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Effects of Lactic Acid on Astrocytes in Primary Culture

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Abstract. Excessive tissue lactic acidosis is considered to be detrimental to the central nervous system (CNS) and may adversely affect recovery from anoxia, ischemia, trauma and epilepsy. Since astrocytes are believed to play a role in pH regulation in the CNS, we studied the effect of this acid on primary astrocyte cultures. Cells exposed to lactic acid showed chromatin clumping, an increase of lipid and dense bodies, a loss of polyribosomal clusters, slightly increased cytoplasmic lucency, swollen mitochondria and tangled intermediate filaments. These alterations progressed with lower pH and longer exposure. Irreversible changes occurred one to two hours after exposure at pH 6; after 30 to 60 minutes (min) at pH 5.5 and after ten to 30 min at pH 5. Comparable results were obtained with the use of other weak acids indicating that the observed changes were due to increased hydrogen ion concentration rather than secondary to lactate per se. Additionally, various concentrations of lactic acid adjusted to identical pH produced similar morphologic alterations. Thus, while lactic acid caused marked and at times irreversible alterations in astrocytes, severe and prolonged acidosis was required to produce such injurious effects. This relative resistance of astrocytes to acidosis is in keeping with their potential role in pH regulation in brain.

Key Words: Astrocytes; Cell culture; Hydrogen-ion concentration (pH); Lactic acid.

INTRODUCTION

The potential deleterious effect of excessive amounts of lactic acid on brain tissue has been emphasized in recent years (1, 2). Such excesses occur in hypoxia, ischemia, trauma and seizures (3–8). Lactic acid may also play a role in Reye's syndrome (9), in thiamine deficiency (10) and in some hereditary enzyme deficiencies (11). The accumulation of such an acid is believed to have a detrimental effect on the nervous system and contribute to poor recovery of neurophysiological (12) and clinical status (13) as well as affecting energy metabolism (14–18).

The morphologic effects of lactic acid on brain have not been well delineated. Degenerative neuronal changes, glial and endothelial swelling have been described in anoxic, ischemic, hyperglycemic animals that had elevated tissue lactate levels (19–22). However, these studies are complicated by the fact that such alterations are not due solely to lactic acid. Moreover, it has not been established whether the harmful effects of lactic acid are caused by lactate itself or by a fall in pH, nor is it precisely known how lactic acid injures nervous tissue.

Most cells in the nervous system appear to be adversely affected by lactic acid. It has been suggested that astrocytes may be especially vulnerable to the deleterious effects of lactic acid since glycogen, the main source of endogenously generated lactic acid, is localized predominantly in astrocytes (23). Not only may this provide a

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source of lactic acid but it may also increase the osmolarity of the cell. It was further suggested that damage to astrocytes or endothelial cells might ultimately be responsible for tissue necrosis.

Accordingly, to explore directly the effects of lactic acid on astrocytes, we have exposed primary astrocyte cultures to lactic acid solutions of different concentrations and pH levels and to other weak acids. In addition, we determined the degree of tolerance or resistance of these cells to lactic acid in order to establish their critical threshold values with acidosis.

MATERIALS AND METHODS

Primary astrocyte cultures were prepared as previously described (24). Briefly, cells were obtained by mechanically dissociating cerebral cortical tissue of neonatal rats. Cultures were incubated in a humidified chamber provided with 5% CO_2 and 95% air. After two weeks (wk), the cells were treated with 0.5 mM dibutyryl cyclic adenosine monophosphate. Based on immunohistochemical identification of glial fibrillary acidic protein (GFAP) and glutamine synthetase, the cultures consisted of about 95% astrocytes.

Experiments were carried out on three- to four-wk-old cells. Cultures were exposed to various experimental solutions for one minute (min) to two hours (h). Two protocols were used. In the first series of experiments, varying amounts of L-lactic acid were added to Dulbecco's modified Eagle medium (DMEM) to achieve concentrations ranging from 15 to 50 mM, resulting in pH levels of 7.6 and 4.4, respectively. In some experiments, acetic acid and citric acid were substituted for lactic acid. In a second set of experiments, the cells were exposed to a phosphate-buffered Ringer's solution (0.01 M) to which lactic acid was added. Because of the lower buffer strength of this solution, far less lactic acid (1.5–13.3 mM) was required to achieve a desired pH (7.4–4.0, respectively). The osmolarity of the various lactic acid solutions used was determined. Based on these values, appropriate solutions of sodium chloride or choline chloride were used as controls.

At the conclusion of the incubation, the plates were fixed with 10% neutral formalin for 30 min and stained with May-Grunwald/Giemsa. For electron microscopy, cultures were fixed with 4% buffered glutaraldehyde for one h at room temperature and post-fixed with 2% osmium tetroxide for another hour. After dehydration with graded alcohols, capsules containing Epon 812 were inverted onto the plates. These were then heated for 24 h at 37°C followed by additional 48 h at 60°C. Ultrathin sections were obtained using a Sorvall MT2-B microtome, stained with uranyl acetate and lead citrate and subsequently examined with a Philips 300 electron microscope.

Reversibility Studies

To establish the limits of tolerance, cells were exposed to lactic acid for variable periods of time and at different pH levels. The media containing lactic acid solutions were then aspirated and replaced by DMEM. After 24 and 48 h, the cells were stained as described above and examined by light microscopy. As another test of cell viability, the ability of the cells to exclude trypan blue after treatment with lactic acid was determined.

RESULTS

Light Microscopic Findings

Control cultures consisted of a monolayered sheet of cells with moderate amounts of amphophilic or basophilic cytoplasm and fine threadlike processes. Nuclei were round to oval in shape, relatively uniform in size and had fine evenly dispersed chromatin (Fig. 1A). Stellate astrocytes with prominent cytoplasmic processes were sometimes seen in clusters. Occasional dense bodies and extracellular debris were present.

Cultures exposed to lactic acid at pH 7.4 for up to two h showed only minimal

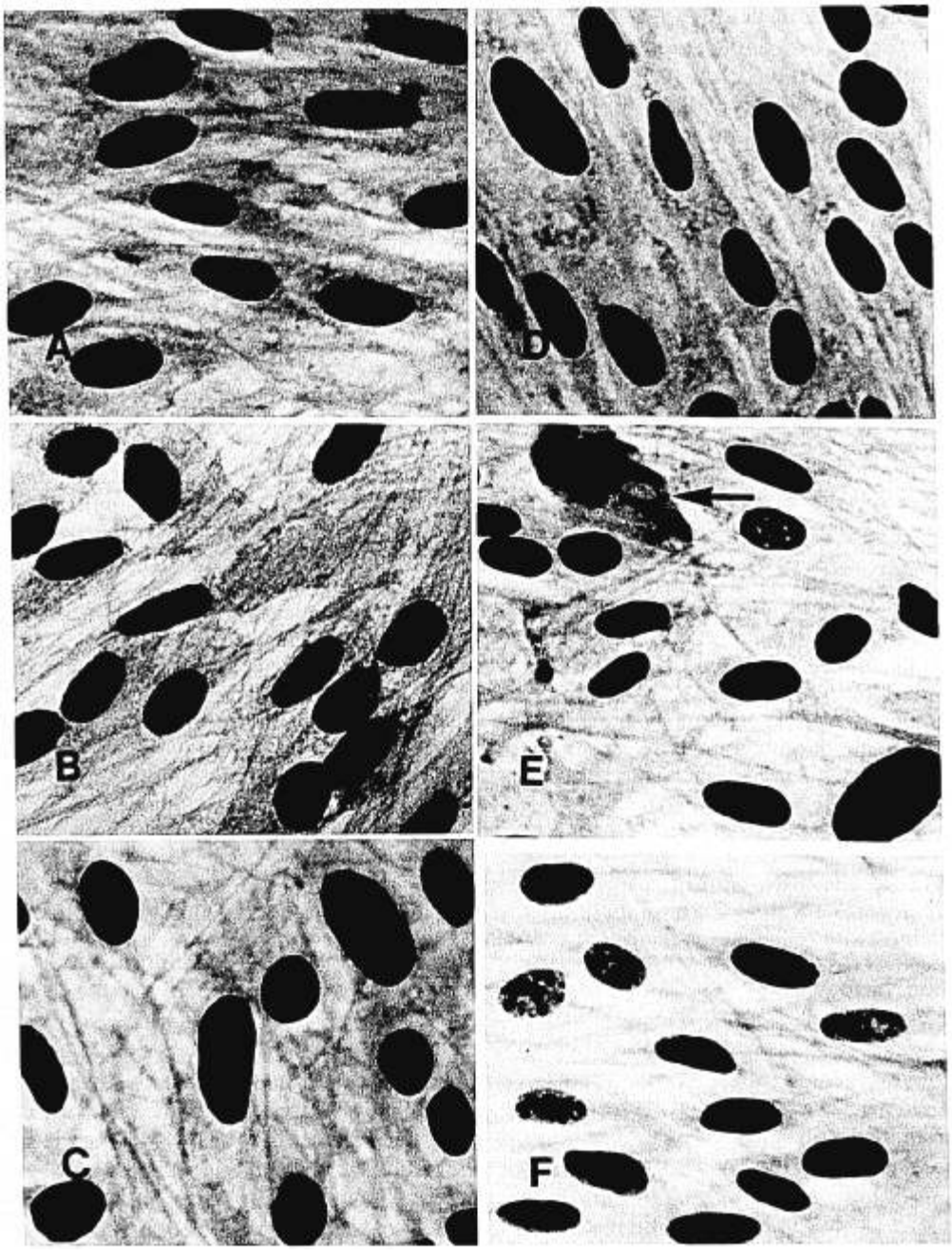

Fig. 1. A. Control culture (untreated) shows a monolayered sheet of cells with regular nuclei and fine, evenly dispersed chromatin. B. Following exposure to lactic acid (pH 7.4) for two h, the cells are essentially similar to the control. C. Nuclear pleomorphism, slight chromatin clumping and pyknosis are noted one h after exposure at pH 6.0. D. Variation in nuclear size and slight chromatin clumping occur at pH 5.5 after only five min. E. Nuclear pleomorphism, pyknosis and moderate chromatin clumping are present at pH 5.0 after 30 min. An occasional cell shows prominent cytoplasmic staining (arrow). F. Smaller nuclei, severe chromatin clumping, nuclear pallor and loss of cytoplasm are noted at pH 4.5 after three min. All ×900.

alterations consisting of a slight variation in nuclear size, minimal to no chromatin clumping and a mild increase in extracellular debris (Fig. 1B). Cells exposed to pH 6.5 for 30 min to two h showed only subtle chromatin clumping. At pH 6.0, slight chromatin clumping was present after ten min. Nuclear pleomorphism and increased chromatin clumping were seen after 30 min while pyknosis was noted after one h (Fig. 1C). Cytoplasmic dense bodies were slightly increased.

At pH 5.5, chromatin clumping was first noted after five min and became more prominent with longer exposure (Fig. 1D). Nuclear pyknosis was present after ten to 30 min, although some pale nuclei were also noted. At pH 5.0, chromatin clumping was seen after three min and nuclear pleomorphism after ten min. Following 30 min of exposure, many cells developed more prominent cytoplasmic staining and slight pallor of nuclei. Some cells also showed nuclear pyknosis (Fig. 1E).

At pH 4.5, nuclear pleomorphism, decreased nuclear size, chromatin clumping and pyknosis were discernible after one min and became marked after three min (Fig. 1F). Some cells with pale nuclei were also seen. Prominent nuclear margins and nuclear pyknosis were noted after ten min and became more conspicuous with longer exposure. At pH 4.0, marked chromatin clumping was observed as early as one min.

Similar changes were observed when the cultures were exposed to acetic acid and citric acid instead of lactic acid at comparable pH levels and duration of treatment.

The nuclear changes are presented in Table 1.

Electron Microscopic Findings

Control cultures consisted of cells with moderately electron dense cytoplasm, numerous bundles of intermediate filaments and a few microtubules. Polyribosomal clusters were abundant. Mitochondria were moderate in number and showed a relatively dense matrix. Homogeneously electron dense bodies were sometimes noted. Lipid droplets were rarely seen. The nuclei were oval with regular borders, delicate limiting membrane and possessed fine, evenly dispersed chromatin granules (Fig. 2A).

Cultures exposed to lactic acid at pH 7.4 showed minimal chromatin clumping and a slight increase in the number of dense bodies within some cells after five min (Fig. 2B). Mild to moderate chromatin clumping was first noticed after ten min of exposure at pH 6.5; after five min at pH 6.0–5.5; and after three min at pH 5.0. Increased mitochondria, lipid and dense bodies as well as prominent Golgi complexes were seen after three min at pH 6.5–5.0 (Fig. 3A). Mitochondrial swelling, slight disaggregation of polyribosomes and subtle cytoplasmic lucency were noted after five min at pH 6.0 (Fig. 3B, C) and after three min at pH 5.5–5.0. Small membrane-bound vacuoles were sometimes present. Tangled cytoplasmic filaments with focal increase in electron density were found after five min at pH 5.5, after three min at pH 5.0 and after one min at pH 4.5 (Fig. 4A, B).

After ten min of exposure at pH 5.0, severe chromatin clumping with nuclear clearing was noted (Fig. 4C). Mitochondria were extremely swollen with focal disruption of the outer membrane as well as fragmentation and loss of cristae. The cytoplasm became more electron lucent, partly as a result of an extensive loss of polyribosomes. The cytosol consisted mostly of irregular tangles of fibrils and microtubules. These changes were noted as early as three min after exposure at pH 4.5 and became even more overt and extensive with time. A detailed account of the ultrastructural changes is given in Table 2.

TABLE 1
Nuclear Changes by Light Microscopy

Exposure time (minutes)	Control	Lactic acid					
		pH 7.4	pH 6.5	pH 6.0	pH 5.5	pH 5.0	pH 4.5
1	uniform fine chromatin	*	*	*	*	*	2+ clumping pleomorphism pyknosis pallor
3	*	*	*	*	*	1+ clumping	3+ clumping
5	*	*	*	*	2+ clumping	2+ clumping	4+ clumping pleomorphism pyknosis
10	*	*	*	2+ clumping	2+ clumping pyknosis	2+ clumping pleomorphism pyknosis	4+ clumping pleomorphism pyknosis pallor
30	*	*	1+ clumping pleomorphism	2+ clumping pleomorphism pallor	2+ clumping pleomorphism pallor	3+ clumping pyknosis pallor	4+ clumping pyknosis pallor
60	*	*	1+ clumping pleomorphism	2+ clumping pleomorphism pyknosis	3+ clumping pleomorphism	4+ clumping	4+ clumping pyknosis pallor
120	*	*	1+ clumping pleomorphism	2+ clumping pleomorphism pyknosis	3+ clumping pleomorphism pyknosis	4+ clumping	4+ clumping

Control—cells treated with choline chloride (pH 7.4).
*, no change; 1+, minimal; 2+, slight; 3+, moderate; 4+, marked.

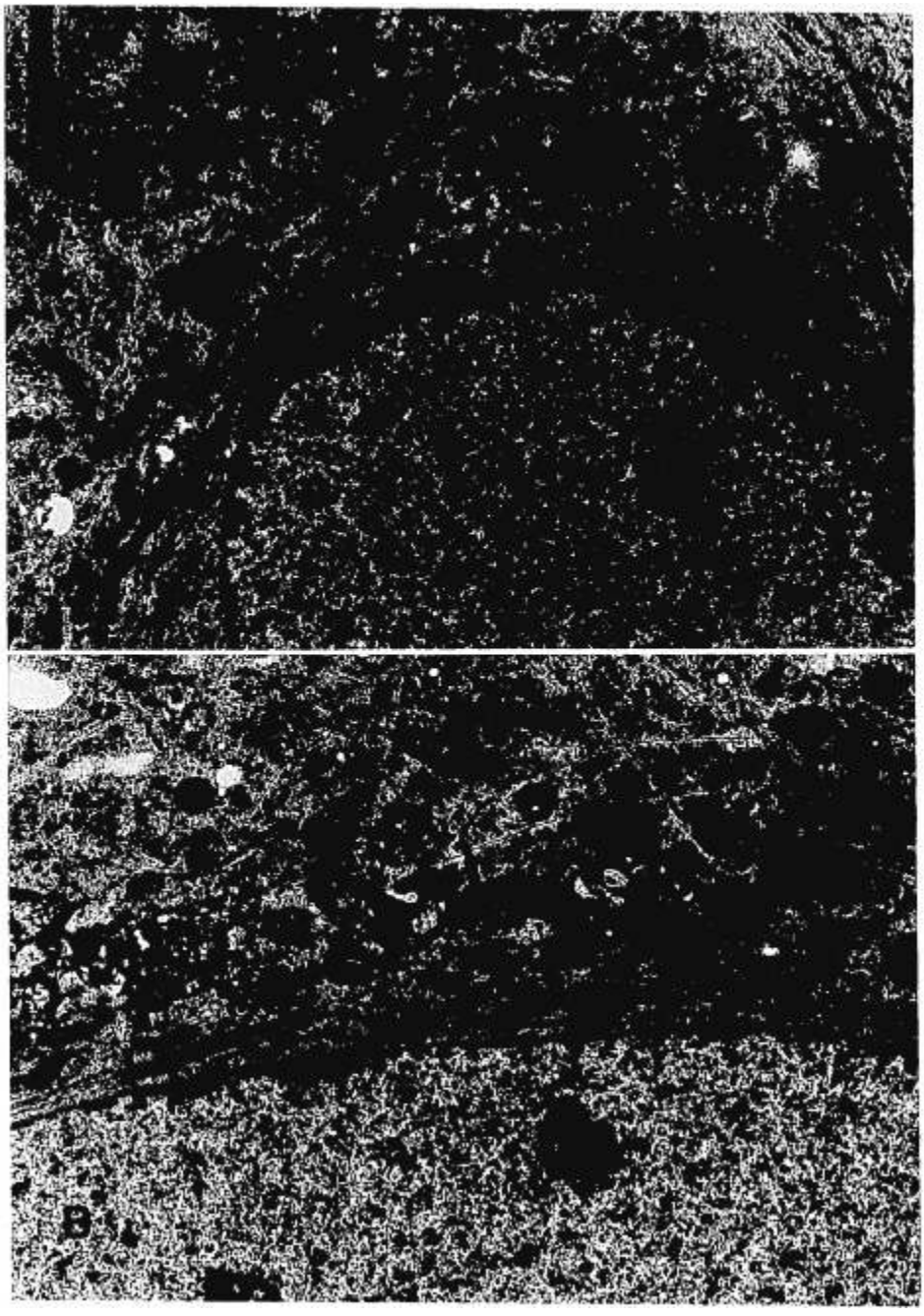

Fig. 2. A. Control culture treated with 25 mM choline chloride at pH 7.4 for three min has a moderately electron dense cytoplasm with bundles of intermediate filaments, abundant polyribosomes, moderate number of mitochondria and few dense bodies. The nucleus contains fine, evenly dispersed chromatin. B. Cell treated with lactic acid at pH 7.4 for five min shows regular nuclear margin without obvious chromatin clumping and slight increase in cytoplasmic dense bodies. Both ×9,800.

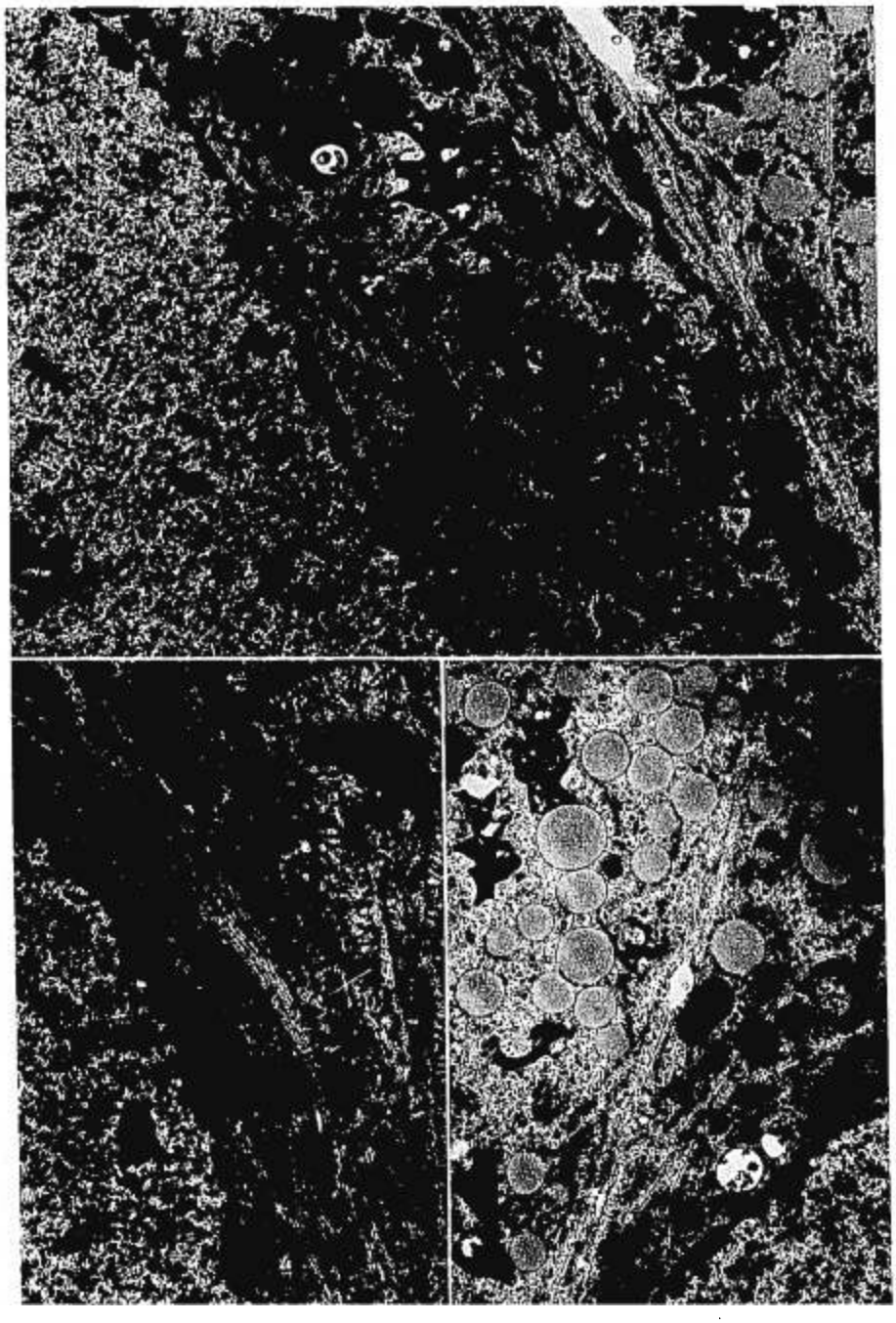

Fig. 3. A. Cell treated with lactic acid at pH 6.5 for five min has a mild increase in mitochondria, lipid and dense bodies. B. After five min of exposure at pH 6.0, mitochondrial swelling (arrows) and mild chromatin clumping are noted. C. Numerous lipid bodies and disaggregated polyribosomes are seen within the more lucent cytoplasm (pH 6.0 for five min). All ×9,800.

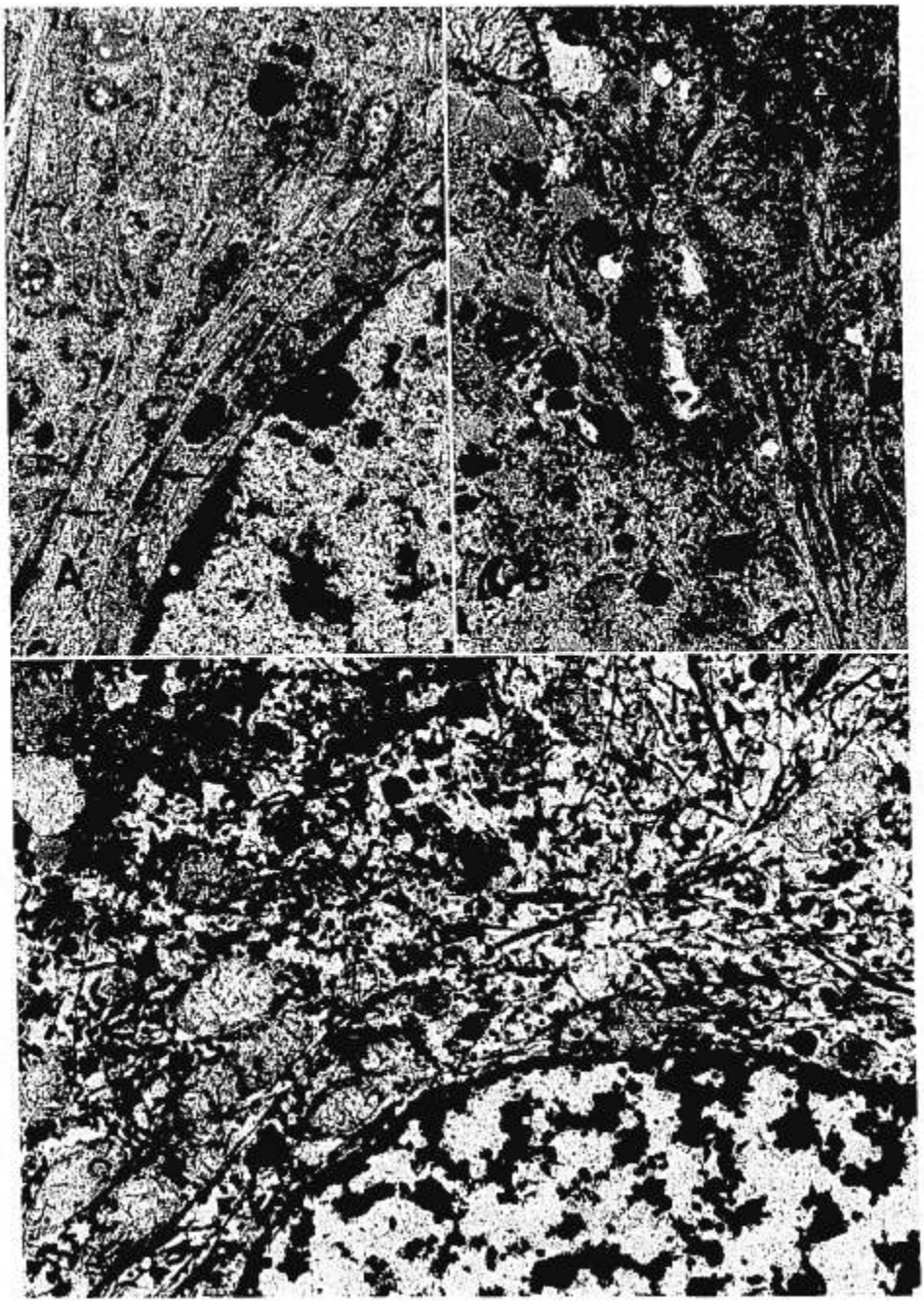

Fig. 4. A. Cell exposed to lactic acid at pH 5.5 for five min demonstrates moderate chromatin clumping, mitochondrial swelling and dense tangled filaments (arrows). B. After ten min at pH 5.5, greater mitochondrial swelling, tangled filaments and many lipid bodies are present. C. Following ten min of exposure at pH 5.0, cytoplasmic lucency and marked chromatin clumping with nuclear clearing are evident. Mitochondria are extremely swollen and contain fragmented cristae. The cytosol consists predominantly of tangled and coalescent filaments. All ×9,800.

TABLE 2
Ultrastructural Changes after Lactic Acid Exposure

Duration	Parameter	Control	Lactic acid pH 7.4	pH 6.5	pH 6.0	pH 5.5	pH 5.0	pH 4.5
One minute	Chromatin	fine	*	*	1+ clumping	1+ clumping	1+ clumping	2+ clumping
	Mitochondria	normal	*	*	*	>	>	>
	Polysomes	abundant	*	*	*	*	*	*
	Filaments	abundant	*	*	*	*	*	tangled
	Golgi body	few	*	*	*	*	>	>
	Lipid body	few	*	>	>	>	>	>
	Dense body	few	*	>	>	>	>	>
Three minutes	Chromatin	*	*	1+ clumping	1+ clumping	1+ clumping	3+ clumping	4+ clumping
	Mitochondria	*	*	>	>	>, pallor	1+ swelling	3+ swelling
	Polysomes	*	*	*	*	2+ loss	2+ loss	4+ loss
	Filaments	*	*	*	*	dense	tangled	tangled
	Golgi body	*	*	>	>	>	>	>
	Lipid body	*	*	*	>	>	>	>
	Dense body	*	>	>	>	>	>	>
Five minutes	Chromatin	*	1+ clumping	1+ clumping	2+ clumping	3+ clumping	3+ clumping	4+ clumping
	Mitochondria	*	*	*	2+ swelling	2+ swelling	2+ swelling	4+ swelling
	Polysomes	*	*	*	2+ loss	2+ loss	3+ loss	4+ loss
	Filaments	*	*	*	dense	tangled	tangled	tangled
	Golgi body	*	>	>	>	>	>	>
	Lipid body	*	*	>	>	>	>	>
	Dense body	*	>	>	>	>	>	>
Ten minutes	Chromatin	*	1+ clumping	2+ clumping	2+ clumping	3+ clumping	4+ clumping	4+ clumping
	Mitochondria	*	*	2+ swelling	2+ swelling	4+ swelling	4+ swelling	4+ swelling
	Polysomes	*	*	1+ loss	3+ loss	4+ loss	4+ loss	4+ loss
	Filaments	*	*	*	*	tangled	tangled	tangled
	Golgi body	*	*	*	*	*	*	*
	Lipid body	*	*	*	*	*	*	*
	Dense body	*	>	>	*	*	*	>

Control—cells treated with choline chloride (pH 7.4); *, similar to control; >, increased in number. 1+, minimal; 2+, slight; 3+, moderate; 4+, marked.

Reversibility Studies

Exposure of cultures to lactic acid at pH 6.0 for one to two h showed mild chromatin clumping. After exposure at pH 5.5 for up to one h as well as at pH 5.0 for up to 30 min, increased cytoplasmic staining, prominent processes and pyknotic nuclei were noted. These changes were reversible since these cultures later appeared morphologically normal 24 and 48 h after transfer to normal media. Trypan blue staining, indicating the inability of non-viable cells to exclude the dye, was seen only occasionally (similar to control).

Longer exposure to lactic acid at corresponding pH levels resulted in marked chromatin clumping, the extent and severity of which was proportional to the level of acidosis and duration of exposure. Such alteration persisted and was accompanied by extensive cytoplasmic degeneration and cell loss when the respective cultures were examined 24 and 48 h after transfer to normal media. Trypan blue staining confirmed the non-viability of most cells following progressive and prolonged acidosis.

DISCUSSION

Lactic acid is a weak acid which can easily permeate the cell membrane to give rise to an intracellular acidosis (25). This study has shown that lactic acid causes degenerative changes in astrocytes. The prominent nuclear changes initially seen were followed by progressive disintegration of cytoplasm. Different concentrations of lactic acid, when buffered to similar pH levels, resulted in identical glial changes thereby indicating that injury to cells was primarily the effect of pH or hydrogen ion concentration rather than of lactic acid, per se. Similar changes were observed using other weak acids at comparable levels. Our findings further concur with the observations of Kalimo et al (19) who suggested that cell damage to neurons and astrocytes of glucose-infused ischemic animals (e.g. chromatin clumping and cytoplasmic clearing) was related to the severity of lactic acidosis and the consequent reduction in intracellular pH.

Several observations deserve emphasis. The presence of numerous lipid bodies suggests that acidosis alone may contribute to the rise in free fatty acids seen in brain following anoxia and ischemia (26–30). Whether the increase in lipid reflects enhanced accumulation or decreased breakdown is not certain. In anoxic and ischemic states, it presumably results from the intracellular shift of Ca^{++} leading to inactivation of phospholipase A (31). Whether acidosis causes a similar Ca^{++} shift in astrocytes or acts through a different process remains to be determined. Nonetheless, this study suggests that astrocytes may be, in part, the cellular source of such lipid.

The significance of the increased lipid bodies in astrocytes is uncertain. It may, however, represent the ultimate source of arachidonic acid (32). If this were the case, it could implicate the astrocyte in the generation of further tissue damage through the formation of prostaglandins and free radicals (33, 34). Of pertinent interest are recent studies indicating that astrocytes are capable of prostaglandin synthesis (35–37) and may even be the chief source of prostaglandins in brain (38). Also, acidosis itself may lead to the generation of free radicals (39).

The mitochondrial abnormality (chiefly swelling) seen early is in keeping with evidence that acidosis per se may be responsible for interference in mitochondrial respiration and suppression of adenosine triphosphate synthesis (15, 16).

Hydropic changes were only minimally observed in our study. Certainly, the marked swelling that occurs *in vivo* in anoxic and ischemic tissue associated with

elevated tissue lactate levels were not seen (19, 20). This suggests that most of the glial swelling *in vivo* may be secondary to other factors such as increased extracellular concentrations of potassium, glutamate and adenosine, rather than lactic acid. While acidosis may possibly potentiate swelling, by itself it was only minimally capable of producing swelling.

Undoubtedly, acidosis results in degenerative changes in astrocytes. Such damage to astrocytes may contribute to the necrosis seen in anoxia and ischemia as previously suggested. Astrocytes are believed to be critically involved in the removal of such potentially toxic agents as ammonia, CO_2 and glutamate, substances that are increased following anoxic and ischemic injury (40). The inability of astrocytes to perform these critical functions may contribute to tissue injury.

On the other hand, the considerable tolerance for acidosis displayed by astrocytes is noteworthy. This tolerance is remarkable in view of the studies of Pulsinelli and Petito (41) who reported a threshold pH level of 6.0 to 6.5 at 20 min for neuronal necrosis to occur. In our studies, astrocytes appeared capable of tolerating pH 5.5 for 30 to 60 min and even at pH 5.0 could withstand at least ten min of acid exposure. The degree of acidosis used in this study is probably greater than usually estimated, although the most severe extent of acidosis has never been accurately determined *in vivo*.

A role of astrocytes in pH regulation has been documented (42–44). Efficient H^+/Na^+ and Cl^-/HCO_3^- antiports have been described in astrocytes (45). Additionally, astrocytes constitute the principal source of glutamine synthetase and also contain carbonic anhydrase, enzymes that are involved in pH homeostasis (46, 47). Thus, the capacity of astrocytes to tolerate a considerable degree of acidosis, as demonstrated in our study, may conceivably allow them to maintain their antiport systems and pH-regulating enzymes, thereby providing a partial protective effect during mild to moderate acidotic states. On the other hand, our studies also indicate that excessive hydrogen ion concentration in the extracellular environment can cause morphological changes in astrocytes. This suggests that subsequent damage to astrocytes due to progressive and prolonged hydrogen ion accumulation may eventually affect their ion transport mechanisms and buffer systems, thereby leading to further impairment of pH homeostasis and irreversible brain injury.

In summary, we have shown that 1) lactic acid is deleterious to astrocytes; 2) that the pathologic alterations are a direct effect of hydrogen ion concentration rather than due to lactate per se; and 3) that astrocytes are capable of tolerating a considerable degree of acidosis before undergoing irreversible change. Their possible protective role in buffering pH changes in brain during hypoxia-ischemia and lactic acidosis remains to be clarified.

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