CALCIUM-CONTAINING STRUCTURES IN VERTEBRATE GLIAL CELLS

Ultrastructural and Microprobe Analysis

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ABSTRACT

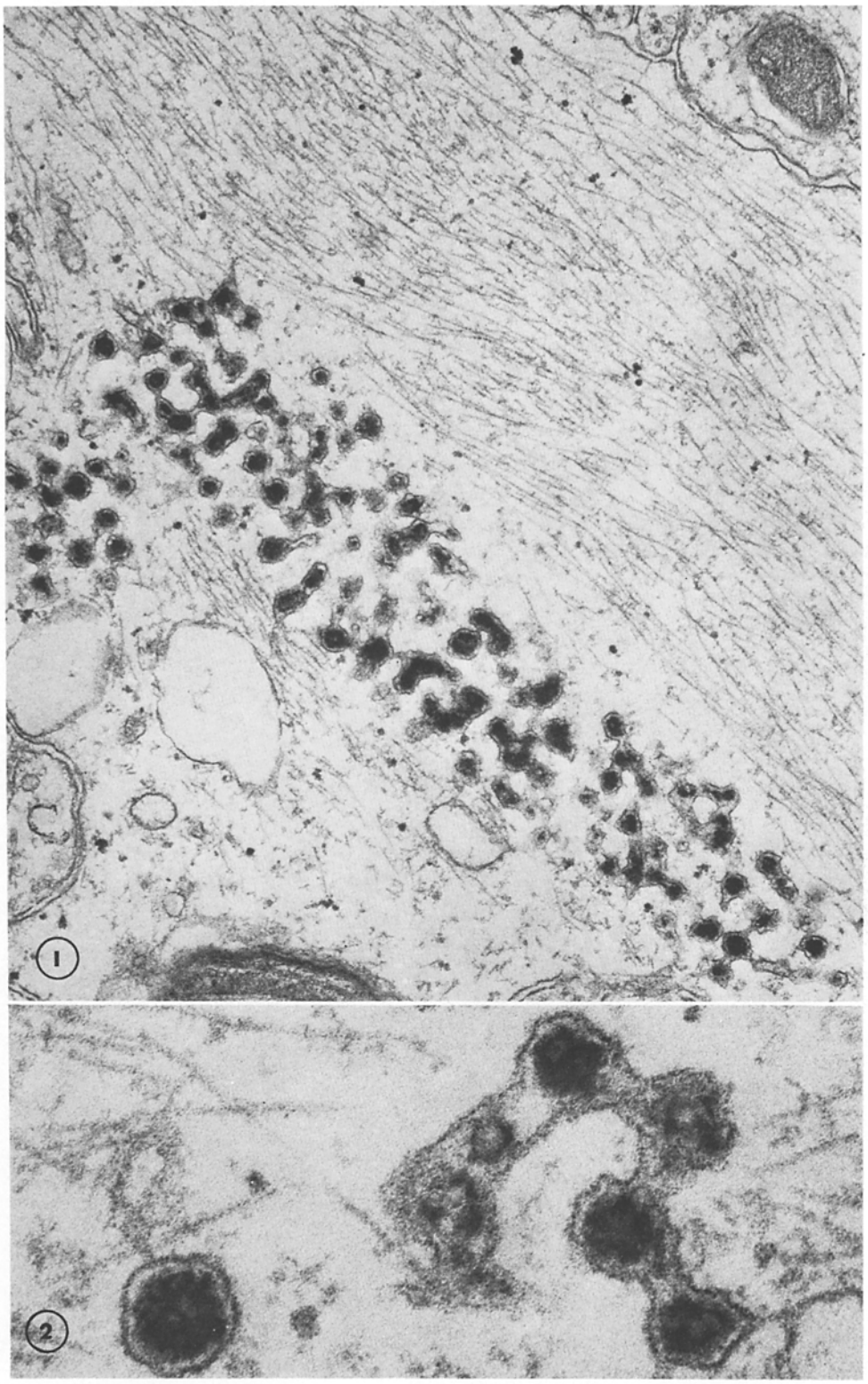
Electron probe microanalysis has revealed that vesicular or cisternal structures containing electron-dense material in frog ependymal glial cells contain deposits of calcium and phosphorus. The so-called "osmiophilic particles" in human astrocytes also contain calcium. It is suggested that these organelles are storage sites of calcium.

There have been several reports describing membrane-bounded, highly electron-dense cytoplasmic structures in glial cells of frog and teleost (1–5); the electron density of these structures has been attributed to osmium fixation. Consequently, some investigators have defined these structures as osmiophilic (2, 3). Other studies have independently reported the presence of "osmiophilic particles" in the cytoplasm of astroglial cells of the central nervous system of man and other mammals in normal as well as abnormal conditions (6–8, see 9 for review). The nature of these structures has not been established. It has been suggested that they may be lipid droplets (1–3, 8, 9), virus particles (6–9), secretory granules (4, 8, 9), or lysosomes (9). During a study on the ultrastructure of the spinal cord of the frog, we observed electron-dense intracytoplasmic structures similar in many aspects to those described previously in man and other mammals. Electron microscope study and electron probe X-ray microanalysis of these structures and of human astroglial dense particles have provided evidence that both of these structures contain Ca. A preliminary report of part of this work has been presented (10).

MATERIALS AND METHODS

Spinal cords from 15 frogs (*Rana pipiens*) were processed for electron microscopy according to various procedures. 12 frogs were perfused by intracardiac catheterization for 15–20 s with frog Ringer's solution and for 20 min with 0.1 M cacodylate buffer (pH 7.4) containing 1% paraformaldehyde and 5% glutaraldehyde at 4°C. 4 mM $CaCl_2$ was added to the fixative before perfusion in seven of these fourteen experiments, whereas $CaCl_2$ was omitted in the remaining experiments. The spinal column was removed from the 12 perfused animals and left overnight at 4°C in fixatives identical to those used for perfusion. Then, under the dissecting microscope, the spinal cords were removed from the spinal columns, bisected longitudinally and sliced. Samples were then postfixed for 2 h either with 2% OsO_4 in 4% dichromate (11) or with 2% OsO_4 in 0.1 M cacodylate buffer, dehydrated in graded ethanols, and embedded in Araldite according to standard techniques (12). Some of the samples were examined without postfixation.

Samples from three spinal cords dissected free from vertebrae were fixed by immersion either for 2–4 h directly in OsO_4 prepared as indicated above or overnight in fixatives identical to those used for perfusion (with or without osmication). For electron microscopy, sections of various thicknesses from gray interference color (less than 600 Å thick [13]) to blue interference color



Unless otherwise stated, all figures were obtained from tissue perfused with $CaCl_2$-containing fixative, postfixed in osmium tetroxide, and stained after sectioning with uranyl acetate and lead citrate.

FIGURE 1 Frog spinal cord: a cluster of intercommunicating vesicular-cisternal structures (VCS) located at the periphery of a filament-containing cell process. × 47,000.

FIGURE 2 Higher magnification of VCS. × 172,500.

(1,900–2,400 Å thick [13]) were mounted on Formvar-coated copper grids. The thinner sections were stained with uranyl acetate and lead citrate (14, 15) and carbon coated.

Unstained, 900–2,400-Å thick sections mounted on Formvar-coated copper grids, with or without carbon coating, were used for electron probe analysis. The analysis was performed on frog's spinal cord tissue fixed by the various aforementioned fixation procedures and on sections from human cerebral white matter fixed by immersion for 2 h in 2% OsO_4 in 4% chromate (11). The human tissue was obtained at biopsy by one of us (N.K.G.) several years ago from a case of subacute sclerosing leucoencephalitis. Clinical, light microscope, and ultrastructural details of this biopsy have been published (7).

Two analytical systems have been used. The majority of the analyses were performed with a Philips EM 301 electron microscope operated at 80 kV with a 300-μm first condenser aperture, between 6 and 16 μA beam current, a probe-forming system including the scanning upper polepiece and equipped with a 30 mm^2 158 eV resolution Kevex Si (Li) detector (Kevex Corp., Burlingame, Calif.) placed 22 mm from the specimen. X-ray counts were processed through either a Kevex 5,000 A and 6,000 multichannel analyzer-data processor (operated at 10 or 20 eV/channel) or an Ortec Delphi II system (Ortec, Inc., Oak Ridge, Tenn.). The high sensitivity of the EM 301-based system permitted the use of low beam currents, short (40–50 s) counting times, and small (600 Å) probe diameters. When analysis of larger areas was desired a probe size of approximately 2,000 Å was used, as indicated in Results. Some analyses were done with a JEM 100 B electron microscope operated at 80 kV with a total beam current of 50 μA and equipped with Edax energy dispersive microanalyzing system operated at 50 eV/channel (Edax International Inc., Prairie View, Ill.). X-ray spectra were obtained over the organelles specifically investigated in this study and, for comparison, over adjacent areas taken at random or over selected areas including myelin, mitochondria, and lysosomes. Areas probed were photographed before and after probing. System peaks present in the spectra included characteristic peaks of Cr (specimen holder), Cu, Si, Cl, and Os: these could be ascribed to grids, instrumental peaks, fixative, and embedding media (16).

RESULTS

Frog Spinal Cord

The tissue studied was obtained from the superficial layer of the spinal cord, close to the entry of the dorsal roots.

MORPHOLOGIC FEATURES OF THE VESICULAR-CISTERNAL STRUCTURES (VCS): A cluster of intercommunicating vesicular-cisternal structures located centrally among the filaments of a glial cell process is shown in Figs. 1 and

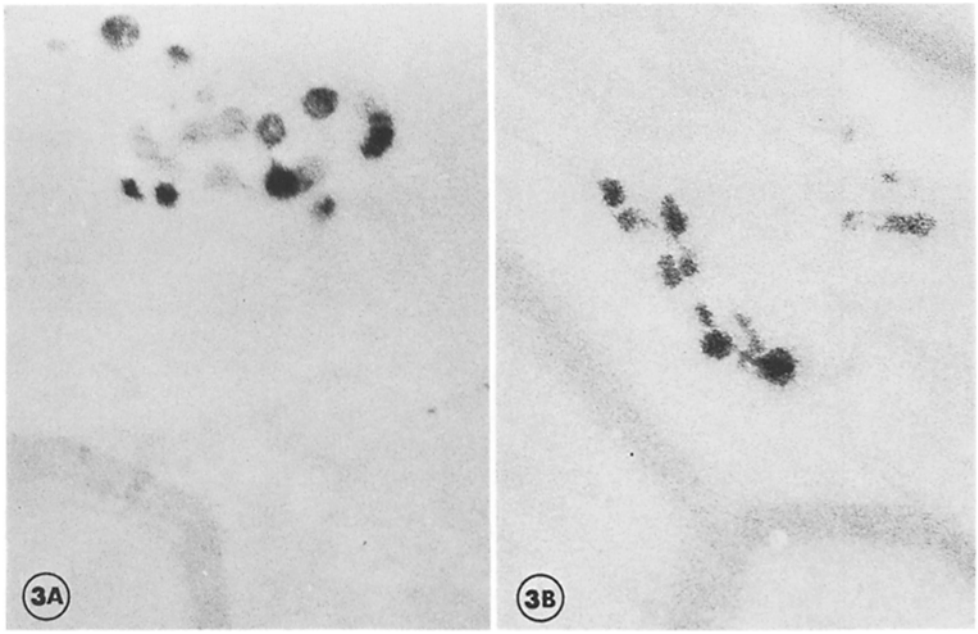


FIGURE 3 VCS in unstained, unosmicated sections 1,000–2,000 Å thick, fixed in aldehyde fixative. A, 4 mM Ca added to the fixative. B, No Ca added to the fixative. × 88,000.

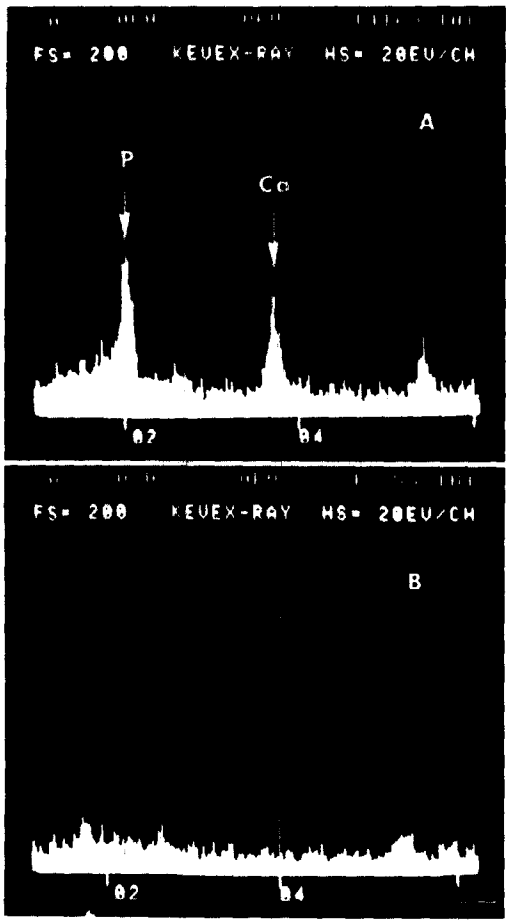


FIGURE 4 X-ray spectra of frog spinal cord. Spectrum A has been obtained over the VCS shown in Fig. 5 A and B. Note the presence of the Ca and P peaks and their absence in spectrum B that has been obtained over the adjacent cytoplasm. Perfusion fixation with aldehyde fixatives. No Ca added to the fixative. No OsO_4 postfixation. Probe diameter 600 Å, counting time 50 s. The unlabeled instrumental peak present in both spectra is due to the chromium in the specimen holder.

2. These clusters were present in all animals examined. However, the number and size of the large aggregates varied considerably from animal to animal. The size of the clusters also varied considerably in the same animal. The largest aggregates measured up to 3 μm × 1 μm. The vesicles measured 750 1,300. Å in diameter; the cisterns were about 500 Å wide (Fig. 2). Most of the vesicles and some of the cisterns contained electron-dense material separated from the limiting membrane of the vesicles and of the cisterns by a 50–110-Å gap (Fig. 2). The electron density of the intravesicular and intracisternal material was variable (Figs. 1, 2); occasionally, the center of the material contained in the vesicle was less electron dense than the periphery, producing a target-like image. Vesicles loaded with electron-dense material could be seen communicating with empty cisterns identical to those of the smooth endoplasmic reticulum. The VCS were not seen to fuse with any other cellular organelles with the possible exception of the smooth endoplasmic reticulum of which the VCS may be a part. Filaments, when present, spread apart around the VCS (Fig. 1). The vesicles of the VCS were somewhat similar to the dense-core vesicles present in many presynaptic endings and other ganglion cell processes; however, dense-core vesicles in presynaptic endings were never seen to form complex aggregates like those formed by the VCS. The overall morphologic features of the VCS remained substantially unchanged with the different methods of fixation, with the exception that in unosmicated specimens the limiting membrane of the VCS was barely detectable (Fig. 3 A, B).

CHARACTERISTICS OF THE ELECTRON-DENSE MATERIAL PRESENT IN THE VCS: Unstained, unosmicated sections, regardless of whether Ca had been added to the fixative, showed that the material contained in the VCS was highly electron dense (Fig. 3 A, B). Although electron-dense VCS were observed in all semithin sections examined, the electron density appeared more intense and widespread in tissues fixed with Ca-containing fixatives.

Postive identification of the elements contained in the VCS was obtained with electron probe analysis. Over 40 areas containing VCS in sections from tissues fixed by different procedures were examined and compared to background. The characteristic Ca Kα and P Kα X-ray peaks were consistently observed over VCS in unstained, unosmicated sections, fixed with Ca-free fixative (Fig. 4 A). Ca was not detected in the adjacent cytoplasm (Fig. 4 B), while a small P Kα peak was occasionally obtained. Electron micrographs of the analyzed VCS before and after analysis are shown in Figs. 5 A and 5 B. A distinct Ca Kα peak was obtained also over VCS in sections fixed by immersion directly in OsO_4 (Fig. 6). Because of the low contrast, myelin was the sole electron-dense structure besides the VCS, in the unosmicated sections. A series of analyses of VCS and myelin

with identical probe parameters (600-Å diameter, 40 s) and in the same section (Table I), shows that Ca Kα peaks are clearly detected during excitation of the VCS, while the Ca peaks over myelin are absent or of borderline significance. The P Kα peaks are highly significant over both myelin and VCS (Table I). With higher currents in larger probes (2,000-Å diameter), significant Ca peaks could also be detected over myelin sheaths.

CELLS CONTAINING THE VCS: Although a systematic and extensive search of the whole spinal cord was not made, the VCS were generally seen at the dorsal surface. In this region, the VCS were located in cell processes which occasionally could be traced up to the surface of the cord where they expanded in end-feet beneath the basement membrane. These cell processes contained 70–100-Å thick filaments and a few microtubules. The cells were also characterized by the presence of membrane-bounded inclusions with a generally amorphous central area that only occasionally contained longitudinally oriented cisterns. These inclusions were similar to those observed in ependymal-glial cells of frog's optic tectum by Potter (17)

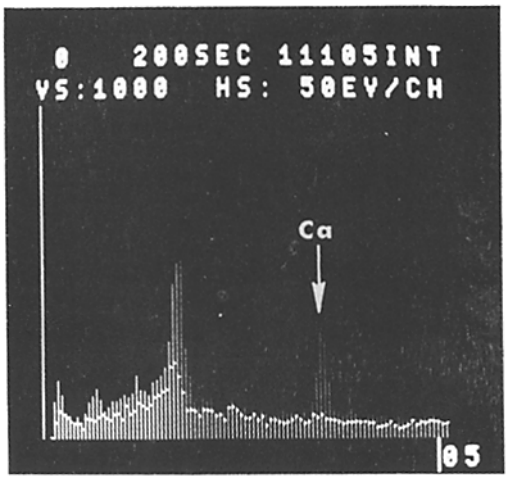


FIGURE 6 Superimposed X-ray emission spectra of VCS (bar display) and adjacent cytoplasm (dot display). The spectrum of the VCS shows a characteristic calcium peak that is lacking in the spectrum obtained over cytoplasm. The unlabeled high peak is that of the overlapping Os-P spectra. Frog spinal cord, immersion fixation with 2% OsO_4 in 4% dichromate (11). Probe diameter 1,000 Å, counting time 50 s.

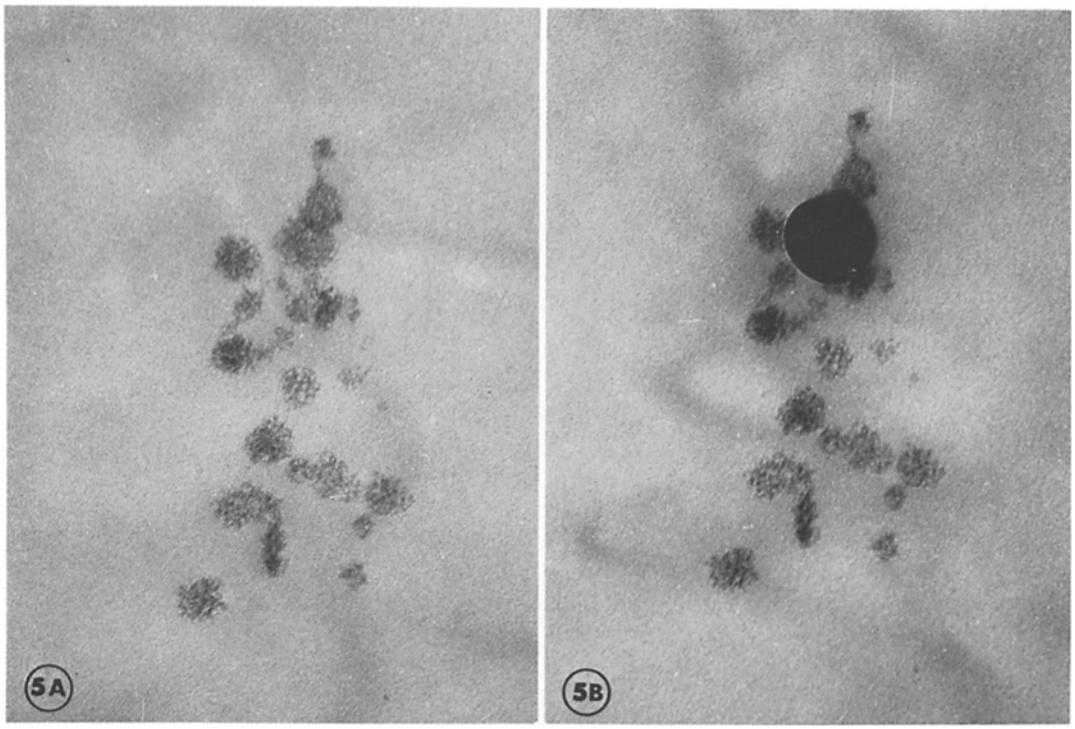


FIGURE 5 Electron micrographs of the VCS from which the X-ray spectra of Fig. 4 A has been obtained. A, Before analysis. B, After analysis. The spot indicates the probe site. Fixation as in Fig. 4, × 83,000.

and might represent modified mitochondria (17). Finally, the cell processes containing the VCS occasionally formed gap junctions (18) with one another. The cells of origin of these processes were apparently located at considerable distance from the surface of the cord and could not be retraced even in semithin cross sections of the whole cord. However, the aforementioned morphological features, namely, the presence of peculiar mitochon-

TABLE I

Electron Probe Analysis of VCS and Myelin Sheaths in Frog Spinal Cord

		Ca counts		P counts	
		P*	P − b‡	P*	P − b‡
VCS	no. 1	537	407	654	532
	2	415	287	621	533
	3	436	276	577	479
	4	752	592	1,078	930
	5	463	332	617	513
Myelin	no. 1	267	53	402	231
	2	204	46	458	337
	3	134	0	296	189
	4	153	5	385	272
	5	121	23	303	245
	6	93	18	316	247

Perfusion fixation in aldehyde fixatives without added Ca. No postfixation.

Probe size: 600 Å. Current: approximately 1 nA. Accelerating voltage: 80 kV. Live counting time: 40 s.

* Number of counts under the characteristic Kα peak integrated over the counting interval.

‡ Background counts integrated over the same interval.

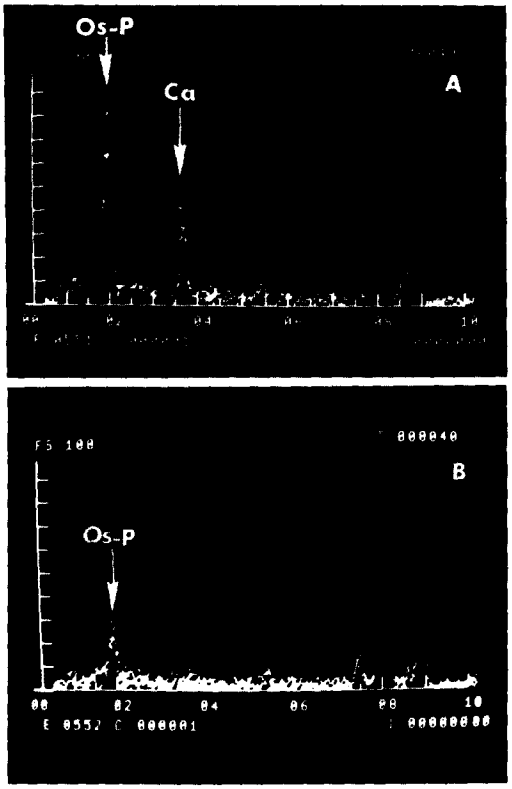


FIGURE 8 X-ray spectra of human white matter. A, The calcium peak, prominent over the dense particle shown in Fig. 9 A, is absent in spectrum B obtained over the adjacent cytoplasm. The peak on the left is that of the overlapping Os-P spectra. Other unlabeled peaks are instrumentation peaks. Immersion fixation with 2% OsO_4 in dichromate (11). Probe diameter 600 Å, counting time 40 s.

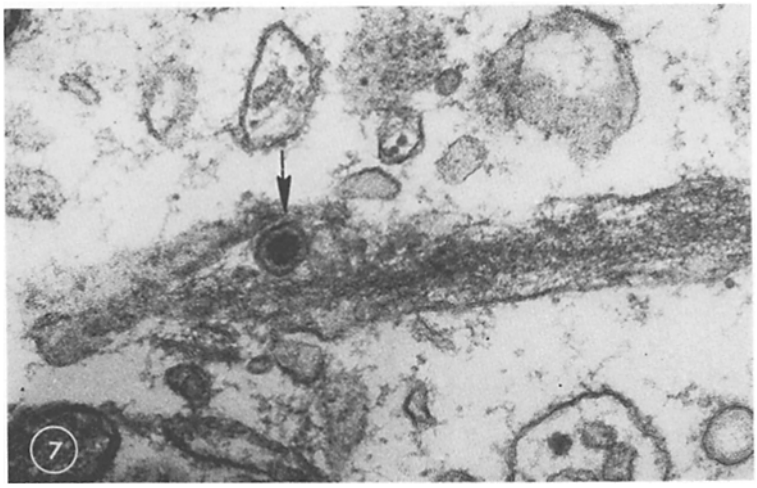


FIGURE 7 Dense particle (arrow) in a process of a human astrocyte. × 64,000.

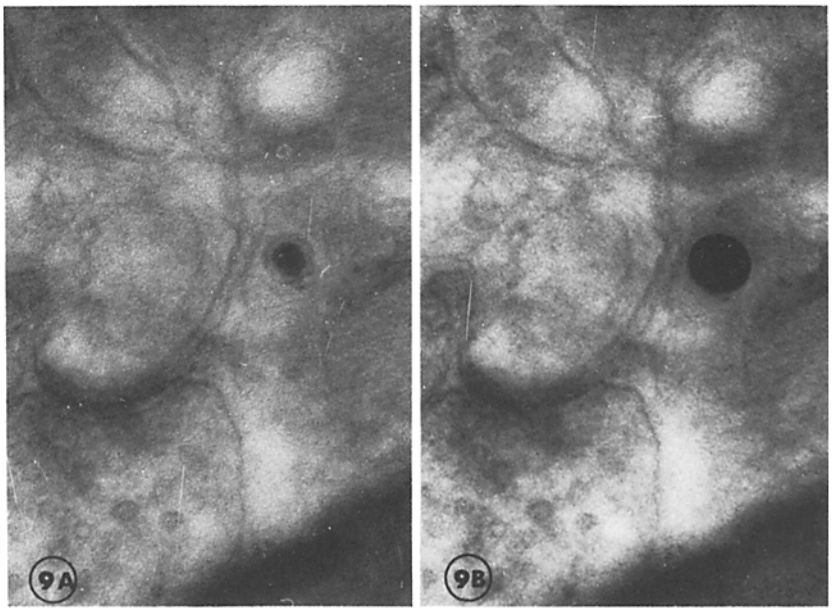


FIGURE 9 Electron micrographs of the astroglial dense particle from which the X-ray spectrum shown in Fig. 8 A has been obtained. A, Before analysis. B, After analysis. × 72,000.

dria, gap-junctions, and formation of end-feet, suggest that the VCS-containing processes originate from ependymal-glial cells (4, 17, 19, 20).

Human Cerebral White Matter

The morphology of the so-called "osmiophilic particles" of astroglial cells has been reported in detail (6–8). Briefly, astrocytic perikarya and processes contained a relatively large number of circular particles with an overall diameter of 800–1,300 Å and a dense core measuring 600–830 Å in diameter (Fig. 7). The core was surrounded by a clear halo. As has been previously reported (7), occasionally the membrane of these particles fused with that of a cistern of smooth endoplasmic reticulum. Some astrocytes contained a very high number of electron-dense particles. However, the particles were never seen to form the complex vesicular-cisternal structures seen in the frog. In unstained sections, the particles remained electron dense.

X-ray spectra obtained over a human astrocyte dense particle and, as control, over adjacent cytoplasm (Fig. 8 A, B) showed a highly significant Ca Kα peak over the dense particle (Fig. 8 A) but not over the adjacent cytoplasm (Fig. 8 B). Electron micrographs of the analyzed dense particle before and after analysis are shown in Fig. 9 A, B. A series of analyses over dense particles, myelin, lysosomes, and mitochondria with identical probe

TABLE II

Electron Probe Analysis of Astrocytic Dense Particles, Myelin Sheaths, Lysosomes, and Mitochondria of Human Cerebral White Matter

		Ca counts	
		P	P − b
Dense particle	no. 1	642	524
	2	488	395
	3	767	666
	4	237	104
	5	498	350
	6	341	203
	7	735	577
	8	811	633
	9	598	358
Myelin	no. 1	369	153
	2	321	161
	3	276	100
Lysosome	no. 1	472	217
	2	360	154
	3	301	94
Mitochondrion	no. 1	210	88
	2	160	18
	3	194	74

Immersion fixation with 2% O_sO_4 in 4% chromate (11). Analytic conditions, P and P − b as in Table I. No Ca/K_α peaks were detected over areas of cytoplasm analyzed as controls in these experiments. No values for P Kα counts are given due to the interference with the overlapping Os Mα peak (16).

parameters (Table II) showed that the Ca Kα counts over the dense particles were significantly higher than those over myelin, lysosomes, and mitochondria.

DISCUSSION

The present finding that VCS containing Ca and P are present in glial cell processes of the frog's spinal cord raises several questions. The first question is whether the VCS also contain Ca in the living cell. All the tissues examined had been prepared in aqueous fixatives; therefore, the possibility has to be considered that Ca present elsewhere in the tissue or present in traces in the fixative (21) is precipitated during fixation to some high affinity material contained in the VCS. This possibility, although not ruled out in the present study, appears rather unlikely, since VCS contained Ca and P in similar ratios regardless of the fixative (with or without Ca) or the method of fixation (perfusion or immersion) used.

It is also likely that Ca and P are the primary constituents of the material contained in the VCS for the following reasons: (*a*) In unstained unosmicated sections, the VCS were by far the most electron-dense structures, and by electron probe analysis they contained the highest concentration of Ca and P. (*b*) The Ca counts appeared to be directly related to the amount of electron-dense material contained in the VCS.

Therefore, the present findings are consistent with the hypothesis that the VCS present in glial cells of frog's spinal cord are Ca storage sites that may be part of the smooth endoplasmic reticulum. This conclusion is warranted also for the electron-dense particles present in human astrocytes, although this does not necessarily imply that the human and frog particles are homologous structures. These particles gave a Ca signal that was far greater than that of any other structure examined, including myelin, mitochondria, and lysosomes.

The presence of membrane-bounded calcium stores inside the glial cells raises the question of the function of calcium. Bianchi and Erulkar (unpublished observations) have carried out calcium ^{45}Ca washout curves from the spinal cord of the frog and found two kinetic components. The fast component ($t_{1/2}$ = 10 min) probably represents free calcium in the interstitial space according to evidence from the washout curve of radioactive sucrose. The slow component ($t_{1/2}$ = 285 min) may reflect "bound" or sequestered calcium. Pending experimental demonstration of Ca movement in and out the VCS, it is tempting to speculate that these structures are stores of bound calcium which may be drawn upon as needed, depending upon the calcium balance within the cord itself. Alternatively, they may act primarily as sinks for maintaining free cellular Ca at physiological levels during periods of high Ca load.

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REFERENCES

1. OKSCHE, A. (Quoted by Zadunaisky et al. 1963.) 1958. Histologische untersuchungen über bedeutung des ependymus, der glia und des plexus choroidei für den kohlenhydratstoffwechsel des Z. N. S. *Z. Zellforsch. Mikrosk. Anat.* **48**:74.
2. ZADUNAISKY, J. A., F. WALD, and E. D. P. DE ROBERTIS. 1965. Osmotic behavior and glial changes in isolated frog brains. *Prog. Brain Res.* **15**:196.
3. ZADUNAISKY, J. A., F. WALD, and E. D. P. DE ROBERTIS. 1963. Osmotic behaviour and ultrastructural modifications in isolated frog brains. *Exp. Neurol.* **8**:2901.
4. KRUGER, L., and D. S. MAXWELL. 1966. The fine structure of ependymal processes in teleost optic tectum. *Ann. J. Anat.* **119**:479.
5. LIEBERMAN, A. R., and C. J. TART. 1971. Ependymoglial cells in the frog's brain. *J. Anat.* **109**:366. (Abstr.).
6. GONATAS, N. K., and G. M. SHY. 1965. Virus-like particles in subacute sclerosing encephalitis. *Nature (Lond.).* **208**:1338.
7. GONATAS, N. K. 1966. Subacute sclerosing encephalitis: electron microscopic and cytochemical observations of a cerebral biopsy. *J. Neuropathol. Exp. Neurol.* **25**:277.
8. GONATAS, N. K., J. MARTIN, AND I. EVANGELISTA. 1967. The osmiophilic particles of astrocytes. Viruses, lipid droplets or products of secretion? *J. Neuropathol. Exp. Neurol.* **26**:369.
9. ANDREWS, J. M., and R. L. ANDREWS. 1973. The significance of dense core particles in subacute

demyelinating disease in an adult. *Lab. Invest.* **28**:236.

10. GAMBETTI, P., and S. D. ERULKAR. 1974. Calcium and phosphorus-containing structures in ependymal glial cells of the spinal cord of the frog. *Anat. Rec.* **178**:359. (Abstr.).
11. DALTON, A. J. 1955. A chrome-osmium fixation for electron miscroscopy. *Anat. Rec.* **121**:281. (Abstr.).
12. GAMBETTI, P., S. DIMAURO, and L. BAKER. 1971. Nervous system in Pompe's disease. Ultrastructure and biochemistry. *J. Neuropathol. Exp. Neurol.* **30**:412.
13. PEACHEY, L. D. 1958. Thin sections. I. A study of section thickness and physical distortion produced during microtomy. *J. Biophys. Biochem. Cytol.* **4**:233.
14. 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.
15. HUXLEY, H. E., and G. ZUBAY. 1961. Preferential staining of nucleic acid-containing structures for electron microscopy. *J. Biophys. Biochem. Cytol.* **11**:273.
16. SOMLYO, A. P., A. V. SOMLYO, C. E. DEVINE, P. D. PETERS, and T. A. HALL. 1974. Electron microscopy and electron probe analysis of mitochondrial cation accumulation in smooth muscle. *J. Cell Biol.* **61**:723.
17. POTTER, H. D. 1971. The distribution of neurofibrils coextensive with microtubules and neurofilaments in dendrites and axons of the tectum, cerebellum and pallium of the frog. *J. Comp. Neurol.* **143**:385.
18. BRIGHTMAN, M. W., and T. S. REESE. 1969. Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* **40**:648.
19. RAMON Y CAJAL. 1952. Histologie du systeme nerveux de l'homme et des vertebres. Maloine (Librairie), S. A., Paris. **1**:554–588.
20. KRUGER, L., and D. S. MAXWELL. 1967. Comparative fine structure of vertebrate neuroglia: teleosts and reptiles. *J. Comp. Neurol.* **129**:115.
21. OSCHMAN, J. L., and B. J. WALL. 1972. Calcium binding to intestinal membranes. *J. Cell Biol.* **55**:58.