Acta Neuropathologica 2, 144—160 (1962)

Section of Neuropathology, Surgical Neurology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda 14, Md., U.S.A.

The Application of Fluorescein Labeled Serum Proteins (FLSP) to the Study of Vascular Permeability in the Brain*

By

IGOR KLATZO, JAIME MIQUEL and RICHARD OTENASEK

With 19 Figures in the Text (4 in colour)

(Received February 22, 1962)

The morphological investigations concerning vascular permeability in the brain and the related problem of the blood-brain-barrier have relied mainly on the use of acid dyes. However, the protein binding affinity of these compounds e.g. trypan blue, has led to some controversial interpretations regarding the extent to which the observed permeability phenomena were attributable to the penetration of the dye-bound protein or of the dye itself (GREGERSEN and RAWSON; TSCHIRGI 1950, 1952; BROMAN 1949, 1955). It may be assumed that this problem could be considerably elucidated if the penetration of these substances could be evaluated separately. With this consideration in mind fluorescein labeled serum protein conjugates (FLSP) as well as free fluorescein isothiocyanate (FITC) tracer were utilized to investigate vascular permeability in the present study.

It is evident from our electrophoretic observations that the remarkable albumin binding capacity of FITC prevented the assessment of the penetration of the free fluorescent marker. However the treatment of the protein conjugates with activated carbon provided protein preparations completely free of the unbound dye and thereby afforded an opportunity for observations on vascular permeability with respect to proteins.

The present study was undertaken on cats which were injected intravenously with fluorescent indicators. Morphological observations on vascular permeability to these compounds were derived from normal brain tissue as well as from areas where the blood-brain-barrier was artifically abolished by the cold injury using the technique previously described (KLATZO et al.).

Material and Methods

Bovine albumin (crystalline and fraction V)[1], bovine gamma globulin (fraction II)[1], cat fibrinogen, and cat serum were conjugated with fluorescein isothiocyanate[1] according to the method originally described by COONS and KAPLAN. In order to remove the free dye the conjugates were subjected to the following procedure. Activated carbon (norit-A)[2] was added to a protein conjugate in the ratio of 1 g of carbon to 1 g of protein. The mixture was magneti-

* From the Section of Neuropathology, Surgical Neurology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service, U.S. Department of Health, Education and Welfare, Bethesda 14, Maryland.

[1] Nutritional Biochemical Corporation.

[2] Fisher Scientific Company.

cally stirred for one hour at 4°C and then left overnight in the refrigerator. The carbon was removed by centrifugation and the entire procedure was repeated once again. After carbon adsorbtion the conjugate was dialysed at 4°C for 2 days against 0.15 M NaCl with the saline changed at least twice daily. The final p_H of the conjugate was adjusted to 7.4 with 0.2 M phosphate buffer.

For electrophoretic analysis of conjugates and of free FITC, 0.006 ml samples containing a conjugate or 0.02% FITC solution in saline were used. Electrophoresis was conducted in M/15 phosphate buffer, p_H 7.4, at 100 V., for 18 hours. The electrophoretic strips were examined and photographed under ultra violet light.

The protein binding capacity of FITC *in vitro* was assessed by mixing equal amounts of cat serum and 0.02% FITC solution and subjecting 0.006 ml samples of this mixture to paper electrophoresis under the conditions as described above.

The protein binding capacity of FITC *in vivo* was investigated by electrophoresis of serum samples derived from cats injected intravenously with the FITC. The animals received 10 ml of solution containing 100 mgm of FITC in 0.15 M NaCl, p_H 7.4. Serum samples were taken at 1 hour, 6 hours, 24 hours, and 48 hours after the injection.

Local brain injury was produced in cats by application of a cold metal plate (−50°C) to the exposed cortex.

Prior to the cold injury the cats were injected intravenously with various protein conjugates (20 ml of 8% protein solution) or with free fluorescent marker (10 ml of 0.5% FITC solution in 0.15 M NaCl, p_H 7.4). The animals were sacrificed after the cold injury at various time intervals either by decapitation or by cutting the major blood vessels in the thorax. Some cats were perfused at the time of sacrifice by the following technique. A cannula was introduced through the left ventricle into the ascending aorta, the descending aorta was clamped, and the right auricle was opened. The perfusate consisted of Ringer's solution, warmed to body temperature, followed by 10% formol-saline. The number of animals used, the type of fluorescent indicator, and the time interval are summarized in Table 1.

Table 1. *Number of cats subjected to cold injury and the time of the sacrifice*

	6 hrs	24 hrs	48 hrs	3 d	5 d	7 d	13 d	
Injected with FITC	6	6	3		1			
Injected with FLA	8;6p*	12	8	10	5	5	4	
Injected with FLG**	4;2p	6	4	4	3		2	
Injected with FLF		4						
Injected with FLS***		3	2	1				
Non-injected (for FAN stain)			4					Total Number
Totals	26	31	21	15	9	5	6	113

* p = perfusion; ** FLG = fluorescein labeled globulin; *** FLS = fluorescein labeled serum.

The brains were removed and sectioned coronally through the area of lesion into blocks 3—4 mm in thickness. For gross observations and photography the specimens were illuminated with a Hanovia U.V. lamp. Superanscochrome Daylight film in conjunction with U.V. 16, Y-70 and M-10 filters was used for color photography while black and white photographs were obtained on Adox KB-14 film using G-15 filter.

The tissue blocks were fixed overnight in 10% formol-saline. Sections 6—10 μ in thickness were cut on the freezing microtome and mounted with 50% glycerol. The details of fluorescence microscopy were as follows: light source—HBO 200 mercury vapor lamp; exciter filter—BG 12; barrier filters—GG 4 and OG 4; condensor—dark field; films—Superanscochrome Daylight and Adox KB-14.

In addition to the unstained preparations for the fluorescence microscopy, adjacent frozen sections were stained with the FAN method (basic fuchsin, amido-black 10 B and naphtol

yellow) introduced by MIQUEL and CALVO. The identity of various cellular elements observed in fluorescence optics was occasionally confirmed either by switching from fluorescence to phase-contrast optics without moving the slide or by staining the preparations with FAN and refocusing on the same microscopic field.

Results

A. Electrophoretic assessment of the fluorescent tracers

The electrophoretic pattern of the free FITC was characterized by four distinct bands which fluoresced bright green under the U.V. light (Fig. 1 A). Conversely, the carbon adsorbed protein conjugates revealed a complete absence of the free FITC pattern, and the only evident green fluorescence corresponded strictly in location to the protein component of the conjugate, indicating a

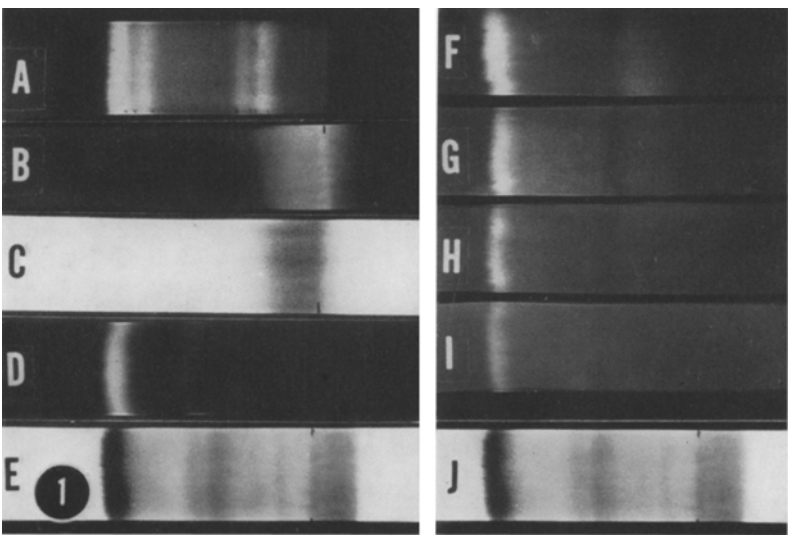


Fig. 1 A—J. A Electrophoretic pattern of free FITC showing 4 characteristic bands. B Bovine gamma globulin conjugate after carbon adsorbtion and dialysis. Electrophoretic pattern of free FITC is not recognizable. The green fluorescence is localized within an area close to the starting line. C Staining of a strip as shown in B with brom phenol blue confirms that the green fluorescent area seen in B corresponds in location to gamma globulin. D Strip representing cat serum mixed with FITC *in vitro*. Green fluorescence of FITC corresponds in location to the albumin band. E Electrophoresis of cat serum, stained with brom phenol blue. F Serum sample taken 1 hour after intravenous injection of FITC. The fluorescence is seen mainly in the albumin area. Smudgy, faint fluorescence extending beyond this band is due to the excess of free dye in the serum (also noticeable in G). G 6 hour sample. H 24 hour sample. I 48 hour sample. The green fluorescence corresponds strictly to the albumin band. J Normal cat serum stained with brom phenol blue

thorough removal of the unbound FITC from the preparation (Fig. 1 B and C). Mixing cat serum with FITC *in vitro* demonstrated a strong and almost selective binding of FITC by the albumin. The electrophoretic strip presented in Fig. 1 D shows this phenomenon clearly. This property of FITC to form a complex with albumin was retained also *in vivo* as is evident from the electrophoretic strips shown in Fig. 1 F, G, H and I. Although the serum samples, obtained one and six hours after intravenous FITC injection, showed some smudgy fluorescence outside of the albumin band (which is undoubtedly due to the excess of free dye in the serum), the 24 and 48 hour samples revealed fluorescence confined only to the albumin fraction and thus presented an electrophoretic picture identical to that which would be obtained by injecting the fluorescein labeled albumin (FLA).

B. Observations on the normal brain

The observations on the normal brain were obtained from 6 control cats injected with FLSP or from operated animals in regions distant to the cold lesion and to the areas of edema.

The various injected tracers showed no difference with regard to their distribution in the normal brain and they were easily detectable by a characteristic bright green fluorescence. This fluorescence was distinct from the autofluorescence of various tissue constituents of the normal brain with the exception of the elastic laminae of the larger blood vessels which appeared greenish in color. With the combination of the barrier filters used (GG4, OG4), numerous cytoplasmic pigment granules (present in the neurons, glia, ependyma, choroidal epithelium, as well as in the perivascular or leptomeningeal macrophages) were easily distinguishable from the tracer by their orange autofluorescence. It should be noted, that with barrier filters which have transmittance limited more to the yellow-red end of the visible spectrum, these pigment granules may appear close in color to that of the fluorescein indicator and thus possibly provide a source of error in interpretation. The normal brain parenchyma generally exhibited a bluish autofluorescence which was more marked in the white matter due to the presence of intensely blue autofluorescent myelinated fibers.

In the animals without intravascular perfusion, the brain tissue with exception of the special areas mentioned below, showed the FLSP confined to the lumina of the blood vessels, regardless of their caliber. No green fluorescence could be detected either in the endothelial cells or in the underlying basement membranes. A different picture was observed in the choroid plexus, area postrema and the tuber cinereum, i.e., regions which are generally credited with special permeability relationships in the CNS. Other similar areas, such as the pineal body and intercolumnar tubercle, were not examined.

The choroid plexus exhibited a distinct green fluorescence of the connective tissue fibers surrounding the blood vessels and underlining the epithelial layer (Fig. 17). The choroid epithelium cells frequently contained orange pigment granules in their cytoplasm but showed no presence of the green fluorescence. The area postrema and the tuber cinereum resembled the choroid plexus in the intense green staining of the perivascular connective tissue fibers of the blood vessels which are characteristically large and sinusoidal in these areas (Figs. 2 and 3). However, in no instance was there any evidence of FLSP passage into the parenchyma outside of the vascular structures.

C. Observations on the area of cold injury

The localized cold lesion used in the present study has been previously described in detail (KLATZO et al.). In brief, the application of a metal plate cooled to $-50°C$ to the exposed cortex of the cat for one minute results in a saucer shaped, sharply demarcated necrosis of the superficial cortical layers, the deeper layers of the cortex usually remaining intact. Within a few hours after cold application the underlying white matter shows evidence of edema, which extends into the white matter of adjacent gyri and approaches the lateral ventricle. The microscopic examination of the area of edema with routine histological methods

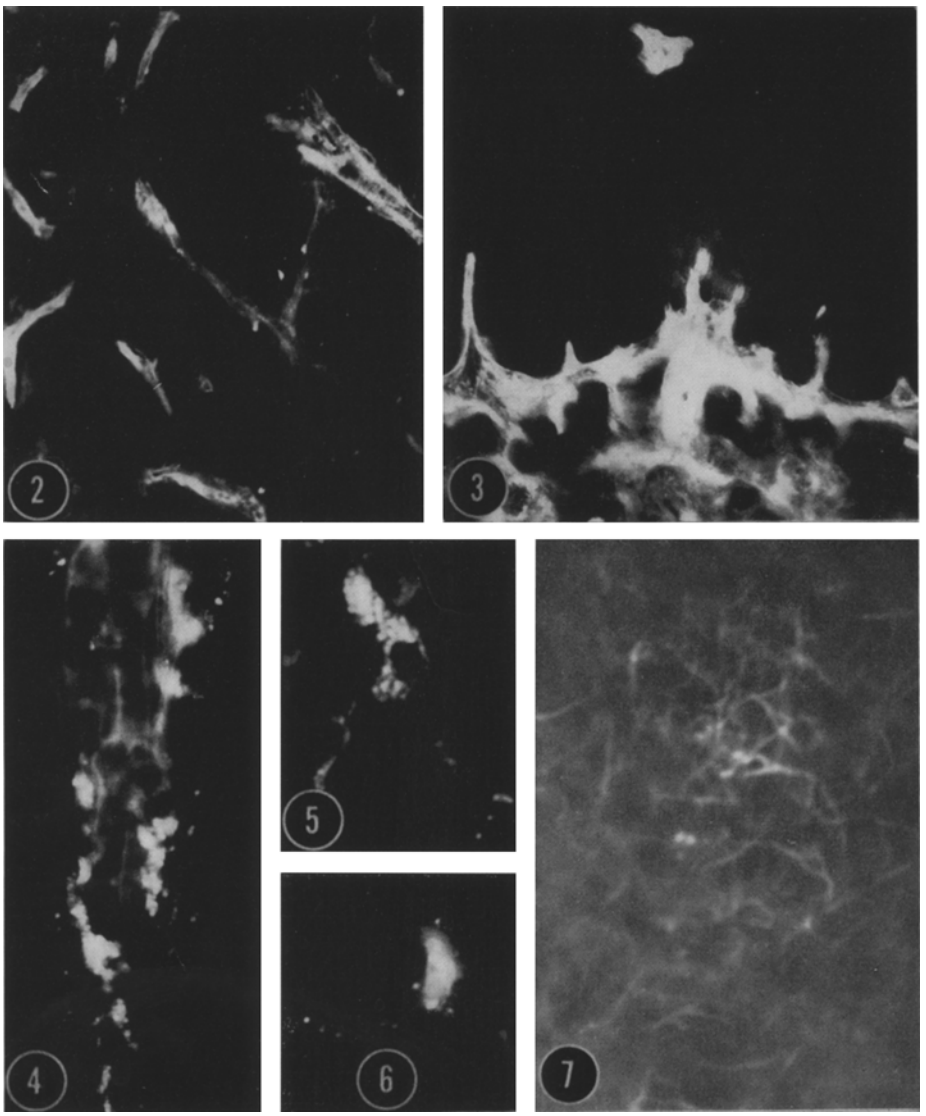


Fig. 2. Area postrema. Cat injected with FLA. Bright green fluorescence of the connective tissue investitures of th prominent blood vessels. × 120

Fig. 3. Tuber cinereum. Cat injected with FLG. Intense green fluorescence of the connective tissue elements which invest the sinusoidal blood vessels. × 120

Fig. 4. White matter of the gyrus subjected to cold injury. Cat injected with FLA and sacrificed 3 days after the operation. Amoeboid microglial cells filled with discrete green inclusions surround a blood vessel. × 340

Fig. 5. Amoeboid type of the microglia filled with FLA inclusions. White matter of the gyrus subjected to cold injury. Cat injected with FLA and sacrificed 24 hours after the operation. × 650

Fig. 6. A "resting" type of the microgliacyte in the white matter, cat injected with FLA and sacrificed 7 days after cold injury. The cell shows a diffuse green fluorescence of the nucleus and the faint outline of the processes. Few green inclusions are visible in the cytoplasm

Fig. 7. Edematous white matter in the cat injected with FLF and sacrificed 24 hours after cold injury. Note the network of filamentous green threads without any spatial relationship either to cellular elements or to blood vessels. × 450

does not reveal any appreciable destruction of tissue elements: the myelinated fibers, although spread wider apart, remain preserved, and no lipoidal debris in the microglia can be detected. The duration of edema in the white matter, as ascertained by the size of the gyrus and by the general histological appearance does not persist beyond 5—7 days, and the final resulting lesion consists of a superficial cortical scar and hypertrophic astrocytes in the area of detumescence.

Observations on the cold lesion with fluorescent tracers revealed no conclusive difference in the morphological appearance among the individual protein conjugates and the FITC with the exception of labelled fibrinogen (FLF), which will be described separately. In view of the remarkable affinity of FITC to the albumin in the serum as shown by the electrophoretic study, it may be assumed that the observations with the FITC demonstrated essentially the penetration of FLA.

Cats sacrificed *6 hours* after the cold injury without intravascular perfusion showed grossly an area of green fluorescence in the white matter of the injured gyrus and extending with a striking predilection into the white matter of the adjacent gyri (Fig. 16). Microscopically the area of cortical necrosis was filled with green fluorescent serum exudate. The neurons within the necrotic lesion and in a zone surrounding this area were conspicuous by their intense, diffuse green fluorescence (Fig. 8). Otherwise, the cerebral cortex adjacent to the necrotic area showed occasional green fluorescent extravasates confined to the immediate vicinity of the blood vessels. The white matter, which showed green fluorescence to the naked eye, revealed a characteristic pattern microscopically which can be best described as a "mottled" appearance. As can be seen from Fig. 18 it was not possible to relate this green "mottling" to outlines of the glial elements and there was no demonstrable relationship between FLSP fluorescence and the traversing parallel nerve fibers which were conspicuous by their blue autofluorescence. In some areas at the periphery of the "mottled" pattern occasional astrocytes were noted to contain the characteristic, pinocytotic FLSP inclusions as described in our previous communication (KLATZO and MIQUEL). These discrete, bright green inclusions were distributed predominantly in the astrocytic expansions, but occasionally they were especially prominent in the vascular foot-plates (Fig. 11).

In the cats which were sacrificed 6 hours after injury and perfused intravascularly, the "mottled" FLSP fluorescence appeared to be reduced in extent and intensity or almost absent. The white matter in the area of edema, which was free of the green "mottling" showed numerous astrocytes and occasional oligodendrocytes with discrete FLSP inclusions conspicuously visible on the blue autofluorescent background (Fig. 19).

In the animals injected with various fluorescent tracers (excluding FLF) and sacrificed *24 hours* after the cold injury the "mottled" green fluorescence appeared to be less pronounced in comparison to that observed in 6 hours non-perfused cats. The remaining "mottled" pattern tended to be more persistent in the immediate subcortical zones of the edematous white matter, whereas the more central areas were usually free of "mottling" and revealed numerous astrocytes and oligodendrocytes with discrete FLSP inclusions. Most of these neuroglial[1] cells with

[1] The term "neuroglia" in this study applies to astrocytes and oligodendrocytes. The term "glia" includes also the microglia cells.

the green inclusions remained practically invisible themselves; nevertheless, the localization of the FLSP inclusions within these cells could be inferred from the

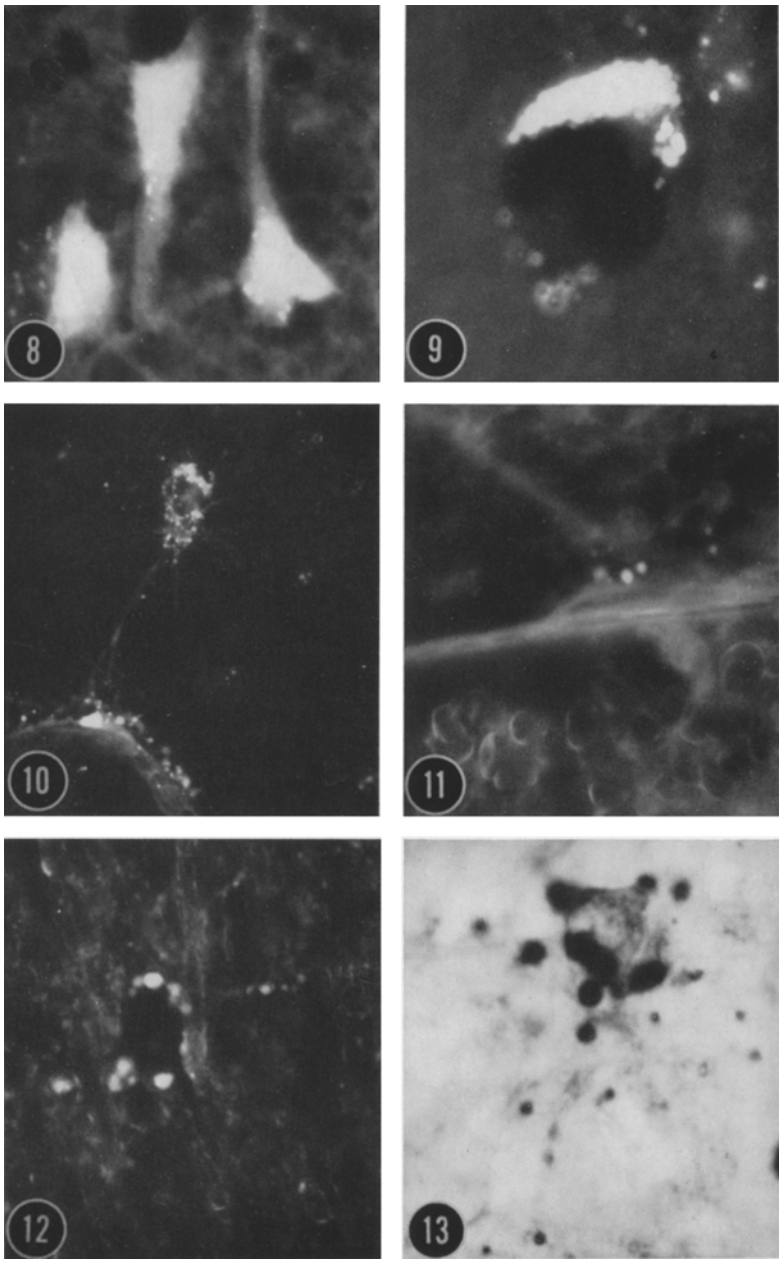


Fig. 8—13

distribution of the inclusions which were outlining the main cellular body and the processes (Fig. 10). The identification of the cellular type was further ascer-

tained by phase-contrast observations on the same cells or by studying the same microscopic field after FAN staining and remounting of the preparation. Some of the astrocytes could be easily recognized without these additional procedures by the faint, diffusely green outline of their cytoplasm. In a few cats in which the cortical lesion was more severe than usual and associated with a very pronounced distention of the affected gyrus, the edematous white matter contained diffusely green and markedly swollen astrocytes. The discrete FLSP inclusions were usually absent in such cells. In addition some of the nerve fibers in these areas appeared diffusely green and markedly swollen. At this time interval after the operation (24 hrs.) there was the first appearance in the edematous white matter of the FLSP inclusions in cells which were classified as being of microglial origin. These cells had usually amoeboid or roundish-oval outlines and were filled with brightly green FLSP inclusions in their cytoplasm (Fig. 5). Staining of the corresponding sections for neutral fat regularly failed to show any sudanophilic material in these cells. The FLSP-containing microglial cells were usually scattered throughout the edematous white matter and were more frequent in the perivascular location (Fig. 4). Occasionally these cells could be seen in the overlying cerebral cortex where they were commonly positioned as satellites applied to the darkly outlined neurons (Fig. 9). Frequently the regions of the cerebral cortex containing scattered FLSP-filled microglia showed small, green fluorescent droplets or granules scattered directly under the pial lining. Sometimes it was impossible to determine the intracellular location of these FLSP granules as they appeared to be lying free in the tissue directly under the pial lining (Fig. 14).

In the cats injected with FLF and sacrificed after 24 hours, the white matter in the area of edema showed two types of appearance with fluorescence optics. In two cats the picture was essentially similar to that observed with other fluorescent tracers, i.e. it consisted of the areas of "mottling" and of the presence of discrete, green inclusions in the glial cells. However in two other cats, the white matter revealed a striking network of green filamentous threads dispersed throughout the edematous area in a most irregular fashion (Fig. 7). These green filaments were occasionally more abundant around the blood vessels, but otherwise they appeared to lay scattered at random and showed no evidence of any spatial relationship to the structural elements of the tissue and particularly to the glial cells.

Fig. 8. Three neurons from the cortex adjacent to the necrotic area showing a diffuse green fluorescence. Cat injected with FLA and sacrificed 24 hours after cold injury. The bright small granules visible in the cytoplasm are orange endogenous pigment. × 580

Fig. 9. Darkly outlined neuron in the cortex from the gyrus adjacent to the cold lesion. A satellite filled with brightly green fluorescent inclusions is seen applied to the upper pole of the neuron. Cat injected with FLA and sacrificed 24 hours after cold injury. × 750

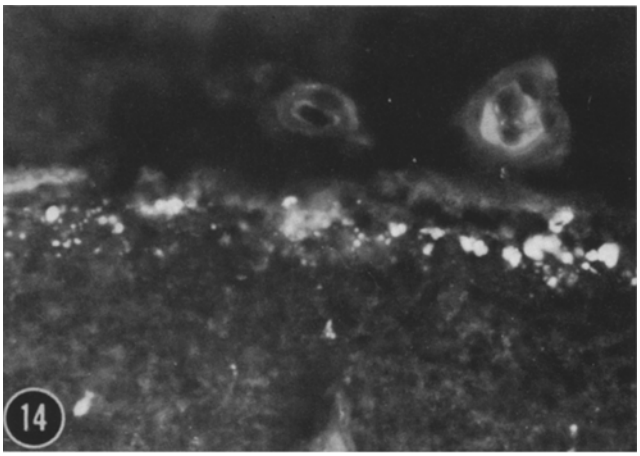
Fig. 10. An astrocyte with discrete FLG inclusions and faintly visible vascular expansion. The granules in the small blood vessel are predominantly orange-yellowish pigment. 48 hours after cold injury, white matter of the injured gyrus. × 320

Fig. 11. A faintly outlined vascular process of an astrocyte with three distinct FLA inclusions at the footplate location. In the lumen of a venule there are numerous blue autofluorescent red blood corpuscles. White matter, 6 hours after cold injury. × 960

Fig. 12. An oligodendrocyte outlined by green FLA inclusions around the nucleus and in the processes 48 hours after the cold injury. × 850

Fig. 13. FAN method. An oligodendrocyte with fuchsin-stained red nucleus and greenish-blue protein inclusions in the processes. Cat without tracer injection; 48 hours after cold injury. Photograph taken with a red filter to emphasize greenish-blue protein droplets. × 840

In the cats sacrificed after *48 hours* the "mottled" green fluorescence in the white matter appeared to be further decreased than after 24 hours, and it was usually confined to the subcortical zones. Otherwise, the white matter in the area of edema contained numerous neuroglial cells studded with discrete FLSP inclusions. The astrocytes predominated but also FLSP-containing oligodendrocytes could be quite frequently recognized (Fig. 12). The amoeboid or oval-shaped



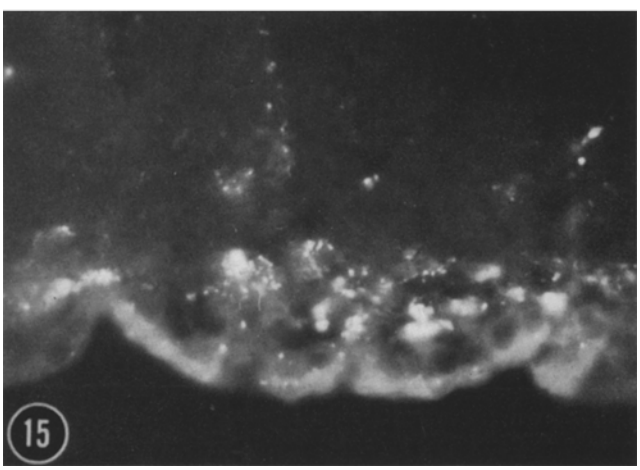


Fig. 14. The pial surface of the gyrus adjacent to the cold lesion showing accumulation of the FLA droplets, 48 hours after cold injury. × 320

Fig. 15. The ependymal surface. The FLA material is visible in the glial cells, astrocytic processes and scattered freely under the ependymal lining. The ependymal cells show yellowish autofluorescence and contain occasional orange pigment granules. Cat injected with FLA and sacrificed 3 days after cold injury. × 400

microglial elements, containing densely packed FLSP granules in their cytoplasm were seen scattered in the white matter as well as in the cortex of the gyrus subjected to cold injury. At this time interval (48 hrs.) there was evident an accumulation of the FLSP material at the subpial and subependymal locations, suggesting a transport of FLSP from the edematous white matter toward the pial and ependymal surfaces. The subpial granules of FLPS, which were observed first

at 24 hours were very conspicuous at 48 hours and were seen extending across the depth of the sulci into the adjacent gyri. The subependymal accumulations were limited to the areas continuous with the white matter exhibiting the extravascular penetration of the FLSP. Similarly to the subpial distribution, it was not always possible to ascertain the intracellular location of the FLSP granules. Frequently they appeared to be within the microglial cells, while sometimes they were present in the long astrocytic processes extending perpendicularly to the ependymal

Fig. 16

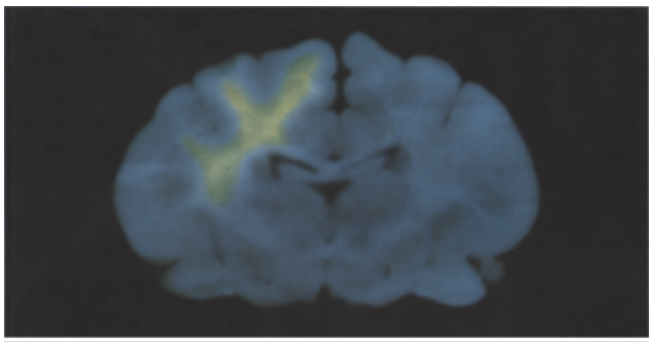


Fig. 17

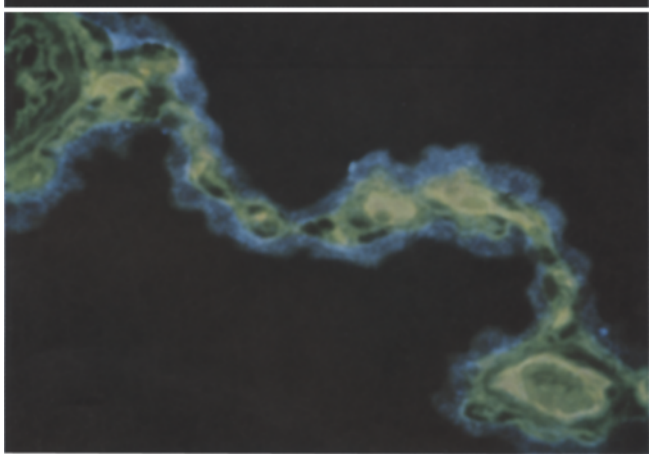


Fig. 16. Coronal section of the cat brain showing green fluorescence in the area of edema localized with striking predilection in the white matter. The injured gyrus is the second left from the midline. Cat injected with FLA and sacrificed 6 hours after cold injury

Fig. 17. Choroid plexus from the lateral ventricle. Normal control cat injected with FLA. Green fluorescence filling the lumina of some of the vascular channels and outlining the stromal connective tissue. Choroid epithelial cells display blue autofluorescence. × 400

lining (Fig. 15). The presence of FLSP within the ependymal cells themselves was not clearly evident. The cytoplasm of the ependymal cells exhibited orange-tinged diffuse autofluorescence with prominent pigment granules. Occasional cells appeared to be faintly diffusely green, but discrete FLSP inclusions were not observed.

In FAN-stained sections derived from animals injected with FLSP as well as from four cats without previous FLSP injection (all animals being sacrificed 48 hours after cold injury), it was possible to demonstrate the greenish-blue

inclusions in the glial cells in locations corresponding to the green fluorecent inclusions observed by fluorescence optics in cats with FLSP injection. There was no difference in appearance between the greenish-blue inclusions in FAN-stained sections derived from FLSP-injected and non-injected animals. The FAN-stained

Fig. 18

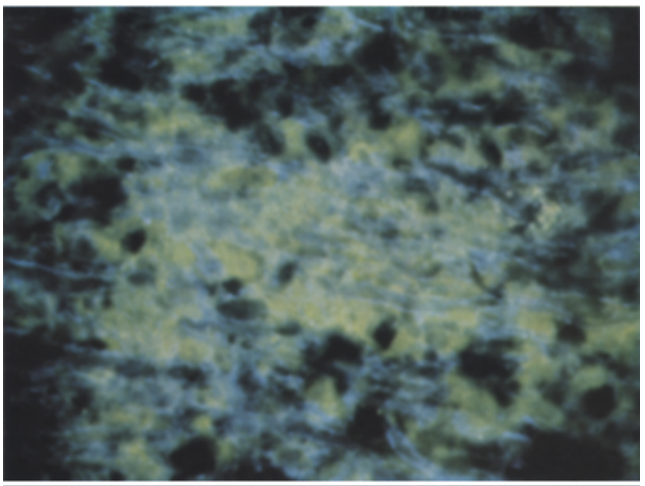


Fig. 19

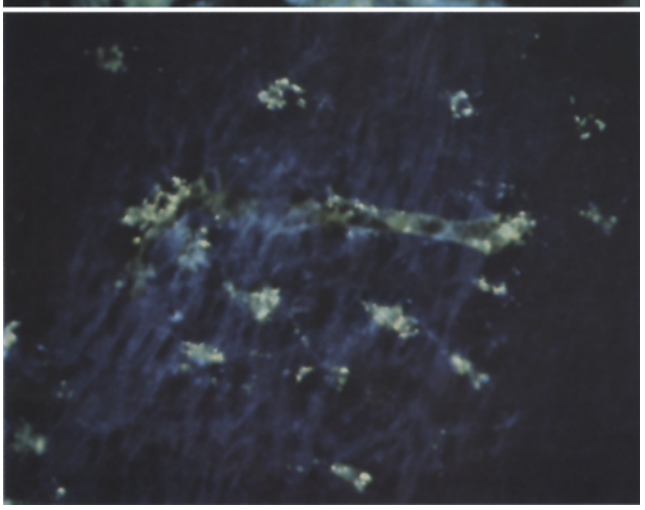


Fig. 18. "Mottled" appearance in the edematous white matter. Cat injected with FLA and sacrificed without perfusion 6 hours after cold injury. The blue autofluorescent nerve fibers are seen running in a parallel fashion × 400

Fig. 19. Cat injected with FLA and sacrificed with intravascular perfusion 6 hours after cold injury. White matter from the area of edema. Discrete FLA inclusions in the neuroglial cells are conspicuous on the blue autofluorescent background of the white matter. × 250

inclusions seemed to be somewhat larger than the corresponding FLSP inclusions, and they were demonstrable in astrocytes, oligodendrocytes (Fig. 13), and in cells which were assumed to be of microglial origin.

In cats sacrificed after *three days* the "mottled" fluorescence in the white matter was seen only occasionally. The FLSP inclusions in the neuroglial cells

were less frequently demonstrable; on the other hand the FLSP containing microglial cells were more common, and the subpial and subependymal accumulations of the FLSP were more conspicuous than after the 48 hour time interval.

In cats sacrificed *5 days* after cold injury there was a further reduction in the amount of demonstrable FLSP. The "mottled" appearance was not seen. Usually only a small number of the glial cells contained FLSP inclusions. Although pial accumulations were now absent, subependymal aggregates of the FLSP were still visible.

In cats sacrificed after *7 days* "mottled" fluorescence was absent. In two animals the area of the white matter subjected to the edematous process still contained a few neuroglial cells with FLSP inclusions. In addition there were scattered green fluorescent cells with outlines of typical "resting" type of microgliacytes (Fig. 6). The nuclei of these cells showed diffuse green fluorescence; whereas the cytoplasm, including processes with characteristic short, "thorny" spikes, was faintly green fluorescent. Occasionally bright green fluorescent inclusions were present in the cytoplasm of these cells. Amoeboid or roundish-shaped microglial forms were not encountered. These two cats also showed the presence of subependymal accumulation of FLSP. In the remaining three cats the green fluorescence was present only in the occasional "resting" microgliacytes as described above.

Cats sacrificed *13 days* after cold injury did not show any green fluorescence outside of the blood vessels, which in some animals revealed a faint green fluorescence of the endothelial lining.

Discussion

Our observations on penetration of the labeled proteins into the brain tissue revealed several characteristic patterns of FLSP distribution which deserve some comment.

The nature of discrete, intracytoplasmic FLSP inclusions can be interpreted as follows. Generally the proteins do not permeate through the cellular membranes of the intact cells, but they can enter the cytoplasm of such cells by the mechanism of pinocytosis. The phenomenon of pinocytosis was studied with regard to various nervous tissue cells grown in tissue culture (KLATZO and MIQUEL) and it was suggested that the formation of discrete intracytoplasmic FLSP inclusions depended on aggregation of protein conjugates taken up in pinocytic vacuoles. It can therefore be concluded that the discrete intraglial FLSP inclusions observed in the present investigation were of pinocytotic origin. The demonstration of the FAN-stained protein inclusions in the animals which were not injected with FLSP suggests that pinocytotic uptake of proteins by the glial cells represents a general phenomenon associated with the leakage of serum proteins into edematous white matter.

With regard to the diffuse green fluorescence displayed by some cells and nerve fibers, it was previously demonstrated (KLATZO and MIQUEL) that the dead or dying cells exposed to FLSP *in vitro* rapidly acquired such fluorescence. Thus it would appear that this type of fluorescence is related to cellular injury, and correspondingly in the present study diffusely green fluorescent cells were found primarily in the area of the cortical lesion (Fig. 8). On the other hand it should be

considered that diffuse cellular staining with FLSP does not necessarily indicate an irreversible change, since the diffusely green and swollen astrocytes and nerve fibers, observed occasionally in severely edematous white matter, showed no evidence of necrobiosis at any later stages. It should be noted that the interpretation of diffuse green fluorescence as an indication of cellular injury presumably does not apply to the fluorescence of the nuclei observed in the resting type of the microglia at the later stages after cold injury (Fig. 6). It would be unlikely that these microgliacytes are injured cells, and it may be possible that cytoplasmic hydrolysis of the conjugate leads to the liberation of free FITC which then enters the nucleus.

In the interpretation of the "mottled" appearance, the most pertinent question is the location of the FLSP in relation to the cellular structures. The electron microscopic studies, which indicate that the size of the extracellular space is extremely small in both normal and the edematous brain (GERSCHENFELD et al.; LUSE and HARRIS; TORACK, NIESSING and VOGELL), suggest that the "mottled" green fluorescence results from the intracellular distribution of FLSP into the glial compartments. However our observations pertaining to the effect of intravascular perfusion ("mottled" appearance of white matter was reduced while neuroglial FLSP inclusions became conspicuous) seem to point to an alternate interpretation. It seems more likely that the FLSP material responsible for the "mottling" was located extracellularly, and was thereby more accessable to the rinsing effect of the perfusion fluid, whereas the FLSP inclusions were unaffected because of their intracytoplasmic location. In this connection it may be speculated that if the extravasated serum proteins are located extracellularly in the edematous white matter, then the size of the protein molecules and the physical laws of diffusion (KLATZO) would necessarily imply that the extracellular space available to the "mottled" FLSP would have to be considerably larger than that allowed by electron microscopists on the basis of 100—200 Å distances separating outer cell membranes.

Another observation favoring the possibility of the extracellular distribution of FLSP in edematous white matter is related to the pattern of FLF penetration observed in two cats sacrificed 48 hours after cold injury. The irregularly scattered filamentous threads of the FLF did not appear to be spatially related to any cellular structures (Fig. 7), and it would be difficult to envisage them located within the glial cells. The filamentous pattern itself could probably be related to the instability of the fibrinogen in the solution. In preparing the FLF the precipitation of the conjugate has been a major problem and it is likely that the filamentous threads scattered in the white matter may represent a precipitated form of FLF. Conversely, it appears that when the FLF remained in a water soluble state, it produced a picture (as seen in the two remaining cats) which was similar to that obtained by using other protein conjugates.

The pinocytotic activity itself, as suggested by uptake of proteins in the edematous white matter, could also be considered more compatible with the existence of an extracellular compartment in which the glial cells could have free space to exercise undulating movements of their membranes.

The virtual absence of an extracellular space as suggested by the electron microscopists has stimulated search for intracellular transport mechanisms in

the brain tissue, and in this connection pinocytosis has been mentioned by a number of investigators (HYDÉN; EDSTRÖM and STEINWALL; POMERAT). However, the acceptance of pinocytosis as the major transport mechanism must meet serious objections based primarily on the present knowledge concerning this phenomenon. Pinocytotic formation of vacuoles in cells in tissue culture as evaluated by the time-lapse-cinemicrography is definitely a slow process. Although the rate of pinocytosis could possibly be different *in vivo*, nevertheless, it would be difficult to imagine that it could be of sufficient speed to meet the requirements for the rapid transfer of substances such as glucose. Furthermore pinocytosis is assumed to be associated with great metabolic activity and it seems unlikely that the glucose taken up by pinocytosis could pass intact through the glial cellular compartments to reach a neuron without biochemical transformation. In this connection it was demonstrated in amoeba that glucose taken up by pinocytosis is for the most part excreted as respiratory carbon dioxide, the remaining 10—15% being incorporated in a more permanent way in the cytoplasm of the amoebae (CHAPMAN-ANDRESEN and HOLTER).

The mentioned reservations concerning the role of pinocytosis for the rapid intracellular transport of metabolites should not detract from its possible importance in other physiochemical processes in the nervous system. Pinocytosis may be of local metabolic importance with regard to relationships between the satellites and the nerve cells. Pictures from the present study, pertaining to the satellites filled with pinocytotic protein inclusions and applied to the darkly outlined neurons (Fig. 9), suggest that the satellites may act as metabolic relay stations providing a proper chemical environment for the neurons. Such an assumption would be in line with Pomerat's concept ascribing to glia a "nurse-like" function (POMERAT). The other important involvement of pinocytosis may be associated with the migratory activity of microglia. LUMSDEN expressed the opinion that microglia may provide an important system for the transport of insoluble materials. In the present study microglial transport was suggested by the presence of numerous FLSP-filled microglial elements chronologically and topographically related to the appearance of the subpial and subependymal accumulations. We propose that Lumsden's idea concerning microglia may be significantly expanded by ascribing to microglia the faculty of transporting soluble substances also, e.g. proteins, which are taken up by pinocytosis. The transport of proteins by the microglia towards the pia and ependyma leads presumably to the release of the proteins at these brain surfaces and it may be suggested that the longer and more conspicuous persistence of the FLSP accumulations at the ependyma may depend either on the more rapid migration of the microglia through the white matter or on the slower rate of FLSP disappearance at the subependymal location.

With regards to vascular permeability in normal brain tissue our observations provided a further support for the assumption that special permeability relationships exist in areas such as choroid plexus, area postrema and hypothalamus. In contradistinction to other regions of the brain, the FLSP at the mentioned locations appeared to render connective tissue investitures of the blood vessels fluorescent, while no penetration of the FLSP into the surrounding parenchyma could be detected. Since the connective tissue investitures of comparable blood

vessels from other regions of the brain did not show any green fluorescence, our observations would indicate that vascular permeability in the "special" areas examined might be significantly different from other regions of the brain. The inability to demonstrate the FLSP in the endothelial cells themselves could be due to the fact that the amount of labeled protein (passing through the endothelial lining) was below that sufficient for microscopic resolution under fluorescence optics.

Summary

1. Vascular permeability in normal and edematous brain tissue was studied by application of the fluorescein labeled serum proteins (FLSP) as well as by the use of free fluorescein isothiocyanate (FITC) marker.

2. The electrophoretic studies demonstrated that the binding capacity of the FITC to albumin was of such degree that morphological observations made after injection of the free tracer can be considered depending primarily on the visualization of the fluorescent serum albumin in the brain tissue.

3. The normal brain, with the exception of the several regions described below, showed no evidence of FLSP passage beyond the vascular endothelial lining.

4. The choroid plexus, area postrema and the tuber cinereum exhibited green FLSP fluorescence in the connective tissue investitures of the blood vessels but no parenchymatous penetration of the FLSP could be detected in the last two mentioned structures.

5. Edema of the white matter, produced by the cortical cold injury in the cat, was associated with the extravascular passage of FLSP which was studied at various stages.

6. The appearance of discrete FLSP inclusions in glial cells is interpreted as resulting from pinocytotic uptake of extravasated serum proteins and the significance of pinocytosis in the nervous tissue is discussed.

7. The involvement of the microglia in the transport of FLSP from the area of edema towards the pial and ependymal surfaces suggests the importance of this glial element with regard to the transport of soluble substances in the brain tissue.

8. It is suggested that the area of edema in the white matter resulting from cold injury may be associated with the presence of an appreciable extracellular compartment.

Acknowledgment. We are indebted to Mr. JOSEPH T. WALKER, Jr. for the histological preparations, and to Mr. FRED MEILLER for assistance in microphotography.

Zusammenfassung

1. Unter Verwendung von fluorescein-markierten Serumproteinen (FLSP) und von freiem Fluorescein-Isothiocyanat (FITC)-Tracer wurde die vasculäre Permeabilität in normalem und ödematösem Hirngewebe untersucht.

2. Die elektrophoretischen Untersuchungen ergaben, daß die Bindungskapazität des FITC an Albumin so stark ist, daß man morphologische Beobachtungen nach Injektion mit der freien Tracersubstanz als hauptsächlich von der Sichtbarmachung des fluoreszierenden Serumalbumins im Hirngewebe abhängig betrachten kann.

3. Mit Ausnahme der unten angeführten Regionen konnte im normalen Gehirn kein Nachweis für die FLSP außerhalb der Gefäßendothelschicht gefunden werden.

4. Das Adergeflecht, die Area postrema und das Tuber cinereum zeigten grüne FLSP-Fluorescenz in den bindegewebigen Hüllen der Blutgefäße; jedoch konnte in den beiden letztgenannten Strukturen kein Eindringen der FLSP in das Parenchym festgestellt werden.

5. Ein bei der Katze durch eine corticale Kälteschädigung verursachtes Ödem der weißen Substanz ging mit extravasalem Durchtritt von FLSP einher, der in verschiedenen Phasen beobachtet wurde.

6. Das Auftreten von geringfügigen FLSP-Einschlüssen in Gliazellen wird auf die pinocytotische Aufnahme von extravasalen Serumproteinen zurückgeführt; die Bedeutung der Pinocytose im Nervengewebe wird erörtert.

7. Die Beteiligung der Mikroglia am FLSP-Transport von der Ödemregion zu den pialen und ependymalen Oberflächen läßt auf die wichtige Rolle schließen, die dieses Gliaelement beim Transport löslicher Substanzen im Hirngewebe spielt.

8. Die Annahme wird vorgebracht, daß das durch Kälteschädigung entstandene Ödem der weißen Substanz mit dem Vorhandensein eines beträchtlichen extracellulären Raumes in Verbindung steht.

References

Broman, T.: Permeability of the Cerebrospinal Vessels in Normal and Pathological Conditions, p. 1—92. Copenhagen: Munksgaard 1949.

—, On the Basic Aspects of the Blood-Brain-Barrier. Acta psychiat. scand. **30**, 115—124 (1955).

Chapman-Andresen, C., and H. Holter: Studies on the Ingestion of 14 C Glucose by Pinocytosis in the Amoeba Chaos Chaos. Exp. Cell Res., Suppl. **3**, 52—58 (1955).

Coons, A. H., and M. H. Kaplan: Localization of Antigen in Tissue Cells. II. Improvements in a Method for the Detection of Antigen by Means of Fluorescent Antibody. J. exp. Med. **91**, 1—13 (1950).

Edström, R., and O. Steinwall: The Blood-Brain Barrier Phenomenon—The Relative Importance of Permeability and Cellular Transport Mechanisms. Acta psychiat. scand. (in press).

Gerschenfeld, H. M., F. Wald, J. A. Zadunaisky and E. D. P. De Robertis: Function of Astroglia in the Water-Ion Metabolism of the Central Nervous System. Neurology **9**, 412—425 (1959).

Gregersen, M. I., and R. A. Rawson: Disappearance of T 1824 and Structurally Related Dyes from the Blood Stream. Amer. J. Physiol. **138**, 698—707 (1943).

Hydén, H.: Biochemical Changes in Glial Cells and Nerve Cells at Varying Activity. Proc. IV. Intern. Congr. of Biochem. Symp. III: Biochemistry of the Central Nervous System, p. 64—89. Ed. by F. Brucke. New York, London, Paris: Pergamon Press 1959.

Klatzo, I., A. Piraux and E. J. Laskowski: The Relationship Between Edema, Blood-Brain-Barrier and Tissue Elements in a Local Brain Injury. J. Neuropath. exp. Neurol. **17**, 548—564 (1958).

—, Discussion Concerning the Problem of the Extracellular Space. Symposium on the Selective Vulnerability of the C.N.S. in Hypoxemia. Baden 1961 (In press).

—, and J. Miquel: Observations on Pinocytosis in Nervous Tissue. J. Neuropath. exp. Neurol. **19**, 475—487 (1960).

Lumsden, C. E.: Histological and Histochemical Aspects of Normal Neuroglia Cells. In: Biology of Neuroglia, p. 141—161. Ed. by W. F. Windle. Springfield, Ill.: Thomas 1958.

LUSE, S. A., and B. HARRIS: Electron Microscopy of the Brain in Experimental Edema. J. Neurosurg. **17**, 439—446 (1960).

MIQUEL, J., y W. CALVO: Un Nuevo Metodo Sencillo y Rapido para la Tincion Histopatologica. Arch. esp. Morfol. **14**, 125—129 (1958).

NIESSING, K., u. W. VOGELL: Electronmicroscopic Investigation into the Structural Change in the Cerebral Cortex in Edema and their Importance in the Problem of Ground Substance. Z. Zellforsch. **52**, 216—237 (1960).

POMERAT, C. M.: Dynamic Neurogliology. Tex. Rep. Biol. Med. **10**, 885—911 (1952).

TORACK, R. M., R. D. TERRY and H. M. ZIMMERMAN: The Fine Structure of Cerebral Fluid Accumulation. I. Swelling Secondary to Cold Injury. Amer. J. Path. **35**, 1135—1147 (1959).

TSCHIRGI, R. D.: Protein Complexes and the Impermeability of the Blood-Brain-Barrier to Dyes. Amer. J. Physiol. **163**, 756—772 (1950).

—, Blood-Brain-Barrier. In: Biology of Mental Health and Disease, p. 34—46. New York: Hoeber 1952.

I. KLATZO, M. D.,
Section of Neuropathology, Surgical Neurology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda 14, Md., U.S.A.