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APPENDIX

The Morphology of Fractions of Rat Forebrain Synaptosomes Separated on Continuous Sucrose Density Gradients

BY V. P. WHITTAKER
Department of Biochemistry, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge, and Department of Biochemistry, University of Cambridge

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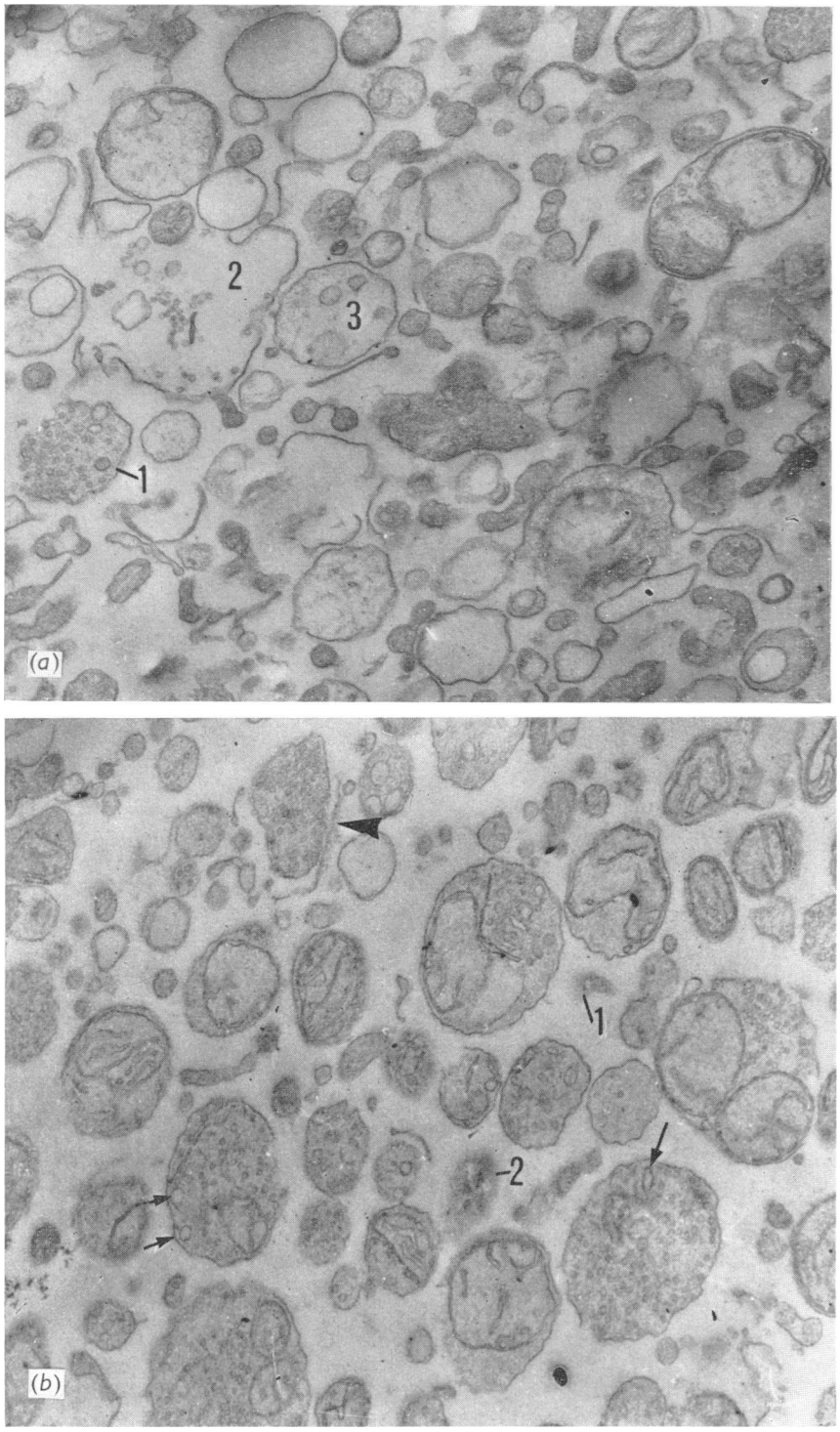
Several attempts have been made to separate synaptosomes (detached presynaptic nerve terminals) into chemically and morphologically distinct fractions by means of density-gradient centrifuging. Variants of the discontinuous sucrose gradients originally employed for this purpose by Hebb & Whittaker (1958) have been used (De Robertis, de Iraldi, Arnaiz & Salganicoff, 1962; Weinstein, Roberts & Kakefuda, 1963); continuous gradients prepared by allowing diffusion to occur in an initially discontinuous gradient (Michaelson & Whittaker, 1963) or by means of a gradient maker (van Kempen, van den Berg, van der Helm & Veldstra, 1965; Fonnum, 1965, 1968; Balázs, Dahl & Harwood, 1966) have also been tried. Morphological studies of the fractions have been mainly qualitative; they have not so far indicated that any clear-cut separation into morphologically distinct types of synaptosome has been achieved.

In the present study, carried out in connexion with the main paper (Fonnum, 1968), fractions of synaptosomes derived from rat forebrain and

EXPLANATION OF PLATE 1

(*a*) Portion of a light synaptosome fraction from the middle of a sucrose density gradient (fraction I6, block A; for sucrose concentration see Table 1), showing a region where empty membranous profiles resembling synaptosome 'ghosts' are concentrated. Profiles 1, 2 and 3 show intermediate stages between synaptosome and 'ghost' profiles. (*b*) Portion of the same fraction (block B) showing well-preserved synaptosomes. Note post-synaptic adhesion (thick arrow) and large vesicles among smaller ones (thin arrows). A third block (C) gave similar profiles. About half the particles in the field cannot be identified as synaptosome profiles, but most of these are quite small. Black patches resembling profile 1 and very small vesiculated bodies (profile 2) are found in serial sections to be grazing sections of larger synaptosomes (C. D. Voorhorst, personal communication). Magnification (*a* and *b*) $\times 30000$.


2
3
1
1
(a)
1
2
(b)

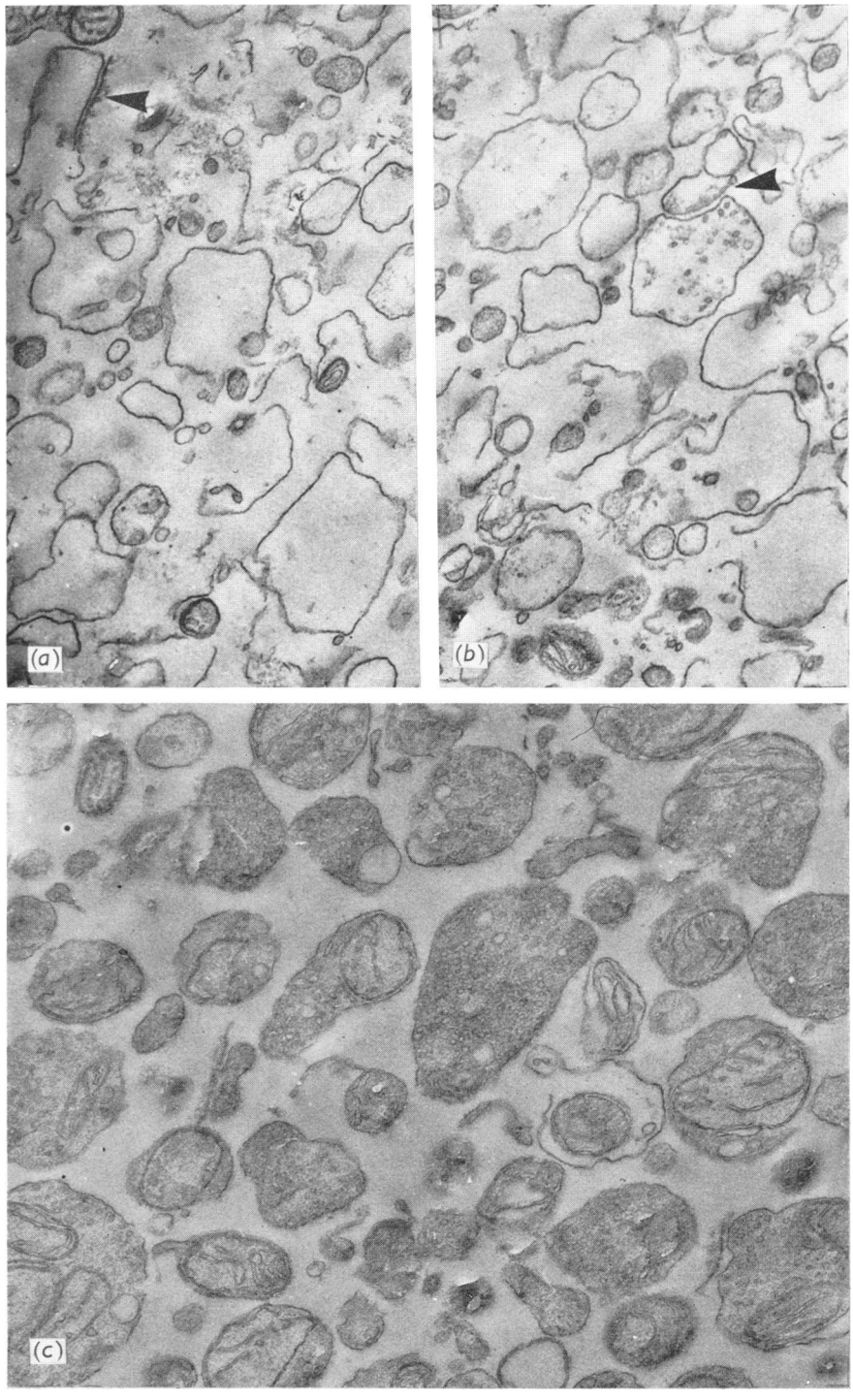

(a)
(b)
(c)

Table 1. *Composition of rat brain synaptosome fractions obtained by continuous-sucrose-density-gradient centrifuging*

For details of preparation see Fonnum (1968). Results are from Expt. I.

Fraction	Concn. of sucrose (M)	Total no. of particles in field	No. identified	Percentage identified		
				As synaptosomes	As myelin	As mitochondria
4	0·98–1·04	285	6	83	17	0
6	1·11–1·17	477	233	96	3	1
7	1·17–1·24	205	62	97	0	3
8	1·24–1·30	356	124	52	0	48
10	1·36–1·44	294	115	44	0	56
11	1·44–1·50	201	92	8	0	92

separated by centrifuging into a continuous density gradient were examined morphologically and the synaptosome profiles were analysed statistically with regard to size and mitochondrial content.

METHODS

Preparation of fractions for electron microscopy. Fractions obtained by centrifuging crude rat forebrain synaptosome preparations into a continuous linear density gradient for 2·5 hr. [for details see the main paper (Fonnum, 1968)] were diluted with an equal volume of water and fixed as described by Whittaker & Sheridan (1965) with 5 vol. of Luft's (1956) permanganate fixative. The fixed particles were sedimented by centrifuging at 5000*g* for 10 min.; the pellets so obtained were dehydrated in acetone and embedded in Araldite; thin sections cut at various depths through the blocks were stained with lead (Millonig, 1961) before viewing at 10000 times magnification in a Siemens Elmiskop I electron microscope at 80 kv. The objective-lens current was measured and used to correct the nominal magnification when necessary by means of a calibration curve.

Evaluation of electron micrographs. The outlines of synaptosomes and, in some fields, those of any mitochondria contained within them were transferred to tracing paper and the tracings cut out and weighed. From a knowledge of the density of the paper/unit area and the magnification factor of the electron micrographs the area (in μ^2) of each organelle was evaluated.

The mean diameters of synaptic vesicles were measured (to 1 μ) directly on the plates by using a projection microscope (Nikon model 6C Shadowgraph; Nippon Kogaku K.K., Japan; agents, Rank, Taylor and Hobson, Leicester) equipped with a movable stage operated by micrometer screws.

RESULTS

Degree of homogeneity of the synaptosome fractions. Synaptosomes were readily identified (Gray & Whittaker, 1962) as membrane-bound sacs of cytoplasm containing numerous synaptic vesicles 300–500 Å in diameter; internal mitochondria were also frequently present. Lengths of post-synaptic membrane were usually seen attached to several profiles in each field; occasionally these took the form of pinched-off dendrite spines (compare Whittaker, 1962). The fractions containing synaptosomes corresponded to a broad hazy band in the density gradient starting just below the myelin band in a sucrose concentration of about 0·9 M and extending to and merging with a band of mitochondria floating in about 1·5 M-sucrose.

All fractions were contaminated to a variable extent by particles and membrane fragments that could not be identified with certainty. These ranged from small dense particles and sacs of microsomal dimensions to empty membrane-bound profiles of about the same size as synaptosomes. Some of the latter may well have been external membranes of inadequately preserved synaptosomes, since intermediate forms containing a few vesicles of adherent post-synaptic thickenings were present (Plates 1*a*, 2*a* and 2*b*), but others could have been derived from dendrites, glial processes or terminal axons. They were most abundant in the lightest fractions (4–6), constituting the predominant structure in some fields. They merit further investigation. Some of the smaller profiles may have been grazing sections of synaptosomes, but, whatever their origin, the contribution of the smaller fragments to the total weight of the fractions must have been quite small.

Other contaminants were myelin fragments and free mitochondria. Myelin contamination was insignificant in fractions denser than 1·1 M-sucrose, but appreciable contamination with free mitochondria occurred in fractions denser than 1·24 M-sucrose.

EXPLANATION OF PLATE 2

(*a*) and (*b*) Portions of other sections of the same material as seen in Plate 1(*a*), showing transitional profiles with post-synaptic adhesions (arrows). (*c*) Portion of dense synaptosome fraction (I8). Note absence of any obvious morphological differences from fraction I6 (Plate 1*b*). Other fractions were qualitatively similar. Magnification (*a*, *b* and *c*) × 30000.

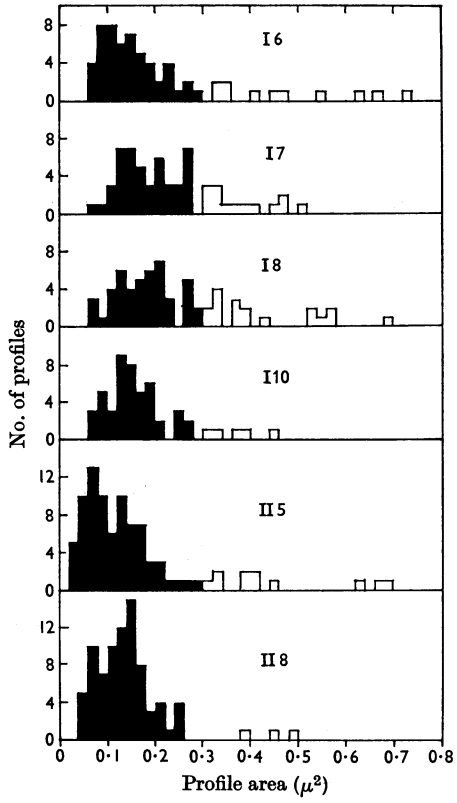


Fig. 1. Frequency of distribution of areas of synaptosome profiles for fractions separated on a continuous density gradient. For sucrose concentrations of fractions see Tables 1 and 2. The main population of synaptosome profiles (of area less than 0·3 μ^2) is shown in black.

Plates 1 and 2 and Table 1 illustrate the degree of contamination of the fractions. Owing to the heterogeneity of the morphologically unidentified material it was difficult to assess the degree of contamination quantitatively. In Table 1, column 3, are given estimates of the total number of particles (irrespective of size) in representative fields and in column 4 the number of particles morphologically identified in these fields. The remaining columns give the proportion of such particles identified as synaptosomes, myelin fragments and mitochondria. As seen in Plates 1(*b*) and 2(*c*), most of the unidentified particles in the middle and lower fractions were quite small, and such fractions constitute fairly homogeneous preparations of synaptosomes.

Morphological variations in synaptosomes. The individual synaptosomes varied considerably in their morphology, but there was no obvious separation of different morphological types into distinct fractions. Profiles differed in size, in vesicle and mitochondrial content and in the type of post-synaptic adhesion, where present. There were differences in mean vesicle diameter between synaptosomes in the same field that were sometimes significant. In addition, many synaptosomes contained relatively large vesicles with clear centres (Plate 1), which appeared to constitute a separate population with a mean diameter of 698 Å, S.D. ± 54 Å (eight observations) as compared with the main population of mean diameter 427 Å. The collapse of such vesicles during fixation might account for the elongated or polymorphic vesicles seen in certain types of ending in glutaraldehyde-fixed tissue (compare Larramendi, Fickenscher &

Table 2. *Mean areas of synaptosome and intraterminal mitochondrial profiles in electron micrographs of density-gradient fractions*

Means are given ± S.D. (numbers of observations in parentheses) and relate to synaptosome profiles of area less than 0·3 μ^2 (black blocks in Fig. 1). Pairs of mean values to which *t* tests were applied are indicated by the same symbols. For sucrose concentrations of fractions of Expt. I, see Table 1. Concentrations for Expt. II were: fraction 5, 1·04–1·12 M; fraction 8, 1·25–1·29 M.

Expt.	Fraction	Percentage of synaptosomes below 0·3 μ^2	Mean profile area (μ^2)		Synaptic-vesicle diameter (Å)
			Synaptosomes	Intraterminal mitochondria	
I	6*	83	0·150 ± 0·057 (52)‡§	0·070 ± 0·032 (26)§	—
	7	92	0·184 ± 0·056 (46)‡	0·081 ± 0·038 (18)	418 ± 60 (117)
	8†	72	0·178 ± 0·059 (46)§	0·068 ± 0·028 (31)	429 ± 44 (77)
	10	90	0·155 ± 0·052 (46)	0·052 ± 0·030 (28)§	428 ± 55 (26)
II	5	89	0·114 ± 0·058 (78)	0·061 ± 0·037 (26)	—
	8	97	0·138 ± 0·056 (82)	0·050 ± 0·025 (37)	—

* Block B, small sample, not significantly different from the larger sample given in Table 3.
† Block A.
‡ Significantly different ($P < 0·01$).
§ Significantly different ($0·01 < P < 0·02$).

Table 3. *Mean areas of synaptosome profiles*

Means are given ± S.D. (numbers of observations in parentheses) and relate to Expt. I (Table 2) and profiles of area less than $0{\cdot}3\,\mu^2$. For sucrose concentrations of fractions see Table 1. Pairs of mean values to which t tests were applied are indicated by the same symbol.

Fraction	Block	Mean profile area (μ^2)	Percentage of total below $0{\cdot}3\,\mu^2$	Synaptic-vesicle diameter (Å)
6	A	$0{\cdot}118 \pm 0{\cdot}062$ (279)*	91	430 ± 51 (32)
	B (first five fields)	$0{\cdot}169 \pm 0{\cdot}063$ (85)†	88	—
	(last four fields)	$0{\cdot}111 \pm 0{\cdot}054$ (56)†	100	—
	(all fields)	$0{\cdot}143 \pm 0{\cdot}063$ (219)*	96	402 ± 53 (131)
	All measurements	$0{\cdot}131 \pm 0{\cdot}061$ (498)‡	93	410 ± 52 (162)
8	A	$0{\cdot}178 \pm 0{\cdot}059$ (46)	72	429 ± 44 (77)
	B (first three fields)	$0{\cdot}153 \pm 0{\cdot}068$ (55)	87	—
	(last three fields)	$0{\cdot}138 \pm 0{\cdot}064$ (57)	93	—
	(all fields)	$0{\cdot}149 \pm 0{\cdot}065$ (150)	88	400 ± 67 (70)
	All measurements	$0{\cdot}156 \pm 0{\cdot}065$ (196)‡	83	416 ± 58 (147)

* † ‡ Significantly different ($P < 0{\cdot}01$).

Lemkey-Johnston, 1967). As far as the small vesicles are concerned, 87% of the variance was estimated to be within individual synaptosome profiles, 9% between synaptosomes in the same field and 4% between different fields of the same block.

Variations in size. In view of the absence of any obvious qualitative differences between the synaptosomes of the various fractions, an analysis of synaptosome size was undertaken. Each fraction (Fig. 1) could be regarded as containing two populations, the main one of profiles less than about $0{\cdot}3\,\mu^2$ in area and forming an approximately normal distribution, and a second population, usually comprising less than 15% of the whole, with areas up to about $0{\cdot}7\,\mu^2$.

A preliminary analysis (Table 2) indicated that the mean areas of the main population of synaptosome profiles derived from fractions from the middle of the gradient were significantly greater (by about 12%) than those from the top and bottom of the gradient. Mean synaptic-vesicle diameters (column 6) showed no significant differences between fractions, suggesting that the differences in mean synaptosome profile areas were not due to changes occurring during preparation for electron microscopy.

In an attempt to investigate the possibility that the differences were simply due to sampling errors, more detailed studies were made on fractions 6 and 8 of Expt. I (Table 3). In fraction 8, there were small differences in mean synaptosome profile areas from one block to another (compare lines 6 and 9) and between different regions in the same block (compare lines 7 and 8), suggesting that some stratification had occurred during preparation, presumably when the fixed material was sedimented for dehydration and embedding. However, these differences were not significant ($P > 0{\cdot}05$). With the blocks derived from fraction 6, stratification was much more obvious. There were significant differences in mean profile area between blocks A and B (compare lines 1 and 4) and at different levels in block B (lines 2 and 3). The fields containing the smaller synaptosomes (block A, lower part of block B) were also those with numerous empty sacs and membrane fragments.

Fraction 6 is clearly more heterogeneous than fraction 8, but in spite of the rather large sampling error the mean of all profiles of fraction 8 is still about 12% greater than the corresponding value for fraction 6. In future work it may be possible to reduce sampling errors by using smaller samples of the original fraction, as advocated by Baudhuin, Evrard & Berthet (1967).

Mitochondrial content of fractions. It was suggested by Weinstein *et al.* (1963) that the densest synaptosomes in their preparations were 'richer in mitochondrial inclusions' than the lighter ones. To investigate this, the mean areas of the profiles of mitochondria contained within synaptosomes, and also the proportion of the total synaptosome profile areas occupied by mitochondrial profiles, were evaluated for the various fractions. As shown in Table 2, there was little difference in the mean areas of the mitochondrial profiles in the various fractions, except that those of the densest synaptosomes had a just significantly lower mean area than those of the lightest.

When the proportion of the total synaptosome profile area occupied by mitochondria was calculated (Table 4A) there was little difference between the lightest and the densest fractions. A similar conclusion is reached if one considers the areas of 100 mean synaptosome profiles (Table 4B) taking the statistics given in Table 2.

Table 4. *Proportion of total area of synaptosome profiles occupied by mitochondria*

The calculations are based on the measurements summarized in Fig. 1 and Table 2.

Expt.	Fraction	No. of synaptosome profiles	Total area of synaptosome profiles (μ^2)	No. of mitochondrial profiles	Total area of mitochondrial profiles (μ^2)	Total area of mitochondrial profiles (% of synaptosome profile areas)
A. All synaptosome profiles						
I	6	63	13·06	37	4·00	31
	7	60	13·72	27	2·76	20
	8	64	15·74	45	3·97	25
	10	51	8·94	33	2·26	27
II	5	89	14·22	36	2·90	20
	8	85	12·41	39	2·12	17
B. 100 mean synaptosome profiles from population less than 0·3 μ^2 in area						
I	6	100	15·0	30	3·50	23
	7	100	18·4	39	3·16	17
	8	100	17·8	67	4·58	26
	10	100	15·5	61	3·17	20
II	5	100	11·4	33	2·04	18
	8	100	13·8	45	2·26	16

Table 5. *Equivalent mean spherical diameters of synaptosomes and their contained mitochondria*

Values relate to synaptosome profiles less than 0·3 μ^2 in area and are calculated from the mean areas given in Table 2.

Expt.	Fraction	Equivalent mean spherical diam. (μ)		Percentage of profiles containing mitochondria		Percentage of synaptosomes containing mitochondria
		Synaptosomes	Mitochondria	Calc.	Found	
I	6	0·555	0·383	72	50	70
	7	0·615	0·409	70	39	56
	8	0·605	0·375	66	67	101
	10	0·565	0·327	59	61	104
II	5	0·485	0·354	76	33	44
	8	0·535	0·321	65	45	70

The diameter of a circular profile of area A is $\sqrt{4A/\pi}$; the diameter of the sphere from which these profiles are derived is larger than the mean profile diameter by the factor $4/\pi$. Thus we may calculate an 'equivalent mean spherical diameter' from the mean profile area $\bar{A}$ by means of the formula $(4/\pi)^{\frac{3}{2}}\bar{A}^{\frac{1}{2}}$. The results are given in Table 5. The values for the equivalent mean spherical diameter of synaptosomes in column 3 are close to the value of 0·5 μ obtained in a slightly different way by Clementi, Whittaker & Sheridan (1966) for synaptosomes isolated from guinea-pig cortex in 0·8–1·2 M-sucrose. The corresponding diameters for mitochondria are about 30% less.

It should be noted that, even if all synaptosomes contain mitochondria, not all synaptosome profiles will contain mitochondrial profiles. If h is the section thickness, then N_m spherical synaptosomes of diameter d_s, each containing a spherical mitochondrion of diameter d_m will give rise (Abercrombie, 1946) to $N_m(d_s+h)/h$ synaptosome profiles and $N_m(d_m+h)/h$ mitochondrial profiles. The percentage of synaptosome profiles containing mitochondria will thus be $100(d_m+h)/(d_s+h)$. This percentage, calculated from the values given in columns 3 and 4 of Table 5, is tabulated in column 5.

The percentage of profiles actually observed to contain mitochondrial profiles within them, shown in column 6, is in good agreement with the calculated values for the denser fractions of Expt. I, but is substantially lower for the lighter fractions, especially in Expt. II. This implies the presence in the lighter fractions of a population of synaptosomes that do not contain mitochondria. If N_0 is

the number of such synaptosomes, the total number of synaptosome profiles is now $(N_m+N_0)(d_s+h)/h$ and the proportion containing mitochondria is $N_m(d_m+h)/(N_m+N_0)(d_s+h)$. Thus the values of column 6 are lower than those of column 5 by the factor $N_m/(N_m+N_0)$, the proportion of synaptosomes containing mitochondria. The factor is obtained by dividing the values of column 6 by those of column 5 and is given (as a percentage) in column 7.

The factor is only equal to the proportion of synaptosomes containing mitochondria if the diameters of both types of synaptosomes are the same. Obviously the smaller the mitochondrion-free synaptosomes are relative to those containing mitochondria the greater the number that would be required to account for the discrepancy between columns 5 and 6 and vice versa.

DISCUSSION

In conclusion, density-gradient centrifuging as used in these experiments did not achieve a dramatic separation of synaptosomes of different sizes or morphological type. There is some evidence that the main population of synaptosomes (i.e. those generating profiles less than $0{\cdot}3\,\mu^2$ in area) consists of two sub-populations, one containing mitochondria and one not. The latter tend to remain in the less dense region of the gradient. [Serial sections confirm that synaptosomes without contained mitochondria are present (C. D. Voorhorst, personal communication).] In this sense it may be true that the denser fractions are 'richer in mitochondrial inclusions' as was thought by Weinstein *et al.* (1963), but the statistical analysis failed to demonstrate that a higher proportion of the total synaptosome volume is occupied by mitochondria in the denser fractions. In addition, the lightest fractions contained considerable numbers of empty membranous sacs similar in dimensions to synaptosomes but of unknown provenance.

The finding (Table 2) that the mean profile areas of synaptosomes migrating to the middle regions of the gradient were about 12% greater than those of either lighter or heavier fractions did not seem to be attributable to variations between samples (though more samples would be needed to establish this with certainty), and suggests the interplay of more than one factor affecting profile area (or synaptosome volume). Possibly the mitochondrion-free synaptosomes of the lightest fractions are smaller than those containing mitochondria, and thus depress the mean value for the total population. The smaller size of the densest synaptosomes may be due to shrinkage brought about by the high osmotic pressure of the densest regions of the gradient; the more readily dehydrated members of the original population may well travel further down the gradient. Further characterization of the fractions will require both better sampling techniques and the use of histochemical methods.

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