MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN RAT SYNAPTOSOME FRACTIONS DURING NEONATAL DEVELOPMENT

NICHOLAS K. GONATAS, LUCILA AUTILIO-GAMBETTI, PIERLUIGI GAMBETTI, and BRENDA SHAFER

From the Division of Neuropathology, Departments of Pathology and Neurology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

ABSTRACT

A biochemical and quantitative morphologic study of presynaptic endings during postnatal development was carried out in subcellular fractions from cerebral cortex of 1, 4, 8, 12, and 18 day old and adult rats. Crude mitochondrial fractions were subfractionated in Ficoll gradients and all resulting fractions were examined in the electron microscope. Presynaptic terminals and other intact processes were counted. Protein content and enzyme activities were assayed in the fractions and in total brain homogenate. In the first and fourth day of life, most of the presynaptic terminals were found in two "light" fractions, between supernatant and 7.5% Ficoll, where they accounted, respectively, for 6 and 22% of all the processes. Progressively with age, more presynaptic terminals were found in the traditional "synaptosomal" fractions between 7.5 and 13% Ficoll. In that region of the gradient, 40, 54, 75, and 89% of the processes were presynaptic endings at 8, 12, and 18 postnatal days and in the adult animal, respectively. A similar shift from the lighter to the heavier fractions was observed in the distribution of choline acetyltransferase and acetylcholinesterase between days 8 and 12. The rate of increase of the specific activity of these two enzymes paralleled that of the percentage of the presynaptic endings after day 8. This study indicates that subcellular fractions can be used to study formation and maturation of synapses during postnatal development.

INTRODUCTION

During fetal and neonatal development of cerebral cortex, behavioral, bioelectric, and chemical changes occur along with progressing structural organization (1–7). In the rat, early postnatal maturation of the cerebral cortex (1–10 days) consists, almost exclusively, of growth and proliferation of neuronal and, to a lesser extent, glial processes (1, 8). According to Eayrs and Goodhead, maximal growth of axons occurs during days 6–19 of postnatal development, while dendritic proliferation takes place during days 18–24 of postnatal development (1).

The proliferation of neuronal processes is accompanied by progressive increase of the number of synaptic junctions which reach adult values around the 20–25th day of postnatal development; a sharp increase of the number of synaptic junctions occurs between days 12–20 of postnatal development (2).

Biochemical studies of synaptosomal fractions isolated from rat brains during the period of perinatal development have shown significant changes in the activities of the sodium-potassium adenosine triphosphatase, acetylcholinesterase,

succinic dehydrogenase, and of the protein, lipid, and acetylcholine contents (9–12); however, the lack of adequate morphologic controls raises doubts on the identity and the purity of these fractions. In the present study a quantitative ultrastructural and biochemical examination was performed on all fractions derived from the crude mitochondrial fraction of rat cerebral cortex homogenates during neonatal development. Also, the sensorimotor cortex of 4- and 15-day old rats has been examined in the electron microscope in order to compare the *in situ* morphology with that of the isolated synapses and other processes.

It has been found that corresponding fractions from animals of different ages are quite dissimilar in morphology and enzymatic activities and contain varying proportions of presynaptic terminals and of other segments of nerve cell processes; in he 1 day old animals, synaptosomes are present only in fractions lighter than the so-called "synaptosome" fraction. In these lighter fractions synaptosomes account for only 6% of the processes while immature neurites account for the remaining 94%. Progressively, more presynaptic terminals appear in synaptosome fractions which by the 18th day have morphology, sedimentation properties, and enzyme activities comparable to the adult fractions.

MATERIALS AND METHODS

Preparation of Subcellular Fractions

Osborn-Mendel white rats were obtained from West Jersey Biological Supply Farms, Wenonah, N. J. Several animals of similar size from a litter were combined for the fractionation. The adults weighed around 200 g. Cerebral cortices were dissected immediately after decapitation and kept at 0°–4°C. Subcellular fractions were prepared as previously described (13). The crude mitochondrial fraction was subfractionated in a discontinuous Ficoll gradient (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). After centrifugation, five distinct bands at the interphase of the different Ficoll solutions plus a pellet were obtained. Fraction 1 was between the supernatant and the 5% Ficoll, fraction 2 between 5 and 7.5%, fraction 3 (which included two bands) between 7.5 and 13%, and fraction 4 between 13 and 20% Ficoll. Fraction 5 was the pellet. Each fraction was removed with a Pasteur pipette, diluted with 0.32 M sucrose, and centrifuged at 40,000 *g* for 30 min; the resulting pellets were resuspended in 0.32 M sucrose with a loose Dounce homogenizer, and samples were taken for electron microscope studies and enzyme determinations.

Assay of Enzyme Activities

Acetylcholineserase (E.C.3.1.1.7) was assayed by the method of Ellman et al. (14), with acetylthiocholine iodide as substrate; the reaction was followed spectrophotometrically at room temperature; a parallel sample containing 1×10^{-4} M 62C47 (Burroughs Welcome & Co., Tuckahoe, N. Y.) was subtracted as a blank. Succinic dehydrogenase (E.C.1.3.9.9.1) activity was determined colorimetrically by the method of Pennington (15). Choline acetyltransferase (E.C.2.3.1.6) was determined by the method of Schrier and Shuster (16) as modified by Schrier[1]; lactate dehydrogenase (E.C.1.1.1.27) was determined according to Kornberg (17), following spectrophotometrically the consumption of nicotinamide adenine dinucleotide at room temperature. All enzymatic activities will be expressed as micromoles substrate per hour per milligram protein. Protein was determined according to Lowry et al. (18), with bovine serum albumin used as standard.

Electron Microscopy

Tissue

Two 4-day old and one 15-day old animals were perfused through the heart with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 2% of 0.1 M $CaCl_2$. A coronal section of the brain corresponding to the level of the infundibulum was removed and the dorsolateral segment of the sensorimotor cortex was sampled for electron microscopy. The tissue was postfixed in Dalton's fixative (19).

Fractions

Small samples from each resuspended fraction were transferred to plastic tubes and fixed for 3–12 hr in the same fixative used for the *in situ* fixation. After fixation, the fractions were centrifuged in a Sorvall RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) (4°C) at 40,000 *g* for 10 min; the supernatant was discarded and the pellet was resuspended in Dalton's fixative for 1–2 hr at 4°C. The fractions were dehydrated in graded ethanol and infiltrated with Araldite. The entire procedure was carried out in the same plastic tube to minimize loss of material. After the fraction was thoroughly suspended in the last mixture of Araldite-accelerator, it was packed into a small pellet by centrifugation for 20 min at 40,000 *g* at 30°C. Polymerization was carried out in the original plastic tube at 60°C for 3–4 days. The plastic tube was sectioned and its Araldite case was removed. The pellets, measuring from 1 to 4 mm in thickness, were cut with diamond knives along the depth to surface axis. Sections were stained with

[1] Schier, B. K., personal communication.

uranyl acetate and lead citrate and examined in a Siemens-Elmiskop I electron microscope at a voltage of 80 kv (20, 21). Pictures were taken in sequence from the entire thickness of the pellet at 4000–8000 magnifications. Electron micrographs, magnified two to three times, were used for the quantitative studies. Broken or empty processes, free mitochondria, and processes less than 1 μ in diameter were not counted. Processes with three or more vesicles, 300–500 A in diameter, were characterized as presynaptic endings (Figs. 5 *a*, 5 *b*); with the exception of day 1, all observations were made in two or three separate experiments.

RESULTS

Electron Microscopy

Sensorimotor Cortex of 4- and 15-Day Old Animals

At day 4, the neuropile was composed of processes 0.5–3 μ in diameter. The width of the extracellular spaces varied from 200 A to 0.5 μ. Most of the processes, which contained neurotubules, were probably growing axons and dendrites (Fig. 1). There were no mature presynaptic

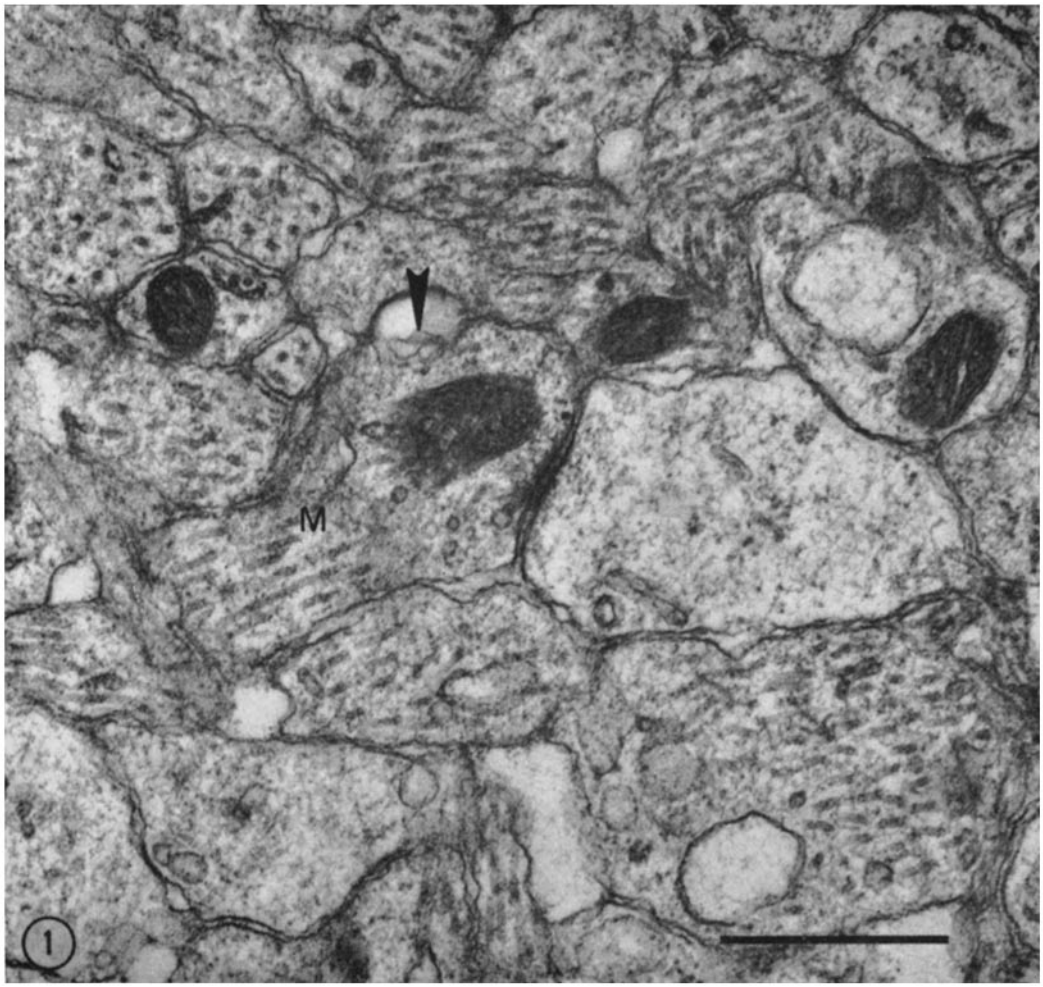

Figure 1 4 day old rat sensorimotor cortex. Fixation by glutaradehyde perfusion. Neuropil is filled with processes containing neurotubules. Arrowhead: immature presynaptic terminal. Note osmiophilia of cleft and a few synaptic vesicles. × 34,000.

terminals; instead, processes, characterized as immature presynaptic terminals, were found (Fig. 1). The immature terminal contained microtubules and three to five synaptic vesicles; the osmiophilic material in the synaptic cleft was well developed, and often, the subsynaptic web was clearly seen in the postsynaptic element. Immature axon terminals made contacts with neuronal perikarya and dendritic processes. Most synaptic complexes seen in the 15 day old neocortex were similar in morphology to those of the adult cortex (Fig. 2). In the 15 day old neocortex, presynaptic terminals did not contain microtubules; 20–40 synaptic vesicles were present in each presynaptic terminal. Flattened synaptic vesicles were rarely observed; dark-core synaptic vesicles were even sparser than flattened synaptic vesicles.

Study of Fractions

FIRST DAY: Examination of fraction 3, which in mature brains contained mainly synaptosomes, revealed free mitochondria, much membranous debris, and very few intact processes which did not contain vesicles. Free mitochondria were concentrated in fraction 4. Presynaptic terminals were found in fractions 1 and 2 which were similar in

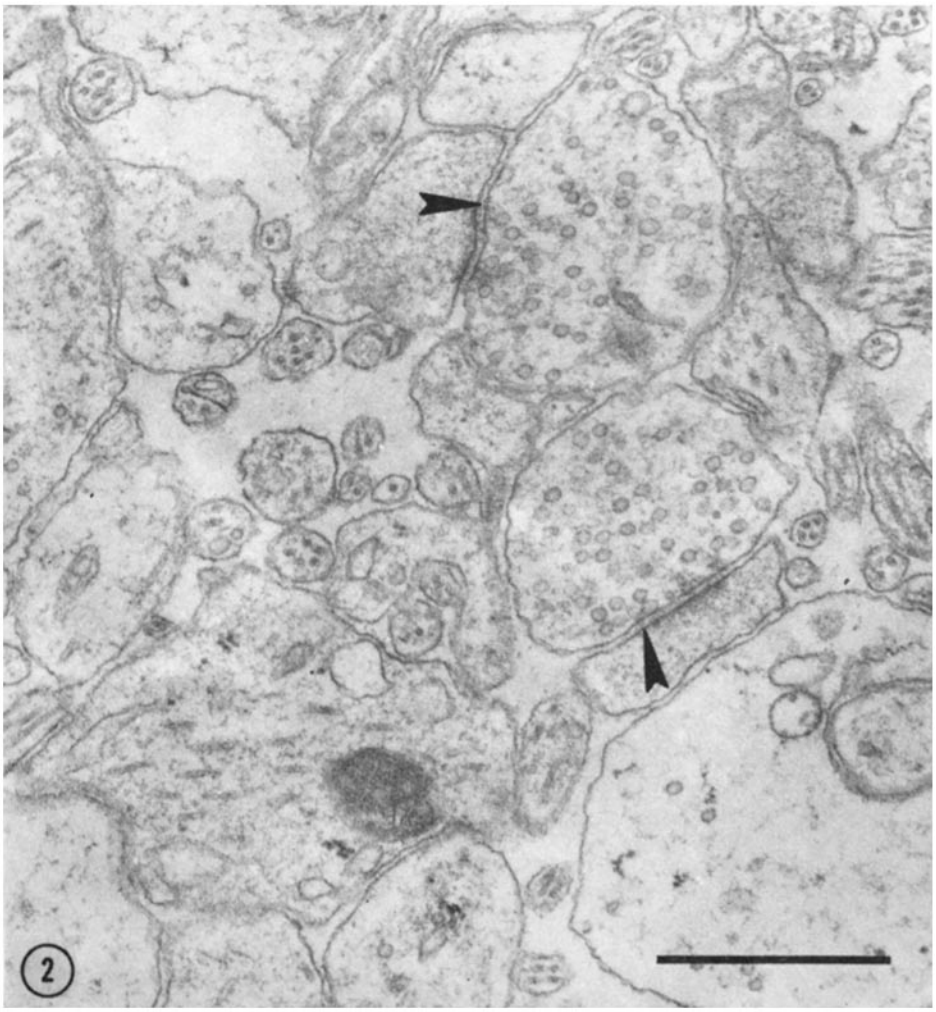

FIGURE 2 15 day old rat sensorimotor cortex. Fixation by glutaraldehyde perfusion. Two fully mature synaptic complexes (arrowheads). The presynaptic endings contain numerous vesicles. × 31,000.

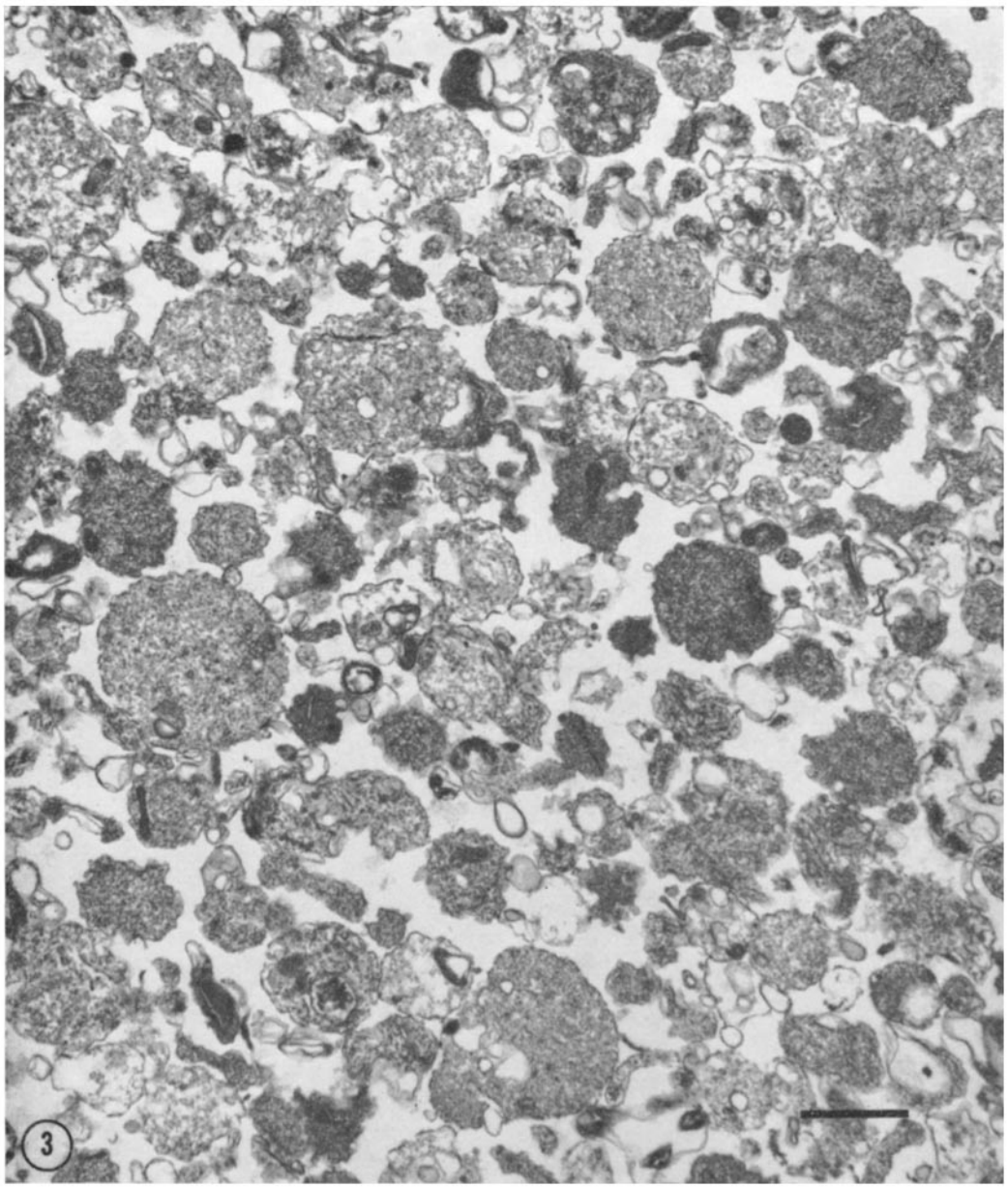

FIGURE 3 1 day old. Fraction 1. × 15,000.

appearance (Figs. 3, 4). Processes measuring 0.5–3 μ were densely packed throughout the thickness of the pellet. Most processes contained amorphous-granular material and a few membrane-bounded vacuoles. Synaptic vesicles of an average diameter of 550 A were seen rarely in these processes (Fig. 3, Table I). Occasionally, dark-core vesicles 800–900 A in diameter were seen in processes containing synaptic vesicles. Synaptic junctions were not seen. A few processes contained either ribosomes or bundles of fibrils 50–80 A thick (Fig. 4). Processes with these fibrils were not seen after the eighth day in any of the examined fractions. Mitochondria within the processes were sparse, and free mitochondria were seen only occasionally. 6% of the processes in fractions 1 and

TABLE I

Rat Synaptosome Fractions during Development

Fractions	Days	Processes without synaptic vesicles	Processes with synaptic vesicles (presynaptic terminals)	Total number of counted processes	Percentage of presynaptic terminals
					%
1 and 2 (between supernatant and 7.5% Ficoll)	1*	797	52	849	6
"	4‡	494	141	635	22
3 (between 7.5–13% Ficoll)	8§	682	463	1145	40.4
"	12§	530	635	1165	54.5
"	18‡	313	958	1271	75
"	Adult‡	185	1428	1613	89

* One experiment.

‡ Two experiments.

§ Three experiments.

Results on days 4, 8, 12, 18, and adult are average of two or three separate experiments. The individual percentage of presynaptic terminals was: 4 days, 21 and 24%; 8 days, 36, 45, and 40%; 12 days, 62, 47, 64, and 47%; 18 days, 83 and 61%; adult 91 and 87%.

2 were presynaptic terminals (Table I); the remaining 94% of processes were identified as immature neurites (dendrites, axons). Most of the presynaptic terminals contained few synaptic vesicles, and probably corresponded to the immature terminals seen *in situ* (Figs. 1, 4).

FOURTH DAY: As in day 1, fraction 3 showed few preserved processes and free mitochondria. The two lighter fractions, 1 and 2, showed a large number of well-preserved processes. In general, fractions 1 and 2 from day 4 resembled the corresponding fractions from day 1; however, significantly more presynaptic terminals were recognized in day 4 than in day 1 (21–24% counted processes were presynaptic terminals) (Table I).

EIGHTH DAY: Examination of lighter fractions 1 and 2 showed processes which appeared empty or contained amorphous-granular material and a few synaptic vesicles (Fig. 5 *b*). Occasional dark-core vesicles were seen either alone or mixed with synaptic vesicles. A rare process contained packed synaptic vesicles as in fractions from older animals. Traces of myelin were first observed in fractions 1 and 2 at this age. Well-differentiated presynaptic terminals were concentrated in fraction 3 which also included many larger processes containing either ribosomes or fibrils and amorphous-granular material (Fig. 5 *a*). Vesicles resembling synaptic vesicles in size and contour were seen frequently in processes which contained amorphous-granular material (arrowhead in Fig. 5 *a*). This finding suggests that the processes with the same amorphous-granular material, but lacking synaptic vesicles, seen in abundance in fractions 1 and 2 of days 1 and 4 (arrowhead, Fig. 4), probably represent immature terminals or growing axons. Fractions 4 and 5 of the gradient contained a mixture of components seen in the lighter fractions, debris, and a large number of free mitochondria. Presynaptic terminals in fraction 3 were 36–45% of all processes (Table I).

12TH DAY: Fractions 1 and 2 were similar to those of day 8, except for the presence of myelin. In fraction 3, most processes identified as presynaptic terminals contained a varying number of synaptic vesicles; however, occasional processes with few synaptic vesicles, probably representing immature terminals, were still seen (Fig. 6 *a*). The other processes had ribosomes or amorphous-

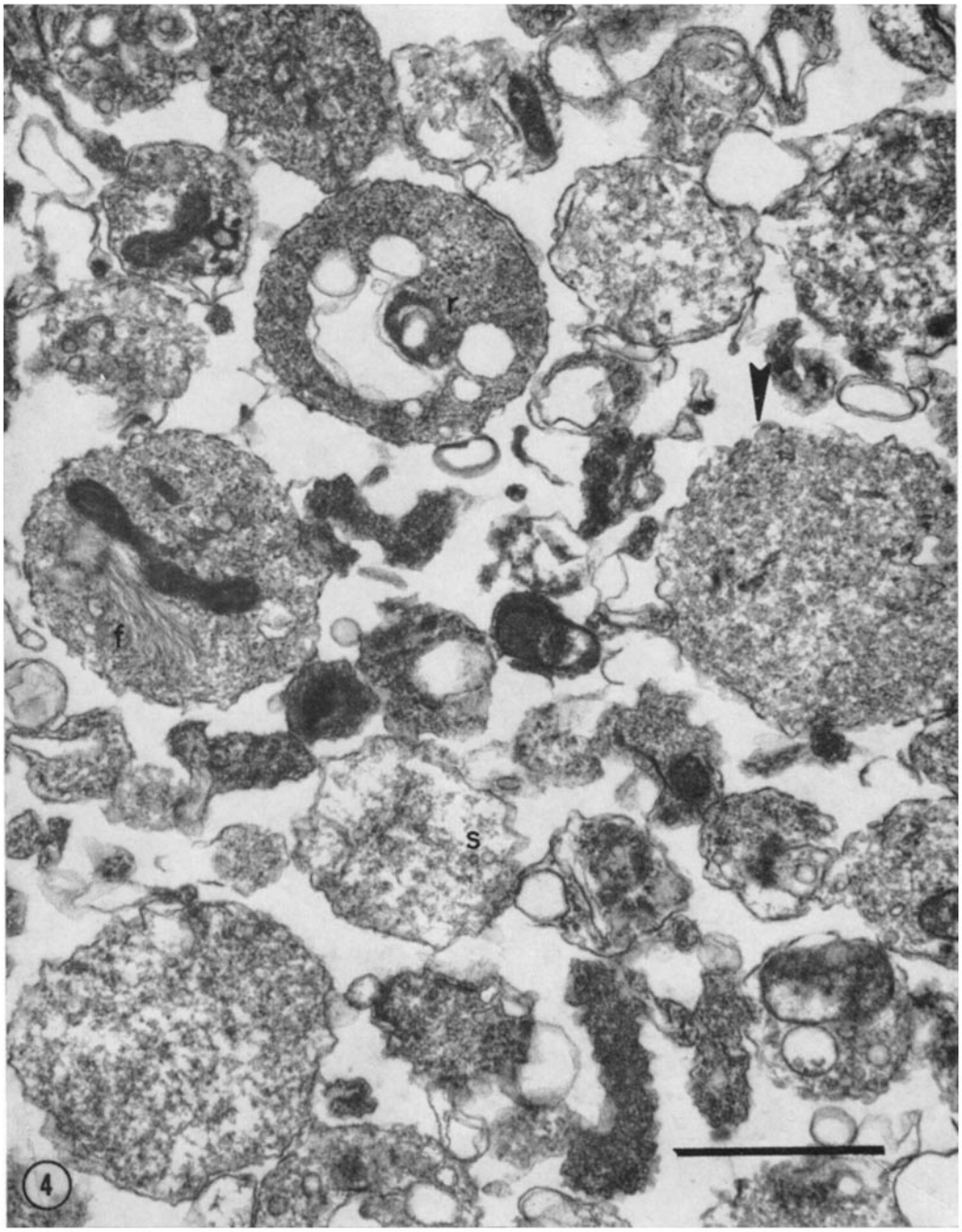

FIGURE 4 1 day old. Fraction 1. *s*, synaptic vesicles; *f*, fibrils; *r*, ribosomes; arrowhead: process with amorphous-granular material. × 31,000.

granular material and probably were fragments of dendrites and growing axons; myelin was not seen in fraction 3. The heavier fraction 4 and the pellet 5 were composed of unidentifiable large processes, free mitochondria, and presynaptic terminals. Presynaptic terminals which were concentrated in fraction 3 accounted for 47–64% of all processes.

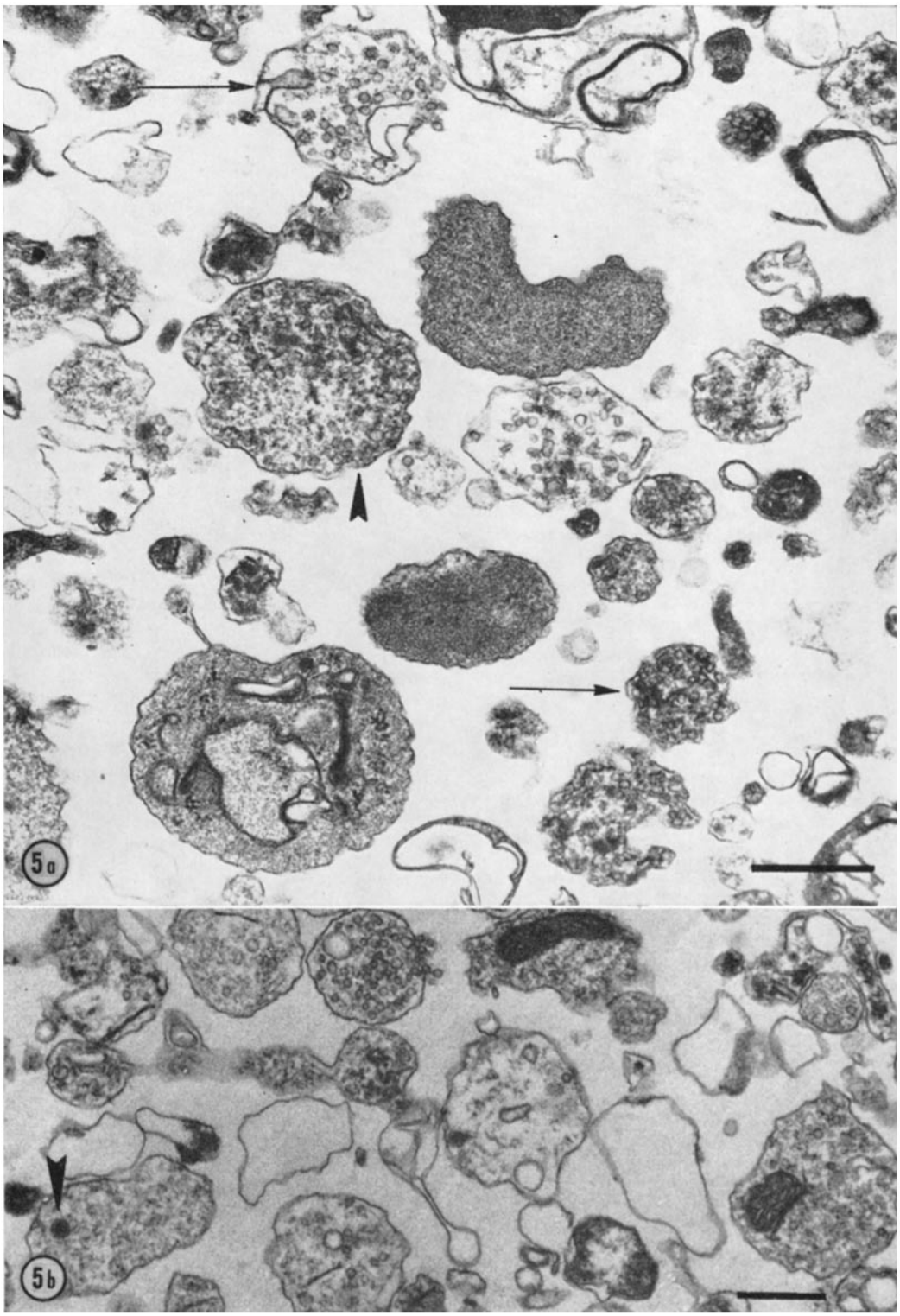

FIGURE 5 (*a*) 8 days old. Fraction 3. Arrows: presynaptic terminals. Arrowhead: process with granular and vesicular contents. × 19,000. (*b*) 8 days old. Fraction 1. Processes with varying number of synaptic vesicles. Arrowhead: dark-core vesicle. × 15,000.

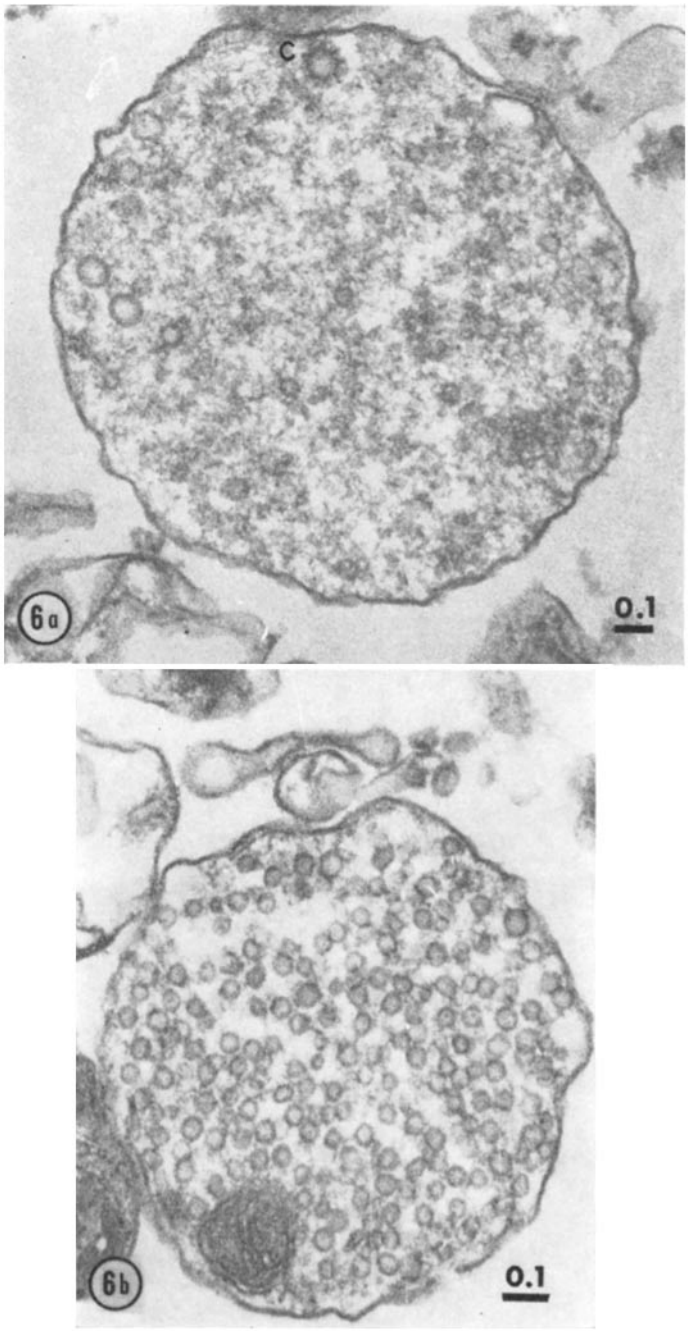

FIGURE 6 (*a*) Day 12. Process with a few vesicles 550–1000 A in diameter. *c*, coated vesicle. × 50,000.
(*b*) Adult. Usual presynaptic terminal filled with synaptic vesicles, 300–650 A in diameter. × 65,000.

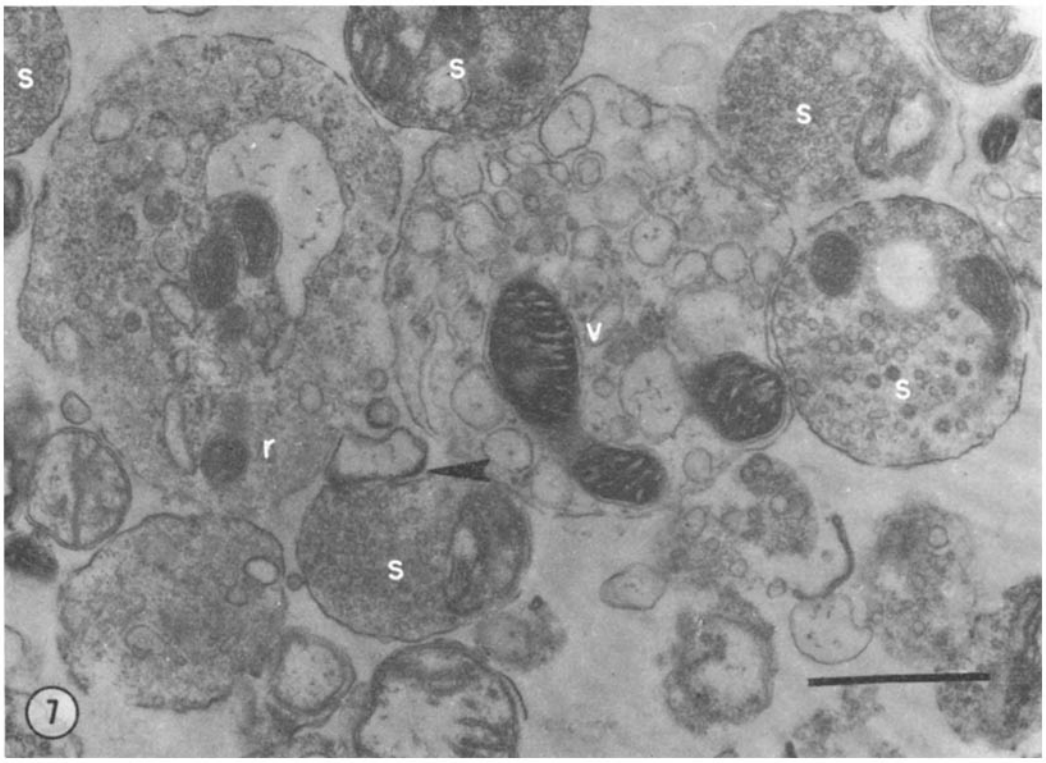

FIGURE 7 18th day. *s*, presynaptic terminals; arrowhead: synaptic cleft; *r*, process containing ribosomes; *v*, process containing large vesicles. × 24,000.

18TH DAY: Fractions 1 and 2 resembled the corresponding fractions of days 8 and 12, except for the presence of larger quantities of myelin. Fraction 3 resembled adult synaptosome fractions. Presynaptic terminals made up 61–83% of counted processes; some of the remaining processes contained endoplasmic reticulum and polysomes (Fig. 7); also, traces of myelin were seen in this fraction.

ADULT: About 90% of the processes in the synaptosome fraction 3 were presynaptic terminals. The other 10% of processes were either postsynaptic elements or isolated processes with ribosomes. Significant amounts of myelin were always present (Fig. 8).

Biochemistry

In the total homogenates of cerebral cortex, the specific activity of all the enzymes studied increased with development (Fig. 9). The activities reported are expressed per milligram of protein; lower but otherwise similar curves were obtained if the activities were expressed per wet weight.

The rates of increase of the enzyme activities were similar; all showed a higher rate of increase between days 8 and 18 (Fig. 9).

The percentage of protein and enzyme activities recovered at different ages in the crude mitochondrial fractions were similar (Fig. 10), indicating that about the same percentage of brain cortex was subjected to subfractionation in the Ficoll gradient.

The enzyme distribution in the Ficoll gradient at different ages correlated fairly well with the morphologic findings (Fig. 11). In adult animals, 40% of both choline acetyltransferase and acetylcholinesterase activities, enzymes known to be localized in high concentrations in synaptosomes (22, 23), were associated with fraction 3 which is composed mainly of synaptosomes (Fig. 11). In contrast, in 1 day old animals, about 40% of these enzyme activities were localized in fraction 1; the percentage of activities recovered in fraction 3 increased progressively with age; also, the

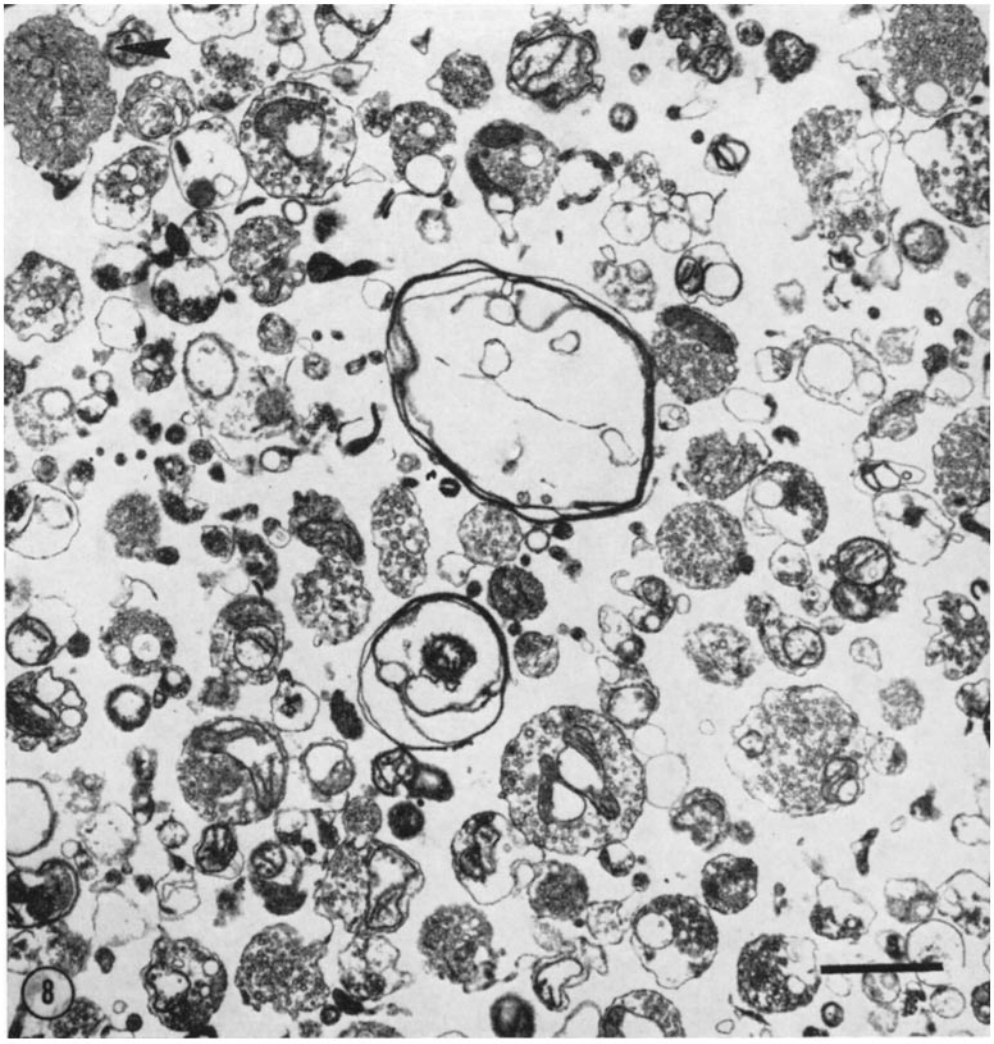

FIGURE 8 Adult. Note myelin contaminants. Majority of processes contain synaptic vesicles. Arrowhead: unidentifiable process with ribosomes. × 16,000.

percentage of protein recovered in this fraction doubled from day 1 to adult (Fig. 11). The distribution pattern of lactic dehydrogenase, a cytoplasmic marker, showed changes with age similar to those of choline acetyltransferase and acetylcholinesterase.

The rate of increase of specific activity of choline acetyltransferase in the synaptosome fraction was similar to that of the percentage of presynaptic terminals from days 8 to 18 (Fig. 12). This correspondence, however, was lacking between days 1 and 8 when the percentage of presynaptic terminals rose sharply while the increase of the specific activity of choline acetyltransferase was negligible. The rate of increase of the specific activity of acetylcholinesterase was low between days 1 and 12 and paralleled the increase of choline acetyltransferase thereafter.

DISCUSSION

In 1- and 4-day old animals, fraction 3, which in the adult contains the bulk of the synaptosomes, is small and contains only 12% of the recovered protein (Fig. 11); this fraction is made up of membranous debris, a few processes, and free mitochondria. At these ages, presynaptic endings

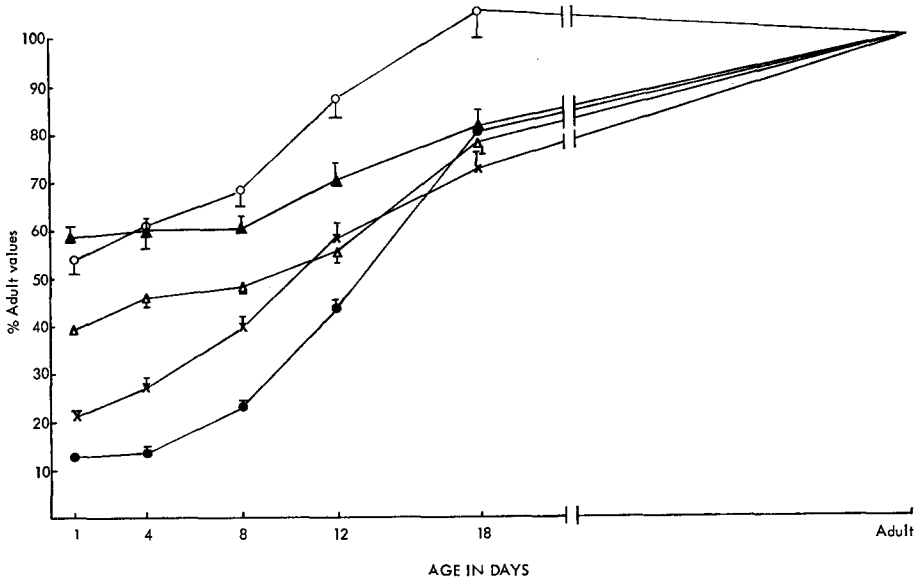

FIGURE 9 Specific activity of acetylcholinesterase, ×; choline acetyltransferase, ●; succinic dehydrogenase, △; and lactic dehydrogenase, ○; in the cerebral cortex of the developing rat expressed as per cent of the adult values. Values are average of three or more experiments. Vertical bars represent SEM drawn as either + or − SEM. Specific activity of the adult rats in micromoles substrate per hour per milligram protein were: acetylcholinesterase, 5.18 ± 0.32; lactic dehydrogenase, 31.1 ± 2.06; succinic dehydrogenase, 3.0 ± 0.09; and choline acetyltransferase, 1.87 ± 0.07 × 10^{-2}. ▲; protein per cent of wet weight (average adult value 13.12 ± 0.34).

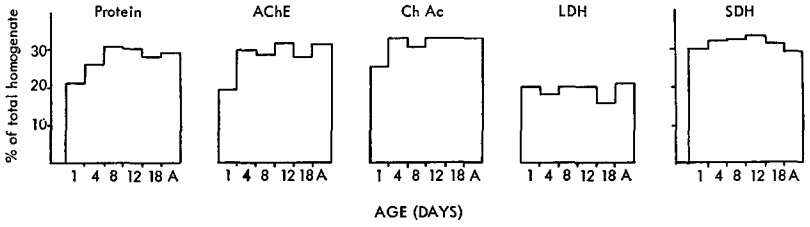

FIGURE 10 Protein and enzyme activities recovered in the crude mitochondrial fractions of different ages. *AChE*, acetylcholinesterase; *Ch Ac*, choline acetyltransferase; *LDH*, lactic dehydrogenase; *SDH*, succinic dehydrogenase. Data are per cent of values in total cortex homogenates.

are seen mainly in the lighter fractions 1 and 2, where at 1 day they represent 6% and at 4 days 22% of the processes. These fractions contain most of the choline acetyltransferase and actylcholinesterase activities (Fig. 11), although at this stage of development their specific activities are very low (Fig. 12). The remaining 94 and 78% of processes in these fractions are unidentifiable by morphologic criteria; however, it is safe to assume that they represent growing neurites and immature presynaptic terminals; it is unlikely that oligodendroglial cell processes are present at this early stage of development because, in the rat, myelination begins around the 10th day of life (24); also, the contamination by astrocytic processes is not significant since processes with characteristic astrocytic fibrils were seen only occasionally (Fig. 4) (25).

In contrast to the nerve endings of the adult, nerve endings from 1- and 4-day old animals contain few synaptic vesicles and amorphous-granular material which is not seen in 18-day old and adult terminals or in the processes of the intact cortex (Figs. 1, 3, 4, 8). This amorphous-granular material could represent poorly preserved synaptic vesicles or microtubules; the latter possibility is

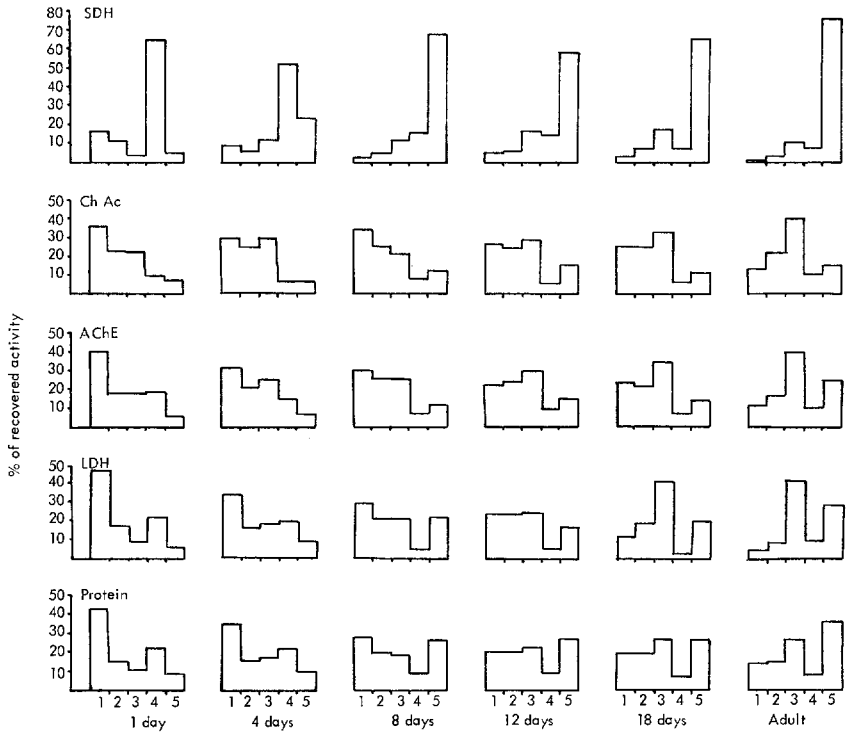

FIGURE 11 Distribution of succinic dehydrogenase (*SDH*), choline acetyltransferase (*Ch Ac*), acetylcholinesterase (*AChE*), lactic dehydrogenase (*LDH*), and protein in the Ficoll gradient at different ages. Values are per cent of recovered activities.

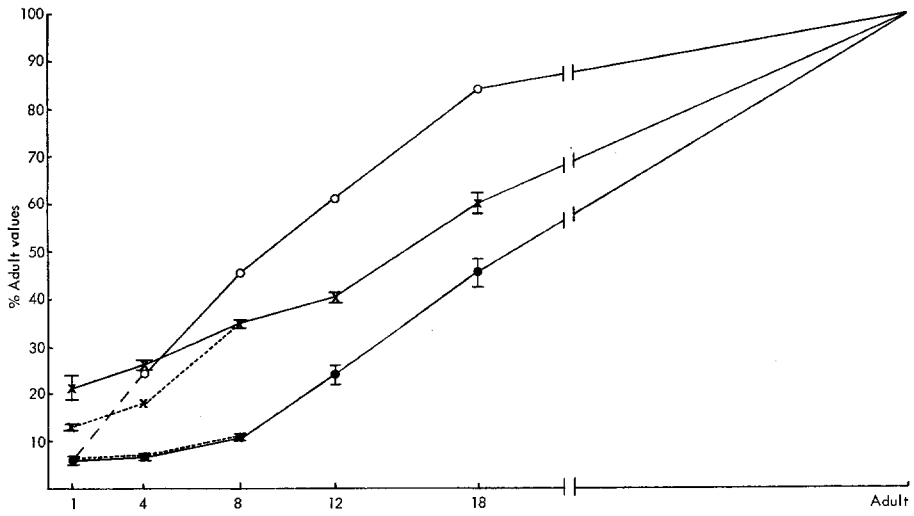

FIGURE 12 Specific activity of acetylcholinesterase, ×; choline acetyltransferase, ●; and percentage of presynaptic endings, ○ in submitochondrial fraction 3 at different ages. In broken lines, values for subfractions 1 and 2. Data are expressed as per cent of adult values (adult values in micromoles substrate per hour per milligram protein) acetylcholinesterase 9.2 ± 0.47 and choline aectyltransferase 4.78 ± 0.48 × 10^{-2}. Vertical bars represent ±SEM.

more likely since microtubules are damaged easily at the low temperatures of cell fractionation and centrifugation (26), and microtubules and microtubular protein are abundant during neonatal development (Fig. 1) (27); the absence of amorphous-granular material in the *in situ* processes also suggests its artifactual nature (Fig. 1).

Subcellular fractions derived from rat brains in the first few days after birth have been studied morphologically by Abdel-Latif et al. and Spence and Wolfe (11, 28). Spence and Wolfe have described a fraction rich in gangliosides which in the electron microscope consisted of membranous arrays and empty processes. The different method of isolation followed by these authors renders comparisons with the present findings difficult; however, they too found that their lightest fraction of the newborn rat contained nerve endings. In contrast to our results, they did not find acetylcholinesterase associated with this fraction. Abdel-Latif et al. indicated that nerve endings from 1 day old rats are similar to those of 25-day old animals (11); however, their illustrations of 1- and 25-day old fractions show significant differences in the number of vesicles per presynaptic terminal, of empty processes, and of well-formed presynaptic terminals between these two ages. As far as we could ascertain, fractions from neonatal rat brains containing neurites and immature presynaptic terminals have not been previously reported.

Beginning at day 8, well-formed nerve endings are seen in fraction 3, and their number in this fraction increases progressively with development. As in younger animals, some nerve endings are present in the lighter fractions 1 and 2; however, these fractions as a whole are morphologically different from those seen in the earlier ages; after the 12th day, many empty processes and myelin are present in these fractions which are similar to the light synaptosome fractions of Whittaker (29). The shift of the synaptosome fraction from the lighter fractions to fraction 3 is reflected also in the enzyme distribution. From the 12th day, choline acetyltransferase and acetylcholinesterase are predominantly found in fraction 3 (Fig. 11). This shift of the synaptosome fractions probably reflects the structural and biochemical maturation of the nerve endings which become heavier and migrate to a denser layer of the gradient. The "lighter" nerve endings in fractions 1 and 2 at days 8, 12, and 18 may be damaged terminals since in these fractions a great number of empty processes and loose membranes are noted; alternatively, the lighter nerve endings may represent a separate population with a chemical composition different from that of the heavier synaptosomes.

Counts of presynaptic terminals at different stages of development have shown a continuous increase in the percentage of morphologically identifiable presynaptic endings during the first 18 days of postnatal development, when 84% of the adult value is reached (Fig. 12). By contrast, the increase of the specific activity of choline acetyltransferase in synaptosomal fractions from days 1 to 8 was negligible, and only after day 8 did it parallel the increase of the percentage of the presynaptic endings (Fig. 12). The discrepancy between morphologic and biochemical observations during the first 8 days of life may be due to the presence in the fractions of immature presynaptic endings endowed with lesser amounts of enzymes. Most of the presynaptic endings of fractions 1 and 2 from days 1 and 4 contained few synaptic vesicles; this finding is in accordance with the observations made in intact neonatal rat cerebral cortex and in the spinal cord of monkey fetus, where paucity of synaptic vesicles was interpreted as indication of synaptic immaturity (30, 31).

Recently a quantitative study of development of synapses in intact rat superficial motor cortex has been reported by Armstrong-James and Johnson (4); the rate of increase of the number of synapses per unit volume of this portion of the cortex correlates well with the present data.

After this manuscript was submitted for publication, a similar study by Piras et al. (32) on rat brain subcellular fractions during development appeared. These authors observed changes in morphologic and enzymatic composition of fractions during development that are similar to those reported here.

This work was supported by United States Public Health Service Grants NS 08075, NS 05572-06, NS 08933-01 from the National Institute of Neurological Diseases and Stroke and FR 07083-03 Institutional grant.

Received for publication 30 July 1970, and in revised form 28 June 1971.

REFERENCES

1. Eayrs, J. T., and B. Goodhead. 1959. Postnatal development of the cerebral cortex in the rat. *J. Anat.* **93**:385.

2. AGHAJANIAN, G. D., and F. E. BLOOM. 1967. The formation of synaptic junctions in developing rat brain. A quantitative electron microscopic study. *Brain Res.* **6**:716.
3. JOHNSON, R., and M. ARMSTRONG-JAMES. 1970. Morphology of superficial postnatal cerebral cortex with special reference to synapses. *Z. Zellforsch. Mikrosk. Anat.* **110**:540.
4. ARMSTRONG-JAMES, M., and R. JOHNSON. 1970. Quantitative studies of postnatal changes in synapses in rat superficial motor cerebral cortex. An electron microscopical study. *Z. Zellforsch. Mikrosk. Anat.* **110**:559.
5. FLEXNER, L. B. 1951–52. The development of the cerebral cortex: a cytological, functional and biochemical approach. *Harvey Lect.* **47**:156.
6. HIMWICH, W. A. 1962. Biochemical and neurophysiological development of the brain in the neonatal period. *Int. Rev. Neurobiol.* **4**:117.
7. CRAIN, S. M. 1952. Development of electrical activity in the cerebral cortex of the albino rat. *Proc. Soc. Exp. Biol. Med.* **81**:49.
8. ALTMAN, J., and G. D. DAS. 1965. Postnatal origin of microneurones in the rat brain. *Nature* (*London*). **207**:953.
9. ABDEL-LATIF, A. A., J. P. SMITH, and E. P. ELLINGTON. 1970. Subcellular distribution of sodium-potassium adenosine triphosphatase, acetylcholine and acetylcholinesterase in developing rat brain. *Brain Res.* **18**:441.
10. BANIK, N. L., and A. N. DAVISON. 1969. Enzyme activity and composition of myelin and subcellular fractions in the developing rat brain. *Biochem. J.* **115**:1051.
11. ABDEL-LATIF, A. A., J. BRODY, and H. RAMAHI. 1967. Studies on Na^+ K^+ ATPase of the nerve endings and appearance of electrical activity in developing rat brain. *J. Neurochem.* **14**:1133.
12. CUZNER, M. L., and A. N. DAVISON. 1968. The lipid composition of rat brain myelin and subcellular fractions during development. *Biochem. J.* **106**:29.
13. AUTILIO, L. A., S. H. APPEL, P. PETTIS, and P. GAMBETTI. 1968. Biochemical studies of synapses in vitro. I. Protein synthesis. *Biochemistry*. **7**:2615.
14. ELLMAN, G. L., K. D. COURTNEY, V. ANDRES, and R. M. FEATHERSTONE. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**:88.
15. PENNINGTON, R. J. 1961. Biochemistry of dystrophic muscle. Mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. *Biochem. J.* **80**:649.
16. SCHRIER, B. K., and L. SHUSTER. 1967. A simplified radiochemical assay for choline acetyltransferase. *J. Neurochem.* **14**:977.
17. KORNBERG, A. 1955. Lactic dehydrogenase of muscle. *Methods Enzymol.* **1**:441.
18. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
19. DALTON, A. J. 1955. A chrome-osmium fixation for electron microscopy. *Anat. Rec.* **121**:281.
20. HUXLEY, H. E., and G. ZUBAY. 1961. Preferential staining of nucleic acid-containing structures for electron microscopy. *J. Biophys. Biochem. Cytol.* **11**:273.
21. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
22. ALDRIDGE, W. N., and M. K. JOHNSON. 1959. Cholinesterase, succinic dehydrogenase, nucleic acids, esterase and glutathione reductase in subcellular fractions from rat brain. *Biochem. J.* **73**:270.
23. DE ROBERTIS, E., A. PELLEGRINO DE IRALDI, G. R. DE LORES ARNAIZ, and L. SALGANICOFF. 1962. Cholinergic and non-cholinergic nerve endings. *J. Neurochem.* **9**:12.
24. DAVISON, A. N., and J. DOBBING. 1968. *In* The Developing Brain, Applied Neurochemistry. F. A. Davis Co., Philadelphia. 253.
25. PETERS, A., and J. VAUGHN. 1967. Microtubules and filaments in the axons and astrocytes of early postnatal rat optic nerves. *J. Cell Biol.* **32**:113.
26. BEHNKE, O. 1967. Incomplete microtubules observed in mammalian blood platelets during microtubule polymerization. *J. Cell Biol.* **34**:697.
27. BARONDES, S., and G. R. DUTTON. 1969. Microtubular protein synthesis and metabolism in developing brain. *Science* (*Washington*). **166**: 1637.
28. SPENCE, M. W., and L. S. WOLFE. 1967. Gangliosides in developing rat brain. Isolation and composition of subcellular membranes enriched in gangliosides. *Can. J. Biochem.* **45**:671.
29. WHITTAKER, V. P. 1968. The morphology of fractions of rat forebrain synaptosomes separated on continuous sucrose density gradients. *Biochem. J.* **106**:412.
30. BODIAN, D. 1966. Development of fine structure of spinal cord in monkey fetuses. I. The motorneuron neuropil at the time of onset of reflex activity. *Bull. Johns Hopkins Hosp.* **119**: 129.
31. BODIAN, D., E. C. MELBY, and N. TAYLOR. 1968. Development of fine structure of spinal cord in monkey fetuses. *J. Comp. Neurol.* **133**:113.
32. PIRAS, M. M., I. SZIJAN, and C. J. GOMEZ. 1970. Enzymatic and ultrastructral changes in subcellular fractions from developing rat brain. *Acta Physiol. Latinoamer.* **20**:74.