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Concentrations of Amino Acids in Extracellular Fluid After Opening of the Blood–Brain Barrier by Intracarotid Infusion of Protamine Sulfate

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Abstract: This article evaluates the influence of an opening of the blood–brain barrier (BBB) on compounds in brain extracellular fluid. The concentrations of amino acids and some other primary amines were determined in dialysates sampled from the right parietal cortex of rats before and after an intracarotid infusion of protamine sulfate. Extravasated plasma proteins were visualized by Evans blue/albumin and immunohistochemistry. CSF albumin—an indicator of blood–CSF barrier opening—was quantified with immunoelectrophoresis. The brains were macroscopically edematous after 10 mg but not after 5 mg of protamine sulfate. The higher dose led to a 50% death rate. The concentrations of amino acids did not change 10 min after the BBB opening. No significant alterations in the amino acid concentrations were observed after the lower dose. The concentrations of glutamate, aspartate, GABA, glycine, taurine, and phosphoethanolamine increased significantly within 50–80 min after the infusion of 10 mg of protamine sulfate. CSF albumin levels were significantly increased 1 h after infusion. We conclude that a dysfunction of the BBB, of a degree known to induce brain edema (10 mg of protamine sulfate), significantly increases the extracellular concentration of excitatory amino acids, GABA, taurine, and phosphoethanolamine in the extracellular space. **Key Words:** Amino acids—Microdialysis—Blood–brain barrier—Brain edema—Protamine sulfate—CSF albumin.
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Introduction of the microdialysis technique opened up the possibility of continuous monitoring of the extracellular compartment in the brain. The technique has been extensively used for studying transmitter release in experimental brain ischemia and trauma (Hagberg et al., 1985, 1987; Globus et al., 1988, 1991; Butcher et al., 1990; Hamberger et al., 1992). In the present study we have investigated if changes in the extracellular environment can be detected in dialysate during the development of vasogenic edema after opening of the blood–brain barrier (BBB). We chose to open the BBB by intracarotid infusion of the polycation protamine sulfate, which gives a transient dose-dependent BBB dysfunction. Whereas 5 mg of protamine infused into a carotid artery increases the albumin passage over the BBB, it does not increase the water content of the brain. Brain edema develops if the dose is doubled (Westergren and Johansson, 1991*b*, 1993). By using these two doses we could thus study possible changes in the dialysate after opening of the BBB per se and during the development of brain edema.

A technical question that this report also will address is whether the trauma of probe insertion induces a damage to the BBB. It has been reported that the BBB is intact after inserting a microdialysis probe (Tossman et al., 1987; Benveniste, 1989). However, such data are contradictory to earlier studies demonstrating that a 50-μm-thick needle will increase the permeability of the BBB for 3 days (Persson, 1976; Persson et al., 1976). To elucidate this point the distribution of extravasated serum proteins around and distal to the probe was evaluated by immunohistochemistry.

MATERIALS AND METHODS

Male Sprague–Dawley rats, weighing 250–300 g, from Møllegaard's Breeding Centre (Copenhagen, Denmark), were housed individually with free access to food and water.

Operative procedures

The experimental protocol was approved by the ethical committee for animal research at Lund University. The rats were anesthetized with an intraperitoneal injection of 20 mg/kg of methohexital (Brietal; Lilly; 50 mg/ml). The head

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Abbreviations used: BBB, blood–brain barrier; HCT, hematocrit; MAP, mean arterial pressure; NMDA, *N*-methyl-D-aspartate.

TABLE 1. *Physiological parameters for basal levels and 1 h after intracarotid infusion of 5 or 10 mg of protamine sulfate*

	Protamine			
	5 mg		10 mg	
	Basal (n = 6)	After infusion (n = 6)	Basal (n = 10)	After infusion (n = 9)
MAP (mm Hg)	115 ± 8	113 ± 15	114 ± 8	105 ± 14
P_aCO_2 (kPa)	5.7 ± 0.4	5.8 ± 1.2	5.6 ± 0.6	5.9 ± 1.3
PO_2 (kPa)	12.8 ± 1.9	12.7 ± 1.8	12.9 ± 2.1	12.1 ± 2.4
pH	7.44 ± 0.03	7.44 ± 0.06	7.45 ± 0.05	7.41 ± 0.09
HCT (%)	51.6 ± 0.9	50.4 ± 1.3	51.1 ± 1.1	51.0 ± 1.5
Glucose (mmol/L)	8.9 ± 1.7	9.1 ± 2.5	9.0 ± 1.7	12.4 ± 4.2[a]

Data are mean ± SD values for the given number of animals (n).
[a] $p < 0.05$ for the difference from basal levels.

was fixed horizontally in a stereotactic frame, the right side of the skull was exposed, and a hole was drilled according to the coordinates for parietal cortex (2.8 mm posterior and 4.1 mm lateral to the bregma). A microdialysis probe (CMA/10; dialysis membrane diameter, 0.5 mm; steel shaft diameter, 0.6 mm; Carnegie Medicin, Stockholm, Sweden), perfused before the implantation with Krebs–Ringer solution (containing 8.6 g of NaCl, 0.3 g of KCl, and 0.5 g of $CaCl_2$/1,000 ml), was implanted into the right parietal cortex (3.5 mm beneath the skull) and fixed to the skull with screws and dental cement. A hole was then drilled between the interparietal and occipital bones, and 50 μl of CSF was withdrawn from the cisterna cerebellomedullaris as described in detail elsewhere (Westergren and Johansson, 1991*a*). The opening was closed with dental cement.

The day after the implantation, the rats were anesthetized with methohexital intraperitoneally, and catheters were inserted in a femoral artery and a femoral vein. After ligation of the right pterygopalatine artery and the distal end of the right external carotid artery, a Portex PE 25 catheter connected to PE 50 tubing was inserted into the right external carotid artery toward the carotid bifurcation and secured to the artery. The wound in the skin was closed, and the catheter was exteriorized on the back of the neck.

BBB opening and microdialysis

The anesthesia was continued with intravenous infusion of methohexital (10 mg/ml), and the rectal temperature was kept between 37 and 38°C with a heating lamp. Heparin was given (270 IU/kg i.v.), and the probe was connected to the microinfusion pump (CMA 100; Carnegie Medicin) and perfused with a sterile Krebs–Ringer bicarbonate solution at a flow rate of 2 μl/min. After collection of two 10-min samples, 2% Evans blue (3 mg/kg) was given intravenously. Ten minutes later, protamine sulfate (grade X; Sigma), dissolved in 0.9% NaCl and filtered through a Millex-HV Millipore filter (pore size, 0.45 μm), was infused into the carotid artery over 30 s in a dose of 5 mg/100 μl or 10 mg/200 μl. Consecutive 10-min samples of the microdialysis perfusate were collected for 110 min, frozen immediately, and stored at −80°C. CSF was taken 1 h after the administration of protamine sulfate. Mean arterial pressure (MAP), blood gases, hematocrit (HCT), and glucose were quantified before, 1 h after the protamine infusion, and before the rats were killed. The CSF albumin content was determined with immunoelectrophoresis.

TABLE 2. *CSF albumin level (mg/ml) before and 1 h after intracarotid infusion of 5 or 10 mg of protamine sulfate*

	Albumin (mg/ml)	
Experimental group	Basal	After infusion
Protamine (5 mg)	0.07 ± 0.02 (6)	0.37 ± 0.15[a] (6)
Protamine (10 mg)		
Survivors	0.07 ± 0.02 (5)	0.72 ± 0.19[a] (5)
Dead	0.06 ± 0.02 (5)	0.99 ± 0.58[a,b] (4)

Data are mean ± SD values (no. of animals).
[a] $p < 0.01$ for the difference from basal level in the same group (by paired *t* test).
[b] $p < 0.05$ for the difference from after infusion level in the protamine (5 mg) group (by Scheffé's post hoc test).

Amino acid analysis

Samples of perfusates were diluted with water 1:8. The amino acids were quantified with liquid chromatography after precolumn derivatization with *o*-phthaldialdehyde (Lindroth and Mopper, 1979). The derivatives were separated on a Nucleosil C-18 (pore size, 5 μm) column (200 × 4.6 mm) using gradient elution with increasing concentrations of methanol (30–100%) in sodium phosphate buffer (50 m*M*, pH 5.40). The system consisted of a Varian model LC 5000 chromatograph and a Schoeffel model FS 970 fluorimeter. Amino acids were quantified by peak height measurements against standard solutions with a Maxima 820 software computer program (Waters, Dynamic Solutions, Milford, MA, U.S.A.).

Morphology and immunohistochemistry

After a 110-min survival period, thoracotomy was performed. A cannula was introduced into the descending aorta through the left ventricle, and the descending aorta was clamped. After an initial flush with physiological saline, 30 ml of 4% purified formaldehyde diluted in 0.1 mol/L of sodium phosphate buffer at pH 7.4 and 37°C was perfused for 15 min. The brains of the rats that died before the end of the scheduled sampling period were perfused immediately after the respiration stopped. The brains were sectioned in 2-mm coronal sections and embedded in paraffin. Sections (5 μm) were stained with acid fuchsin/cresyl violet or hema-

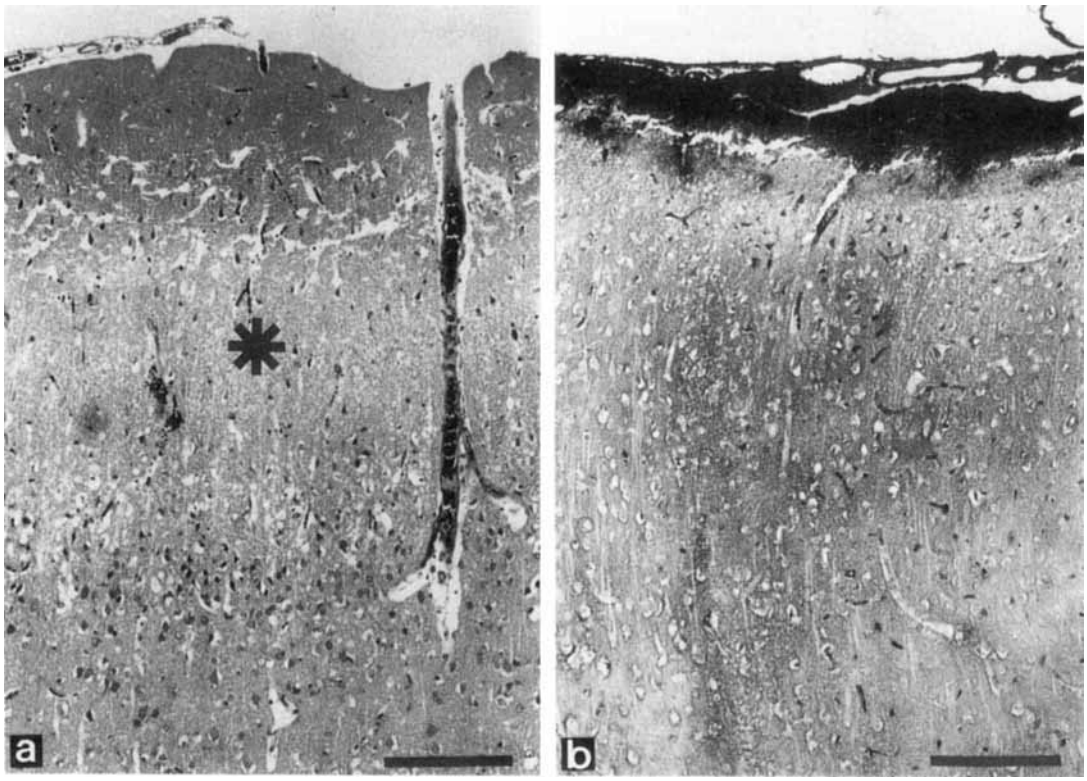

FIG. 1. a: Parenchymal lesions around the probe 24 h after probe insertion. The superficial cortical layers are necrotic (star). Acid fuchsin and cresyl violet. Bar = 210 μm. **b:** The immunoreactivity for albumin was most pronounced in the subpial regions and in the leptomeninges in the area surrounding the probe.

toxylin/eosin. Endogenous serum albumin and fibrinogen as indicators of BBB opening and the glial fibrillary acidic protein, which indicates gliosis, were identified with immunohistochemistry. The sections were incubated with rabbit specific antisera to rat albumin (Cappel, West Chester, PA, U.S.A.) and glial fibrillary acidic protein (Dakopats A/S, Glostrup, Denmark). Bound antibodies were visualized by the avidin–biotin peroxidase method (Hsu et al., 1981) using a Vectastain (Burlingame, CA, U.S.A.) ABC kit and 3,3′-diaminobenzidine tetrahydrochloride as a chromogen.

Statistics

The comparison within the groups was made with paired *t* test (physiological parameters and CSF). One-way ANOVA with Scheffé's post hoc procedure was used for group comparison of physiological parameters and CSF albumin, and Walsh's test was used for evaluation of changes in concentrations of amino acids.

RESULTS

MAP, HCT, and blood gases were stable throughout the experiments (Table 1). As in our earlier studies (Westergren and Johansson, 1991*b*), the blood glucose level was significantly higher in the rats given 10 mg of protamine sulfate, presumably a stress phenomenon.

All rats survived after infusion of 5 mg of protamine sulfate, whereas five of the rats infused with 10 mg of protamine died 50 (n = 1), 60 (n = 1), or 80 min (n = 3) after the carotid infusion. Signs of medullary compression indicating increased intracranial pressure were observed in these rats. With the lower dose, the right hemispheres were lightly to moderately stained by extravasated Evans blue/albumin. In rats given 10 mg of protamine, the coloring was more intense. Furthermore, the right hemisphere was macroscopically enlarged. CSF albumin content increased after the protamine infusion in both groups but significantly more after 10 mg than after 5 mg of protamine (Table 2).

Albumin and fibrinogen leakage was pronounced around the dialysis probe in all rats (Fig. 1). The histological examination showed neuronal necrosis, bleedings, and an acute inflammatory reaction in all animals in the areas of the probe with no difference between rats given 5 and 10 mg of protamine sulfate. Severely shrunken nerve cells with a diffuse immunopositivity for albumin and fibrinogen were found in superficial layers of the damaged cortex, whereas some histologically well-preserved neurons in deeper layers showed a granular immunoreactivity in the cytoplasm.

Amino acid analysis

The basal levels of amino acids in the dialysate (Table 3) were in the range of those reported previously

TABLE 3. *Levels of amino acids in the dialysate from the right parietal cortex before intracarotid infusion of protamine sulfate*

Amino acid	Basal (nmol/ml)
Aspartate	0.3 ± 0.2
Asparagine	0.4 ± 0.1
Glutamate	0.3 ± 0.2
Serine	2.5 ± 0.5
Glutamine	17.2 ± 13.3
Glycine	1.8 ± 1.1
Threonine	1.9 ± 0.2
Tyrosine	0.7 ± 0.1
Phosphoethanolamine	0.3 ± 0.1
Taurine	1.2 ± 0.3
α-Aminobutyric acid	0.2 ± 0.1
GABA	0.02 ± 0.02
Phenylalanine	0.4 ± 0.1
Valine	1.0 ± 0.3
Isoleucine	0.6 ± 0.2
Leucine	0.8 ± 0.3

Data are mean ± SD values from 16 animals.

for rat parietal cortex (Tossman et al., 1987; Butcher et al., 1990). After opening of the BBB no change was observed in the dialysate during the whole sampling time in rats given 5 mg of protamine sulfate, and no immediate changes were seen after 10 mg of protamine. However, after some latency that varied between those rats that survived the whole sampling period and those that died, some changes started to occur. Glutamate reached a steady-state level of 23 times and aspartate of eight times baseline 80 min after the protamine infusion, when phosphoethanolamine content was also significantly increased (five times baseline). Significantly increased levels of taurine and GABA were obtained 30 min later (Table 4).

In those rats that developed more severe edema and did not survive the whole sampling time, changes occurred earlier and were significant for glutamate, serine, glycine, phosphoethanolamine, taurine, and GABA 50 min after infusion. The levels increased further and became significant also for aspartate in those rats that survived 80 min, when the level of glutamate was 35 times the basal level, and those of aspartate 15, glycine five, phosphoethanolamine seven, taurine 10, and GABA 75 times higher than basal levels (Table 5). In these more severely edematous rats, the increase in glutamate content did not precede other changes and the increase in taurine content appeared earlier and was more pronounced than in the rats that survived the whole experimental period (Figs. 2 and 3).

Morphology and immunohistochemistry

The histopathological examination showed multifocal plasma protein immunoreactivity in the right hemisphere in all rats. It was more extensive for albumin than for fibrinogen. After 5 mg of protamine sulfate, moderately extensive spongiotic lesions were found in two and minor lesions in one of six animals. The nerve cells were severely shrunken and eosinophilic within these lesions. However, they showed no albumin immunoreactivity. There were no "cytolytic" nerve cells, i.e., nerve cells with an attenuation of the peripheral cytoplasm and a wrinkled central nucleus. There were no inflammatory reactions or bleedings.

After 10 mg of protamine, the spongiotic lesions were much more extensive, particularly in those rats that died before 110 min. Still, the vast majority of the shrunken nerve cells were albumin negative. There was no inflammatory reaction, but small bleedings were found in the two animals with the most widespread lesions. Astrocytes with a strong immunopositivity for albumin and fibrinogen were found especially in loose, edematous parts of the white matter. The blood vessels in edematous brain tissue often appeared collapsed or incompletely perfused.

DISCUSSION

After intracarotid infusion of 10 mg of protamine sulfate, the mortality was higher than in an earlier study in conscious rats (Westergren and Johansson, 1991*b*), which may reflect that the BBB is more vulnerable in anesthetized animals (Johansson, 1978; Jo-

TABLE 4. *Amino acids in the dialysate from the right parietal cortex before (basal level) and after intracarotid infusion of 10 mg of protamine sulfate in rats that survived the whole sampling time*

Amino acid (nmol/ml)	Basal	Interval samples (min after protamine infusion)				
		20–30	30–40	40–50	70–80	100–110
Aspartate	0.3 ± 0.2	0.4 ± 0.3	0.8 ± 0.8	0.6 ± 0.4	2.3 ± 2.8[a]	2.3 ± 2.5[a]
Glutamate	0.5 ± 0.3	1.2 ± 1.2	5.1 ± 5.4	4.7 ± 5.9	11.7 ± 12.3[a]	11.9 ± 13.3[a]
Glycine	1.8 ± 1.2	2.1 ± 1.5	2.2 ± 1.5	4.3 ± 2.9	2.8 ± 2.9	4.4 ± 2.7
Serine	3.1 ± 1.2	3.0 ± 2.0	3.1 ± 1.8	3.5 ± 1.9	4.0 ± 3.2	4.2 ± 3.0
Phosphoethanolamine	0.4 ± 0.4	0.5 ± 0.3	0.6 ± 0.4	0.4 ± 0.3	2.1 ± 2.0[a]	2.2 ± 1.9[a]
Taurine	1.6 ± 1.1	1.7 ± 0.9	1.8 ± 1.2	3.1 ± 3.2	7.3 ± 11.4	10.0 ± 11.4[a]
GABA	0.05 ± 0.05	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.05	1.1 ± 1.7	1.7 ± 2.3[a]

Data are mean ± SD values from five animals.
[a] $p < 0.05$ for the difference from the basal level (by Walsh's test).

TABLE 5. *Levels of amino acids in the dialysate from the right parietal cortex before (basal level) and after intracarotid infusion of 10 mg of protamine sulfate in rats that did not survive the whole predetermined sampling time*

Amino acid (nmol/ml)	Basal	Interval sampled (min after protamine infusion)			
		20–30	30–40	40–50	70–80
Aspartate	0.2 ± 0.1	0.6 ± 0.2	0.9 ± 0.6	1.3 ± 1.1	2.9 ± 1.7[a]
Glutamate	0.4 ± 0.2	2.2 ± 1.6	3.8 ± 4.0	6.5 ± 7.1[b]	14.1 ± 6.8[a]
Serine	3.3 ± 1.2	3.6 ± 1.5	4.2 ± 1.4	5.0 ± 1.2[b]	6.4 ± 2.3[a]
Glycine	1.3 ± 1.2	3.3 ± 2.9	3.9 ± 2.4	4.5 ± 2.4[b]	6.5 ± 2.6[b]
Phosphoethanolamine	0.5 ± 0.6	1.3 ± 0.8	1.7 ± 1.4	2.4 ± 1.6[b]	3.4 ± 2.1[a]
Taurine	1.7 ± 1.3	7.0 ± 4.8	7.8 ± 7.2	10.1 ± 8.1[a]	17.4 ± 7.0[a]
GABA	0.05 ± 0.05	0.2 ± 1.9	0.7 ± 1.3	2.1 ± 2.8[b]	3.7 ± 1.4[a]

Data are mean ± SD values from five (≤ 50 min) or three (70–80 min) animals.
[a] $p < 0.01$, [b] $p < 0.05$ for the difference from the basal level (by Walsh's test).

hansson et al., 1978) or that the insertion of the microdialysis probe could have increased the vulnerability.

The concentration of amino acids in the extracellular fluid, as measured in the dialysate, did not change immediately after opening of the BBB in spite of the considerable concentration gradient for amino acids between blood and extracellular fluid (Hamberger et al., 1983). This may reflect that the BBB around the dialysis probe was affected by the trauma of dialysis probe insertion, as indicated by the intense protein extravasation around the probe in the immunohistological sections. Furthermore, radioactive inulin, which does not pass the intact BBB, enters the dialysate to the same extent after probe insertion alone as after combined probe insertion and opening of the BBB with protamine sulfate (authors' unpublished data).

In addition to the "elevated baseline hypothesis," there are alternative explanations for the lack of detectable changes in the dialysate after opening of the BBB. We do not know to what extent the mechanisms

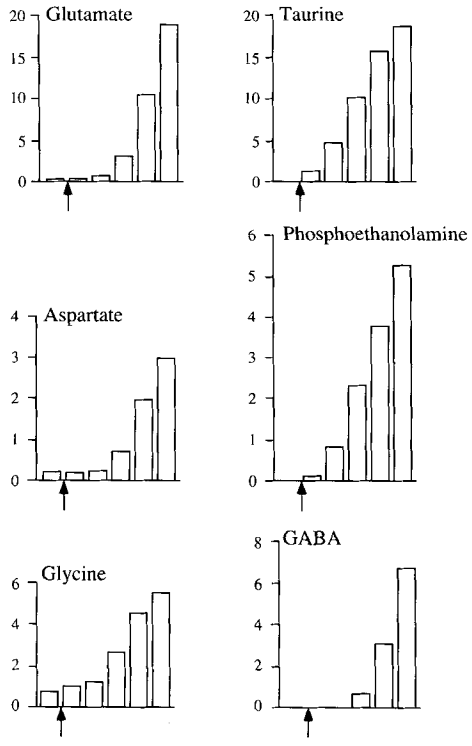

FIG. 2. Concentrations (nmol/L) of glutamate, aspartate, taurine, phosphoethanolamine, GABA, and glycine before and in successive 10-min samples after intracarotid infusion of 10 mg of protamine sulfate (arrow) in a rat that developed severe brain edema and died 50 min after the infusion.

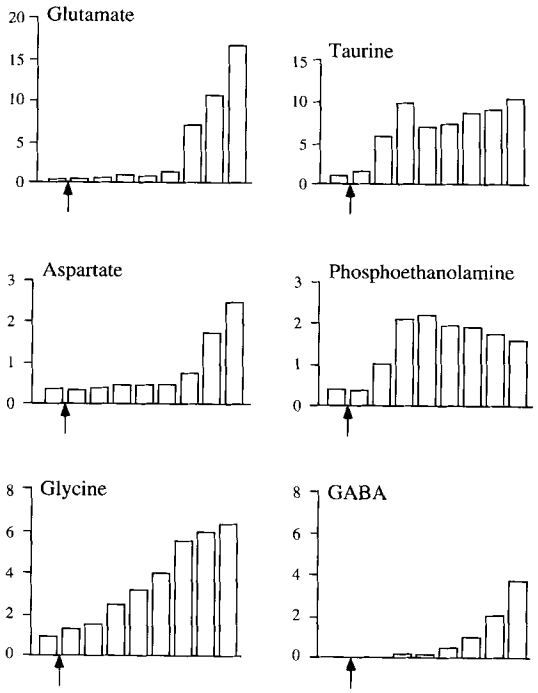

FIG. 3. Concentrations (nmol/L) of glutamate, aspartate, taurine, phosphoethanolamine, GABA, and glycine before and in successive 10-min samples after intracarotid infusion of 10 mg of protamine sulfate (arrow) in a rat that survived for 80 min.

regulating the fluxes of amino acids between blood and brain are affected. Some amino acids are metabolized in the endothelial cells. For those that enter the brain proper, the distribution in the extracellular space, metabolic pools, and the entry in glial cells and neurons varies (Lefauconnier, 1992). With an intact BBB, amino acids that have transmitter functions, such as glutamic acid, aspartic acid, glycine, and GABA, have little access to the brain from blood, and there is evidence for a net efflux from the brain of glutamate and aspartate under physiological conditions. Furthermore, active cellular uptake processes are likely to modify the "signal" on its way from passing over the endothelial cells to the entry over the dialysis membrane. Consequently, it is difficult to say what "basal levels" for extracellular amino acids really reflect, and it seems unlikely that the amino acid content in the dialysate is significantly related to their passage over the BBB.

Concomitant with the development of edema, marked changes in concentrations of glutamate, aspartate, glycine, taurine, GABA, phosphoethanolamine, and serine occurred in the extracellular fluid when brain edema was induced. This may be caused by an increased release of amino acids from glial cells or neurons or to an insufficient "buffering" capacity. The uptake mechanisms might be sufficient to normalize extracellular amino acid concentrations in the early phase after the BBB opening but be insufficient in a later phase when the cellular metabolism might be disturbed by the developing edema. Speaking against the possibility of influx from the blood is the transient nature of the BBB opening, with the largest leakage occurring in the early phase after protamine infusion (Westergren and Johansson, 1990).

N-Methyl-D-aspartate (NMDA) induces release of taurine and phosphoethanolamine from the hippocampus in vivo (Lehmann et al., 1985). In the present study, the increase in glutamate level preceded that for taurine in the dialysate in survivors, whereas the taurine level increased earlier in the rats that developed more severe edema and died 50–80 min after protamine infusion. Taurine and phosphoethanolamine are involved in the osmoregulation of the brain (Thurston et al., 1980; Wade et al., 1988; Lehmann et al., 1991). Consequently, glutamate-independent mechanisms are probably, at least in part, responsible for the taurine release in severe brain edema (Figs. 2 and 3).

Swollen astrocytes are common in brain edema (Kimelberg and Ransom, 1986), and swollen perivascular astrocytic end-feet have been observed after opening of the BBB by acute hypertension (Nag et al., 1977). Glutamate induces acute neuronal (Choi et al., 1987; Choi, 1988) as well as glial (Chan and Chu, 1989) swelling. The acute neuronal swelling has been attributed to interaction with the NMDA receptor (Choi et al., 1987; MacDermott and Dale, 1987; Choi, 1988). So far, no NMDA receptors have been identified on glial cells (Ballanyi et al., 1989; Usowicz et al., 1989; Cornell-Bell et al., 1990). Whereas the NMDA antagonist MK-801 does not reduce the edema caused by intracarotid infusion of 5 mg of protamine followed by glutamate infusion as well as the edema after 10 mg of protamine sulfate, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*f*)quinoxaline (NBQX) reduces both types of edema (Westergren and Johansson, 1992, 1993). However, to what extent the increase in amino acids in the dialysate is related to astrocytic and/or neuronal dysfunction remains unclear.

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