METHOD FOR COMBINED ULTRASTRUCTURAL AND BIOCHEMICAL ANALYSIS OF NEURAL TISSUE

W. ARNOLDO LEVY, IVAN HERZOG, KUNIHIKO SUZUKI, ROBERT KATZMAN, and LABE SCHEINBERG

From the Saul R. Korey Department of Neurology, and the Departments of Neurological Surgery and Pathology (Neuropathology), Albert Einstein College of Medicine of Yeshiva University, New York

ABSTRACT

No significant change was found in the electrolytes and lipids of the brain analyzed after glutaraldehyde fixation by perfusion of laboratory animals; such fixation also satisfactorily preserves neural tissues for electron microscopy. The brains of normal and tumor-bearing C3H mice, Wistar rats, and New Zealand rabbits were studied. Little difference was found in the dry weight and the content of sodium, potassium, total lipid and lipid fractions, and in the sulfate space ($S^{35}O_4$) between specimens from unperfused and perfused animals, whether normal or tumor-bearing. The results suggest the possibility of using selected regions of the nervous system, dissected after fixation, for chemical study and at the same time characterizing similar regions morphologically with the electron microscope.

The importance of the fixation procedure in the results of electron microscopic study of normal and pathologic neural tissues is now well known. Recently, perfusion technics with OsO_4 (1) or formalin (2) have been used with advantage for fixing these complex and labile structures *in situ*. New fixatives, such as acrolein (3), glutaraldehyde, and other dialdehydes (4, 5) have been found to preserve the cell structures most satisfactorily and, to some extent, several enzymatic activities detectable histochemically. In the work reported here, glutaraldehyde perfusion has been utilized as a method of fixation of the nervous system of mice, rats, and rabbits. We have determined quantitatively the degree to which water and electrolyte exchange can occur, and the lipid structural components be retained, after perfusion fixation.

MATERIALS AND METHODS

The animals were normal and tumor-bearing C3H mice,[1] Wistar rats,[2] and New Zealand rabbits.[3] The tumor tissue for intracerebral implantation in the respective species was mouse mammary carcinoma and rat Walker carcinosarcoma, both carried subcutaneously, and rabbit Brown-Pearce carcinoma carried in the anterior chamber of the eye. The technic used has been described in our earlier reports (6, 7). It was essentially the same for the three species.

[1] Obtained from Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine.
[2] Obtained from Dr. O. Seguira, Sloan-Kettering Institute, New York.
[3] Obtained from Dr. H. Greene, Department of Pathology, Yale University Medical School, New Haven, Connecticut.

The control animals were anesthetized with Nembutal, sacrificed, and drained free of blood. Tissue samples were obtained as in the perfused animals.

Perfusion

All animals were given an injection of labeled sodium sulfate ($S^{35}O_4$) 4 to 6 hours before perfusion. The mice received an injection of 80 μc, rats, 200 μc, and rabbits, 500 μc. In one group of studies, a biopsy specimen was obtained from the rabbit before perfusion.

The perfusate was a 3 per cent glutaraldehyde solution in 0.1 M sodium phosphate buffer, pH 7.3, containing 0.01 per cent $CaCl_2$ (8, 8 a). The osmolality of the perfusate was approximately 650; the sodium content, 205 mEq/liter. The volumes of perfusate and duration of perfusion were determined by trial and error on the basis of the satisfactory quality of the electron micrographs and the possibility of performing biochemical analyses.

	Weight _gm_	Perfusate _ml_	Perfusion _min_
Mouse (descending aorta unclamped)	20–30	6	4
Rat	150–200	30	100
Rabbit	2500–3000	50	10

Na^{24} and K^{42}, 5 to 15 $\mu c/100$ ml, were added to the perfusate of a group of mice, rats, and rabbits, respectively.

After intraperitoneal Nembutal anesthesia, the chest was opened in the midline, the pericardium and right auricle were incised, and blood samples obtained. The left ventricle was then incised; the ascending aorta was threaded with a glass cannula connected to a plastic tube, the descending aorta being clamped just above the diaphragm. Perfusion began within 1 minute of the chest incision.

For a successful perfusion, care should be taken not to bypass the brachiocephalic trunk or tear the aorta, and a clamp should be properly placed on the descending aorta.

The tumor-bearing animals were perfused as soon as signs of tumor growth became evident, usually 9

to 15 days after implantation in mouse and rabbit and 14 to 18 days in the rat.

Tissue Sampling

On removal, the brains were placed in a humid chamber (90 to 100 per cent saturation). Mouse cerebral tissue samples were usually taken from whole brain. Rat and rabbit samples of gray and white matter, weighing 15 to 60 mg, were obtained under the dissecting microscope at a sevenfold magnification. The samples of white and gray matter from tumor-bearing animals were taken from areas adjacent to the lesion and at a distance from it.

Biochemical Analyses

Wet weights were obtained in the humid chamber in order to reduce evaporative loss of water from the small tissue samples. The samples were then dried at 100°C for 12 hours; the difference between wet and dry weights is expressed as a percentage.

Sodium and potassium contents were determined by internal standard flame photometry on nitric acid digests of tissue samples (9); Na^{24} and K^{42} activity, in a well-type gamma scintillation counter; S^{35} in a gas-flow counter.

The "uncorrected sulfate space" was calculated from these data, according to the formula of Barlow *et al.* (10). If the sulfate space is a measure of brain extracellular space, the calculation should be corrected for the incorporation of S^{35} into organic compounds. However, it has been found that approximately one-third of the sulfate is organically bound within 6 hours of perfusion, in both the plasma and normal and edematous brains (11, 12). The sulfate space is therefore not appreciably altered by this correction.

The lipid determinations included total lipid, gangliosides, cholesterol, phosphatides, and cerebrosides. The samples were homogenized with 19 volumes of chloroform-methanol (2:1, v/v) (11), and filtered through a sintered glass funnel to remove the insoluble residue. The extract was partitioned with ⅕ volume of 0.88 per cent potassium chloride, and the lower phase was washed twice with the theoretic upper phase. The initial upper phase was combined with the washings of the lower phase, and dialyzed against water. The sialic acid content was determined

FIGURE 1 Normal mouse; cerebral cortex; glutaraldehyde fixation. Note closely packed neuropil. × 7000.

FIGURE 2 Tumor-bearing mouse; cerebral cortex adjacent to tumor; glutaraldehyde fixation. Mitochondria (*M*), Golgi apparatus (*G*), and endoplasmic reticulum (*ER*) well preserved. × 19,000.
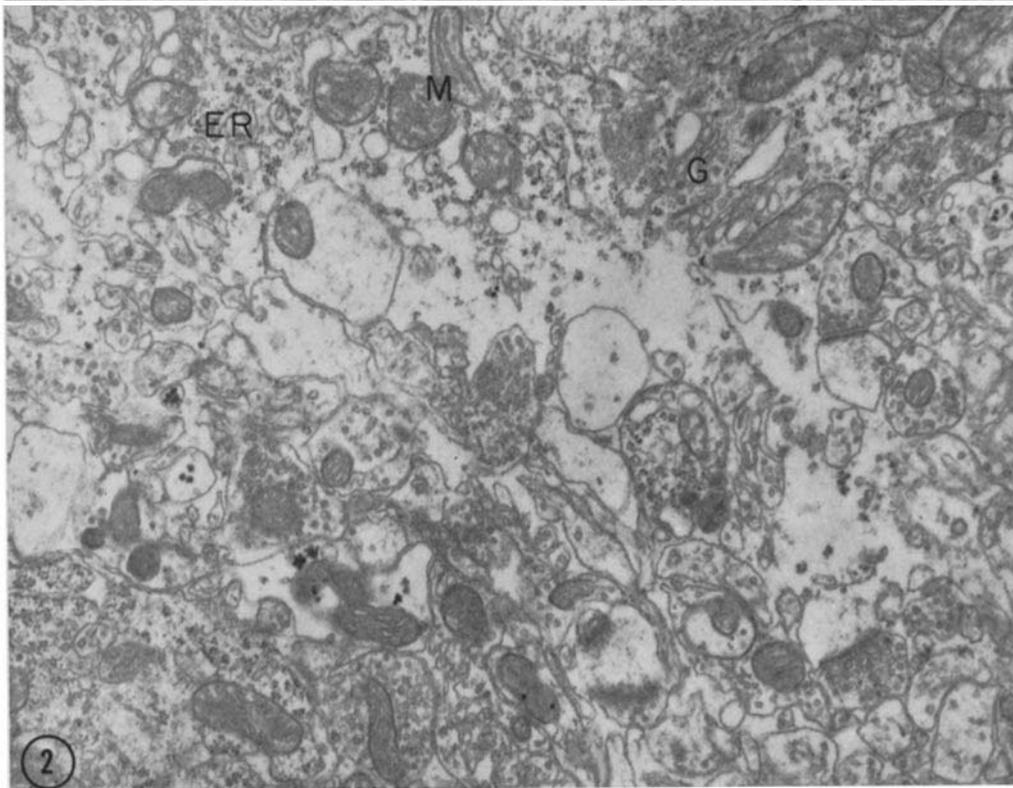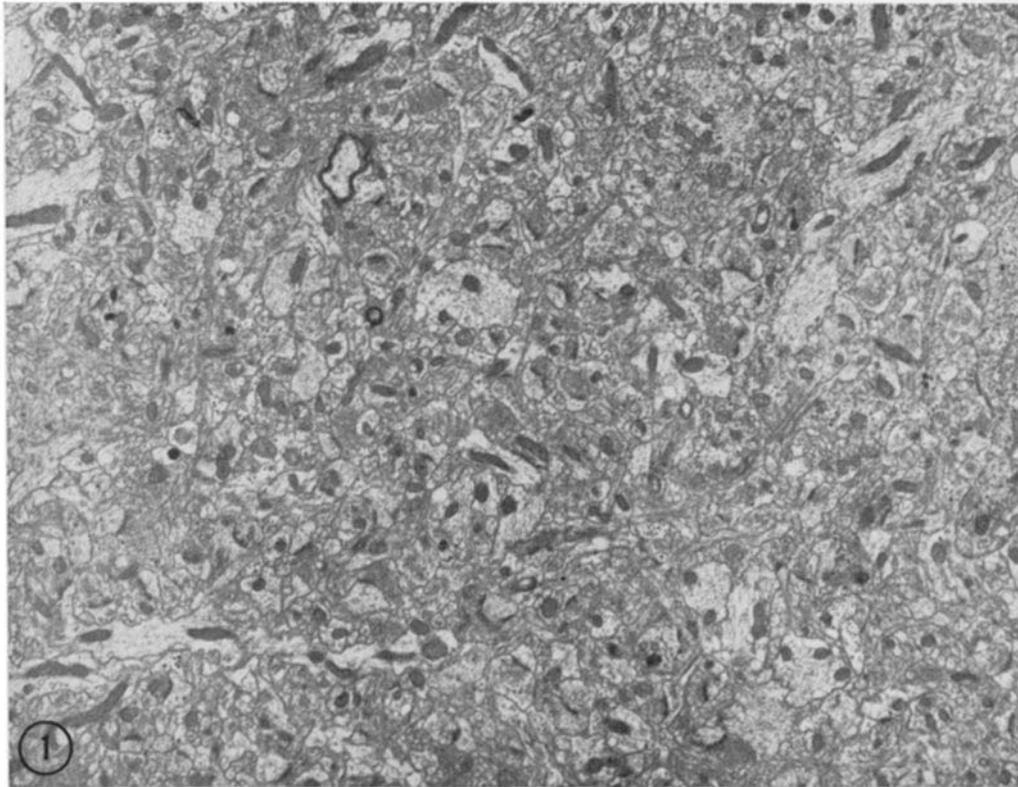
by the thiobarbituric acid method (13), and the gangliosides were calculated from it by a conversion factor of 4.35.

The amounts of cholesterol, cerebrosides, and phosphatides were estimated on the lower phase after separation of the proteolipids and drying and filtration. The cholesterol was determined by the method of Searcy and Bergquist (14); the cerebrosides were calculated by a conversion factor of 4.7 from the total lipid hexose determined by the orcinol method (15) after hydrolysis of the total lipids in ethanolic sulfuric acid. The phosphatides were estimated by a conversion factor of 25 from the phosphorus content determined by the method of Chen and coworkers (16).

Electron Microscopy

The samples initially fixed by glutaraldehyde perfusion were washed in phosphate buffer with 10 per cent sucrose, postfixed for 90 minutes in osmium tetroxide, dehydrated with acetone, embedded in Epon 812, and sectioned on a Porter-Blum microtome with glass knives. The electron microscope used was a Hitachi HS7.

RESULTS

Morphologic

In all successfully perfused animals, the cortical vessels were free of blood. The brains were yellow and firm and were easily sectioned with a razor blade without crushing of tissue. Dissection of the gray and white matter under the dissecting microscope was accomplished without difficulty. This is one of the advantges of glutaraldehyde perfusion-fixation for small animals, in which the amount of white matter is minimal. The edematous tumor-adjacent areas were neither watery nor soft and were also easily dissected.

Electron Microscopic

The electron micrographs of perfused normal cerebral cortex and white matter of mice, rats, and rabbits showed good, uniform preservation of entire tissue blocks. The cellular elements were tightly fitted together, and the extracellular space was not distended (Fig. 1). There was no disintegration of tissue elements, nor shrinkage of cells. The limiting membranes of the cellular components were unbroken and sharp; the internal cellular structure was well preserved, with dense cytoplasm and mitochondria. The endoplasmic reticulum did not have distended or disrupted sacs, and the mitochondrial cristae and double limiting membranes were sharp. One or two mitochondria of every second or third neuron or astrocyte showed an artifactual ballooning generally occupying one-third of the organelle (Figs. 3, 4). Within this area there was no matrix, but the double limiting membrane was intact. The Golgi complex was well preserved, but occasionally an artifactual ballooning, closely resembling that of the mitochondria, was seen (Fig. 5). The nucleus was not surrounded by a halo, and the nuclear chromatin was dense and evenly dispersed. Compact, fine granules (presumably RNA) were present in the nucleolus. Ballooning of the synaptic vesicles was not observed. The neuroglial cell processes contained densely arranged organelles.

The deep white matter was well preserved. The myelinated nerve fibers were close together, with evenly spaced myelin lamellae (Fig. 7). "Explosions" within the myelin sheaths were not seen. The axonal filaments and mitochondria were evenly distributed.

The edematous tissue was also well fixed. In the cortex, distended vacuoles or clear spaces surrounded by an intact membrane were frequently found (Figs. 2, 4, 6). In the white matter, the extracellular edema was delineated by well pre served cellular elements (Fig. 8). The artifactual ballooning of mitochondria and Golgi apparatus was easily distinguishable from the intracellular vesicles of edematous tissue (Fig. 2).

FIGURE 3 Normal rat; cerebral cortex; glutaraldehyde fixation. Cellular organelles well preserved; minute vacuoles present in Golgi apparatus (G), and mitochondria (M); distended, membrane-lined vesicle (V). × 21,500.

FIGURE 4 Tumor-bearing rat; cerebral cortex; glutaraldehyde fixation. Intracellular edema; structures within edematous cell and neuropil well preserved. × 9500.
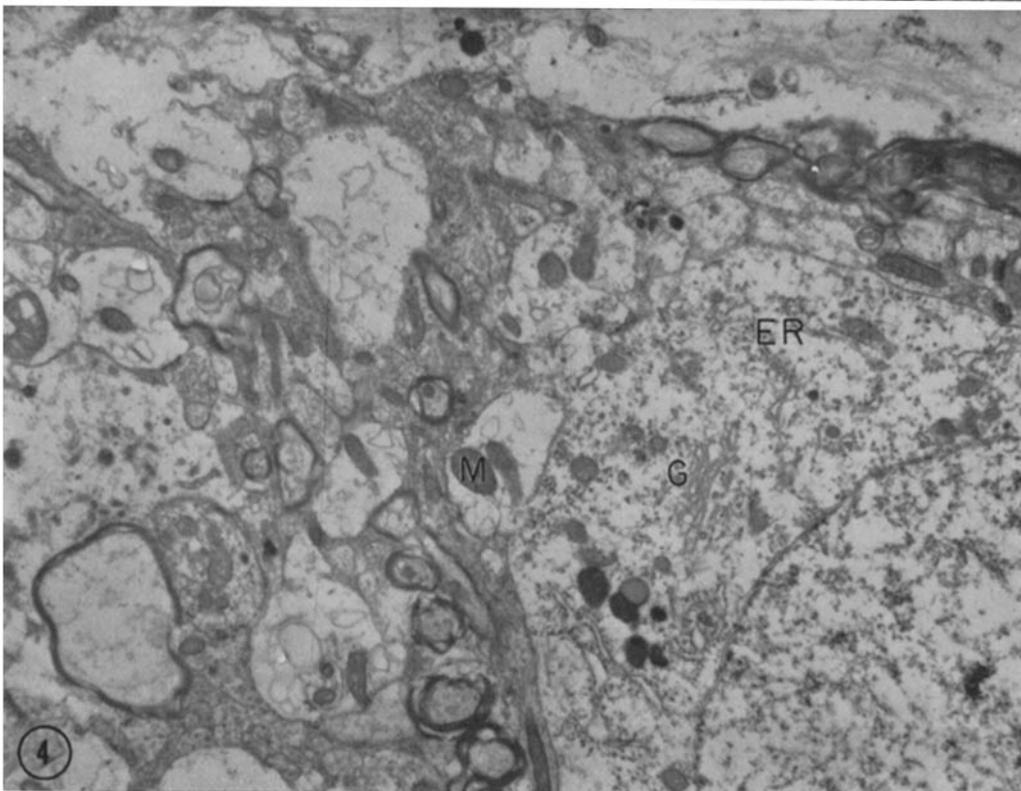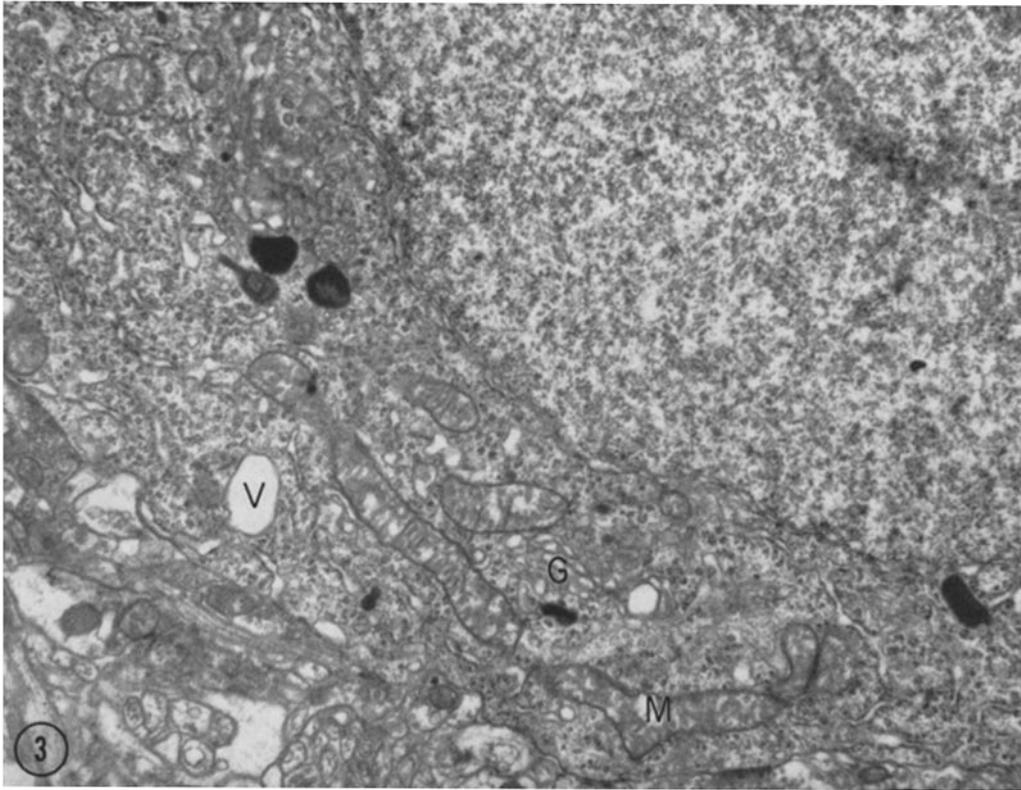
Biochemical

Judging by the results of analyses of samples from normal and tumor-bearing animals, glutaraldehyde perfusion of these small animals *in vivo* had little effect on the chemical parameters studied (Tables I through VI). Since the osmolality of the perfusate (650 osmolals) is high, dehydration might be expected. This is in fact observed with increasing volumes of the perfusate and duration of perfusion (Table 1). However, with the volumes and perfusion times routinely used, the dehydration was minimized, and there was no consistent difference in the average (per cent) dry weight of similar specimens obtained from perfused and non-perfused animals (Tables I and II). The sodium content did not increase with perfusion, although the perfusate contained 205 mEq/liter of Na, nor was there any consistent loss of $S^{35}O_4$, or total lipid or lipid fractions from the cerebral tissues (Table IV). However, the value for potassium in the perfused specimens was, on the average, 4.5 per cent less than in control specimens and was above the variation to be expected from the errors in the method.

Although the net sodium content was unchanged, it seemed desirable to determine whether Na exchange had occurred. This was done by adding Na^{24} to the perfusate (Tables V and VI). The relative activity (or Na^{24} space) of the brain was found to be less than 2 per cent, and less than 3.5 per cent of the sodium was labeled by the procedure. Similarly, the uptake of K^{42} from the perfusate was small (Table VI).

DISCUSSION

Detailed investigation of diseases of the nervous system has made clear the need for adequately fixed tissues which can be used for both electron microscopy and biochemical studies. In order to preserve the ultrastructure of deeper portions of the brain, perfusion fixation has been widely adopted. The osmium tetroxide perfusion technic of Palay and coworkers (1) gives excellent electron microscopic results, but the presence of osmium

tetroxide in the tissues precludes biochemical analyses. Another good fixation method for electron microscopy of neural tissues is the formalin perfusion-fixation technic of Gonzalez Aguilar, and De Robertis (2). Their report gave only the water content of the fixed tissues; whether or not biochemical analyses are possible remains to be seen.

The perfusion method we have used is based on the work of Sabatini *et al.* (4), who introduced glutaraldehyde as a first-stage fixative. Glutaraldehyde had been found by leather chemists to be a useful tanning agent. Sabatini *et al.* found that this molecule penetrates tissue rapidly and fixes the tissue sufficiently for routine histologic and histochemical procedures as well as for electron microscopic cytochemistry. By the use of osmium tetroxide as a second-stage fixative, the tissue then meets the criteria of preservation for electron microscopy previously established by Palade (8, 17) and Palay *et al.* (1). Originally, Sabatini *et al.* (4) immersed their tissue fragments in glutaraldehyde; but perfusion of glutaraldehyde through the blood vessels also fixes tissue, as shown by Webster and Collins (18) for the peripheral nervous system. The advantage of perfusion fixation for the central nervous system lies in the uniform preservation of such structures as the basal ganglia and deep white matter. This is especially important in pathologic processes, particularly in regions where preexistent injury and edema may lead to factitious disruption of cell membranes if fixation is incomplete. One disadvantage of glutaraldehyde perfusion is the presence of occasional minor artifacts, such as the sporadic ballooning of a single mitochondrion or of vesicles in a Golgi complex. However, they were easily identified and differentiated from the changes produced by the pathologic process.

The glutaraldehyde-perfused tissue was firm, even though the perfusion lasted only 5 minutes. It was therefore easy to handle and dissect, and we found that minute lesions were easily identified. Suitable samples for chemical determinations

---

FIGURE 5 Normal rabbit; cerebral cortex; glutaraldehyde fixation. Slight vacuolation within Golgi apparatus (*G*) and mitochondria (*M*). × 21,000.

FIGURE 6 Tumor-bearing rabbit; cerebral cortex; glutaraldehyde fixation. Perivascular edema within cellular processes limited by well-outlined membranes. × 14,500.
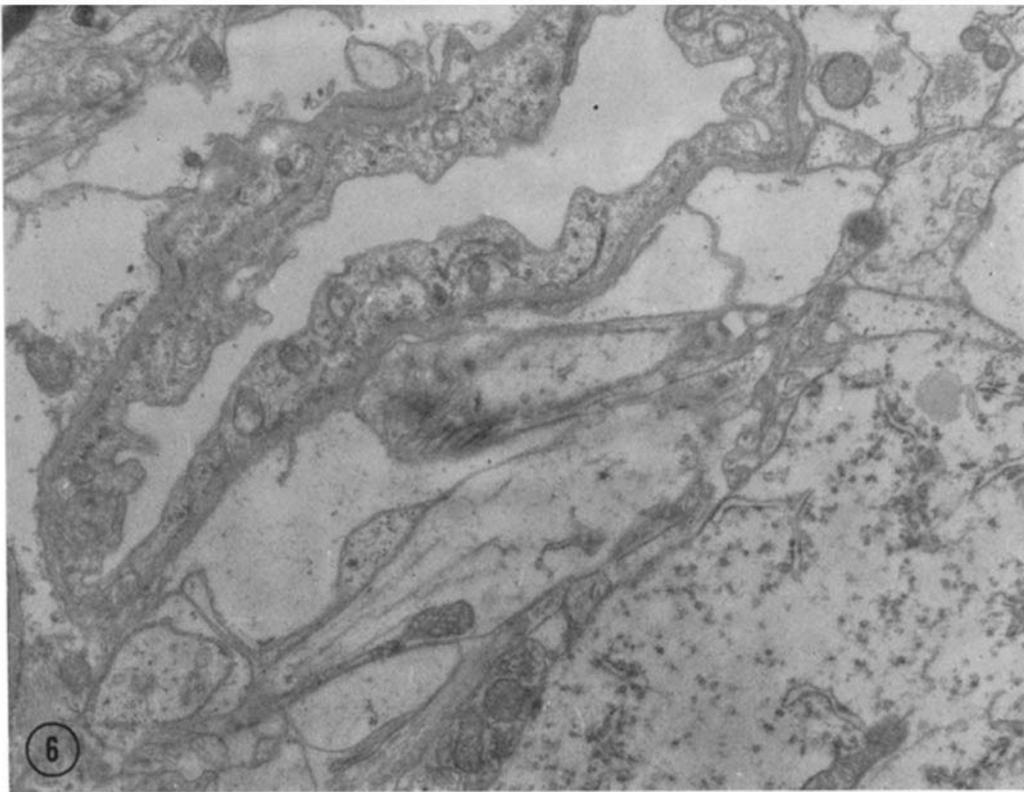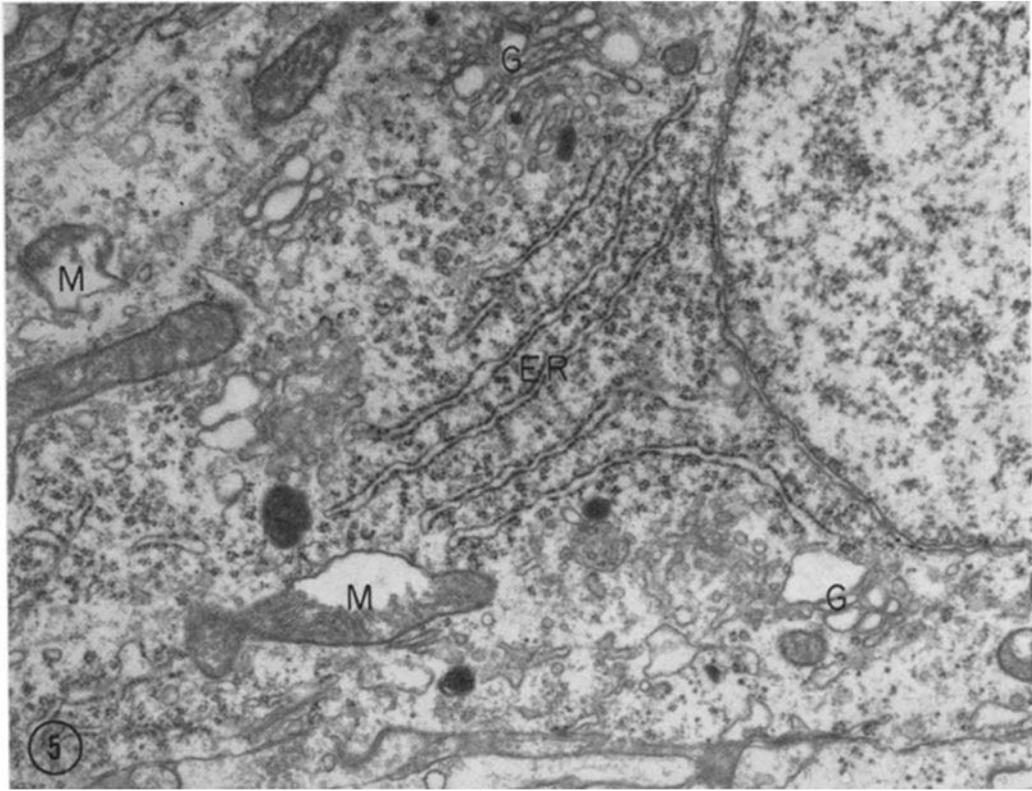
TABLE I

Effect of Varying Perfusion Conditions on Cerebral Tissue of Normal Mice

Perfusate	Perfusion	Dry wt	Sodium-potassium	Sulfate space*
ml	*min*	*per cent*	*molar ratio*	*per cent*
0	0	22.0	0.51	3.1
2	2	23.5	0.54	—
4	2	22.5	0.51	4.6
6	2	24.0	0.54	—
6	4	23.0	0.50	4.9
8	4	24.5	0.54	5.6
10	2	26.5	0.52	—
10	4	25.0	0.53	3.2

* Expressed by the formula: per cent sulfate space (uncorrected)

$$= \frac{(\text{CPM/gm wet tissue}) \times 100.}{\text{CPM/ml plasma}}$$

could therefore be selected, which were weighed and analyzed immediately; the samples for electron microscopy were immersed in osmium tetroxide.

Our most important finding is that glutaraldehyde perfusion caused little change in the water, electrolyte, and lipid contents of normal and pathologic tissues. It is evident from the tables that the standard perfusion did not change brain water content, although prolonged perfusion may have a deleterious effect (Table 1). The perfusate used contained glutaraldehyde and buffer concentrations optimal for tissue preservation. The total osmolality of the perfusate was 650, a markedly hypertonic solution. Approximately half of the osmolality can be accounted for by the buffer, half by the glutaraldehyde; nevertheless, dehydration did not occur. If the capillary wall were impermeable to this molecule, there would be appreciable changes in the water content, even within a few minutes. Brain water has been shown to exchange within seconds with labeled plasma water (19). Moreover, half of the change in water

content produced by hypertonic urea, a partially permeable solute, occurs within 15 minutes (20). The absence of rapid dehydration thus suggests that the capillary wall is freely permeable to glutaraldehyde and that the significant tonicity of the fluid is due to the buffer which is approximately isotonic. In favor of this assumption is the observation that the tissues hardened appreciably during brief glutaraldehyde perfusion, and thus the glutaraldehyde must have diffused rapidly.

The lack of change in tissue electrolyte concentrations (Na, K, and SO_4) may be related to the normal slow exchange of these ions between blood and brain. The uptake of isotopes of these electrolytes by cerebral tissue has been shown to be slower than by other tissues (9). The half-time of the exchange between brain Na and parenteral Na^{24} is about 1½ hours (21); for K, 36 hours (22). The uptake of $S^{35}O_4$, which some authors consider a measure of brain extracellular space, reaches a peak in about 2 hours and then levels off, the brain concentration being only 2 to 6 per cent of the blood level (10).

The exchange of these isotopes during perfusion is shown for Na^{24} and K^{42} influx in Table VI; for $S^{35}O_4$ loss, in Tables II and III. Part of the apparent uptake of Na^{24} and K^{42} may represent perfusate remaining in intravascular spaces. However, even if this is not taken into account, the values obtained for K^{42} are less than those observed in dog brain after 15 minutes of intravenous perfusion (23); for Na^{24}, the uptake is similar to that observed *in vivo* in the rat by Woodbury (21). Thus the blood-brain barrier to Na^{24} and K^{42} exchange remains intact during glutaraldehyde perfusion. Similarly, $S^{35}O_4$ was not washed out except in the single instance shown in Table II, in which the $S^{35}O_4$ content of perfused samples of mouse brain adjacent to implanted tumors appeared to be decreased. In those brain areas, breakdown of the blood-brain barrier was shown by dye uptake (6).

The failure of glutaraldehyde perfusion to increase the permeability of brain to these ions

FIGURE 7 Normal rabbit; white matter; glutaraldehyde fixation. Axon and myelin lamellae well preserved. × 30,000.

FIGURE 8 Tumor-bearing rabbit; white matter; glutaraldehyde fixation. Edema; distended intracellular spaces outlined by well preserved membranes. × 7000.
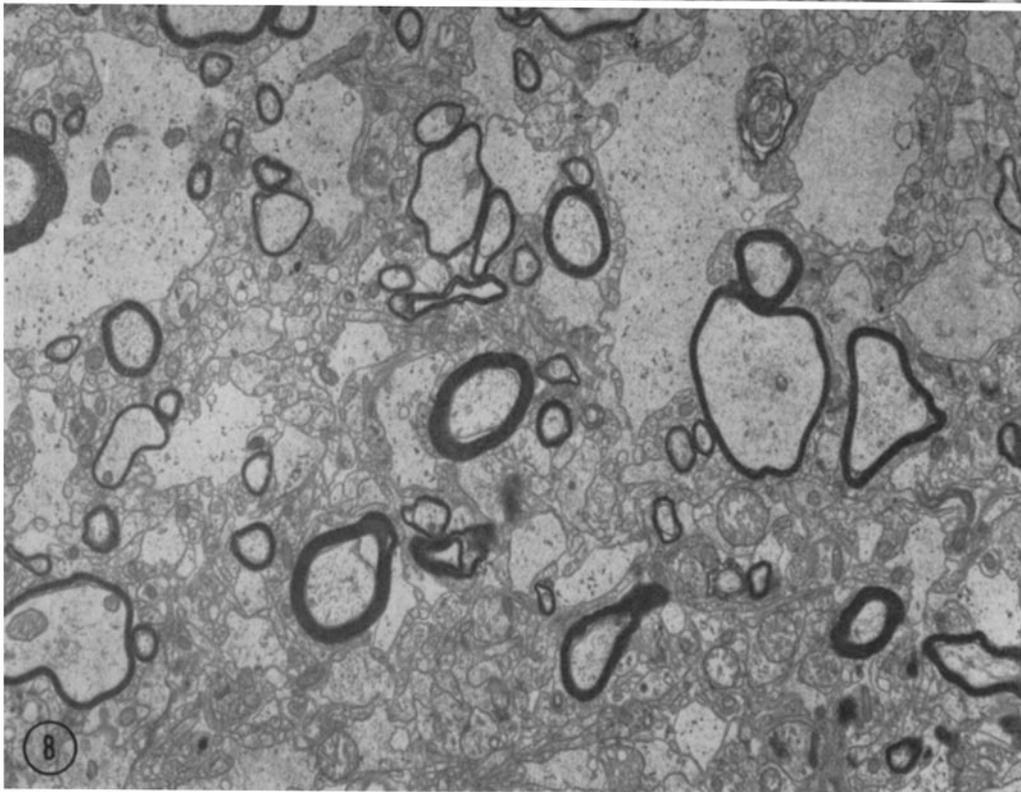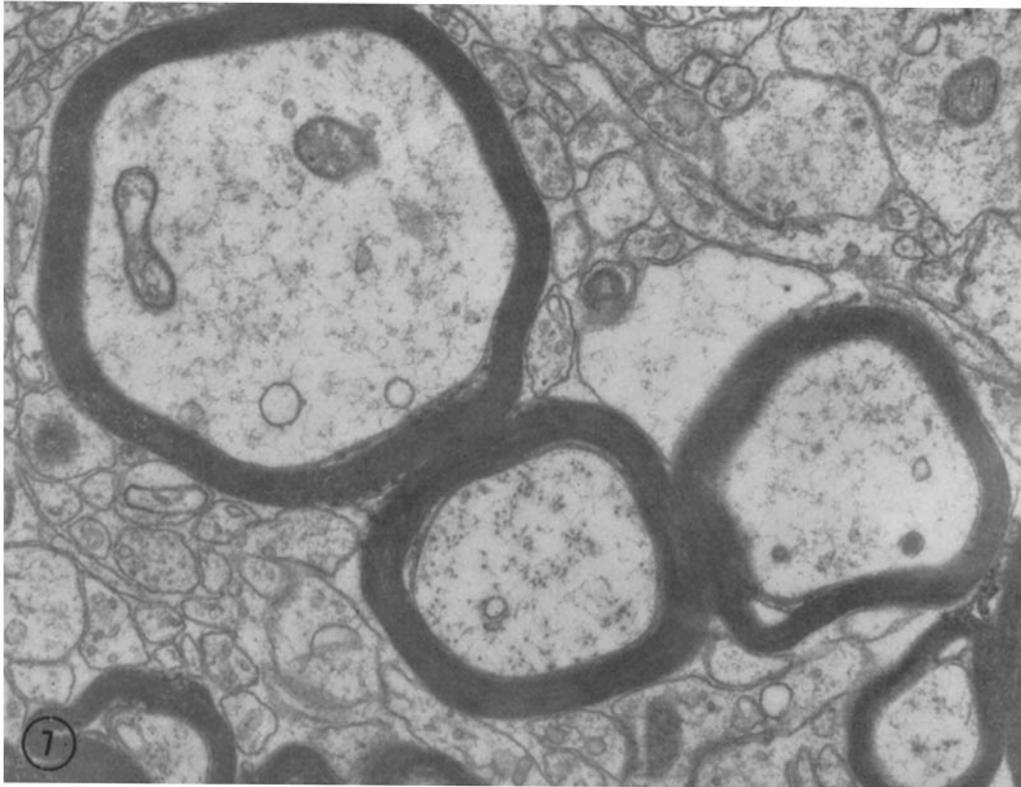
W. A. Levy, I. Herzog, K. Suzuki, R. Katzman, and L. Scheinberg *Neural Tissue* 127

TABLE II

Effect of Perfusion on Water and Electrolyte Content of Cerebral Tissue

	Control*	Perfused‡
Dry weight, *per cent*		
Mice	22.0 (20.4–24.2)	22.9 (21.8–24.0)
Rats		
Gray matter	24.2 (19.0–29.1)	24.0 (16.0–32.0)
White matter	26.5 (22.1–33.3)	28.0 (21.3–37.4)
Rabbits		
Gray matter	19.2 (16.4–22.5)	18.5 (16.1–20.5)
White matter	28.3 (22.4–33.2)	29.6 (26.5–32.4)
Sodium, *mEq/kg wet wt*		
Mice	51 (43–63)	50 (44–58)
Rats		
Gray matter	56 (46–68)	57 (45–80)
White matter	51 (46–82)	55 (45–64)
Rabbits		
Gray matter	60 (48–69)	61 (48–77)
White matter	57 (49–67)	57 (43–65)
Potassium, *mEq/kg wet wt*		
Mice	102 (93–128)	100 (93–112)
Rats		
Gray matter	107 (98–108)	93 (88–105)
White matter	96 (92–104)	91 (82–103)
Rabbits		
Gray matter	91 (79–102)	91 (79–102)
White matter	83 (75–94)	82 (73–99)
Na:K, *molar ratio*		
Mice	0.51 (0.35–0.60)	0.50 (0.45–0.60)
Rats		
Gray matter	0.51 (0.47–0.58)	0.53 (0.51–0.86)
White matter	0.53 (0.47–0.61)	0.61 (0.51–0.86)
Rabbits		
Gray matter	0.64 (0.51–0.79)	0.66 (0.51–0.84)
White matter	0.71 (0.58–0.92)	0.69 (0.53–0.80)
Sulfate space, *per cent*		
Mice	3.1 (1.6–5.4)	4.9 (2.3–9.0)
Rats		
Gray matter	7.1 (4.3–11.1)	6.0 (5.2–8.1)
White matter	7.5 (5.5–14.3)	4.8 (3.4–6.7)
Rabbits		
Gray matter	3.3 (1.8–5.2)	2.8 (0.7–9.5)
White matter	2.3 (0.4–4.3)	1.7 (0.7–6.0)

* Mice, 14 control, 40 perfused; rats, 4 control, 7 and 8 perfused for gray and white matter, respectively; rabbits, 11 control, 18 perfused.

‡ Perfusate: 6 ml for 4 min. for mice; 30 ml for 5 min. for rats; 100 ml for 10 min. for rabbits.

TABLE III

Effect of Perfusion on Water and Electrolyte Content of Cerebral Tissues of Tumor-Bearing Animals*

	Adjacent to tumor		Distant from tumor	
	Control‡	Perfused‡	Control‡	Perfused‡
Dry weight, *per cent*				
Mice	19.3 (17.4–21.9)	20.7 (18.0–24.7)	21.3 (20.7–24.0)	22.5 (21.5–23.0)
Rabbits				
Gray matter	17.7 (15.4–19.8)	18.3 (12.8–20.1)	17.7 (16.2–19.8)	18.2 (15.8–21.6)
White matter	22.8 (17.7–28.6)	22.3 (16.6–25.9)	26.1 (20.8–30.3)	25.2 (19.4–33.2)
Sodium, *mEq/kg*				
Mice	71 (62–84)	76 (54–98)	57 (49–75)	59 (38–71)
Rabbits				
Gray matter	70 (48–101)	63 (54–92)	64 (56–73)	64 (50–78)
White matter	70 (59–93)	72 (49–87)	64 (52–78)	67 (42–82)
Potassium, *mEq/kg*				
Mice	77 (68–89)	95 (64–106)	95 (84–104)	96 (68–110)
Rabbits				
Gray matter	85 (75–95)	76 (54–96)	87 (67–95)	80 (55–103)
White matter	68 (59–75)	57 (32–80)	70 (58–85)	65 (46–81)
Na:K, *molar ratio*				
Mice	0.92 (0.67–1.11)	0.86 (0.66–1.21)	0.60 (0.49–0.81)	0.60 (0.52–0.78)
Rabbits				
Gray matter	0.83 (0.58–1.06)	0.88 (0.57–1.13)	0.74 (0.62–1.03)	0.82 (0.56–1.15)
White matter	1.05 (0.89–1.48)	1.28 (0.62–2.60)	0.93 (0.64–1.34)	1.06 (0.62–1.46)
Sulfate space, *per cent*				
Mice	19.2 (14.0–24.0)	11.7 (6.5–19.6)	7.7 (3.0–11.0)	5.8 (2.1–10.7)
Rabbits				
Gray matter	6.8 (2.4–14.0)	5.3 (2.8–8.5)	3.0 (2.2–3.9)	3.0 (0.8–6.3)
White matter	8.2 (2.9–13.9)	9.2 (1.6–20.0)	3.2 (0.8–8.3)	3.6 (1.1–10.7)

* 6 ml of perfusate for 4 min. and 100 ml for 10 min. for mice and rabbits, respectively.
‡ Mice, 10 control, 9 perfused; rabbits, 12 control, 17 perfused.

TABLE IV

Effect of Perfusion on Lipid Content of Whole Brain Tissue

	Total lipids	Proteolipid proteins	Gangliosides	Lipid fractions		
				Cholesterol	Phosphatides	Cerebrosides
	per cent of wet weight			*per cent of total lipids*		
Mice						
Control	8.22	0.585	0.187	21.0	56.5	17.8
Perfused	6.63	0.562	0.207	20.9	58.8	15.7
Rats						
Control	8.07	0.354	0.183	15.8	59.7	18.3
Perfused	9.20	0.433	0.202	16.8	57.9	20.1
Rabbits						
Control	7.65	0.562	0.222	19.4	62.8	15.7
Perfused	7.75	0.558	0.196	19.7	60.7	17.3

TABLE V

Example of Na^{24} Uptake from Perfusate by Mouse Intracerebral Tumor and Cerebral Tissues

	Perfusate	Tumor	Gray matter		White matter	
			Adjacent	Distal	Adjacent	Distal
Activity, CPM/*ml* or *gm* wet wt	178,000	30,800	1531	3050	2420	2070
Relative activity, $\left(\dfrac{\text{CPM}/gm\ wet\ wt}{\text{CPM}/ml\ perfusate}\right) \times 100$	100	17.30	0.85	1.71	1.36	1.1
Sodium, $\mu Eq/gm$ wet wt	207	61	65	101	46.5	71.5
Specific activity, CPM/μEq Na	0.86	0.28	0.013	0.017	0.029	0.016
Relative specific activity*		32.5	1.51	1.98	3.37	1.86

* Ratio of specific activity of brain to specific activity of perfusate.

might seem anomalous, in view of the widespread alteration in protein that is taking place. However, it might be argued that denaturation of membrane protein might reduce rather than increase membrane permeability, provided the lipid structure of membranes is not disrupted. The electron micrographs demonstrate the integrity of the lipid structure of membranes after glutaraldehyde perfusion. Mueller et al. (24) have found that the electrical resistance is exceedingly high in a membrane model consisting of a bimolecular layer of brain lipids; these membranes permit little water and electrolyte to pass. Only upon addition of specific protein is the electrolyte resistance reduced and permeability increased. Denaturation of this protein then reduces permeability.

Although glutaraldehyde, like formaldehyde, is a tanning agent which reacts with many functional groups of proteins, producing cross-linking and rendering them inert to digestion with trypsin, not all functional groups are so altered (25, 26). Thus, formaldehyde-treated toxins may lose their potency yet serve as excellent antigens. With glutaraldehyde, Sabatini et al. (4) have shown a wide range of preservation of enzyme activity, from acetylcholinesterase to cytochrome oxidase. Such preservation of the adenosine triphosphatases, enzymes intimately related to Na and K

transport, may also play a role in the conservation of these electrolytes during perfusion.

The integrity of the lipid structure of membranes after glutaraldehyde perfusion is consistent with the finding that the perfusion did not alter the concentration of complex lipids. In particular, cholesterol, which is the brain lipid most easily extracted, was unchanged. Although glutaraldehyde is a small organic molecule, it is in fact relatively insoluble in oils and organic solvents. Moreover, it apparently did not react chemically with the lipids, at least to the extent of changing their solubility characteristics during a chloroform-methanol extraction. Whether other chemical changes occurred cannot be determined from the present data.

The glutaraldehyde-perfusion method used gives great promise as a useful tool for biochemical study carried out in conjunction with ultrastructural analysis. It permits analysis of water, electrolytes, and total lipids as well as various histochemical procedures, as described by Sabatini *et al.* (4, 5). But it must be emphasized that the chemistry of glutaraldehyde is as yet poorly understood, and that before tissue perfused with this fixative is used for the analysis of any other chemical moiety, the effect of glutaraldehyde on such a chemical must be specifically evaluated.

REFERENCES

1. PALAY, S. L., McGEE-RUSSELL, S. M., GORDON, S., and GRILLO, M., Fixation of neural tissues for electron microscopy by perfusion with solutions of osmium tetroxide, *J. Cell Biol.*, 1962, **12**, 385.
2. GONZALEZ AGUILAR, F., and DE ROBERTIS, E., A formalin-perfusion fixation method for histophysiological study of the central nervous system with the electron microscope, *Neurology*, 1963, **13**, 758.
3. VAN DUIJN, P., Acrolein-Schiff, a new staining method for proteins, *J. Histochem. and Cytochem.*, 1961, **9**, 234.
4. SABATINI, D. D., BENSCH, K., and BARRNETT, R. J., Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation, *J. Cell Biol.*, 1963, **17**, 19.
5. SABATINI, D. D., MILLER, F., and BARRNETT, R.

J., Aldehyde fixation for morphological and enzyme histochemical studies with the electron microscope, *J. Histochem. and Cytochem.*, 1964, **12**, 57.
6. ALEU, F. P., EDELMAN, F. L., KATZMAN, R., and SCHEINBERG, L. C., Ultrastructural and biochemical analysis in cerebral edema associated with experimental mouse gliomas, *J. Neuropath. and Exp. Neurol.*, 1964, **23**, 253.
7. SCHEINBERG, L. C., SUZUKI, K., EDELMAN, F. L., and DAVIDOFF, L. M., Studies in immunization against a transplantable cerebral mouse glioma, *J. Neurosurg.*, 1963, **20**, 312.
8. PALADE, G. E., The fixation of tissues for electron microscopy, *in* Proceedings of the Third International Conference on Electron Microscopy, 1954, London, Royal Microscopical Society, 1956, 129.
8 *a*. HARKIN, J., personal communication.
9. KATZMAN, R., Electrolyte distribution in mam-

malian central nervous system. Are glia high sodium cells? *Neurology*, 1961, 11, 27.

10. BARLOW, C. F., DOMEK, N. S., GOLDBERG, M. A., and ROTH, L. J., Extracellular brain space measured by S^{35} sulfate, *Arch. neurol.*, 1961, 5, 102.

11. KATZMAN, R., ALEU, F., and WILSON, C., Further observations on triethyltin edema, *Arch. neurol.*, 1963, 9, 178.

12. KATZMAN, R., GONATAS, N., and LEVINE, S., Electrolytes and fluids in experimental focal leukoencephalopathy, *Arch. neurol.*, 1964, 10, 58.

13. WARREN, L., Thiobarbituric acid assay of sialic acids, *J. Biol. Chem.*, 1959, 234, 1971.

14. SEARCY, R. L., and BERGQUIST, L. M., A new color reaction for serum cholesterol, *Clin. Chim. Acta*, 1960, 5, 192.

15. SVENNERHOLM, L., Quantitative estimation of cerebrosides in nervous tissue, *J. Neurochem.*, 1956, 1, 42.

16. CHEN, P. S., TORIBARA, T. Y., and WARNER, H., Microdetermination of phosphorus, *Anal. Chem.*, 1956, 28, 1756.

17. PALADE, G. E., A study of fixation for electron microscopy, *J. Exp. Med.*, 1952, 95, 285.

18. WEBSTER, H. DE F., and COLLINS, G. H., Comparison of osmium tetroxide and glutaraldehyde perfusion fixation for the electron microscopic study of the normal peripheral nervous system, *J. Neuropath. and Exp. Neurol.*, 1964, 3, 109.

19. BERING, E. A., Water exchange in the brain and cerebrospinal fluid. Studies on the interventricular instillation of deuterium (heavy water), *J. Neurosurg.*, 1954, 11, 234.

20. REED, D. J., and WOODBURY, D. M., Effect of hypertonic urea on cerebrospinal fluid pressure and brain volume, *J. Physiol.*, 1962, 164, 252.

21. WOODBURY, D., Fine structure of the neuropil in relation to neuroglia cells, *in* Biology of Neuroglia, (W. Windle, editor), Springfield, Illinois, Charles C Thomas, Publisher, 1958, 99–119.

22. KATZMAN, R., and LEIDERMAN, P. H., Brain potassium exchange in normal adult and immature rats, *Am. J. Physiol.*, 1953, 175, 263.

23. RUDOLPH, G. G. and OLSEN, N. S., Transfer of potassium between blood, cerebrospinal fluid and brain tissue. *Am. J. Physiol.*, 1956, 186, 157.

24. MUELLER, P., RUDIN, D. O., TI TIEN, H., and WESCOTT, W. C., Reconstitution of excitable cell membrane structure *in vitro*, *Circulation*, 1962, 26, 1167.

25. FRENCH, D., and EDSALL, J. T., The reactions of formaldehyde with amino acids and proteins, *in* Advances in Protein Chemistry, New York, Academic Press, Inc., 1945, 2, 277–335.

26. FEIN, M. L., and FILACHIONE, E. M., Tanning studies with aldehydes, *J. Am. Leather Chemists' Assn.*, 1957, 52, 17.