THE ENZYMATIC RESPONSE OF ASTROCYTES TO VARIOUS IONS *IN VITRO*

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ABSTRACT

The effect of environmental ion concentration on the enzyme activity of astrocytes was investigated in tissue cultures of rat cerebral cortex. It was found that the oxidative enzymatic activity (succinic dehydrogenase, DPN-diaphorase, and several other enzymes) of astrocytes depended on the concentration of NaCl in the environment. This response was not specific for NaCl, but was also elicited by $MgCl_2$ and LiCl; the response was less consistent, and often questionable for KCl. However, only NaCl could elicit enzymatic changes in astrocytes at concentrations known to be present in a living organism. Astrocytes were the only cells which responded this way; it appeared that the foot-plates were particularly involved in the response since increase of enzyme activity occurred earlier in the foot-plates than in the perikarya. It was concluded that astrocytes are metabolically involved in the maintenance of the ionic and osmotic environment of the central nervous system, particularly in regard to the active transport of sodium.

Astrocytic hypertrophy and its extreme stage of swollen or gemistocytic astrocytes is one of the most universally observed cellular reactions in pathological nervous tissue. Yet, it is not known what specific factors are responsible for initiating this response of astrocytes. With enzyme histochemical methods, it has been demonstrated that swollen hypertrophic astrocytes, in a variety of pathological conditions, develop a marked increase of succinic dehydrogenase (7) and of several other oxidative enzymes (5, 8, 13, 38, 10, 11, 32, 25, 26). This extremely strong enzyme reaction in hypertrophic astrocytes was in striking contrast to the very weak reaction in normal astrocytes (27, 9, 11).

The present study was undertaken in search for factors which trigger this enzymatic response of astrocytes. Observations in tissue cultures indicated that astrocytes are particularly sensitive to the ion concentration of the surrounding fluid. The effects of ionic or osmotic changes on cell morphology (30) and differentiation (3) have been studied before, but there are no data on their effect on enzyme activity in various cell types. Our material seemed of considerable interest, in view of recent claims that glia cells (39, 34) serve as compartments for the movement of fluids between blood and nerve cells, being homologous to the extracellular space of other organs, and that they might be involved in the regulation of the ionic environment of nervous tissue (6). Katzman (16, 17), continuing this line of thinking, claimed that glial cells might represent "high sodium" cells, as suggested by the binding of Na^+ to acidic lipids. Giacobini's (14) observation of very strong carbonic anhydrase activity in glial cells seems to support Tschirgi's concept of a secretory system in glia cells (37). Kock, Rank, and Newman (18) reported increased

Na^+ in glial tissue of gray matter which had been depleted of neurons.

MATERIALS AND METHODS

TISSUE CULTURE METHODS: Explants of newborn rat cerebral cortex were grown in plasma clots affixed to 12 × 50 mm coverslips in roller tubes. The cultures were fed in a nutrient medium containing 50 per cent balanced Gey's solution, 45 per cent human ascitic fluid, and 5 per cent embryonal extract, the medium was enriched to contain 300 mg per cent glucose (Pomerat and Costero, 29). The ionic composition of the stock ascitic fluid and embryonal extract was determined by spectrophotometric analysis. The concentration of NaCl, KCl, $MgCl_2$, and LiCl was varied in the Gey's solution which was mixed with an equal quantity of a stock solution of ascitic fluid and embryonal extract. The final ionic concentrations are given in Table I. In the first series (Table I), NaCl was varied in 17.2 mEq/liter increments from 0 to 292.4 mEq/liter, later, only "elevated NaCl" and "low NaCl" were used for routine testing. The elevated $MgCl_2$, LiCl, and KCl concentrations (Table I) were in the range between the minimum that would produce an enzymatic response of the astrocytes and the maximum that was compatible with survival of the cultures; this had been determined in other explorative series. All of the ion concentrations used for these experiments were tolerated by the tissue cultures, at least for 8 days, but there was usually retardation of outgrowth with elevated salt concentrations.

Although some of the cultures for each of the salts were studied after 4 days, most of the cultures were routinely grown for 8 days with renewal of the culture medium on the fourth day. At the end of the experiment, the unfixed cultures were incubated for 2 hours at 38° in 10 ml of histochemical media for one of the following enzymes: succinic dehydrogenase (SD), DPN-diaphorase, TPN-diaphorase, glutamic dehydrogenase, lactic dehydrogenase, malic dehydrogenase, and glucose-6-phosphate dehydrogenase. The methods complied essentially with Pearse (28), however, several modifications are being used in this laboratory (12). The medium for SD was used for routine evaluation because the enzymatic changes were most marked for this enzyme. The cultures were fixed in formalin and mounted in glycerin-gel.

CONTROLS: Conventionally, Gey's balanced salt solution is considered a "normal" ionic environment for cultures of brain tissue. However, if compared with Na^+ levels in normal brain tissue (22), the balanced solution contains a higher concentration of Na^+. The absence of or very slight SD reaction observed in astrocytes of normal brain is much more similar to the intensity of reaction observed in astrocytes cultured in "low NaCl" media than to the moderate reaction seen in astrocytes cultured in balanced solution. Since the precise concentration of Na^+ in the various cellular compartments of the brain is not known, we have not identified any given concentration of Na^+ as "normal." For our studies, the "low NaCl" cultures were considered as controls and "low NaCl" concentration was used with the other ion variation series. Trowell (36) noted that the optimal concentration of NaCl for survival of lymphocytes is 0.4 per cent, a preference which is not shared by other tissues.

TABLE I

mEq/liter of Ions That Were Varied in Culture Media

	NaCl	KCL	$CaCl_2$	$MgCl_2$	LiCl
NaCl varied in 17.2 mEq increments	0 to 292.4	5.03	4.9	2.06	0
(Balanced Gey's solution)	138.0	5.03	4.9	2.06	0
Elevated NaCl	205.0	5.03	4.9	2.06	0
Low NaCl	98.3	5.03	4.9	2.06	0
Elevated KCl	98.3	58.8	4.9	2.06	0
Elevated $MgCl_2$	98.3	5.03	4.9	20.2	0
Elevated LiCl	98.3	5.03	4.9	2.06	49.5

RESULTS

Arrangement and Identification of Cells in Cultures

Increased concentration of NaCl in the tissue culture media, as defined in the next paragraph, had two effects on astrocytes: (*a*) the astrocytes almost always remained in the explant, in contrast to the low NaCl cultures in which many outgrowing cells were identified as astrocytes,

and (*b*) to elicit an increase of SD and other enzymes which was proportional to the increase of NaCl concentration (compare Figs. 1 to 5 and description in next paragraph).

Astrocytes grown in elevated NaCl, LiCl, or $MgCl_2$ (Table I) showed no tendency to emigrate from the explant into the adjacent clot. Instead, they aggregated at the surface of the explant (Figs. 3 and 4); some attached their perikarya flat to the surface, while others sent a short husky process to the surface where it enlarged, forming a foot-plate.[1] These foot-plates were seen only in tangential views of the explant (Fig. 6), and the cells were easily distinguished from all others by their extremely strong SD reaction (Fig. 4). The presence of a foot-plate served as a criterion for positive identification of astrocytes, as no other type of glia cell is known to have processes which terminate in foot-plates. Often, such cells became atypical and lost all their processes except the extremely hypertrophic foot-plates (Figs. 7 and 8), or they developed clasmatodendrosis (Fig. 5). These changes were consistent with those known to develop in extremely hypertrophic astrocytes in neuropathological material (19), that is, a marked increase in oxidative enzyme activity, thickening, shortening, and reduction in number of processes, and a relative hypertrophy of the process connected with the foot-plate. Undoubtedly, these cells were *viable*, since they managed to increase their enzyme supply over a period of 8 days. On the other hand, since astrocytic hypertrophy is considered a pathological change *per se*, it was not surprising to observe retardation of outgrowth of all cell types under conditions which produced astrocytic hypertrophy. Retardation of outgrowth as a result of postmortem storage has been observed by Hogue (15), but this phenomenon evidently is not related to our observations.

Cells with strong SD reaction were never seen in cultures grown in low NaCl media (Table I). In the explants of such cultures, astrocytes, if present, were not discernible from the rest of the tissue. In such cultures, there was a generous emigration of cells into the adjacent clot and the impression was given that lowered NaCl stimulated emigration of astrocytes and other cells from the explants; outgrowth of astrocytes is known to be retarded under "normal" culture conditions (23). Astrocytes among the outgrowing cells had all their characteristic morphological features (see below) except foot-plates, probably because of lack of an appropriate structure for attachment. These cells had only a very slight reaction for SD (Figs. 1 and 2); they were identified as astrocytes by the number, length, and gradual tapering of their processes, their large cytoplasmic bodies, and their vesicular nucleus (Fig. 2); occasionally, they were multinucleated (Fig. 1), as is known for astrocytes (but not for oligodendroglia) in brain tissue.

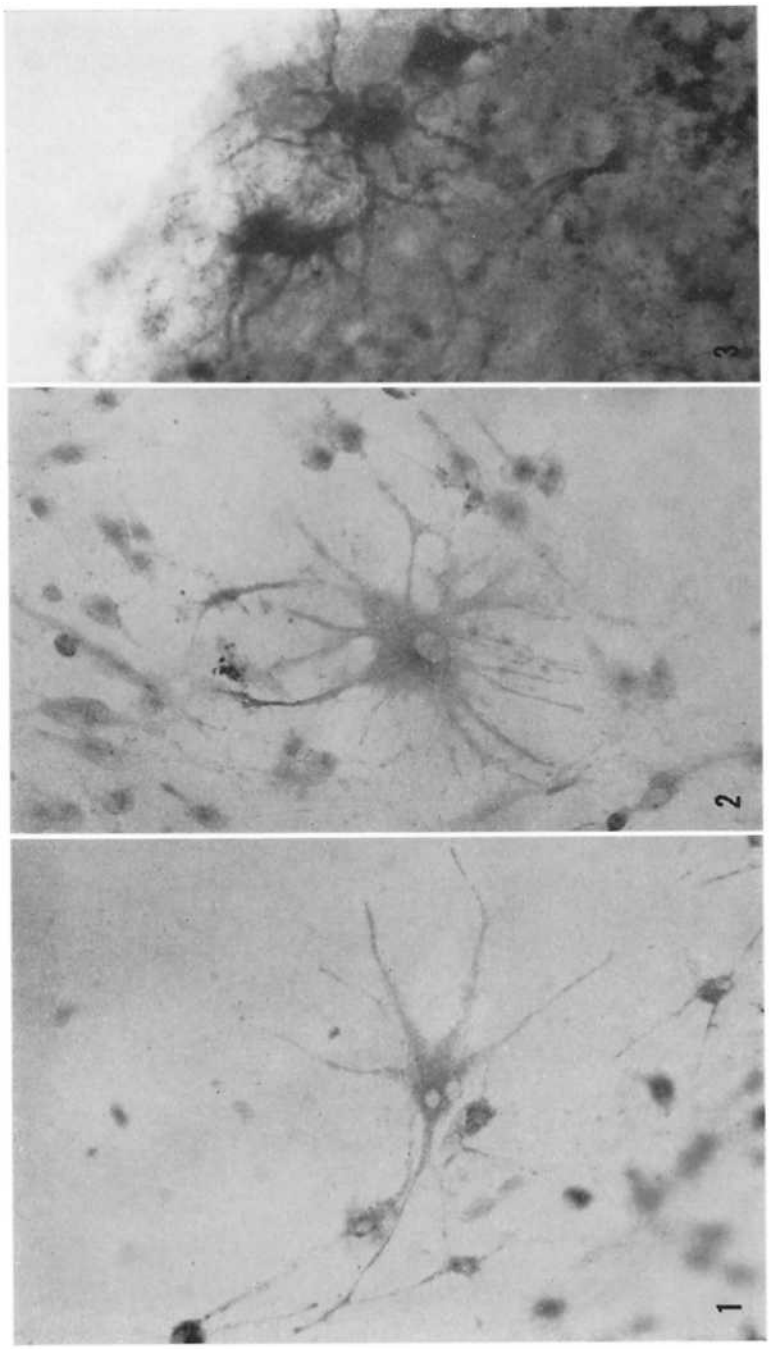
It was not a major problem to distinguish astrocytes from oligodendroglia and other cell types, because under conditions which elicited an enzyme increase in astrocytes the attached foot-plate could often be seen and served as positive identification; also, our studies were made well before the known onset of myelination (9) in rat cortex, during which time oligodendroglia cells are known to differentiate.

Nerve cells were identified by their large nuclei and their nucleolus, sometimes by their spindle or pyramidal shape, and mode of branching, and size of their dendrites (Fig. 9) which usually did not taper gradually from the perikarya—as astrocytic processes do—and, sometimes, by the presence of an axon hillock with axon, the latter being thinner than the dendrites. In nerve cells, there was a moderate reaction for SD which was not influenced by the composition of the media and which was less than in hypertrophic astrocytes (compare Figs. 9 and 4) but more than in astrocytes from low NaCl media (compare Figs. 9 and 1).

The Succinic Dehydrogenase Response of Astrocytes to Variations of NaCl, $MgCl_2$, LiCl, and KCl in the Culture Media

In a series of cultures in which Na^+ in the media was varied from 0 to 292.4 mEq/liter (Table I), there was a gradual increase of SD in astrocytes with increase of NaCl in the media. In media containing 34 to 86 mEq/liter Na^+, the astrocytes in the explant did not show enough SD reaction to permit identification; however, among the

[1] This peripheral arrangement of cells suggested that they made an effort to form a limiting membrane encapsulating the explant like a small "organ." Their arrangement resembled that of astrocytes covering the pial surface of brain tissue, however, the fact that the arrangement was observed in opposite portions of the explant indicated that the cells had actively acquired the peripheral position and were not merely persisting subpial astrocytes.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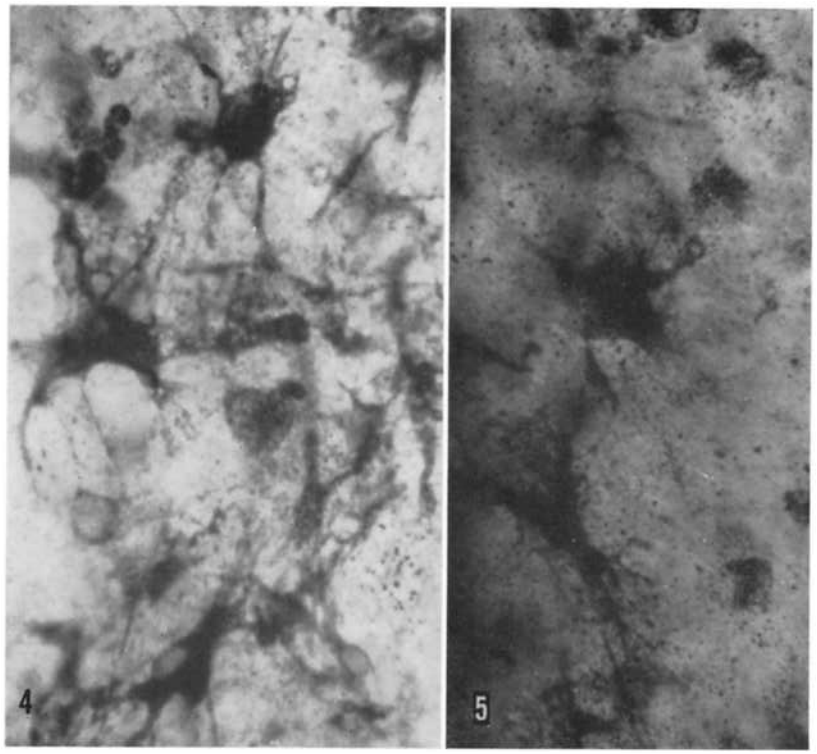


FIGURES 1 through 5 All five pictures illustrate astrocytes grown for 8 days at various concentrations of NaCl. All cultures were incubated for the same length of time in the medium for succinic dehydrogenase (SD). In addition to the increase of the enzyme reaction, one can also recognize the shortening, thickening, and rarefaction of processes, which is typical for astrocytic hypertrophy. Fig. 1: 51.6 m.eq/liter Na^+, binucleated astrocyte; Fig. 2: 68.8 m.eq/liter Na^+; Fig. 3: 172 m.eq/liter Na^+, astrocytes at periphery of explant, moderate reaction in the cytoplasm; Fig. 4: 205 m.eq/liter Na^+ ("elevated NaCl"), intense reaction throughout cytoplasm; Fig. 5: 292 m.eq/liter Na^+, sublethal concentration, clasmatodendrosis. All about × 600.

outgrowing cells, there was a very slight SD reaction (Figs. 1 and 2) in numerous cells that were identified as astrocytes (see above). If Na^+ in the culture media was 138.0 mEq/liter (balanced Gey's solution), astrocytes with a moderate SD reaction could be identified, even in the explants (40). In the explants of the "elevated NaCl" cultures (Table I and Figs. 3, 4, and 5), the excessively strong SD reaction in the hypertrophic astrocytes was in marked contrast with the moderate reaction in any other cell type. Extremely high sublethal concentrations of 292 mEq/liter produced an excessive enzyme reaction in astrocytes, but also advanced clasmatodendrosis (Fig. 5). While one could histochemically distinguish that a stepwise increase of NaCl in the media produced a proportional increase of SD in the astrocytes (Figs. 1 to 5), the difference in the SD reaction was much more striking if astrocytes grown in low NaCl (98.3 mEq/liter) media were compared with those grown in elevated NaCl media (205 mEq/liter). Ninety-five unscreened cultures that had been grown in either elevated or low NaCl media were coded to permit blind identification. Eighty-one per cent of these were correctly identified. Further study showed that if these cultures had been screened to eliminate those with generalized poor growth, the identifications would have been 92 per cent correct.

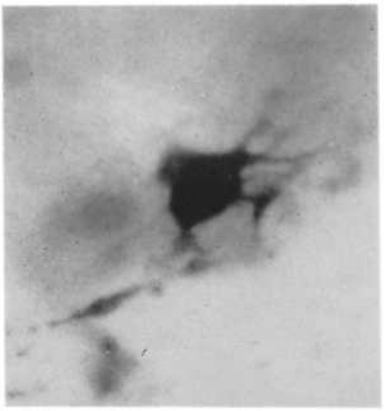


FIGURE 6 Astrocytes with short process terminating in a foot-plate. 20.2 mEq/liter Mg^{++}. × 550.

Cultures grown in media with markedly elevated $MgCl_2$ and LiCl (Table I) developed astrocytes with increased SD which were indistinguishable from those produced by elevated NaCl (Fig. 10).

The astrocytic response to KCl was much more difficult to evaluate than the other studies. The maximum K^+ that would permit survival of the cultures was 58.8 mEq/liter (Table I). No astrocytes were seen in the explants that were cultured in media in which the K^+ was markedly less than 58.8 mEq/liter. While the cultures grown in the elevated KCl medium did show scattered cells with a strong SD reaction, only a few of these could be absolutely identified as astrocytes; the reaction varied consistently and foot-plates were rarely seen. Thus, if there was an enzymatic astrocytic response to elevated KCl, it was much weaker than the response to the other salts, was erratic, and was elicited only by an extreme increase.

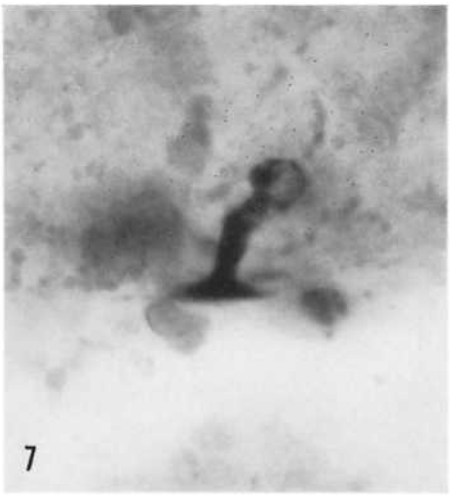
While this material indicated that the enzymatic response of astrocytes was not specific for NaCl, it may well be of physiological significance that NaCl was the only one of the salts studied that could elicit the response at concentrations which can be found in a living organism. Doubling or tripling of KCl or $MgCl_2$, or adding a few mEq/liter of Li^+, had no recognizable effect on the growth rate of cultures and never resulted in any increase of SD in the astrocytes.

After careful and thorough study, it was concluded that the presence of hypertrophic astrocytes in the explants was dependent on only the concentration of salts in the medium; it was not influenced by size or growth rate of the explants, the presence or absence of degenerative changes, the amount of dead tissue in the culture, or the penetration of the histochemical substrate. Since all of the explants were very thin and not an organ-type culture, penetration of the histochemical substrate was not a problem.

Time Sequence of Histochemical Changes

In preliminary studies (not reported in this paper), some cultures were kept in elevated NaCl media for 1 day. No astrocytes with increased enzyme could be seen in 1-day cultures. Exposure to elevated ionic concentrations for approximately 4 to 8 days was required to produce a marked enzymatic response.

It could definitely be observed that the enzyme reaction increased first in the foot-plates; a gradient between foot-plate and perikaryon was often seen in cultures that had been exposed to increased ion concentration media for 4 days. After 8 days of exposure, the distribution of



FIGURES 7 and 8 Atypical astrocytes which lost all their processes except the hypertrophic one which connects with the foot-plate. Remnants of other processes out of focus in Fig. 7. Eight days in 205 mEq/liter Na^+. × 650.

activity was more uniform throughout the cell and its processes. The time involved for accumulation of enzyme in these cells indicated that this must have been a phenomenon of synthesis of enzyme in the cell, rather than a phenomenon of ionic activation or inhibition of already available enzyme. (Figs. 11 and 12).

Enzyme Systems Involved in the Astrocyte Responce to NaCl

All of the observations described above were of the histochemical reaction for SD. The following enzymes were also studied: TPN- and DPN-diaphorase, glucose-6-phosphate dehydrogenase, malic dehydrogenase, lactic dehydrogenase, and glutamic dehydrogenase. A marked difference in enzyme activity between astrocytes grown in low NaCl media and those grown in high NaCl media was apparent for all the enzymes studied, but the degree of difference varied with each enzyme, being highest for SD and decreasing for the others in the order listed above. Astrocytes grown in low NaCl media showed a moderate reaction for glutamic and lactic dehydrogenase, but the reaction was still much stronger in cultures grown in elevated NaCl media. It was of interest to note that the extent of response of the various enzyme systems to varied salt concentrations was very similar to that observed in experimental brain lesions (32, 26).

DISCUSSION

Marked activity of oxidative enzymes has been observed in a variety of biologically active or proliferating cells, such as osteoclasts (7, 4), phagocytes (21), foreign body cells (2), and macrophages (33). An increase of enzyme activity in oligodendroglia occurs during the period of myelination, both *in situ* (9) and *in vitro* (40). Thus, the increased activity of oxidative enzymes in hypertrophic astrocytes might not be considered a specific change. However, our data

indicated that the increase in oxidative enzyme activity in astrocytes depended on the salt concentration of the environment, and this was considered a specific metabolic response which was seen only in astrocytes.

The response was not specific to NaCl, but was also elicited by $MgCl_2$ and LiCl. However, the response to the latter salts was only of theoretical interest because the concentrations required to elicit enzymatic changes in astrocytes were not compatible with a living organism. Only NaCl elicited enzymatic changes of astrocytes in concentrations which are conceivable for brain *in vivo*.

The Na^+ concentrations which increased astrocyte enzyme activity in cultures were within the range of increased Na^+ reported for edematous brain (35) in which hypertrophic, high-enzyme astrocytes are known to develop. In contrast, the Na^+ levels of low NaCl culture media were within the normal range for brain (65 to 90 mEq/liter wet weight/hemispheres; 22). These media did not elicit an increase of SD activity in astrocytes, agreeing with the observation that there is very little SD in astrocytes of normal brain tissue. Moderate enzyme reaction was found in astrocytes grown in a normal balanced saline medium (40). It was felt, thus, that the increased enzymatic activity produced *in vitro* represented a phenomenon closely related to, if not identical with, that of astrocytic hypertrophy in pathological tissue.

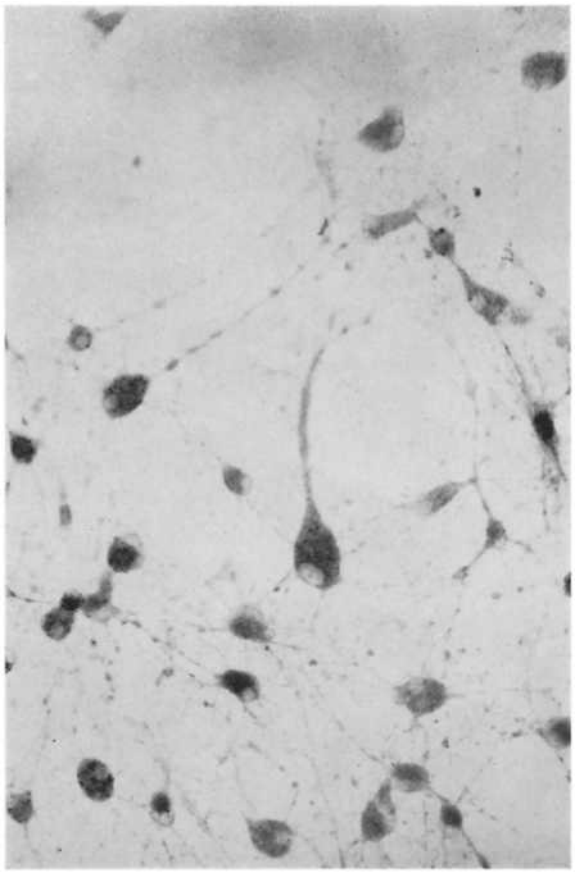


FIGURE 9 One nerve cell and oligodendroglia (presumably) with a moderate reaction for SD. 86 mEq/liter Na^+, 8 days. × 500.

In view of these observations, we considered the astrocytic response to NaCl of great potential significance for the interpretation of brain edema and astrocytic hypertrophy. Whether the effect of NaCl on astrocytes is a specific response to Na^+ or an osmotic effect cannot be decided without knowledge of the mechanism involved. In brain tissue, water content and ionic concentration are

interdependent (22). A cell sensitive to Na^+ concentrations would undoubtedly also be involved in the osmotic regulation of tissue fluids.

The response to KCl was markedly weaker than that to other ions. This suggested that the response of astrocytes might not be a general osmotic phenomenon but one more specific for certain ionic movements in the tissue. There was some similarity between this datum and the observations by Alexander and Myerson (1) who found, in a variety of lesions, accumulation of Na^+, Mg^{++}, and Ca^{++} in scavenger cells, but not of K^+, which was decreased in damaged tissue.

NaCl in nerve tissue. Indeed, only astrocytes are morphologically adapted to this function. A cell responsible for the removal of sodium would have to have a morphological system to channel sodium ions out of the tissue. Such a system is present in the vascular or pial process and the foot-plates of astrocytes which cover about 85 per cent of the capillary walls in rat brain (24). This interpretation of the significance of foot-plates would be in keeping with the observation that the increase of enzyme activity seems to develop first in the foot-plates.

Reports of interchangeability of astrocytes and

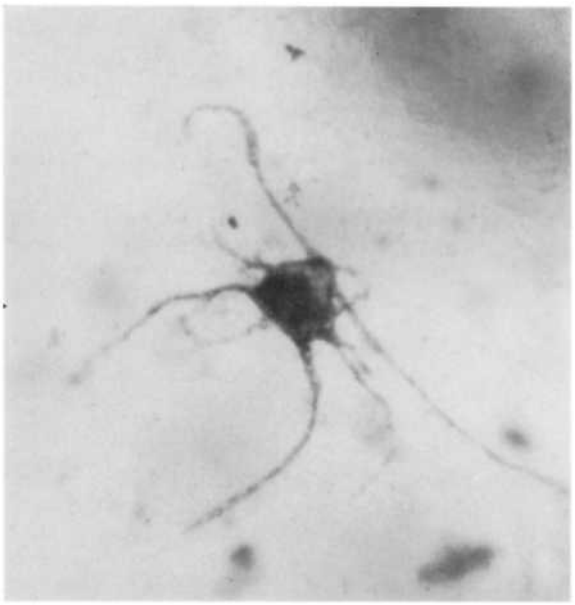


FIGURE 10 Astrocytic enzyme response to 20.2 mEq/liter Mg^{++}. × 500.

The precise mechanism of the enzymatic response of astrocytes to changes of the ionic environment is still obscure, yet it seems logical and in agreement with present metabolic concepts that a higher rate of sodium removal from brain tissue would require more energy, and thus increased oxidative metabolism. One approach which needs to be developed is a method for analytical measurement of the enzyme response of astrocytes to the ion concentration of their environment.

Our data are consistent with the theory that astrocytes are involved in the maintenance of the osmotic and ionic balance, particularly that of oligodendroglia (19, 20) are not supported by the present data; likewise, they are difficult to reconcile with experience in neuropathological conditions (31). No data are available where such claims had been based on the demonstration of cytoplasmic processes and foot-plates, without which cellular identification is futile.

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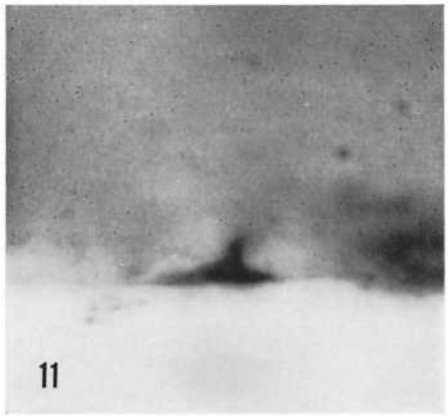

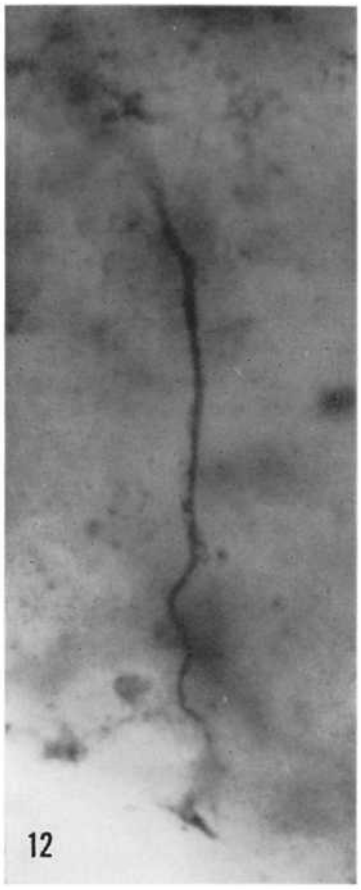

FIGURES 11 and 12 Increase of SD in foot-plates only after exposure to elevated salt concentration for 4 days Fig. 11: 205 mEq/liter Na^{+}. × 800. Fig. 12: 20.2 mEq/liter Mg^{++}. × 650.

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