 min at  $25^\circ\text{C}$  in a medium described by Marchbanks (11). 50 mM unlabeled acetylcholine, 0.1 mg/ml eserine, and 10  $\mu\text{Ci}$  of acetylcholine- $^{14}\text{C}$  were added and the incubation was continued for 30 min. The material was then spun down, resuspended in chamber buffer, and separated by free-flowing electrophoresis for 45 min. Relative positions of the chamber electrodes are indicated by + and -. The output fractions were analyzed by liquid scintillation counting techniques. *A* represents the disintegrations per minute of the collected output fractions; *D* represents the effect of the addition of 100  $\mu\text{M}$  hemicholinium No. 3 to the incubation medium, on the disintegrations per minute. *B* represents the specific activities of the samples, and *C* the effect of 100  $\mu\text{M}$  hemicholinium No. 3 on the specific activities. Specific activities could not be calculated over the full range because protein content was too low to measure accurately beyond tube 48 or 49.

synaptosomal band (positive electrode side of the protein peak); the larger peaks indicate the position of bulk unseparated material. It can be seen that the specific activity curves increase substantially in the vicinity of the synaptosome peak. In every experiment hemicholinium reduced the uptake of the labeled acetylcholine into the material making up the two peak regions.

Fig. 10 shows the distribution of choline- $^{14}\text{C}$  taken up by the major constituents of the  $\text{P}_2$  fraction. The specific activity curves indicate two peaks: the synaptosomal peak to the positive electrode side of the protein peak (tube 56) and the mitochondrial peak to the negative electrode edge of the main protein peak (tube 47). Again, hemicholinium was found to reduce the uptake of choline in each experiment. Fig. 10 represents the

best separation in a series of five with similar results.

### Neuraminic Acid

Table II indicates the distribution of *N*-acetylneuraminic acid (NANA) in the electrophoretic subfractions of the synaptosomal-mitochondrial preparation ( $\text{P}_2$ ). The results are taken from one of two experiments which provided identical information concerning the relative distribution of material. The highest specific concentration and relative specific concentration of NANA occurred in the synaptosomal band, which in all the "marker" studies was consistently the band with highest mobility. The values corresponding to tube 48 in Table II are comparable to those obtained by De Robertis (2), Sellinger and Borens (5), and Lapetina et al. (27) for ultracentrifugal synaptosomal preparations.

### ATPase Activity

Fig. 11 represents the distribution of ouabain-resistant ATPase and Na,K-ATPase in the electrophoretic subfractions of the ( $\text{P}_2$ ) mito-

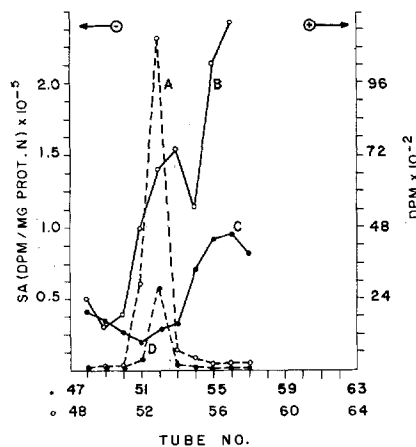


FIGURE 10 Distribution of choline- $^{14}\text{C}$  in the electrophoretic output fractions of a  $\text{P}_2$  preparation separated under the conditions shown in Fig. 1. The procedure was similar to that described in Fig. 9 except that eserine and "cold" choline were not added to the incubation medium. *A* represents the disintegrations per minute of the electrophoretic output fractions; *D* represents the effect of 100  $\mu\text{M}$  hemicholinium No. 3 on the disintegrations per minute curve. *B* represents the specific activities of the samples, and *C* the effect of 100  $\mu\text{M}$  hemicholinium No. 3 on the specific activities. Electrode positions indicated as in Fig. 9.



TABLE II  
Distribution of *N*-Acetylneuraminic Acid in the Electrophoretic Subfractions of the Mitochondrial-Synaptosomal Preparation ( $P_2$ )

Tube No.	Concentration	Specific concentration	Relative specific concentration
	$\mu\text{g}$	$\mu\text{g}/\text{mg protein}$	$\% \text{ NANA}/\% \text{ protein}^*$
44 ‡	1.23	6.40	0.443
45	1.42	9.60	0.663
46	2.26	15.8	1.09
47	2.50	24.6	1.63
48	1.48	31.8	2.27
49	0.474	29.7	2.01
50	0.190	23.8	1.41

\* Relative specific concentration indicates the amount of NANA in a given tube, as per cent of total NANA in the whole output array, divided by protein content of the same tube expressed as per cent of total protein in the whole output array.

‡ Tube in which protein peak occurred.

chondrial-synaptosomal preparation. Again the distribution is consistent with the location of the mitochondria to the negative electrode side of the main protein peak, as evidenced by high ouabain-resistant ATPase activity. The high Na,K-ATPase activity to the positive electrode side of the main protein peak is indicative of the location of synaptosomes. Comparison of the levels of ATPase activity in tubes 43 and 48 with the results of Whittaker (28) and Hosie (29) suggest that the separation of synaptosomes and mitochondria is equivalent to that obtained by ultracentrifugal methods.

#### Succinic Dehydrogenase

The cumulative 140 min oxygen uptake of the electrophoretic subfractions of the ( $P_2$ ) mitochondrial-synaptosomal preparation is given in Fig. 12. The specific activity curve suggests that mitochondria are located to the negative electrode side of the main protein peak.

#### Choline Acetylase Activity and Bound Acetylcholine Content

Preliminary experiments, carried out before the electrophoretic separations described above, had confirmed Whittaker's observations on the distribution of bound acetylcholine in ultracentrifugal subfractions of the crude mitochondrial-synaptosomal fraction of guinea pig brain homogenates (1).

The maximum relative specific activities found for acetylcholine (percent of total brain acetylcholine contained in a given fraction, divided by per cent of total weight) were 15.95 for the upper layer of fraction D<sub>1</sub> (synaptic vesicles) and 4.25 for fraction B (synaptosomes).

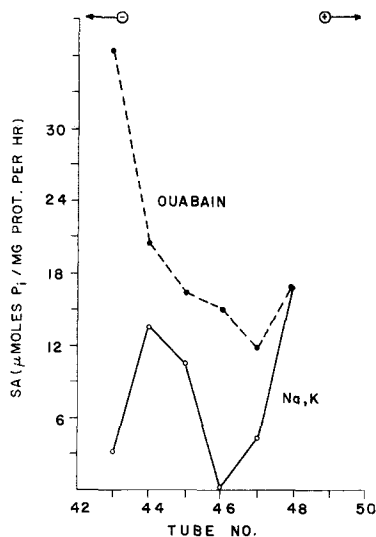


FIGURE 11 Distribution of ouabain-resistant ATPase activity (Ouabain) and Na,K-ATPase activity (Na,K) in the electrophoretic subfractions of the ( $P_2$ ) mitochondrial-synaptosomal preparation. ATPase activity was measured by the method of Post and Sen (16).

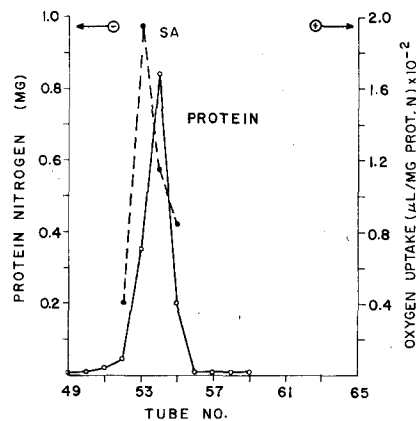


FIGURE 12 Distribution of succinic dehydrogenase activity in the electrophoretic subfractions of the ( $P_2$ ) mitochondrial-synaptosomal preparation. Cumulative oxygen uptake at 140 min was measured by means of the Warburg apparatus as outlined by Umbreit et al. (17). The tubes above No. 55 showed too little activity and too little protein for accurate measurement.

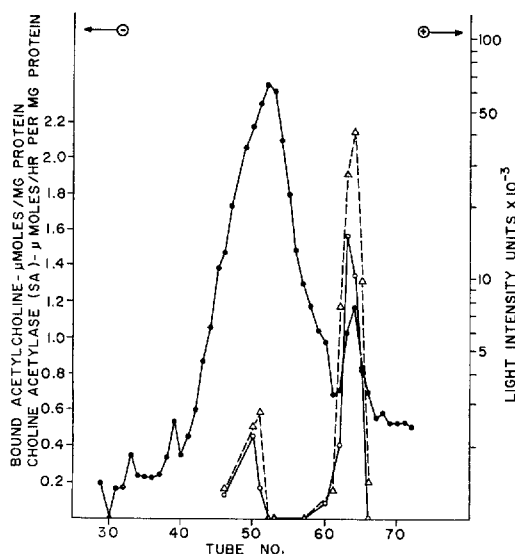


FIGURE 13 Distribution of bound acetylcholine ( $\Delta$ ) and of choline acetylase activity ( $\circ$ ) in the electrophoretic subfractions of the ( $P_2$ ) mitochondrial-synaptosomal preparation from guinea pig brain. Values are expressed in relation to the protein content of each subfraction, and are superimposed on the curve showing nephelometric estimation ( $\bullet$ ) of distribution of total particulate material in the same experiment.

The distributions of choline acetylase activity and of bound acetylcholine content among the electrophoretic output fractions of the ( $P_2$ ) mitochondrial-synaptosomal preparation are shown in Fig. 13. About 75% of the identified choline acetylase activity and of the bound acetylcholine measured in the various fractions was contained in the synaptosomal peak. In the experiment illustrated, the total recoveries for all fractions, in relation to the initial amounts in the equivalent volume of crude brain homogenate, were 44% for choline acetylase activity and 49% for bound acetylcholine. The loss presumably reflects a combination of losses during the initial centrifugal preparation of the crude mitochondrial-synaptosomal fraction, losses by inactivation during electrophoresis, and distribution of small unmeasurable amounts among other tubes in the output array. The peak concentration of bound acetylcholine (21.5  $\mu\text{g}/\text{mg}$  protein, in tube 64 of Fig. 13) compares very favorably with that found in Whittaker's fraction B in the preliminary experiments, when allowance is made for the conversion of protein content to equivalent fresh weight.

### 5'-Nucleotidase Activity

Measurement of 5'-nucleotidase activity did not prove very satisfactory as an index of biochemical specificity of the fractions. Preliminary investigations on rat brain homogenates showed an activity of 9.6  $\mu\text{moles}$  hydrolyzed/hr per ml of homogenate. Guinea pig brain showed a considerably lower activity, amounting to about 4.5  $\mu\text{moles}/\text{ml}$  per hr. In the experiment corresponding to Fig. 13, measurable activity was found only in tubes 62-66, with a peak concentration in tube 63. The total recovery in these tubes amounted to only 18.5% of the original input, with 5.6% in tube 63. The reasons for the poor recovery have not yet been ascertained.

### DISCUSSION

By means of continuous free-flow electrophoresis, preparations of synaptosomes have been obtained which compare favorably with fractions isolated by ultracentrifugal techniques. Examination by electron microscopy, and measurements of several independent biochemical markers, indicate that the preparation obtained electrophoretically is at least as pure as the best centrifugal preparations described in the literature. A significant advantage of the electrophoretic method is that the total preparation time is at least 3 hr shorter than that required for the ultracentrifugal methods.

On the other hand, this electrophoretic procedure has distinct limitations. Because of the low volume of sample which can be isolated per hour, and the intrinsically short period of viability of certain biological materials such as synaptic vesicles, purity of the preparations is obtained at the expense of the yield. For preparations of greater stability, this limitation would not apply. There is little probability that the yield can be improved substantially, except by increase in the scale of the electrophoretic apparatus. The limiting feature is the ratio of linear flow rate of chamber buffer to rate of lateral displacement of particles in the electrical field. Optimization of this ratio limits the rate of sample injection relative to the buffer flow rate, while the rate of electrophoretic migration is limited by the optimal composition of the buffer as explained below, and by the capacity of the refrigerating system which cools the electrophoretic chamber. The method is therefore of major value for rapid preparation of small samples.

A second constraint on the method is the prob-

lem of compatibility of the chamber buffer with the biological particles undergoing separation, and with the electrical current requirements of the separator. The buffer must not only be of suitable tonicity to maintain the integrity of the particles, but must also have an appropriate ionic composition to minimize clumping. At the same time, the conductivity of the buffer must conform to the requirements for regulation of current flow across the chamber. These two limiting features require a rather extensive set of preliminary tests before optimal conditions can be defined for a particular biological preparation.

A third limitation on the convenience of the method is the need for some "internal marker" to which output fractions can be related. As in column chromatography, the whole output spectrum may shift by three or four tubes because of variations in operating conditions from one run to another. This is illustrated by the difference between Fig. 1 and Fig. 13. This means that even for a well-studied spectrum it is necessary to measure either the protein content, turbidity, or other appropriate index of every output tube in each experiment, in order to locate precisely the position of the desired fraction.

Another advantage of the present method, apart from speed of preparation, is the opportunity which it provides for accurate measurement of the surface electrical properties of separated particles. Other investigators (4, 5) have used electrophoretic methods to prepare synaptic vesicles from the brain but their techniques, although empirically useful as preparative methods, have deficiencies which restrict the theoretical usefulness of their measurements. For example, the mobilities of synaptosomes, synaptic vesicles, and mitochondria reported by Vos et al. (4) are in the same relative order as reported here, but their absolute values are only about one-half as large as ours. There are probably three reasons for the discrepancy: (a) different buffer and temperature conditions were used, (b) Vos et al. used a continuous sucrose density gradient which introduced unknown viscosity factors into the net mobility, and (c) electro-osmotic effects and electrode polarization were ignored in their calculations. These factors can be taken into account with the present method, so that valid calculations of surface electrical properties of the various types of particle can be made. It is interesting to note that the surface charge density values in Table I are similar

in magnitude and sign to that calculated by Abramson et al. (30) for the guinea pig erythrocyte. Knowledge of the surface electrical properties permits one to develop theoretical approaches to an analysis of electrostatic interaction between particles and membranes. An analysis of this type has been undertaken (26), and will be published elsewhere.

Further advantages of the present electrophoretic method over earlier ones include superior regulation of temperature in the separation chamber, a shorter time requirement for separation, and continuous operation. These features raise the possibility that purer preparations may be achieved by recycling individual fractions through the separation chamber. The potential advantages are obvious. Attempts are currently being made to apply this approach to the isolation of cell membrane fragments.

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