# SELECTIVE VULNERABILITY OF THE BLOOD-BRAIN BARRIER IN CHEMICALLY INDUCED LESIONS*

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There is a recent tendency to consider the blood-brain barrier (BBB) as a complex of multiple systems regulating the exchange of metabolic material between brain and blood by a variety of excluding or even facilitating mechanisms (5, 27, 38, 42). This non-unitary concept would imply that injurious agents of a certain nature and intensity which would not indiscriminately abolish all functions of the barrier may selectively interfere in a differential manner with individual mechanisms (40).

The purpose of the present study was to approach the problem of selective vulnerability of the BBB by application of special technics based on the principle of concurrent use of two different blood-borne tracers, which could be individually visualized in thin brain sections. This was accomplished by selecting tracer compounds of contrasting color fluorescence or by combining fluorescent and radioactive tracers. For damaging the BBB itself we used the technic of unilateral chemical injury to the cerebral vasculature, originally developed by Broman and Olsson (6, 7), and modified by Steinwall (37). This procedure, which depends on unilateral perfusion of the internal carotid artery with a BBB damaging agent, seemed to offer special advantages for our study. By perfusing at a pressure slightly higher than the arterial systolic pressure the blood is completely expelled and the walls of the cerebral vasculature are exposed directly to the undiluted perfusate. This facilitates controlling the intensity of vascular damage by varying the concentration of the injurious agent and/or the duration of the intravascular perfusion. Furthermore, a variety of chemical agents can be tested and the unilateral character of the lesions allows for a direct comparison with the unaffected hemisphere. The control of intensity of the BBB damage appeared to be of critical importance for the present investigation since it could be assumed that a differentiated effect would be more likely demonstrable in a partial dysfunction than in a complete disruption of the barrier. The noxious agents employed were those discussed earlier by Steinwall (38) with regard to their BBB-damaging potency and presumed mode of action.

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## MATERIAL AND METHODS

*Experimental Procedure:* The experiments were performed on adult rabbits under Nembutal (pentothal sodium) or urethane anaesthesia. Unilateral BBB injury was produced by intravascular application of noxious solutions according to the technic described by Steinwall (37). A polyethylene catheter was secured in the left common carotid artery after ligation of its proximal part and external branches in order to direct the injected solutions into the internal carotid artery. A trephine opening was made over the left cerebral hemisphere exposing the intact dura. To test the perfusion system, physiological saline was injected under a pressure which would just expel the blood from pial vessels visible through the dura. The damaging agent was then injected for 30 to 35 sec. under similar conditions. The tracers for assessment of the BBB behavior were given intravenously prior to or immediately after the carotid perfusion. The animals were sacrificed by exsanguination after a time interval of 6 to 30 minutes.

*Barrier damaging agents* were 0.03 to 0.08 mM $HgCl_2$ in physiological saline, 3.5 to 5 per cent penicillin G. 12 to 18 per cent Urokon (sodium acetrizoate) and 5 per cent NaCl.

With regard to the tracers used for the indication of the BBB damage our study comprised 4 series of experiments, as summarized in Table 1.

In *Series 1*, fluorescein labeled albumin (FLA) was employed for the microscopic assessment of the extravasation patterns for a single protein tracer. FLA was prepared by conjugation of crystalline bovine albumin* with fluorescein isothiocyanate according to a procedure previously described (23). The rabbits received 10 ml/kg of FLA. Formalin-fixed frozen sections were examined and photographed under the fluorescence microscope.

*Series 2* was designed to ascertain a differential penetration of albumin and $\gamma$-globulin tagged with markers contrasting in color in various chemical BBB lesions (see table 1). The green fluorescent $\gamma$-globulin (FLGG) was prepared similarly to FLA by conjugation of crystalline bovine $\gamma$-globulin† with fluorescein isothiocyanate. As a tracer contrasting in color, albumin conjugated with red fluorescent Rhodamine B (RLA) according to the procedure of Silverstein (35), was employed first. In later experiments RLA was replaced by the Evans blue-albumin complex (EBA) because of our accidental discovery that this compound displays a vivid red fluorescence when viewed in thin sections under the fluorescence microscope. It was also found that the Trypan blue, chemically an isomer of Evans blue, exhibits a similar appearance, but that the red fluorescence of the Trypan blue was of lesser intensity. For our experiments the EBA was prepared by dissolving 2 gm of the dye and 10 gm of bovine albumin in 100 ml of physiological saline. Complete binding of Evans blue with albumin under the present experimental conditions was evident from the following observations: 1. trichloracetic acid precipitation of the serum from rabbits injected with double standard dose of EBA gave a supernatant without discernible dye, and 2. in agar-gel electrophoresis of the similar rabbit serum the dye migrated entirely with the albumin fraction (fig. 1). This strong and direct binding of Evans blue to albumin when mixed *in vitro* or injected *in vivo* (1, 34) provides a valuable tool for the localization of albumin in fluorescence microscopy. The red fluorescence seems to persist for weeks in tissue kept in formalin fixative (own observations).

To assess the behavior of the same protein tagged with different markers in 2 rabbits EBA was given together with FLA.

In *Series 3* and *4* a special technic was developed for the comparison of distribution of fluorescent and radioactive tracers in the same thin section. The tracers used were as follows: FLA, 10 ml/kg; EBA, 2.5 ml/kg; sodium fluorescein (NaFl) in 10 per cent solution, 0.5 ml/kg; $C^{14}$ sucrose‡, $C^{14}$ inulin‡, and $C^{14}$ methyl-O-glucose*, 250 to 350 $\mu$C/kg of

* Nutritional Biochemical Corporation.

† Nutritional Biochemical Corporation.

‡ New England Nuclear Corporation.

TABLE 1

| Series | Tracers | BBB-damaging agents, conc. | No. of experiments |
|---|---|---|---|
| 1 | FLA | $HgCl_2$, 3-5 × $10^{-5}$ M<br>Penicillin G, 3.5-5% | 22 (14 / 8) |
| 2 | RLA + FLGG | $HgCl_2$, 6 × $10^{-5}$ M<br>Urokon, 16% | 3 (2 / 1) |
| | EBA + FLGG | $HgCl_2$, 6 × $10^{-5}$ M<br>Urokon, 12-14% | 9 (6 / 3) |
| | EBA + FLA | $HgCl_2$, 6 × $10^{-5}$ M | 2 |
| 3 | $C^{14}$ sucrose + NaFl | $HgCl_2$, 5 × $10^{-5}$ M | 1 |
| | " + EBA | $HgCl_2$, 6 × $10^{-5}$ M | 1 |
| | $C^{14}$ inulin + NaFl | $HgCl_2$, 5 × $10^{-5}$ M | 3 |
| | " + FLA | $HgCl_2$, 6 × $10^{-5}$ M | 2 |
| 4 | $C^{14}$ methyl-O-glucose + NaFl | $HgCl_2$, 4-5 × $10^{-5}$ M | 4 |

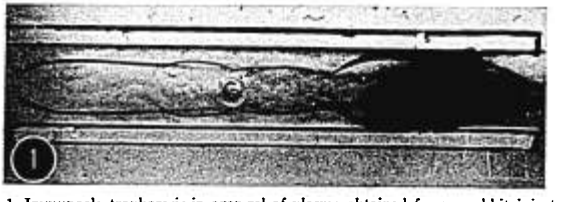

FIG. 1. Immunoelectrophoresis in agar-gel of plasma obtained from a rabbit injected intravenously with EBA (5 ml of 2% Evans blue/kg of body weight) and goat antiserum against rabbit plasma. Rabbit plasma was placed in the circular basin and antiserum in the horizontal slits. Evans blue is visible in the albumin region of the rabbit plasma as a diffuse dark smudge and also as black crescent lines produced by precipitation of EBA with antibody. For technical details of the procedure see Grabar and Williams (18). We are indebted for this assay to Dr. Gunilla Berglund, Department of Microbiology, University of Gothenburg, Sweden.

each. One fluorescent and one radioactive tracer were given intravenously in various combinations (see table 1) at the time of the noxious intracarotid perfusion. Following the sacrifice, the brain was immediately removed and sliced into coronal blocks. The blocks were placed on metal holders covered with a layer of 5 per cent gum tragacanth (in saline), and rapidly frozen in dry ice. Sections 15 $\mu$ in thickness were cut in a cryostat set at 15°C. and mounted on coverslips. A rapid thawing and drying of the sections (within several seconds) was accomplished by transferring the coverslips from the cryostat to a hot plate set at 60°C. The localization of a fluorescent tracer was carried out by placing the coverslips with the mounted sections on the black paper and by illuminating them in the dark room by an oblique beam of light from a HBO 200 mercury vapor lamp provided with U.G. 5 Zeiss (Schott) filter. For color photography U.V. 16, Y 70 and M 10 gelatin filters were placed in front of the lens of the Leica Macrophotographic

Camera unit and 200 Anscochrome Daylight film was used. Black and white photography of the fluorescence was carried out with G15 yellow filter and Adox KB 14 film. Following the photographic recording of the fluorescence, the sections were subjected to radioautography. The coverslips were attached to regular slides with the aid of Vikem Vinyl Protective Coating Spray*, covered with Anseo Non-Screen Film and fastened in Kodak X-ray exposure holders. The radioautographic exposure was carried out in the refrigerator at 4°C for approximately 4 to 10 days. Following the exposure, the films were developed using Kodak X-ray Developer and Fixer. Examination of fluorescence photographs and radioautographs from the same section provided the comparison in distribution of fluorescent and radioactive tracers.

## RESULTS

*Series 1: BBB Damage Traced With FLA Alone.* The intensity of the BBB damage was generally proportional to the concentration of the chemical agent (the application time being kept constant at 30 sec.), and also to the time elapsed between the intracarotid perfusion and the sacrifice. Nevertheless, considerable variations were observed in individual rabbits. Furthermore, in a given animal the pictures of abnormal FLA penetration from the cerebral vessels were not uniform, but were frequently expressed in several characteristic patterns which were sometimes recognizable in adjacent areas in the affected hemisphere.

The earliest or slightest demonstrable injury appeared to be represented by a picture in which the penetration of FLA extended through the layers of a vessel wall, but not beyond into the neural parenchyma (fig. 2). In this type of injury the blood vessel walls revealed green fluorescent lines outlining the margins of cellular components and in general corresponding topographically to the structure described by electron microscopists (19) as the basal membrane system, a homogeneous substance which separates the cellular confines of vessel wall components and forms a distinct layer against glial elements.

In unaffected blood vessels throughout the brain, with exception of a few "special" areas (23), the green fluorescent conjugate appeared to be strictly confined to the vascular lumen without any evidence of penetration into the endothelium or into the vascular wall proper.

Another pattern of BBB injury was related to the presence of green fluorescent globules situated in the close proximity of a blood vessel (figs. 3 and 4). These globules varied in size from very small ones at the limit of microscopic resolution to large ones reaching approximately 15 $\mu$ in diameter. They were found most commonly around the capillaries, small arterioles, or venules. The proteinaceous nature of the globules was indicated by the FAN stain (29) which possesses a strong histochemical affinity to proteins. Although the presence of indicator globules around a blood vessel sometimes was the only sign of vascular injury, more often the globules were associated with a diffuse infiltration of the adjacent parenchyma by the indicator (fig. 5).

The diffuse infiltration or "parenchymatous" infiltration, as it was called in a previous study (24), corresponded topographically to what is defined in the

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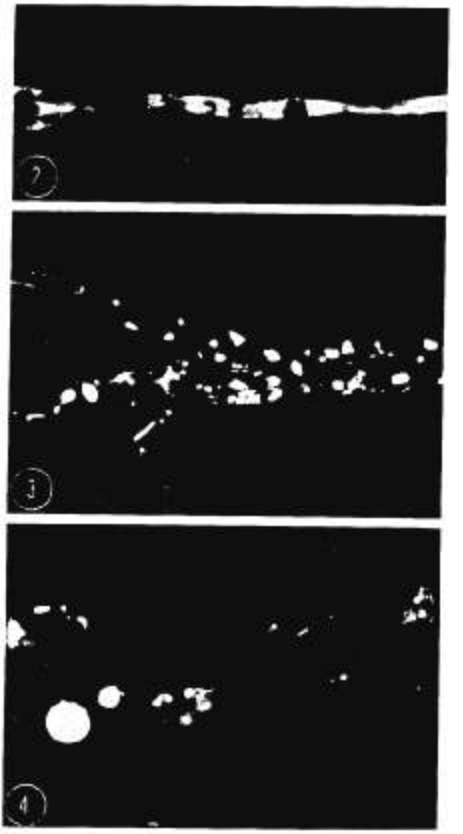

FIG. 2. Series 1. An arteriole in the cortex of the hemisphere perfused with $HgCl_2$. The green fluorescent FLA is visible in the lumen of the vessel and also in delicate lines separating individual cellular components of the vascular wall. The neural parenchyma outside of the vessel displays a faint bluish autofluorescence; × 650.

FIG. 3. Series 1. A branching blood vessel showing a perivascular penetration of the FLA. Green fluorescent globules of the indicator are located in the immediate vicinity of the vessel; × 420.

FIG. 4. Series 1. A small venule with perivascular globules of FLA; × 720.

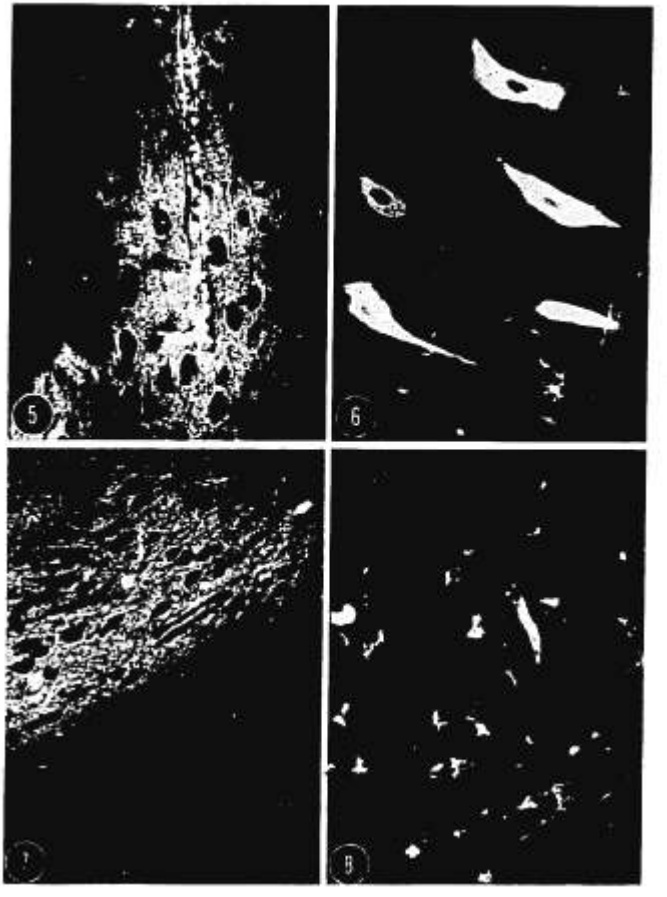


FIG. 5. Series 1. A damaged blood vessel in the cerebral cortex surrounded by a diffuse "parenchymatous" spread of the FLA into the neuropil. The neurons stand out as dark silhouettes. The vessel wall is studded with FLA globules; × 240.

FIG. 6. Series 1. The neurons from the basal ganglia on the perfused side displaying a green fluorescent cytoplasm and dark nuclei. The surrounding parenchyma shows only a non-specific autofluorescence; × 680.

FIG. 7. Series 1. The "parenchymatous" spread of the FLA in the cerebral cortex stops in a straight line at the border of corpus callosum; × 210.

FIG. 8. Series 1. The white matter on the perfused side. The FLA appears to be localized in the astrocytes and nerve fibers; × 220.

light microscopy as neuropil. The neurons within an area of such infiltration by FLA were usually conspicuously outlined as dark silhouettes (figs. 5 and 7). Although the "parenchymatous" spread of the protein tracer in most instances was confined topographically to the vicinity of visibly affected blood vessels, sometimes such penetration was very extensive, spreading diffusely throughout large areas of the gray matter, particularly in the cerebral cortex. Generally, the white matter appeared to be resistant to such invasion of the tracer and the demarcation between the diffusely green fluorescent gray matter and the blue autofluorescent white matter corresponded in most instances strictly to the anatomical borders between these structures presumably without any relationship to the areas of blood supply (fig. 7).

In regions where the extravasation of the FLA was particularly intense some of the neurons acquired a bright green fluorescence. Commonly, only the neuronal cytoplasm was green fluorescent, the nucleus remaining dark. However, in some instances of apparently more severe cellular damage the whole nerve cell appeared to be invaded by the tracer. Interestingly, some rabbits with the BBB injury produced either by mercuric chloride or penicillin revealed areas where the parenchymatous infiltration was almost absent whereas the neurons showed bright green fluorescence conspicuous on the dark background (fig. 6). Usually the blood vessels in the vicinity of such neurons revealed some evidence of an increased permeability to FLA, expressed either by green fluorescent staining of their walls or by the presence of perivascular globules.

In some rabbits where the intense BBB injury was due to longer time interval between perfusion and sacrifice the brains showed evidence of an abnormal penetration of the tracer into the white matter associated with the appearance of developing edema. Usually the green fluorescence in the edematous white matter presented a picture which has been previously described as the "mottled appearance" (23). This "mottled" pattern of the tracer distribution, similarly to the "parenchymatous" infiltration, could not be related morphologically in the fluorescence optics to any specific cell structures. On the other hand, in some areas the tracer appeared to be selectively localized in the cellular elements in the edematous white matter, the nerve axons and astroglia appearing conspicuously green fluorescent against the dark background (fig. 8).

*Series 2: BBB Damage Traced Simultaneously With Two Contrasting In Color Protein Indicators.* All the rabbits which received the red fluorescent RLA or EBA combined with green fluorescent FLGG revealed microscopically a dissociation in passage of respective protein conjugates from the injured vessels in the affected hemisphere. Due to the fact that the red fluorescence of Evans blue was stronger and more distinct in color than that of the somewhat orange Rhodamine B marker the dissociation features were more pronounced when the combination of EBA and FLGG was used, although the microscopic pictures observed with RLA and FLGG administration were of a basically similar nature.

The mixing of red and green fluorescent conjugates in the circulating blood accounted for a predominantly yellow fluorescence visible in the lumina of the

blood vessels throughout the brain. On the other hand, the sites of abnormal penetration of the indicators from the injured vessels were characterized by various shades of color ranging from pure red to yellow which undoubtedly was due to proportional differences in concentration of the two component tracers. This differentiation in color based on relative concentration of fluorescent conjugates was very conspicuous with regard to the "globular" pattern of penetration. Figure 9 clearly shows this phenomenon. Otherwise, the pictures indicated that the albumin tracer usually penetrated out of the damaged vessel more easily than the labeled γ-globulin (fig. 10). The diffuse "parenchymatous" penetration was as a rule purely red in color and only severely injured blood vessels were occasionally surrounded by diffuse exudates fluorescent in orange-yellowish shade. The neurons were often penetrated only by the red albumin conjugate. A special feature of the Evans blue marker, not seen with FLA, was the occasional staining of the neuronal nuclei, with or without discernible red fluorescence of the cytoplasm. As in the experiments with the FLA, the EBA tracer could be seen in some areas conspicuously localized in the neurons, whereas the surrounding neural parenchyma remained darkly autofluorescent (fig. 11).

Two rabbits in which FLA and EBA were used together revealed no demonstrable separation of the tracers, the only exception being that the nerve cell nuclei in some areas of abnormal penetration appeared deeply red fluorescent whereas the neuronal cytoplasm displayed the color corresponding to the mixture of both indicators, *i.e.*, yellowish-orange.

*Series 3: BBB Damage Traced With Radioactive Sucrose Or Inulin And Fluorescent Indicators.* The features of dissociation were also demonstrable in the distribution of concurrently applied fluorescent and radioactive indicators. There were, however, considerable individual variations, and in some animals the differences in distribution were too small to be certain. The latter applies especially to the two rabbits given $C^{14}$ sucrose with EBA or NaFl. In these two animals the areas of fluorescence and radioactivity matched each other closely, although there was an impression that the distribution of $C^{14}$ sucrose in areas of abnormal penetration was somewhat more extensive than that of EBA or NaFl.

Pictures of clear-cut differences in distribution were observed in 3 out of 5 rabbits which received fluorescent indicators combined with $C^{14}$ inulin. Out of two rabbits injected with FLA and $C^{14}$ inulin one showed no evidence of any extravasation of FLA, whereas there were extensive areas of radioactivity in the left hemisphere perfused with Urokon. The second rabbit revealed areas of $C^{14}$ inulin extravasation additionally in the right hemisphere, whereas the areas of green fluorescence were strictly confined to the left side (figs. 12 and 13). More extensive distribution of $C^{14}$ inulin than NaFl was demonstrable in one rabbit. In two other rabbits which received NaFl and $C^{14}$ inulin the differences in distribution were too slight to be recognized with certainty.

*Series 4: Mercurial BBB Damage Traced Concurrently With $C^{14}$ Methyl-D-Glucose And NaFl.* These experiments carried out in 4 rabbits revealed the

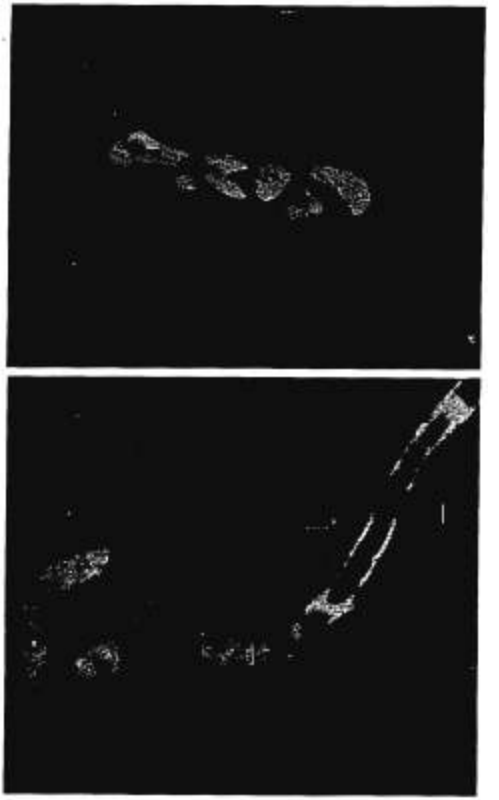

Fig. 9. Series 2. A small blood vessel containing a yellow mixture of EBA and FLGG. The perivascular globules display a range of colors depending on relative proportion of albumin versus γ-globulin indicator. A faint penetration of only EBA can be seen in the adjacent neuropil; × 640.

Fig. 10. Series 2. A damaged capillary showing a similar dissociation of albumin and γ-globulin indicators as in Fig. 9; × 650.

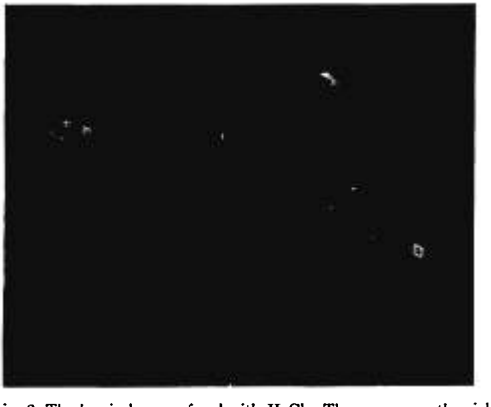

FIG. 11. Series 2. The hemisphere perfused with $HgCl_2$. The neuron on the right shows penetration by EBA. The small blood vessel on the left is stained yellow by the mixture of two indicators. The intervening neural parenchyma displays a blue autofluorescence; × 860.

following findings: Brains of two rabbits showed several circumscribed areas of green fluorescence on the side of $HgCl_2$ perfusion. The radioautographs in these two animals presented a pattern of a practically symmetrical uptake of $C^{14}$ methyl-O-glucose in both hemispheres. In two other rabbits abnormal penetration of NaFl could not be seen. On the other hand, in both of these animals there was a distinctly reduced passage of the $C^{14}$ methyl-O-glucose on the side of $HgCl_2$ perfusion (figs. 14 and 15). Characteristically, the decreased passage of $C^{14}$ methyl-O-glucose involved also a small portion of the contralateral cerebral cortex adjacent to the midline, which receives a collateral circulation from the anterior cerebral artery (fig. 15).

## DISCUSSION

*Ad Series 1.* In the past, in numerous studies on the BBB damage it has been generally assumed that the abnormal staining of the brain tissue by a dye indicator signifies the disturbance of the barrier in this area. However, the recent investigations on experimental edema due to a cold lesion (2, 25, 40) have clearly demonstrated that the extravasated serum components, as well as introduced tracers spread extensively *via* extravascular migration, the BBB in the edematous white matter remaining undisturbed. In this respect the present technic of direct chemical injury in animals sacrificed shortly after BBB injury provides a special advantage for elucidation of events related to disturbances of the barrier alone without confusing pictures due to subsequent migration of a tracer.

Although the observed patterns of abnormal FLA penetration are difficult to interpret, several conjectures seem to be justified. Thus, it may be assumed that the picture showing the penetration of FLA confined to the vascular wall alone is related to the earlier stages of increased permeability of the barrier. Such assumption is supported by the fact that, although this picture was frequently observed with other patterns, in a number of animals the abnormal penetration of FLA confined to the vessel wall represented the only evidence of BBB disturbance in the affected hemisphere. It is interesting to note that Lampert and Carpenter (28) in their electron microscopic investigation on vascular permeability in experimental allergic encephalitis showed with exquisite clarity that in affected blood vessels thorotrast penetrated between the endothelial cells and accumulated within the basement membrane, *i.e.*, in structures outlined by FLA in the present investigation. This may indicate the actual route of serum proteins penetration through the injured vessels, although the other possibility of transcellular passage should be kept in mind. In this respect an increased pinocytotic activity within endothelium was described in experimental brain injury by Raimondi *et al.* (32). Under normal conditions, serum proteins as well as free or protein-bound Trypan blue (8) seem to be efficiently barred at the innermost endothelial lining.

The appearance of perivascular globules has been observed in a variety of conditions associated with BBB injury (4, 8, 22). Their proteinaceous nature can be conjectured from the histochemical staining reactions. The exact

locations of these globules in the tissue, however, awaits further clarification by electron microscopy.

Equally obscure in regard to compartmental localization remains the "parenchymatous" spread of FLA. It is also puzzling that, while in extravascular migration of serum proteins during the development of edema the white matter serves as the preferential pathway, there seems to be a striking affinity for gray matter in spreading of FLA from the ventricular CSF (24) or in the presently described chemical, direct BBB injury (see fig. 7).

In several rabbits, sacrificed after some delay, there was also evidence of FLA penetration into the white matter which revealed edematous features. Concerning eventual further study on this type of edema it might be mentioned that Floodmark and Steinwall (17) have shown that the mercurial BBB damage is poorly reversible and gradually progressive in nature, while the injury produced by organic acids like penicillin G and Urokon is often reversed within a few hours.

The entry of the protein tracer into the neurons undoubtedly denotes a toxic injury to these cells. This has been evident from a study on pinocytotic uptake of fluorescein conjugated protein in nervous tissue grown *in vitro* (21), as well as from the observations *in vivo* pertaining to neurons located in areas of severe traumatic injury (23). With regard to the intracellular penetration there seems to be a noticeable difference between fluorescein isothiocyanate or Rhodamine conjugates and Evans blue or Trypan blue bound albumin. Whereas the former tracers invade primarily the cytoplasm of the damaged cells and only in instances of a more severe injury spread into the nucleus, the latter occasionally show a striking affinity for the nucleus with rather minimal penetration into the cytoplasm.

*Ad Series 2:* The regularly observed dissociation of passage of labeled albumin and $\gamma$-globulin through the walls of injured blood vessels deserves several comments. It was apparent that tagged albumin penetrated more easily and more extensively than the $\gamma$-globulin. Especially, the diffuse parenchymatous spread and penetration into neurons appeared to be features confined only to the albumin tracers. It can be assumed that the observed patterns of dissociation were related primarily to the behavior of the protein component of an indicator and not to the free markers. Otherwise it would be difficult to explain why similar pictures were obtained using albumin labeled with different markers or why albumin and globulin tagged with the same fluorescein isothiocyanate differed so much in the penetration (24). A certain release of free dye has been postulated by Clasen *et al.* (10) and Cutler *et al.* (13); it appears, however, that marker-protein dissociation played no significant role in our observations.

The simplest explanation for the observed differential penetration of albumin and $\gamma$-globulin could be related to the relative difference in molecular size of these two proteins. If the penetration of proteins into the neural parenchyma were primarily based on passive diffusion, it would be expected that a molecularly smaller compound would penetrate more extensively. There are,

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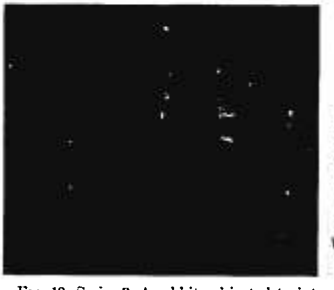

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FIG. 12. Series 3. A rabbit subjected to internal carotid perfusion with $HgCl_2$ on the left side and injected intravenously with FLA and $C^{14}$ inulin. The extravasation of the FLA remains confined strictly to the left hemisphere.

FIG. 13. The autoradiograph from the section illustrated in Fig. 12. The abnormal penetration of $C^{14}$ inulin is present also in the right hemisphere.

FIG. 14. Series 4. A rabbit subjected to a slight BBB damage on the left side with $HgCl_2$ and injected intravenously with sodium fluorescein and $C^{14}$ methyl-D-glucose. There is no detectable abnormal penetration of the fluorescent tracer.

FIG. 15. Autoradiograph from the section shown in Fig. 14. There is a striking reduction in radioactivity involving the whole left hemisphere and the most mesial portion of the cerebral cortex (which normally receives a rich collateral blood supply from the opposite side).

however, some data which indicate that the differences in passage of proteins into the nervous tissue may be related to some more complex mechanisms. The investigations on passage of various substances from the cerebrospinal fluid into the periventricular brain tissue by Draskoci *et al.* (14), Felberg and Fleischhauer (16), Edström and Steinwall (15), and Klatzo *et al.* (24) have implied the possibility of an active transport mechanism being involved. Such a mechanism has been more clearly established with regard to passage of substances from the cerebrospinal fluid into the choroid plexus (3, 30, 33, 43), and it is interesting to note that Smith *et al.* (36) in their observations on the transport of proteins by the isolated choroid plexus found a striking difference between γ-globulin and albumin suggesting an active transport mechanism in regard to the latter. Concerning the penetration of serum proteins in BBB damage it is conceivable that a biological transport through cell membranes could contribute to the separation. Such a transport system directed from brain to blood may have a "scavenger" excretory function as well as preventing the entry from blood to brain of organic acid tracers (15). Overloading or a toxic influence may interfere with functioning of such a system in a selective fashion, and this may be expressed in a differential penetration of indicators.

The dissociation of the tracers contrasts with their uniform rate of spreading in developing edema (26, 40). This difference may be related to the fact that in edematous tissue the tracers migrate extravascularly, presumably *via* an extracellular compartment (25), the BBB remaining undisturbed, whereas in chemical damage due to intravascular perfusion the picture of tracer penetration directly reflects disturbances in barrier function.

*Ad Series 3:* The purpose of this series was to assess comparatively the penetration of $C^{14}$ labeled sucrose and inulin as representative of tracers characterized by being: a. lipid-insoluble and thus poorly permeating cell membranes, b. non-metabolizable, c. electrically inert, and d. not engaged in active or "carrier mediated" transport.

Although the results were not entirely uniform, there appeared to be a general tendency to indicate that radioactive inulin and sucrose showed a more extensive penetration than concomitantly given fluorescent tracers. The possibility that radioautography represents a more sensitive method of tracer detection than evaluation of fluorescence should be kept in mind. This factor, however, appears to be very unlikely since, in general, the borders of fluorescent and radioactive areas were sharp and matched each other closely. In instances where the dissociation was observed (figs. 12 and 13), the radioactivity was very distinct in areas which showed no trace of fluorescence.

The observation that $C^{14}$ inulin penetrated more widely than the albumin tracers may, as is true of albumin-globulin, be related to differences in molecular weight. If this factor were operative, there should be even more pronounced dissociation when comparing sucrose and EBA, but this was not the case in our observations. Further strong contradiction to the relevance of the diffusion factor comes from the observation indicating more extensive spread of inulin in comparison with sodium fluorescein, since the diffusibility of the former due

to its molecular weight and elongated shape is much lower than that of the latter compound. The explanation of the observed dissociation here again could be related to a selective interference with some particular barrier mechanisms.

*Ad Series 4:* Glucose constitutes one of the brain nutrients which is physiologically transferred from blood to brain by a specific transport mechanism (11). The possible involvement of a disturbed blood-brain transfer of glucose and other nutrients in BBB physiopathology was outlined by Steinwall (38, 39) on the basis of preliminary experiments indicating that a slight mercurial damage of the barrier could induce a *decreased* influx of blood-borne $C^{14}$ glucose into the affected hemisphere as compared with the control side. By increasing the intensity of damage this difference became less apparent and eventually a reversed picture was observed in a severe BBB injury, *i.e.*, more radioactivity was present in the damaged hemisphere (Steinwall, unpublished data).

In the present study $C^{14}$ methyl-O-glucose was substituted for $C^{14}$ glucose. It was considered that this substitution represents a refinement in our experimental assay since methyl-O-glucose is known to share with glucose its specific (carrier-mediated) transport mechanisms elsewhere in the body (12, 20), without being further metabolized. This last feature makes that glucose analog an especially suitable indicator of the transfer events by avoiding any possible criticism that one may be tracing metabolic products rather than the glucose molecule itself.

Assuming that methyl-O-glucose and glucose share the same transport mechanism in the brain, our observations demonstrated that, glucose unlike sucrose, is largely transferred through the intact blood-brain interface. This transfer can be inhibited by a slight BBB damage due to mercuric chloride. The inhibitory effect of mercuric ions on various glucose-transporting mechanisms elsewhere in the body has been demonstrated by Passow *et al.* (31). Under the present experimental conditions this inhibition seems to be best seen when the mercurial injury is very weak as can be judged by comparison with conventional BBB indicators; *e.g.*, Figure 15 shows a distinct disturbance of blood-brain uptake of $C^{14}$ methyl-O-glucose at such slight noxious influence that it failed to produce any discernible extravasation of sodium fluorescein. Thus, it is possible that the decreased uptake of glucose or other brain nutrients may represent one of the most sensitive responses of the transfer-controlling machinery to certain noxious agents at the blood-brain interphase.

## SUMMARY

1. Vulnerability of the blood-brain barrier to unilateral intracarotid perfusion with chemical injurious agents was studied with the application of various fluorescent and radioactive tracers.

2. Evans blue when bound to albumin fluoresces brightly red in formalin-fixed frozen sections as viewed under ultraviolet light provides a useful and convenient tool for microscopic localization of this tracer.

3. Using fluorescent labeled albumin as a single indicator, several patterns

of its abnormal penetration through the damaged cerebral vessels have been elucidated and discussed.

4. Tracing the BBB damage by concurrent intrasystemic administration of albumin and γ-globulin labeled with contrasting fluorescent color markers revealed dissociation features in their extravascular penetration. The albumin indicator spreads, as a rule, more extensively.

5. Differences in extent of penetration were also observed when fluorescent albumin or sodium fluorescein was concurrently used with radioactive inulin or sucrose.

6. A pronounced inhibition of $C^{14}$ methyl-O-glucose brain uptake was observed on the side of a slight mercurial blood-brain barrier damage, which otherwise failed to produce any abnormal penetration of sodium fluorescein.

7. The features of selective vulnerability of the blood-brain barrier are discussed with regard to possible mechanisms involved.

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## REFERENCES

1. Allen, T. H., and Orahovats, P. D.: Spectrophotometric Measurement of Traces of the Dye T-1824 by Extraction with Cellophane from Both Blood Serum and Urine of Normal Dogs. Am. J. Physiol., 154: 27, 1948.
2. Bakay, L.: The Movement of Electrolytes and Albumin in Different Types of Cerebral Edema. *In:* Biology of Neuroglia. Progress in Brain Research. Vol. 15, p. 155, Amsterdam-London-New York, Elsevier Publ. Co., 1965.
3. Becker, B.: Cerebrospinal Fluid Iodine. Am. J. Physiol., 201: 1149, 1961.
4. Brightman, M. W.: Early Effects of Intensive X-ray Irradiation of the Diencephalon of the Rat. Exper. Neurol., 1: 97, 1959.
5. Broman, T.: On Basic Aspects of the Blood-Brain Barrier. Acta Psychiatr. Neurol. Scand., 30: 115, 1955.
6. ——, and Olsson, O.: The Tolerance of Cerebral Blood Vessels to a Contrast Medium of the Diodrast Group. Acta Radiol., 30: 326, 1948.
7. ——, and ——: Technique for the Pharmaco-Dynamic Investigation of Contrast Media for Cerebral Angiography. Acta Radiol., 45: 96, 1956.
8. ——, Grøntoft, O., and Steinwall, O.: Comparative Intravital and Post-Mortem Studies on Chemically Induced Blood-Brain Barrier Damage Tested with Trypan Blue. Acta Neurol. Scand., 41: 527, 1965.
9. ——, Branemark, S. I., Johansson, B., and Steinwall, O.: Intravital and Post-Mortem Studies on Air Embolism Damage of the Blood-Brain Barrier Tested with Trypan Blue. Acta Neurol. In Press.
10. Clasen, R. A., Cooke, M. P., Pandolfi, S., Boyd, D. J., and Carnecki, G.: Vital Staining Mechanisms in Damaged and Edematous Central Nervous System. J. Neuropath. & Exper. Neurol., 22: 344, 1963.
11. Crone, C.: Facilitated Transfer of Glucose from Blood to Brain. J. Physiol. (London), 181: 103, 1965.
12. Csaky, T. Z.: Active Intestinal Transport of 3-O-Methylglucose. *In:* 4th Intern. Congr. Biochem. Intern. Abstr. Biol. Sci. Suppl. p. 80. Vienna, 1958.
13. Cutler, R. W. P., Watters, G. W., and Barlow, C. F.: $I^{125}$ Labeled Protein in Experimental Brain Edema. Arch. Neurol., 11: 225, 1964.
14. Draskoci, M., Feldberg, W., Fleischhauer, K., and Haranath, P. S. R.: Absorption

of Histamine into the Blood Stream on Perfusion of the Cerebral Ventricles, and Its Uptake by Brain Tissue. J. Physiol., 150: 50, 1960.

15. EDSTRÖM, R., AND STEINWALL, O.: The Blood-Brain Barrier Phenomenon—The Relative Importance of Permeability and Cellular Transport Mechanisms. Acta Psychiatr. Neurol. Scand., 37: 1, 1961.
16. FELDBERG, W., AND FLEISCHHAUER, K.: Penetration of Bromphenol Blue from the Perfused Ventricles into the Brain Tissue. J. Physiol., 150: 451, 1960.
17. FLODMARK, S., AND STEINWALL, O.: Reversible Blood-Brain Barrier Alteration Induced by Certain Organic Acids and Indicated by Means of EEG and Dye Tests. Acta Physiol. Scand., 58: 386, 1963.
18. GRABAR, P., AND WILLIAMS, C. A.: Méthode Immuno-Électrophorétique de Mélanges de Substances Antigéniques. Biochem. Biophys. Acta, 17: 67, 1955.
19. HAGER, H.: Elektronenmikroskopische Untersuchungen über die Feinstruktur der Blutgefässe und perivasculären Räume im Säugetiergehirn. Acta Neuropath., 1: 9, 1961.
20. JORGENSEN, C. R., LANDAU, B. R., AND WILSON, T. H.: A Common Pathway for Sugar Transport in Hamster Intestine. Am. J. of Physiol., 200: 111, 1961.
21. KLATZO, I., AND MIQUEL, J.: Observations on Pinocytosis in Nervous Tissue. J. Neuropath. & Exper. Neurol., 19: 475, 1960.
22. ——, ——, TOBIAS, C., AND HAYMAKER, W.: Effects of Alpha Particle Radiation on the Rat Brain, Including Vascular Permeability and Glycogen Studies. J. Neuropath. & Exper. Neurol., 20: 459, 1961.
23. ——, ——, AND OTENASEK, R.: The Application of Fluorescein Labeled Serum Proteins (FLSP) to the Study of Vascular Permeability in the Brain. Acta Neuropath., 2: 144, 1962.
24. ——, ——, FERRIS, P. J., PROKOP, J. D., AND SMITH, D. E.: Observations on the Passage of the Fluorescein Labeled Serum Proteins (FLSP) from the Cerebrospinal Fluid. J. Neuropath. & Exper. Neurol., 23: 18, 1964.
25. ——, WIŚNIEWSKI, H., AND SMITH, D. E.: Observations on Penetration of Serum Proteins into the Central Nervous System. *In:* Biology of Neuroglia. Progress in Brain Research. Vol. 15, p. 73. Amsterdam-London-New York, Elsevier Publ. Co., 1965.
26. ——, ——, STEINWALL, O., AND STREICHER, E.: Dynamics of Cold Injury Edema. *In:* Brain Edema. Edit. F. Seitelberger and I. Klatzo. Vienna, Springer, In Press.
27. LAJTHA, A.: The Brain Barrier System. *In:* Neurochemistry (2nd ed.) p. 399. Edit. K. A. C. Elliott, I. H. Page, and J. H. Quastel. Springfield, Ill., Thomas, 1962.
28. LAMPERT, P., AND CARPENTER, S.: Electron Microscopic Studies on the Vascular Permeability and the Mechanism of Demyelination in Experimental Allergic Encephalomyelitis. J. Neuropath. & Exper. Neurol., 24: 11, 1963.
29. MIQUEL, J., AND CALVO, W.: Un Nuevo Metodo Sencillo y Rapido Para la Tincion Histopathologica. Arch. Esp. Morfol., 14: 125, 1958.
30. PAPPENHEIMER, J. R., HEISEY, S. R., AND JORDAN, E. F.: Active Transport of Diodrast and Phenolsulfonphthalein from Cerebrospinal Fluid to Blood. Am. J. Physiol., 20: 1, 1961.
31. PASSOW, H., ROTHSTEIN, A., AND CLARKSON, T. W.: The General Pharmacology of the Heavy Metals. Pharmacol. Rev., 13: 183, 1961.
32. RAIMONDI, A. J., EVANS, J. P., AND MULLAN, S.: Studies on Cerebral Edema III. Alterations in the White Matter: An Electron Microscopic Study Using Ferritin as a Labeling Compound. Acta Neuropath., 2: 177, 1962.
33. RALL, D. P., AND SHELDON, W.: Transport of Organic Acid Dyes by the Isolated Choroid Plexus of the Spiny Dogfish S. Acanthias. Biochem. Pharmacol., 11: 169, 1961.
34. RAWSON, R. A.: The Binding of T-1824 and Structurally Related Diazo Dyes by Plasma Proteins. Am. J. Physiol., 138: 708, 1943.

35. Silverstein, A. M.: Contrasting Fluorescent Labels for Two Antibodies. J. Histochem. Cytochem., 5: 94, 1957.
36. Smith, D. E., Streicher, E., Milkovic, K., and Klatzo, I.: Observations on the Transport of Proteins by the Isolated Choroid Plexus. Acta Neuropath., 3: 372, 1964.
37. Steinwall, O.: An Improved Technique for Testing the Effect of Contrast Media and Other Substances on the Blood-Brain Barrier. Acta Radiol., 49: 281, 1958.
38. ——: Transport Mechanisms in Certain Blood-Brain Barrier Phenomena—A Hypothesis. Acta Psychiatr. Neurol. Scand., 36, Suppl. 150: 314, 1961.
39. ——: Blood-Brain Barrier Dysfunction: Some Theoretical Aspects. Acta Neurol. Scand., 40, Suppl. 10: 25, 1964.
40. ——, and Klatzo, I.: Double Tracer Methods in Studies on Blood-Brain Barrier Dysfunction and Brain Edema. Acta Neurol. Scand., 41, Suppl. 13: R31/1, 1964.
41. Streicher, E., Ferris, P. J., Prokop, J. D., and Klatzo, I.: Brain Volume and Thiocyanate Space in Local Cold Injury. Arch. Neurol., 11: 444, 1964.
42. Tower, D. B.: Molecular Transport Across Neural and Non-Neural Membranes. *In:* Properties of Membranes and Diseases of the Nervous System. New York, Springer, p. 1, 1962.
43. Welch, K.: Active Transport of Iodide by the Choroid Plexus of the Rabbit in Vitro. Am. J. Physiol., 202: 757, 1962.