QUALITATIVE AND QUANTITATIVE MORPHOLOGICAL CHANGES IN SUBCELLULAR FRACTIONS FROM MOUSE CEREBRAL CORTEX DURING POSTNATAL DEVELOPMENT

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ABSTRACT

Discontinuous Ficoll-sucrose gradients were used to prepare subcellular fractions from mouse cerebral cortex at various stages of postnatal development. Representative samples of each subcellular fraction were obtained by sedimentation in an analytical ultracentrifuge and each fraction was examined quantitatively and qualitatively by electron microscopy. The amount of synaptosomal material was determined for each fraction on the basis of volume percentage, obtained from a series of contiguous micrographs, to circumvent any sampling error. This allowed an accurate appraisal of synaptosomal distribution during neural development and a direct comparison of the Ficoll-sucrose gradient fractions to the original crude mitochondrial preparations. The distribution of synaptosomal material was shown to be quantitatively altered during neural development, and maturation-dependent changes, at a qualitative level, were described. In addition, the relationship between neural maturation and the relative proportion and distribution of subcellular particles which contain processes tentatively identified as growth cones were characterized.

INTRODUCTION

On a cellular level, the development of the mammalian nervous system involves an extremely intricate series of events. This process includes the proliferation of neuroblasts and glioblasts, the migration of cells to various locations, as well as cellular differentiation which results in mature neurons and glia (18, 19). In addition, neural development includes the formation of functional synaptic contacts at a cellular level. All of these processes, which occur at various rates and times in different anatomical regions of the nervous system (18), complicate any analysis of any one event on a molecular level. One method which has been employed to approach an understanding of these phenomena has involved the separation of various subcellular fractions before chemical and morphological analysis. In particular, subcellular fractionation techniques have been successfully used to obtain preparations enriched in synaptic structures. These isolated fractions have been used to measure maturation-dependent changes in enzyme activities, protein and lipid contents, and binding of neurologically active chemical compounds (1, 11, 15). Interpretation of such results depends on a careful evaluation and description of the contents of these fractions (16, 22). However, the primary condition for quantitative electron microscopy of cellular fractions is that the sample which is ex-

amined morphologically must be statistically representative of the fraction. The biases which can be introduced into a sample during preparation have been recognized and discussed in some detail (6, 8, 9). Procedures commonly used for concentrating subcellular fractions, before analysis by electron microscopy, do not provide representative samples of the contents of each fraction. The present study was designed to evaluate, at both a qualitative and quantitative level, the morphology of subcellular fractions obtained from mouse cerebral cortex during various stages of postnatal development.

MATERIALS AND METHODS

Isolation of Fractions

Cerebral cortices of 5-, 12-, 22-day old and adult Swiss-Webster albino mice were quickly hand dissected free from underlying white matter and placed in 9 vol of ice-cold 0.32 M sucrose, 10 mM Tris-HCl (pH 7.4). Preliminary experiments established that there were no significant morphological differences in purity of fractions from brain tissue previously homogenized in sucrose solutions with or without 10 mM Tris-HCl. Differential centrifugation as described by Cotman and Matthews (10) was used to remove large cellular debris and nuclei, and to subsequently obtain a crude mitochondrial pellet. The mitochondrial pellet was then resuspended in the buffered sucrose and layered on a prechilled discontinuous Ficoll gradient (lot no. 523, Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). With the following modifications, the gradient and centrifugation conditions are those of Autilio et al. (4). The Ficoll solutions were made up (wt vol) in 0.32 M sucrose in 1 mM Tris-HCl (pH 7.4), and the 10% Ficoll step was omitted. The gradients were centrifuged for 30 min at 64,000 *g* in the SB-269 rotor of the International Ultracentrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.). After centrifugation the fractions at the interfaces were isolated and removed with the aid of a mechanical centrifuge tube slicer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Following the nomenclature of Gonatas et al. (16) the material collected from the interface between the supernate and 5% Ficoll was designated fraction I; between 5% and 7.5%, fraction II; between 7.5% and 13%, fraction III; between 13% and 20%, fraction IV.

Preparation of the Fractions for Electron Microscopy

The fractions were fixed in ice-cold 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Representative samples of the fractions for quantitative electron microscope analysis were prepared using a technique developed by Grove, et al. (17). After the fractions had been in the fixative for at least a half hour, they were thoroughly mixed and an aliquot of 0.5 ml (0.1–0.3 mg protein) was loaded into the standard 12-mm, 4°-sector cell of the analytical ultracentrifuge. As a cushion, 0.05 ml of the dense fluorocarbon oil FC43 (Beckman Instruments, Inc.) was added. The fractions were then layered by centrifuging at 20,000 rpm at 20°C for 30 min in the Beckman model E Analytical Ultracentrifuge. The resulting thin layers (30–60 μm) could be easlly poured into glass vials. Although the layers would often break into three to four segments, the composition of these fragments have been shown to be essentially identical (17). Subsequent changes of solutions were carefully performed with Pasteur pipettes to avoid further disruption of the layers. The subcellular material was rinsed briefly with 0.1 M phosphate buffer (pH 7.4) and treated for 1 h with 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4). The pellets were then dehydrated through ethanol and embedded flat in a soft Epon mixture according to Luft (seven parts of A: three parts of B) (21). Silver to gray sections were cut parallel to the direction of sedimentation and mounted on Formvar-coated grids. They were then stained for 10 min in saturated uranyl acetate in 50% ethanol followed by 1 min in Sato's lead salt solution (23). The sections were examined with a Hitachi Hu-12 electron microscope operated at 75 kV.

Quantitation

A series of micrographs was taken to encompass the entire thickness of each layer at a magnification of 10,000 and enlarged 2.0–2.5 times photographically. A membrane-bound structure was defined to be a synaptosome only if it contained three or more well-defined vesicles 30–50 nm in diameter. In this study we considered only the presynaptic components. The fractional area (A_a) occupied by synaptosomes was determined by placing a grid of 957 regularly spaced dots over the micrographs. The number of dots which fell upon synaptosomes was divided by the number of dots which fell upon any electron dense object in the micrograph. Therefore, the somewhat variable space between the structures in each layer could be ignored. Because the structures in the micrograph are representative of the entire layer, the area fraction measured from the micrographs is equivalent to the volume fraction occupied by synaptosomes ($A_a = V_v$) (13). The volume fraction has been expressed as a percentage, and used as a measure of the concentration of the synaptosomes in each subcellular fraction.

Protein was isolated from each Ficoll fraction by

precipitation with 5% trichloroacetic acid and measured by the colorimetric assay of Lowry et al. (20).

RESULTS

The geometry of the analytical ultracentrifuge cell results in a particulate layer which is stratified on the basis of the sedimentation coefficient of the subcellular particles. This avoids the complicating effects of convective forces along walls and rounded bottoms found in pellets produced in conventional centrifuge tubes in either the swinging bucket or angle rotors of high speed centrifuges. Layers were then sectioned in a plane parallel to the direction of sedimentation to control for all of the heterogeneity present in the fraction. The importance of this method of surveying the contents of a fraction is demonstrated in Fig. 1. Fig. 1 *a* is a low magnification micrograph encompassing the entire thickness of the layer prepared from the synaptosomally enriched fraction (fraction III) prepared from adult mouse cortex. While at this magnification it is not possible to easily identify individual structures, the variation and the distribution of the different sized structures is quite evident. The differences are even more apparent when Fig. 1 *b* is compared to Fig. 1 *c*: these micrographs were taken of regions at the top and bottom of the particulate layer. Similar heterogeneity was observed with the comparable Ficoll fraction from cortical tissue obtained from 5-day old mice. In fact, the heterogeneity of this fraction was even more pronounced (Fig. 2 *a* and *b*). Since electron microscope techniques limit the amount of material which can be analyzed, it is necessary to control for all distribution variations introduced into the subcellular fractions at any stage of processing for electron microscopy. Therefore, the descriptions and quantitations which follow are based upon contiguous micrographs which spanned the entire thickness of the particulate layers prepared in the analytical ultracentrifuge which minimizes any sampling bias.

Despite the heterogeneity inherent in each subcellular fraction, there were obvious morphological differences in the comparable fractions obtained from adult and neonatal cortical tissue. The fractions obtained from adult mice were qualitatively quite similar to those reported previously by other workers (10, 14). Therefore, the following descriptions will emphasize differences observed between mature and neonatal fractions. For the reasons discussed above, the micrographs presented are for qualitative purposes only; the relative concentrations of the synaptic components in the entire fractions will be presented later. In order to facilitate size comparisons, this series of micrographs is presented at a constant magnification ($\times$ 20,000).

Fraction I obtained from adult cortex was composed primarily of free myelin and myelinated axon fragments (Fig. 3 *a*). In contrast to this adult fraction, there was very little recognizable myelin present in fraction I prepared from 5-day old mouse cortex. In addition, the myelin present in the fraction from the 5-day animal consisted primarily of from two to three lamellae, while the adult myelin contained up to 12 lamellae. Similar preparations from 12-day old animals also displayed small quantities of myelin. However, by 22 days the development of myelin resembled that which was observed in the adult fraction I. Unlike the adult fraction, the preparation from 5-day tissue (Fig. 3 *b*) was composed primarily of numerous cellular fragments from 0.2 to 4.0 μm in diameter. These cellular fragments frequently contained ribosomes, neurotubules, neurofibrils, and mitochondria. (Fig. 3 *b*). A major difference in fractions I prepared from adult and 5-day old cortical tissue was the common occurrence of synaptosomes in the younger preparations.

The adult fraction II (Fig. 4 *a*) contained smaller myelin fragments and small membrane bound vesicles about 1 μm in diameter. Synaptosomes and mitochondria were rarely observed in this adult fraction. In contrast, the material in fraction II obtained from 5-day old cortex (Fig. 4 *b*) was very similar to that found in the 5-day fraction I.

In the adult, fraction III is commonly accepted to be the synaptosomally enriched fraction. An example is shown in Fig. 5 *a*, where synaptosomes exhibiting varying electron density can be seen. In the adult preparation, synaptic vesicles comprised the larger part of their contents, while mitochondria filled much of the remaining space. Fraction III from 5-day cortex was similar to that obtained in the more mature preparation in that a significant number of synaptosomes were observed (Fig. 5 *b*). However, the average number of synaptic vesicles contained in the younger synaptosomes was considerably fewer. The major distinction of fraction III from 5-day old cortex was the presence of subcellular particles containing numerous vesicles larger than synaptic vesicles. The possible significance of this finding will be discussed later.

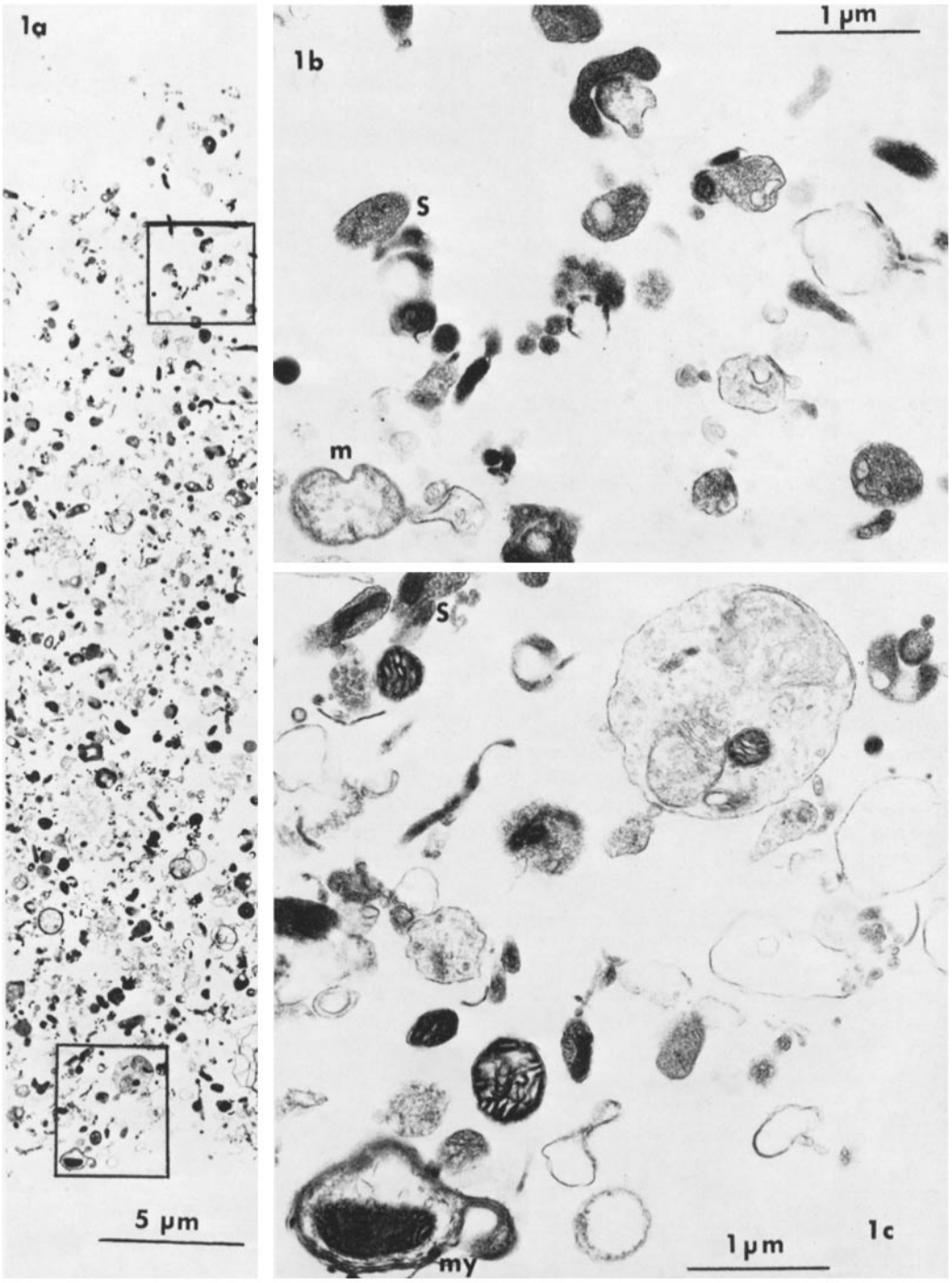

FIGURE 1 Fraction III from mature mouse cerebral cortex. 1 *a* shows the entire thickness of the sedimented layer. × 4,000. 1 *b* was taken from the top of the layer as noted. × 20,000. 1 *c* is from the bottom region as noted in 1 *a*. × 20,000. (*S*) indicate synaptic vesicles, (*m*) mitochondria, and (*my*) myelin.

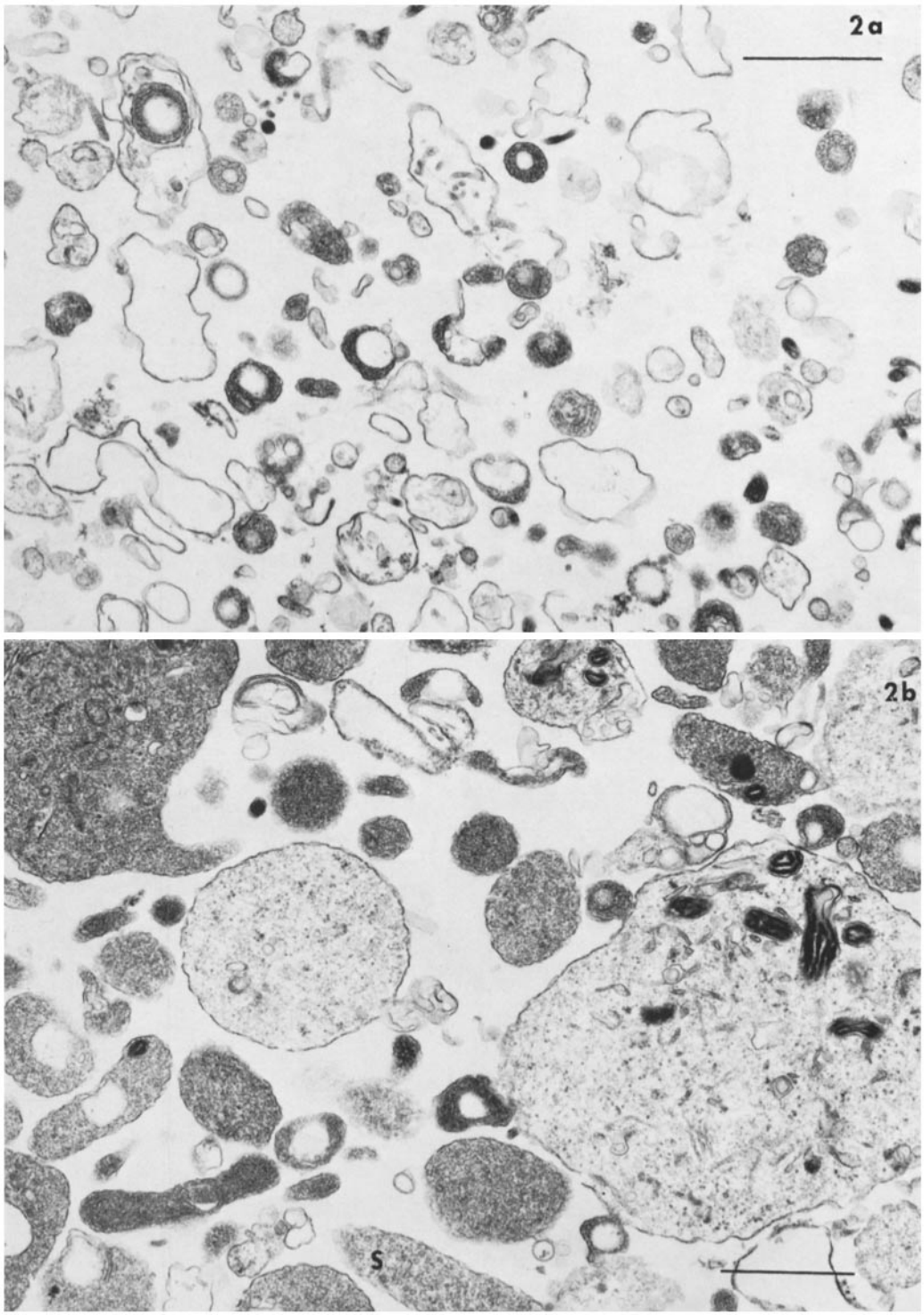

FIGURE 2 Fraction III from 5-day old mouse cerebral cortex. 2 *a* is from the region near the top of the layer while 2 *b* is from an area near the bottom. (*S*) synaptic vesicles. Both × 20,000.

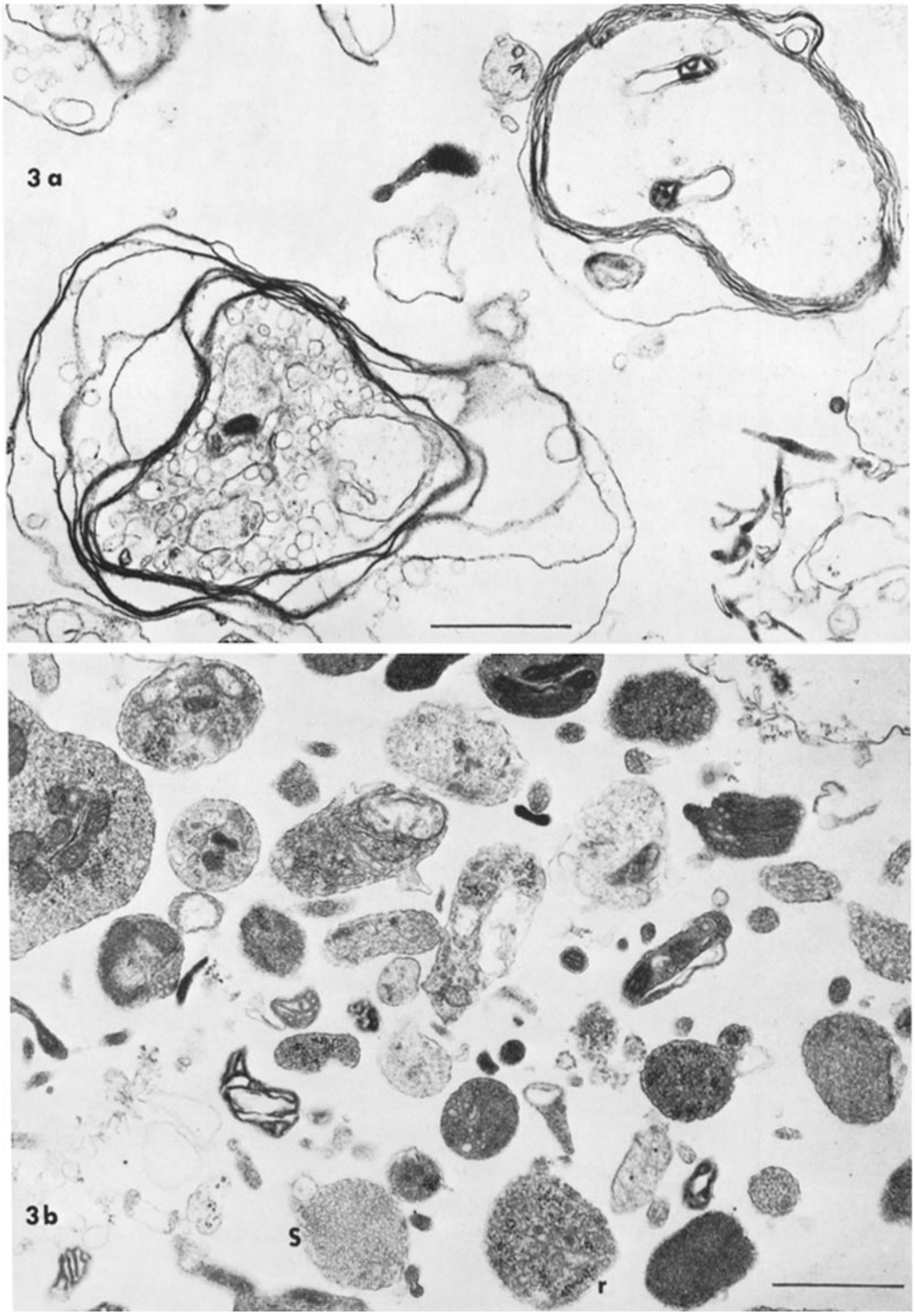

FIGURE 3 Fractions I from mature cortex (3 *a*) and 5-day old cortex (3 *b*). (*S*) synaptic vesicles, (*r*) ribosomes. Both × 20,000.

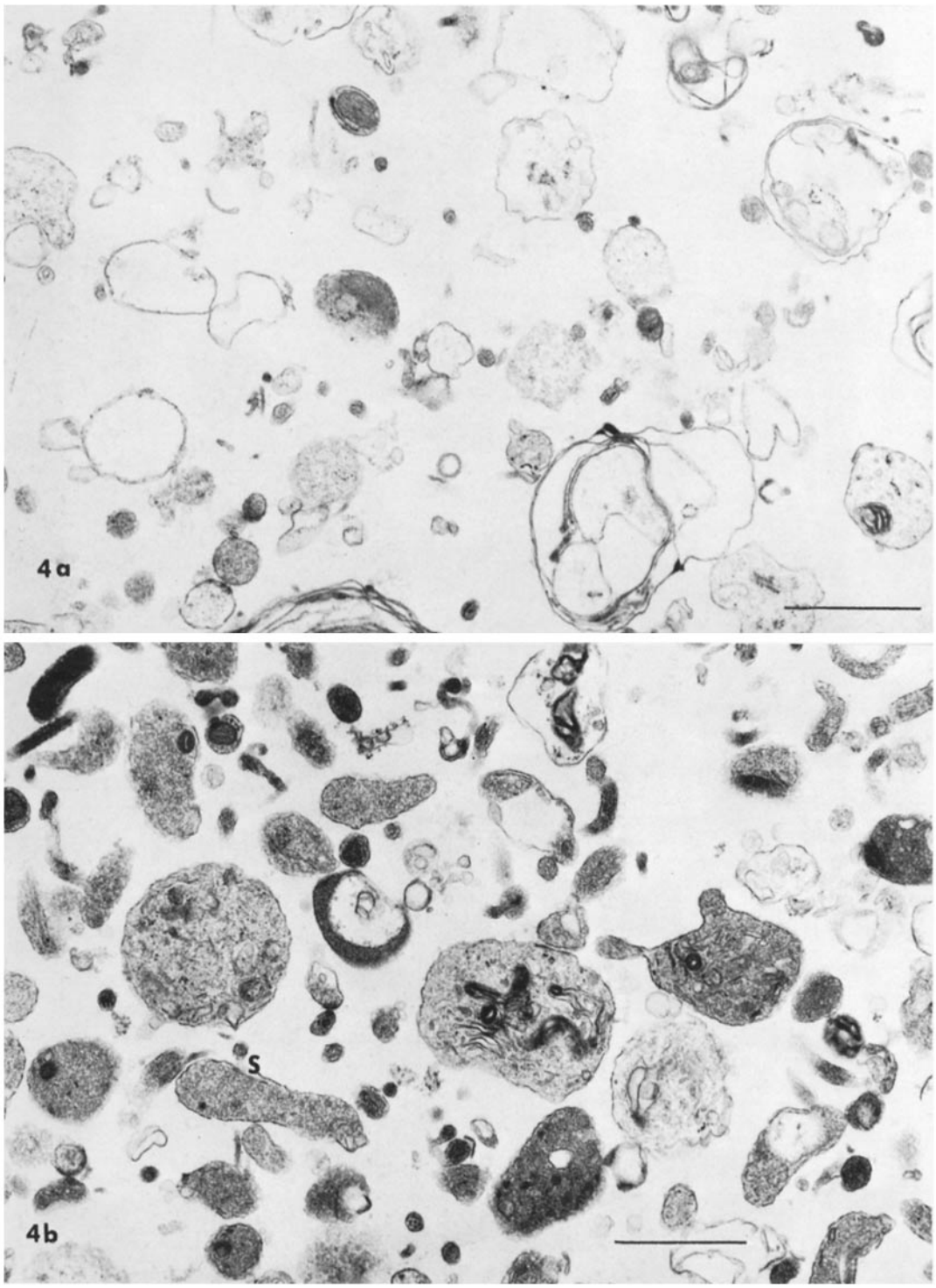

FIGURE 4 Fractions II from mature cortex (4 *a*) and 5-day old cortex (4 *b*). (*S*) synaptic vesicles. Both × 20,000.

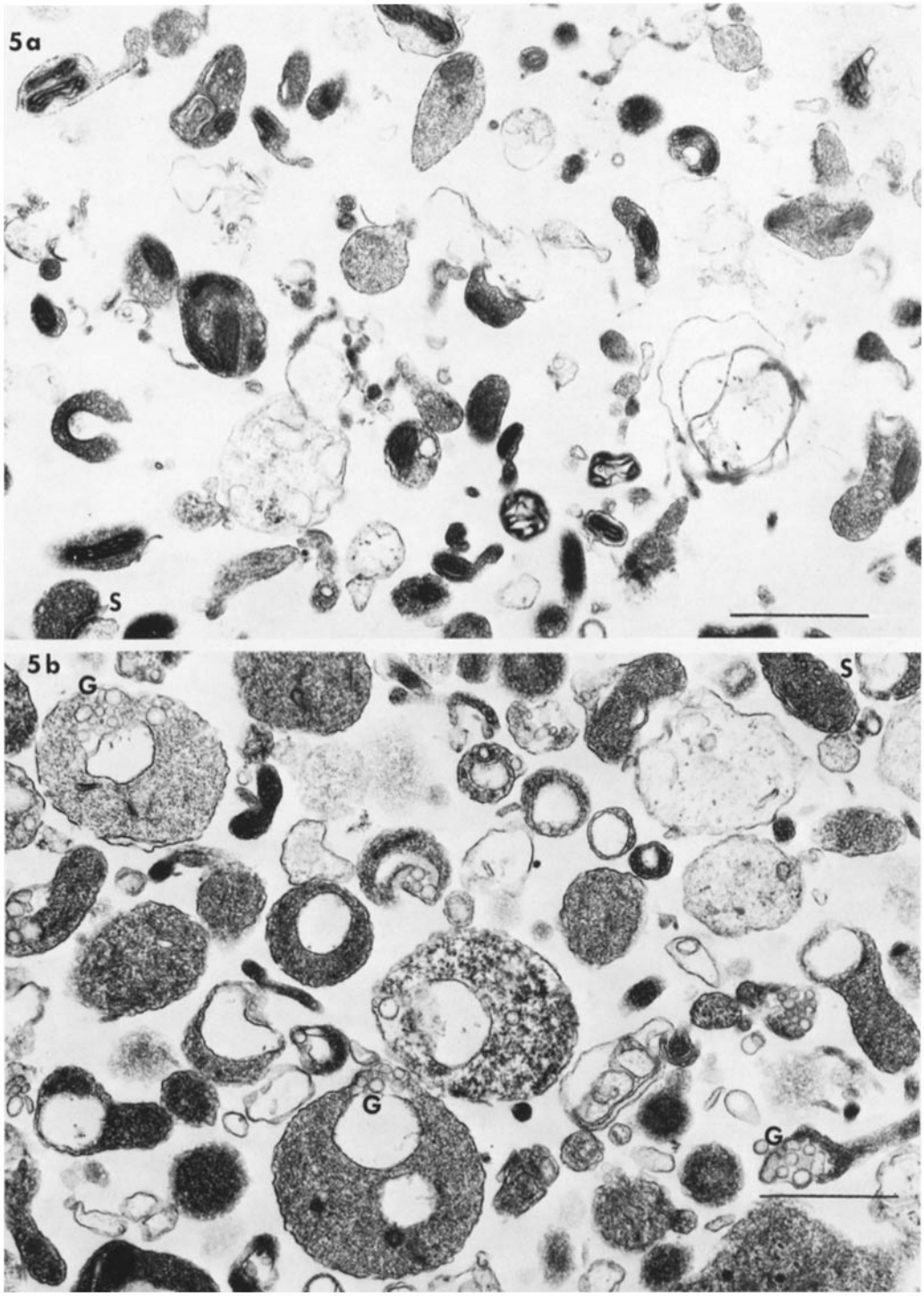

FIGURE 5 Fraction III from mature cortex (5 *a*) and 5-day old cortex (5 *b*). Note synaptosomes (*S*) and growth cones (*G*). Both × 20,000.

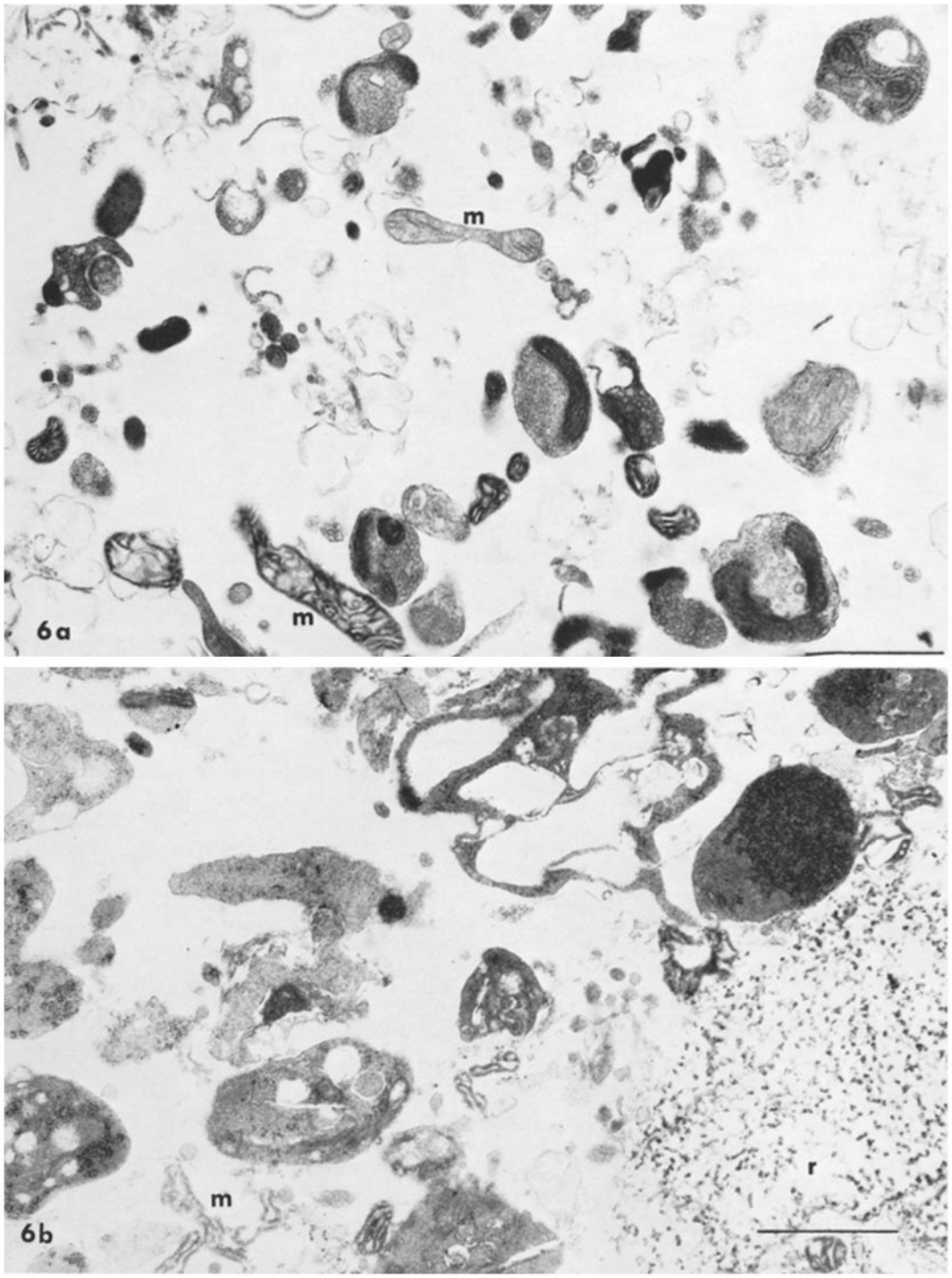

FIGURE 6 Fraction IV from mature cortex (6 *a*) and 5-day old cortex (6 *b*). Note ribosomes (*r*) and mitochondria (*m*). Both × 20,000.

Striking differences were found when the fourth fraction from adult and neonatal cortex were compared. Micrographs of the adult fraction always contained synaptosomes (Fig. 6 *a*); in contrast, synaptosomes were seldom encountered in preparations from 5-day cortex (Fig. 6 *b*). The most obvious difference between these preparations was the presence of a significant number of free ribosomes and unidentified cell processes in the young fraction. Systematic examination of all of the other fractions from each of the four ages did not reveal any comparable accumulation of ribosomes. This is similar to the observation reported by Gomez et al. with subcellular fractions obtained from rat brain (15).

In order to evaluate the use of these fractions for studies of synaptogenesis during postnatal development, we determined the relative concentration of synaptosomes in each fraction taken from the Ficoll gradients. The results are expressed as the V_v as described in Materials and Methods. From the results presented in Table I it can be seen that content of synaptosomes in the adult fraction III have been enriched almost 3.5-fold by fractionation of the crude mitochondrial preparation (P_2). Synaptosomes from neonatal cortex were not so effectively purified by the same procedure and fractions II and III were both enriched about 1.5-fold. It is evident that the concentration of morphologically recognizable synaptosomes varies in each fraction as a function of age (Table I). It

TABLE I

Concentration of Synaptosomes in the Fractions Prepared from Mouse Cortex during Postnatal Development

Fraction	Age of mice			
	5 days	12 days	22 days	Adult
P_2	11	—	—	17
I	11	21	<5	<1
II	16	8	15	<5
III	16	37	38	57
IV	<5	6	16	17

Particulate layers of the fractions were prepared in an analytical ultracentrifuge. The values are presented as volume percentages of the layer occupied by synaptosomes, determined as described in Materials and Methods. All quantitations represent the average of two or three series of micrographs encompassing the thickness of different regions of the particulate layer. In all, 236 micrographs were quantitated.

TABLE II

Relationship between Protein and Synaptosome Content of Subcellular Fractions

	Fraction	*a* Synaptosome content per fraction	*b* Protein per fraction	*c* Relative synaptosome content	*d* Synaptosome distribution
		V_v%	mg	$a \cdot b$	%
Adult	I	<1	0.29	<0.3	<1
	II	<5	0.56	<2.8	<3
	III	57	1.52	86.6	91
	IV	17	0.29	5.0	5
5-day	I	11	1.76	19.3	42
	II	16	0.94	15.1	33
	III	16	0.64	10.2	22
	IV	<5	0.26	<1.3	<3

is important to note that the total amount of material in each fraction does not bear any constant relationship to the proportion of synaptic material present.

A measure of total synaptic material can be obtained by multiplying the V_v by the amount of protein recovered in the fraction ($V_v \cdot \mu$g protein). This relationship may be used to determine the distribution pattern of synaptosomes within the Ficoll gradient, as shown in Table II. On the basis of these data, it is evident that the synaptosomes from 5-day old cortex display a much greater heterogeneity in their sedimentation properties than the synaptosomes from adult cortex. During neural development more synaptosomes were recovered from the denser regions of the gradient, until in the adult, over 90% banded at the 7.5–13% interface (fraction III).

During the progress of this investigation, subcellular particles containing numerous closely packed vesicles larger than synaptic vesicles were readily observed in subcellular fractions obtained from neonatal mouse cortex (Fig. 5 *b*). The morphology of these structures corresponds to that which has been described as growth cones at the growing ends of axons and dendrites of young spinal cord, cortex, and cerebellum of several mammalian species (6, 12). While the function of these vesicles remains obscure, they do not appear to be the artifact of any particular conditions of chemical fixation (12). When the same procedures used to quantitate synaptosomes were applied to

TABLE III

Concentration of Growth Cones in Subcellular Fractions Prepared from Mouse Cortex during Postnatal Development

Fraction	Age of mice			
	5 days	12 days	22 days	Adult
I	4	<1	0*	0
II	5	<1	0	0
III	20	4	<1	0
IV	<1	<1	<1	0

Concentrations are expressed as the volume percentage of the particulate layer occupied by cell processes containing identifiable growth cones, determined as described in the text.

* 0, no growth cones were observed upon examination of electron micrographs from these preparations.

cellular processes exhibiting three or more closely packed vesicles 60–120 nm in diameter immediately subjacent to the plasma membrane, the results presented in Table III were obtained. Processes tentatively identified as growth cones were most concentrated in fractions from neonatal cortex. The concentration decreased rapidly with age until they were rarely found in fractions from 22-day old mice. The other striking feature is their restricted distribution in the gradient (Table III). In terms of total recovery from the gradients, well over half of the growth cones were found in fraction III. The V_v of 20% in this fraction from the 5-day old animal represents an enrichment of nearly threefold from the crude mitochondrial fraction. This gives hope that experimental conditions may be devised to isolate and purify these organelles and their functional and biochemical properties determined.

DISCUSSION

A number of methods have been used to quantitate subcellular fractions from brain tissue. As has been comprehensively discussed elsewhere (8), the commonly used procedures all suffer from a lack of specificity when applied to synaptic structures. Total protein and lipid determinations are convenient but little better than light scattering with regard to the information that is provided concerning the identification of the substituents of subcellular fractions. Unfortunately, no unique enzyme markers have been demonstrated for mammalian synaptosomes. The only specific and positive marker for synapses is their distinctive morphology. For these reasons, electron microscopy is necessary at the present time to characterize the contents of each subcellular fraction.

Unfortunately, there are practical limitations on the amount of material which can be adequately handled with the electron microscope. Cotman has estimated that a volume of less than 0.0008 mm^3 is usually examined (8). Morphological quantitation is time consuming and further limits the volume of material that can be considered. If the sample preparation is not physically small enough to allow analysis of all of the biases in distribution, examination of volumes that are large by standards of electron microscopic techniques may not accurately reflect the overall composition of the fraction. This is clearly illustrated in Fig. 1 and Fig. 2 where the variation in components, at various depths in the subcellular layers, is readily seen. In our experiences and that of others (5, 9), samples pelleted in round bottom tubes in either fixed-angle or swinging bucket centrifuge rotors vary not only when one scans from the top to the bottom of the pellet, but also as one scans across the pellet from the center towards the periphery. The latter variation is not present in subcellular layers prepared with the analytical ultracentrifuge (Grove et al., 17). By using suitably thin layers, the work of morphological quantitation is greatly reduced while the precision is greatly enhanced.

One source of difference between our quantitative results and those previously published (10, 14, 16) is the method of subcellular fraction preparation. However, the major source involves the method of quantitation itself. Previous reports have expressed the number of synaptosomes as a percentage of the total number of subcellular processes while we have presented our results in terms of fractional volumes. In one study, cellular processes smaller than 1 μm were not included, nor were broken or empty processes and free mitochondria considered (16). In contrast, we have measured the volume occupied by all electron-dense objects. The use of fractional volumes may be more meaningful since it does not exclude significant amounts of material that can be stained and visualized in each sample.

Studies have been carried out using phosphotungstic acid in ethanol to identify "presynaptic grids" (2). However, the work of Armstrong-James and Johnson (3) with intact developing rat cortex has shown that a greater proportion of presynaptic endings can be detected by counting processes that

contain three or more synaptic vesicles. Because synaptic vesicles may not uniformly fill synaptosomes, it seems unavoidable that some sections should pass through synaptosome structures without displaying this minimum number of synaptic vesicles. By definition, such structures would be counted as nonsynaptic material. This probably does not represent much of an underestimate of synaptic content of subcellular fractions of mature mouse brain tissue. However, without extensive serial sectioning, this seems to be an unavoidable bias against the younger fractions which contain, on the average, fewer vesicles (Fig. 5 *b*). Theoretically, a distribution function describing the location of synaptic vesicles within processes might be derived and used to predict the relative number of undetected synaptosomes. On the other hand, if the functional maturity of the synapse is related to the number of vesicles, a more meaningful value may only include those synapses which can be readily recognized.

The present study provides the morphological quantitation for correlative studies of synaptogenesis using subcellular fractions. In general agreement with the findings in rat cortex (16, 22), we find that synaptosomes from immature mouse cortex sediment to the less dense areas of the discontinuous Ficoll-sucrose gradients. We have determined the synaptosomal volume percentages of all four fractions from 5-, 12-, 22-day old and adult brain tissue. Although synaptosomes from neonatal animals are quite heterogeneous in their sedimentation properties and are found in layers I, II, and III, there is an enrichment of 1.5-fold in fractions II and III. In the adult, the synaptosomes appear to be more homogeneous and the enrichment in fraction III is 3.5-fold.

Because there are relatively few glial cells present in neonatal cortex, it has been suggested that much of the unidentified material in newborn subcellular fractions may represent growing neurites and immature presynaptic terminals (16). However, it is difficult to account for the possible presence of fragments of the perikarya of immature neurons. Nonetheless, a certain proportion of these subcellular structures can be more positively identified as neurites. Structures (growth cones) at the tips of extending neurites which were originally observed by light microscopy and described by Cajal (7) have been more recently shown by Bodian (6) and DelCerro and Snider (12) to have a distinctive ultrastructure. Unlike neonatal synaptosomes, subcellular structures resembling growth cones were quite homogeneous in their sedimentation properties. In addition, they were found most frequently in preparations from neonatal cortex (Table III). Many of these subcellular structures also contained synaptic vesicles which would identify the process as a developing axon. However, it is known that all growing axons do not exhibit growth cones with this distinctive ultrastructure (24) and growth cones have been reported to be associated with known dendrites in the intact cerebellum (12).

Despite the limitations described above, a quantitative morphological study of the changes in the contents of the subcellular fractions during neural development would be valuable to subsequent studies on synaptogenesis. For instance, quantitative and qualitative biochemical changes during maturation could be normalized. Specific activities based on total protein content of a fraction may be less relevant than those based on total synaptosomes or synaptosomal volume (Table II). This is particularly critical when pure subcellular preparations are not available.

This investigation was supported by Public Health Service research grant NS 06853 from the National Institute of Neurological Diseases and Stroke.

Received for publication 27 February 1973, and in revised form 10 May 1973.

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