Preparation of Purified Myelin of Rabbit Brain by Sedimentation in a Continuous Sucrose Gradient[1] (34447)

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Myelin has been purified in high yield by a single centrifugation of a rabbit brain homogenate in the B-IV zonal rotor. The present report contains data showing that myelin prepared by this method is of high purity, as judged by enzymatic and chemical assays and by electron microscopic examination.

Previously reported methods of preparing myelin involved repeated centrifugations, usually in a single solvent or in a discontinuous gradient (1–6). Since in these methods the sample accumulated as a pellet or as a cohesive layer at an interface, the contamination with other materials could not be avoided. We found that myelin can be prepared relatively free of recognizable contamination when a well-homogenized tissue sample is sedimented to equilibrium in a continuous sucrose gradient. Myelin membranes have the highest lipid content of any membrane in the homogenate and are, therefore, the most buoyant membrane fraction. The myelin membrane fragments are heterogeneous in size and, when such membrane fragments are sedimented for short periods, they tend to distribute according to size. However, when they are centrifuged for long periods in a continuous sucrose gradient, 10–35% (w/w), the myelin distributes according to its density at approximately 15% sucrose.

Advantages of the zonal centrifuge over conventional methods using tubes have been discussed (7–8). We emphasize here the advantages of quantity and quality; an entire rabbit brain may be processed with a good resolution of the myelin in a single centrifugation.

In our procedure freshly removed rabbit brain, approximately 8 g, was washed twice with 10% sucrose in 0.01 *M* Tris–HCl, pH 8.0, then homogenized in a Teflon pestle glass homogenizer (1.5-mm clearance) in three 8-ml portions of 10% sucrose cooled in ice water. In order to assure complete homogenization, 9–10 down and up cycles with the rotating pestle were necessary. The homoggenate was added immediately to the B-IV rotor and spun for 2 hr at 40,000 rpm in the system shown in Table I. All operations were carried out at 2° or in an ice bath.

The gradient was removed from the rotor at 3000 rpm by displacement with cold 50% sucrose. The effluent was continuously monitored at 400 nm in a 1-mm flow cell in a Beckman DB spectrophotometer and collected in 13-ml fractions. Every fifth fraction from 20 through 120 was taken for electron microscopy. An equal volume (13 ml) of distilled water was added to each and the tubes were centrifuged for 2 hr at 35,000 rpm in a No. 40 rotor of a Beckman L-1 centrifuge. The pellets were resuspended in 2% osmium tetroxide and allowed to fix for 30 min. They were dehydrated with ethanol and

TABLE I. System.

Solution[a]	Sucrose (%, w/w)	Vol (ml)
Overlay	5	50
Sample	8	30
Upper	10	100
Gradient	10–35	1500
Cushion	50	65

[a] For a general description of the method, see Anderson (2).

[1] A preliminary report of this work was presented at the Annual Southeastern Sectional Meeting of the Society for Experimental Biology and Medicine, November, 1968.

embedded in Maraglas. Silver–gold sections were cut at several levels in the blocks with a diamond knife on a Porter-Blum ultramicrotome and examined with an RCA-EMU-3G electron microscope.

Every fifth to tenth fraction throughout the gradient was assayed for 10 enzymes, which assist in identifying the subcellular components (Table II). The fractions from the gradient were also assayed for RNA, DNA, and sialic acid. These chemical studies were limited to those that might show contamination in the myelin fraction. RNA was isolated from the fractions and analyzed by the method of Burka and collaborators (9, 10), an assay procedure specifically developed to study the concentration of RNA in membranes. DNA was determined by the diphenylamine method (11) on the precipitated, dialyzed fractions. Sialic acid was measured by the thiobarbiturate method (12).

TABLE II. Subcellular Components.

Enzyme	Monitor for
1. Leucylglycinase[a]	soluble and microsomes
2. Leucyl β-naphthylamidase[b]	soluble and microsomes
3. Acetylcholinesterase[c]	synaptosomes and synaptic membranes
4. Cytochrome oxidase[d]	mitochondria
5. Glucose-6-phosphatase[e]	plasma membranes and microsomes
6. K^+-activated phosphatase[f]	plasma membranes and synaptic membranes
7. K^+-activated ATPase[g]	plasma membranes and synaptic membranes
8. Acid phosphatase[h]	lysosomes
9. Alkaline phosphatase[i]	microsomes and plasma membranes
10. Inosine diphosphatase[j]	plasma membranes

[a] Binkley, Leibach, King, Arch. Biochem. Biophys. **128**, 397 (1968).

[b] Goldbarg and Rutenburg, Cancer **11**, 283 (1958).

[c] Nabb and Whitfield, Arch. Environ. Health **15**, 147 (1967). Ellman *et al.*, Biochem. Pharmacol. **7**, 88 (1961).

[d] Schael, Tipton, and Anderson, J. Biol. Chem. **22**, 317 (1969).

[e] Allen, Methods Enzymol. **3**, 140 (1957). Nordlie and Arion, Methods Enzymol. **9**, 619 (1966).

[f] Albers *et al.*, Proc. Natl. Acad. Sci. U.S. **53**, 557 (1965).

[g] Wallach and Kamat, Methods Enzymol. **8**, 164 (1966).

[h] Bessey, Lowry, Brooks, J. Biol. Chem. **164**, 321 (1946); (at alkaline pH).

[i] Bessey, Lowry, Brooks, J. Biol. Chem. **164**, 321 (1946); (at acid pH).

[j] Plant, Methods Enzymol. **6**, 231 (1963).

Figure 1 shows the slope of the sucrose gradient and the turbidity of the fractions at 400 nm obtained after centrifugation at 40,000 rpm for 2 hr. The first 25 fractions contain the low molecular weight soluble material and were not characterized by electron microscopy. Figures 2–9 are electron micrographs which illustrate the types of subcellular components observed in different regions of the sucrose gradient. Figures 2–6 are representative of fractions 25–50 and show myelin fragments, membrane bound vesicles, and occasional mitochondria. In other sections not shown, fragments of axoplasm were seen embedded within myelin rings. The lamellar organization of myelin as seen in tissue sections is apparent in many areas and is indicated by arrow A. The alternating dense bands and the interperiod lines are easily seen in the original plates. Many of the myelin lamellae have split. The break occurs between major dense bands for the most part, but some splitting of the interperiod lines can also be seen. It appears in many places that some of the vesicles have arisen from these myelin strands, as shown by arrow B. A similar origin for others is suggested by their location between myelin strands (arrow C). The majority of the vesicles do not clearly show such a relationship, however, and many lie free in the supporting medium. Figure 7 illustrates the appearance of fractions 55–85, which consist primarily of membrane-bound vesicles varying in size between 300 to 4000Å. Some of these vesicles probably originate from myelin. None have distinguishing features such as those described by De Robertis for synaptic vesicles and membranes (13, 14). In addition there are numer-

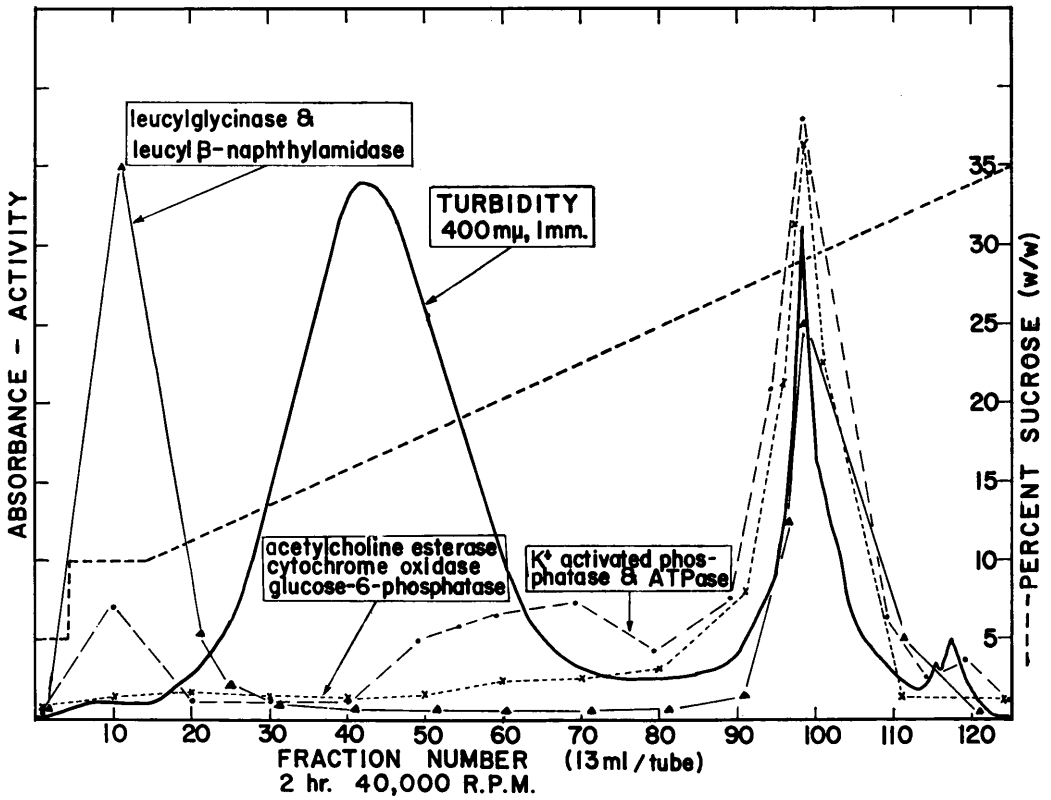


FIG. 1. Sucrose gradient pattern of whole rabbit brain homogenate: (—), absorbance (turbidity); (- - -), sucrose gradient; (x, ▲, ●), enzyme activities.

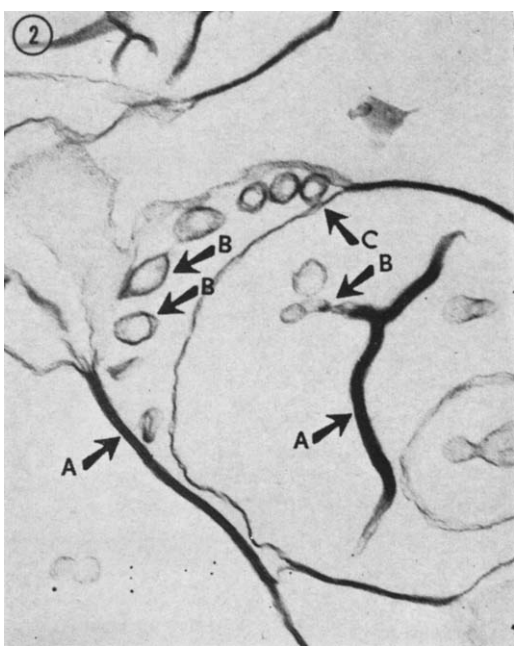


FIG. 2. Fraction 30; 51,000 magnification. A, lamella structure of myelin; B, splitting of lamella to form vesicles; C, vesicles formed between myelin layers.

ous small (150–300 Å) dense particles, probably glycogen, and occasional myelin fragments and mitochondria. Fractions 90 to 110 have the appearance shown in Figs. 8 and 9. Mitochondria, synaptosomes, lysozomes, myelin fragments, vesicles, and rough endoplasmic reticulum are easily recognizable. Fractions 110–120 contain collagen, nuclei, blood vessels, and cell debris.

The 10 enzymes studies are listed in Table II together with the subcellular components these enzymes are thought to monitor. References to the assay procedures of the enzymes are also included in Table II. The results of the first seven enzyme studies are plotted in graph form in Fig. 1. The patterns of certain enzyme activities were similar and accordingly have been plotted together as a single line. In that portion of the gradient considered to contain myelin as judged from the electron micrographs, *i.e.*, fractions 25–50, enzyme activities are barely detectable except for a low level of K^+-activated phosphatase in fraction 50. The very low level of cytochrome ox-

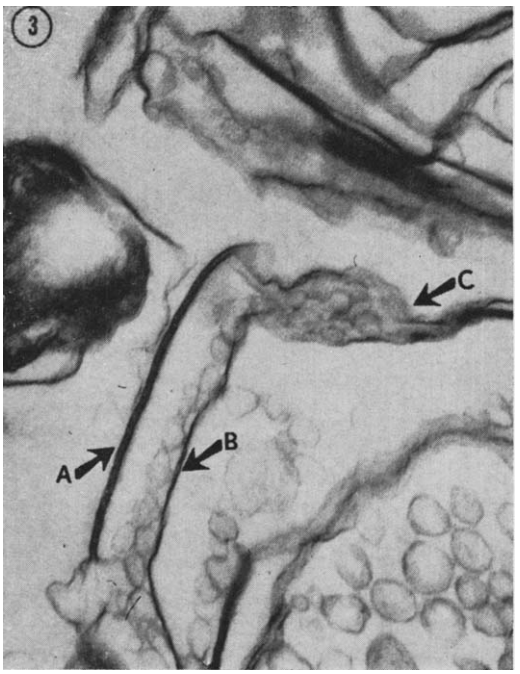


FIG. 3. Fraction 40; 51,000 magnification.

idase activity is consistent with the electron microscopic finding of rare mitochondria. The absence of leucylglycinase and leucyl

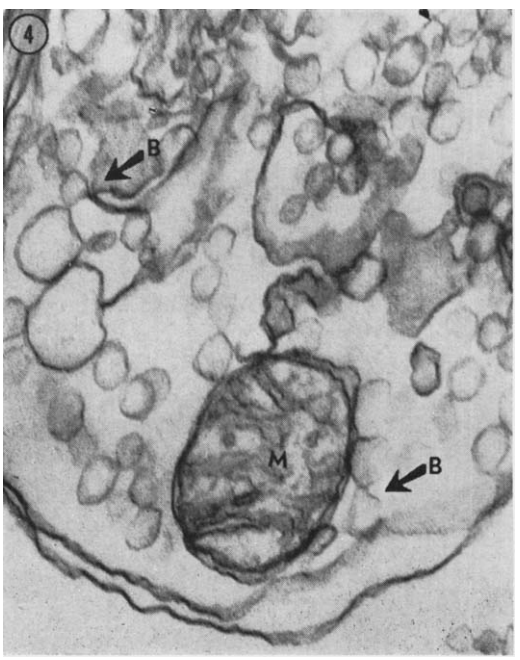
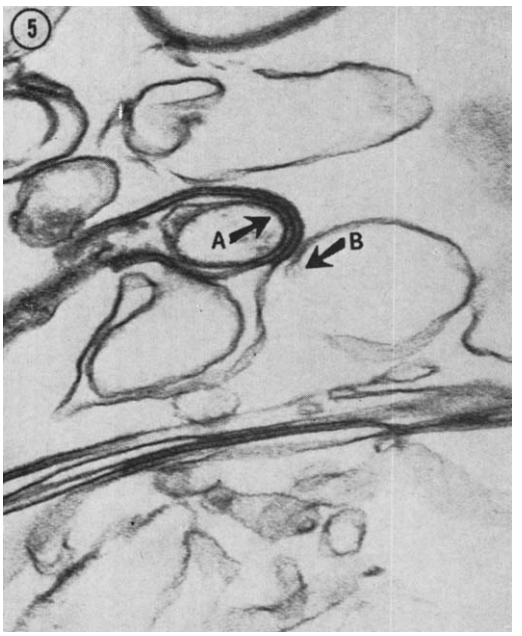
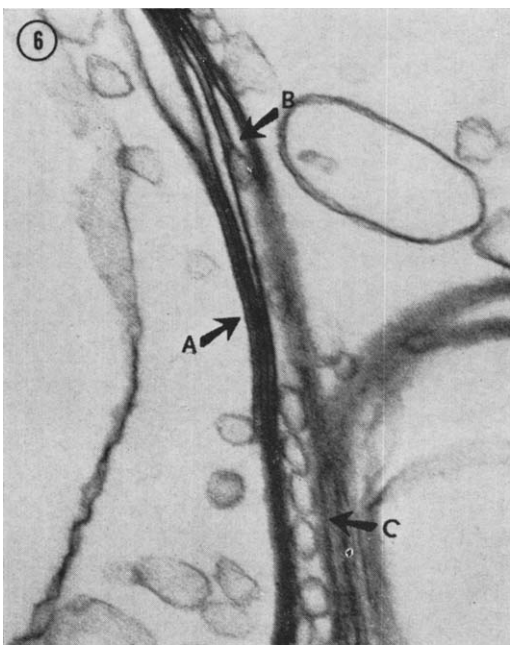


FIG. 4. Fraction 50; 51,000 magnification. M, occasional mitochondrian.



FIGS. 5 and 6. Fraction 50; Fig. 5., 76,500 magnification; Fig. 6, 90,000 magnification.



β-naphthylamidase activity in the myelin fraction is contrary to the findings of Adams *et al.* (15). All enzyme activities were at peak levels in fractions 90–110, which is consistent with the electron micrographs of this

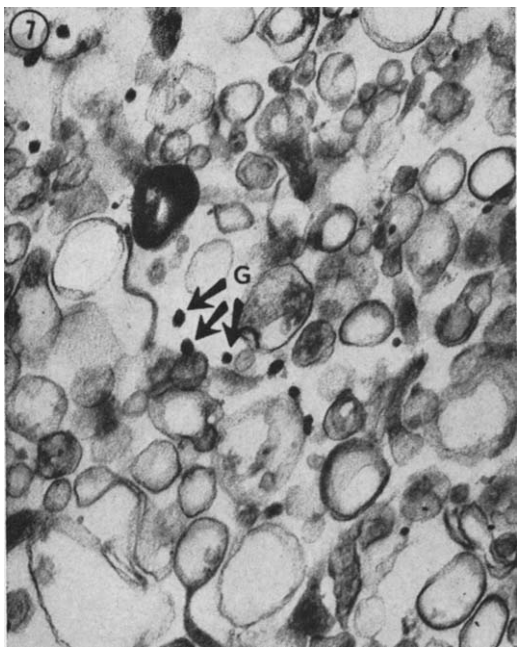


FIG. 7. Fraction 60; 51,000 magnification. G, glycogen.

region of the gradient. The low to moderate level of activity of acetylcholinesterase, glucose-6-phosphatase, K^+-activated phosphatase and ATPase in fractions 50–80 suggest that the vesicles seen in these fractions contain microsomes, synaptic membranes, and plasma membranes. Acid phosphatase, alkaline phosphatase, and inosine diphosphatase were clearly detectable only in the mixed organelle fraction (fractions 90 to 110).

Tubes 25 to 50, which include the myelin fraction, contained 1–2 μg of RNA/ml. This was rather invariable and unrelated to the myelin concentration. Tube 100, the mixed organelle fraction, contained 30 μg of RNA/ml. The myelin fractions, therefore, contain approximately 5% of the RNA content of the mixed organelle fraction (fractions 90 to 110), which suggests possible contamination of the myelin fraction with mitochondria, microsomes, and ribosomes and is in agreement with the traces of enzyme activities discussed above. No DNA was found in the myelin fractions. However, 5 μg of DNA/ml was found in the top of the gradient, tube 21, where DNA would be expected. Sialic acids were not found in the myelin region of the gradient, which is consistent with the virtual absence of enzyme activities associated with plasma membranes in this region.

It is apparent that fractions 25–40 constitute the highest purity myelin by all criteria used and that fractions beyond this point through number 50 are relatively pure, especially by electron microscopy.

These studies indicate our myelin to be of a purity comparable to that reported by Autilio *et al.* (16). These authors prepared their myelin from white matter of brain, in a procedure involving five centrifugation steps before sucrose gradient centrifugation which, under their conditions, did not reach equilibrium. The myelin layers were removed from the centrifuge tubes with a Pasteur pipette and then shocked with distilled water and washed before electron microscopy. It should be noted that in our procedure the isolated sucrose gradient fractions were neither shocked nor extensively washed. This was done to evaluate contamination of the myelin fractions before lysing any organelles which might be present. As discussed above, the electron micrographs showed only an occasional mitochondrian. It can be assumed that shocking in distilled water would remove the fragments of axoplasm that were also observed.

Summary. Purified myelin was prepared in high yield by a single centrifugation in the L-4 zonal centrifuge. A whole rabbit brain (8 g) homogenate in 7% sucrose was layered over a 10–35% sucrose gradient in the B-IV rotor and sedimented for 2 hr at 40,000 rpm. The gradient was removed by pumping 50% sucrose into the rotor with the recovery of 120 fractions of 13 ml each. Every fifth to tenth fraction was examined under the electron microscope and was assayed for RNA, DNA, sialic acids, and 10 enzymes. The latter included cytochrome oxidase (CyOx) to monitor for mitochondria, K-activated ATPase (K*ATP) and acetylcholinesterase (AChE) for synaptic membranes, leucylglycinase for soluble and microsomes, acid phosphatase for lysosomes, and glucose-

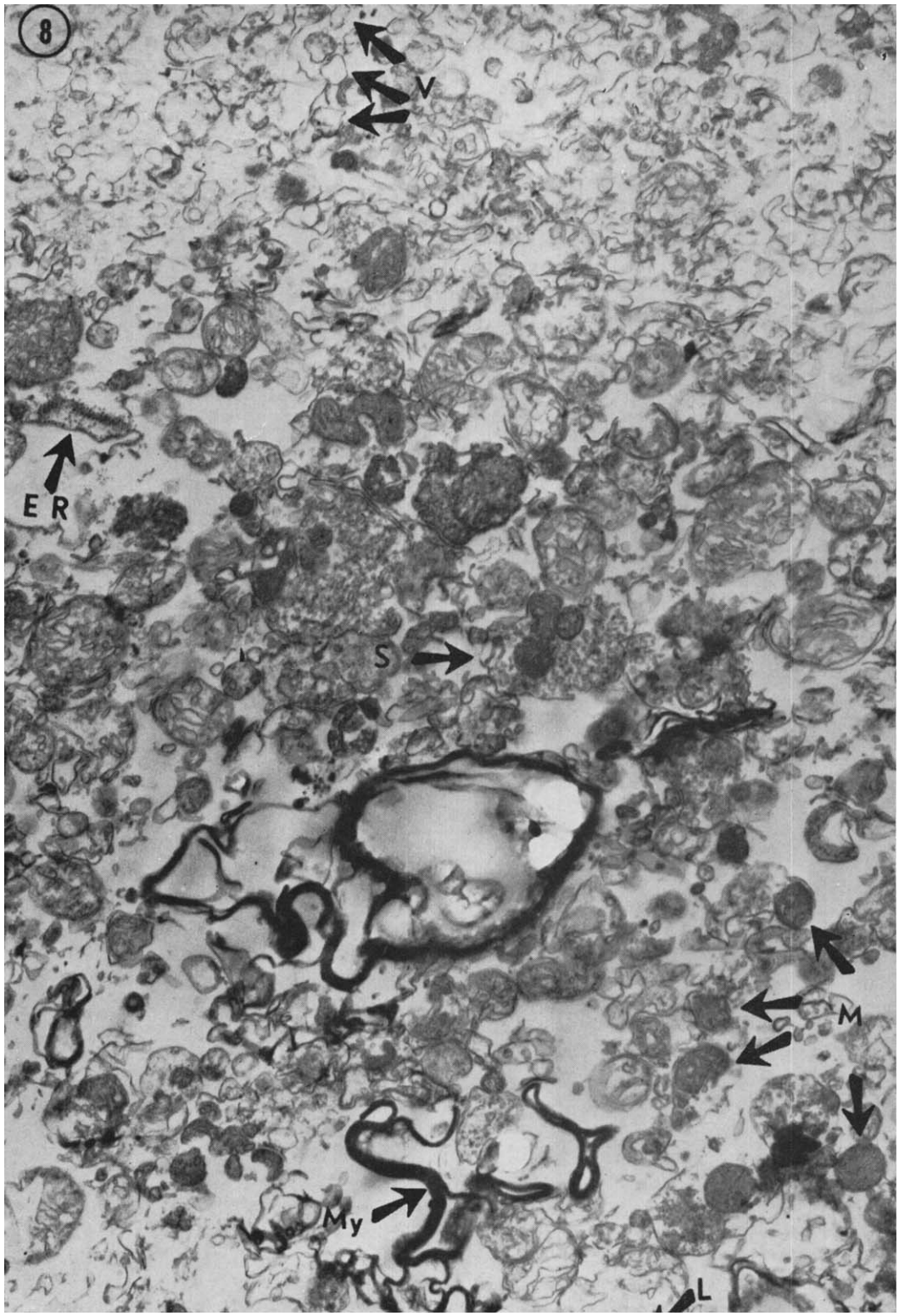


FIG. 8. Fraction 108; 20,800 magnification. ER, endoplasmic reticulum; M, mitochondria; L, lysosome; S, synaptosome, My, myelin; and V, vesicles.

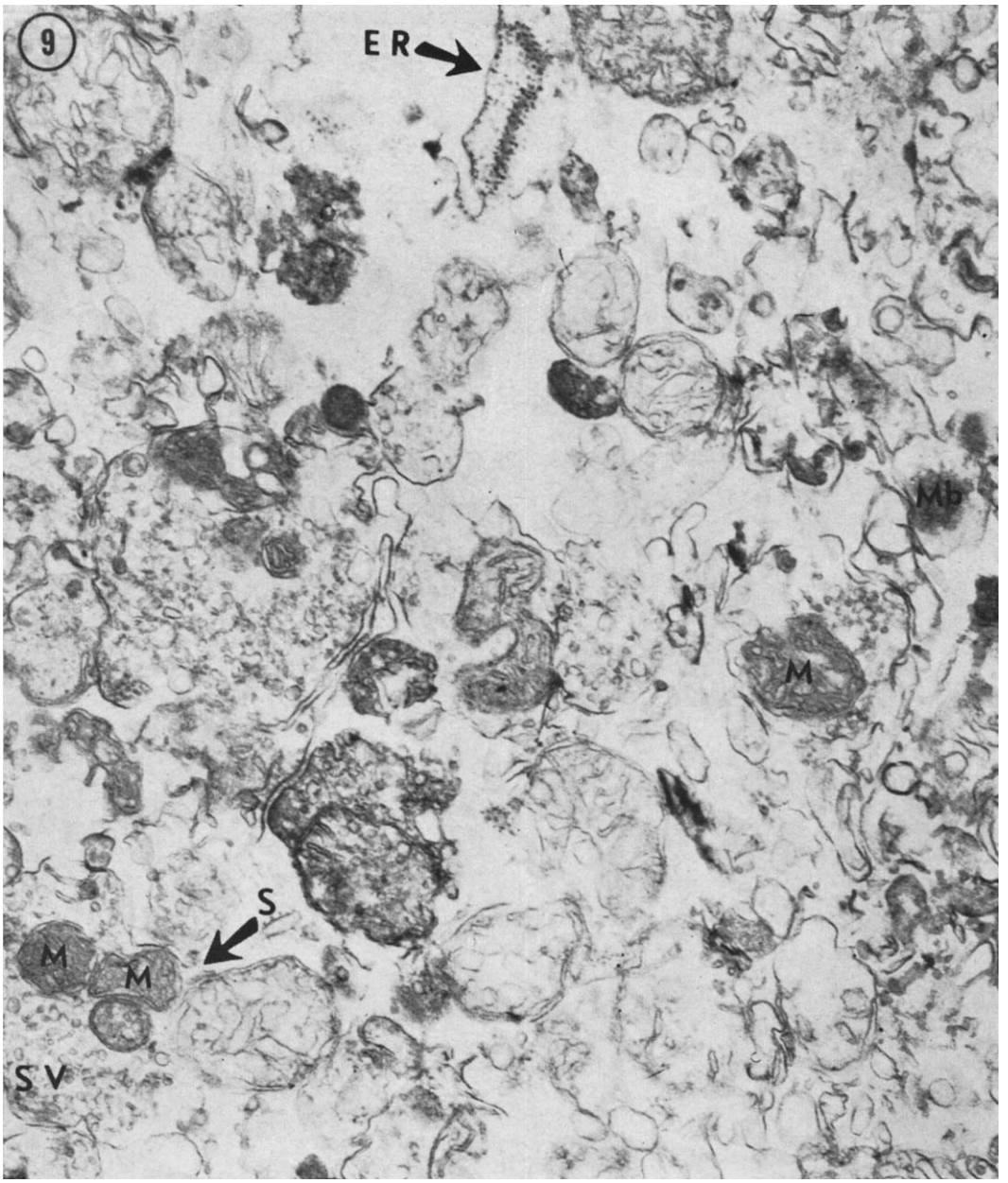


FIG. 9. Fraction 108; 76,500 magnification; SV, synaptic vesicles; Mb, microbody; ER, M, and S same as in Fig. 8.

6-phosphatase (G-6-Pase) and inosine diphosphatase for plasma membranes. Fraction 25–50 contained typical myelin fragments, rare mitochondria, and numerous membrane-bound vesicles which arose from splitting of the myelin lamellae. The chemical and enzyme analyses showed only a very low level of CyOx activity. Fractions 55–85 consisted primarily of vesicles varying in size from 300–4000 Å. Low to moderate levels of G-6-Pase, AChE, and K*ATP were present. All chemical and enzyme analyses gave high

yields in fractions 90–110, in keeping with the presence of all subcellular constituents in these fractions.

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