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Taurine in Toad Brain and Blood Under Different Conditions of Osmolality: An Immunohistochemical Study*

Claude F. Baxter,[1,2] Roger A. Baldwin,[1] Peimin Lu,[3] Humi Imaki,[3] and John A. Sturman[3,4]

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The concentrations of taurine in blood and brain regions of the toad *Bufo boreas* have been measured. Most of these values are considerably lower than those found in mammals. Using an antibody prepared against conjugated taurine, the distribution of taurine in three brain regions of the toad has been visualized. The possible osmoregulatory functions of taurine have been investigated by making toads hyper- or hypo-osmotic in vivo. Induction of hypoosmolality is accompanied by a massive taurine tide in blood plasma, but has no immediate effects upon the taurine concentrations in the brain areas studied. However, histochemical visualization indicates a marked redistribution of taurine between cellular components and extracellular space of brain tissues. This may indicate that taurine has an osmoregulatory function in brain tissue under hypo-osmotic conditions. Hyperosmolality results in no elevation of the taurine concentration in blood plasma of toads, but rather in a very gradual decline of total plasma taurine content over a prolonged time period. Histochemical studies reveal little change in frontal cortex after 1 hour but deeper staining of many neurons in optic lobe accompanied by greater staining in the extracellular fluid. By 3 hours there is a depletion of taurine from all compartments of cerebral cortex tissues. No evidence of any prolonged direct osmoregulatory role for taurine is indicated under hyperosmotic conditions. A possible indirect osmoregulatory function of taurine is discussed.

KEY WORDS: Taurine; osmolality.

INTRODUCTION

Only a few vertebrate species are osmoconformers, i.e. they acclimate to changes in their environment by establishing an iso-osmotic equilibrium between the altered external salinity and their internal salt concentration. The western toad *Bufo boreas* is an osmoconformer (1), and, in its native habitat, acclimates to large changes in the environmental osmolality. Thus it is possible to study the effects of altered blood plasma osmolalities upon biochemical parameters in tissues of the nervous system under conditions that approximate osmotic changes in the natural habitat of this specie. Past studies have shown that one of the early changes induced by an altered plasma osmolality in vivo is an elevation or decrease in many of the non-peptide linked amino acid concentrations in brain tissues. Most of the more abundant amino acids increase in concentration under hyperosmotic conditions and decrease under hypoosmotic conditions (for summaries of various studies see 2). These

[1] Neurochemistry Laboratory, V.A. Medical Center, Sepulveda, CA 91343.
[2] Department of Psychiatry and Biobehavioral Sciences, NPI and BRI, School of Medicine UCLA, Los Angeles, CA 90024.
[3] Department of Developmental Biochemistry, Institute for Basic Research in Developmental Disabilities, Staten Island, NY 10314.
[4] Address reprint requests to: Dr. J. A. Sturman, Department of Developmental Biochemistry, Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314. Tel: (718) 494–5219, Fax: (718) 698–3803.
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changes in the amino acid concentrations in response to osmotic change have been reported also under less physiological conditions in mature rodents (3–5). The overall observations have led to the concept that, during early stages of acclimation to an altered osmotic environment, amino acids play a major role in assuring an iso-osmotic balance between brain tissues and their external environment.

The in vivo studies cited thus far provide evidence that taurine, in brain tissues of mature toads and rats, has no major function as an osmolyte in the maintenance of osmotic equilibrium between the external environment (blood plasma) and most tissues of the central nervous system (CNS) (2–6). Yet other in vivo studies with infant mice, kittens, and pregnant rats provide evidence that the contribution of taurine as an osmolyte in the CNS is significant (7–9). More recent experiments using tissue culture and perfusion techniques suggest that taurine may function to maintain osmoregulatory equilibrium between extracellular and intracellular tissue compartments (10–12). Taurine has been implicated also to act as a neurotransmitter, neuromodulator (13,14), and as a required factor for the development of various areas in the CNS, the visual system in particular (15,16). A number of theories have been advanced to account for the mechanisms by which taurine exerts some of its effects (17,18) but hard evidence to substantiate such mechanisms remains elusive.

If altered plasma osmolalities in vivo can cause the redistribution of taurine in specific brain tissues in a predictable manner, then a new tool would be available for the study of mechanisms through which taurine may exert its effects upon the CNS, especially if such redistribution can be visualized.

During the past decade, polyclonal antibody techniques have been developed for the histochemical localization and visualization of amino acids. These techniques utilize immunogens in which amino acids are covalently bound with glutaraldehyde to bovine serum albumin or poly-L-lysine (19–22). Antibodies against these immunogens recognize specific amino acids that have been fixed in tissues with glutaraldehyde. Monoclonal antibodies specific for taurine can be produced in a fashion that is similar to that outlined above (23,24). Their use, in conjunction with the peroxidase-conjugated avidin/DAB method of indirect labeling to localize taurine concentration has permitted us to test whether osmotic changes in vivo can induce a predictable redistribution of taurine within brain tissues. We report here that by altering osmolality in vivo, a marked redistribution of taurine within specific brain tissues of the toad *Bufo boreas* can be demonstrated. Under hypoosmotic conditions, such redistribution in brain tissue is accompanied by a large transient taurine tide in blood plasma. Hyperosmotic conditions result in an increase and then a depletion of taurine in the cortical brain areas after 3 hours but no significant changes in the taurine concentration of blood plasma in short-term (4h) acclimation experiments.

EXPERIMENTAL PROCEDURE

Adult toads were collected in March and April in the San Fernando Valley and housed under laboratory conditions in large plastic tanks containing a mixture of moist sand and peatmoss with continual access to water. The environmental temperature was 20° to 23°C and the light/dark cycle was 12/12. Acclimation to these conditions was for a minimum of 2 months. During this time period toads were fed mealworms *ad libitum* except that they received no food for 3 days prior to testing. After cannulating the cloaca to remove accumulated urine, body weight of the toads used ranged from 75 to 110 g.

Toads were made hypoosmotic by first injecting them subcutaneously with a solution of arginine vasotocin (10 μg/ml at a level of 2 μg/100 g body wt) and then, 15 minutes later, by infusing a large bolus of a 25 mOs hypotonic solution (45% of body weight) into the pleuroperitoneal (pp) cavity. The completion of this infusion was taken as zero time for the experimental data presented. Three different groups of controls were used: 1) toads receiving no treatment, 2) toads injected with arginine vasotocin only and 3) toads injected with a saline solution isotonic with normal toad blood. Since no significant differences were found in these three groups they are reported as one control group.

For short term experiments only (up to 12 hours) toads were made hyperosmotic by pp injection of a bolus of 2.5 M NaCl (3.5% of body weight). Toads were then placed into one gallon battery jars containing 400 mOs NaCl. The jars were tilted so that the free moving animals could be in or out of the saline solution. Control animals were kept in similar jars containing fresh water.

For histochemical localization of taurine, brain tissues were obtained from experimental animals 1/2, 1 and 3 hours after initiating hyper- or hypoosmotic treatment. All toads were anesthetized by injecting tricaine methane sulfonate (MS222, 1 μmol/gm body wt) into the subdermal lymph space and then subjected to intracardiac perfusion for 15 min with a solution containing 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After perfusion the brains were removed from the skull and stored in the 0.1 M phosphate buffer (pH 7.4) at 4°C until processed. Blood samples were obtained from toads that had not undergone intracardiac perfusion. Instead, blood was drawn from the heart ventricle into heparinized tubes. (The low heparin concentration used did not alter plasma osmolalities). Plasma was separated by centrifugation, deproteinized (3 pts plasma to 1 pt 1M 5-sulfosalicylic acid containing norleucine as an internal standard) and analyzed for taurine and osmolarity. Analytical methods for amino acid quantitation have been described previously (25,26). Samples were hydrolyzed and reanalyzed if the small taurine peak was partially obscured by a very large peak of glycerophosphoethanolamine (26). In a few experiments when insufficient sample was available, the taurine peak was quantitated graphically using the Braswell method (27). In a comparison of samples analyzed graphically for taurine before hydrolysis, and directly after hydrolysis, results were similar (± 10%) with the graphic method providing slightly higher results.

Plasma volume was measured using the Evans Blue dye (T1824) technique (28), modified for use with toads. Briefly: the dye (10 mg/

ml) was injected into the exposed toad heart at a dose level of 10 μg/gm original body wt. (29). Prior and subsequent blood samples were obtained by heart puncture. The passive flow of blood through the puncture needle was sufficient to fill a microhematocrit tube. The 60 μl of blood needed for analysis was obtained during a single contraction of the heart. Ten minutes after dye injection (to allow for mixing and equilibration), blood samples were drawn every 2 minutes for a total of 6 samples. There was no loss of blood from the puncture site if the 25 gauge needle was withdrawn while the ventricle was contracted. Blood samples were processed as previously described (28) and as required for plasma volume measurements. Plasma osmolality was measured using a Westcor vapor pressure osmometer (30).

Immunogens were synthesized by the method of Campistron et al. (22) starting with 100 mg of taurine and coupling with glutaraldehyde to bovine serum albumin (BSA) or poly-L-lysine (PL; MW 30,7000–70,000, Sigma Chemical Co., St. Louis, MO).

Rabbits were immunized by first injecting 500 μg of immunogen emulsified in Freund's complete adjuvant, followed every 4 weeks by injection of 500 μg immunogen emulsified in Freund's incomplete adjuvant. Injections alternated taurine-glutaraldehyde-BSA and taurine-glutaraldehyde-PL. Two rabbits were injected subcutaneously and two intramuscularly. Blood was collected 7 and 21 days after each injection and serum frozen in 1 ml batches.

The serum was characterized for titer and cross-reactivity using the ELISA method (31). High, stable titers of reactivity against taurine-glutaraldehyde-BSA were reached after 3 injections (> 1:664,000 dilution). The serum had a small reactivity to GABA-glutaraldehyde-BSA and to β-alanine-glutaraldehyde-BSA. This reactivity was removed by preadsorption with both conjugates and resulted in little loss of taurine-glutaraldehyde-BSA activity.

Tissue was embedded in paraffin and serial 6 μm sagittal sections of frontal cortex, optic lobe, and thalamencephalon were cut and mounted on glass slides. Sections were deparaffined and dehydrated by warming at 60° for 1 hour, and then 5 minutes in a histoclear bath, followed by graded ethanol washes. Staining with taurine antisera (1 to 2000 dilution) was carried out at 4° overnight and visualized using the peroxidase-conjugated avidin/DAB method (31,32) (Dako Corp., Carpinteria, CA). Sections from each group were processed simultaneously with the same reagents. Control sections were included in every batch, those in which the antisera was preadsorbed with the immunogen prior to processing, and others replacing the primary antiserum with preimmune serum at similar dilutions. Control slides showed no visible staining after processing, whereas slides prepared with the taurine antisera showed brown reaction products. Counterstaining was with hematoxylin. Photomicrographs were taken on a Zeiss III photomicroscope using Kodak 25 color reversal film.

Portions of thalamencephalon were postfixed in 2% osmium tetroxide, dehydrated in graded acetone and embedded in Epon 812. Thin sections of silver interference color were collected on uncoated copper grids, stained with uranyl acetate and lead citrate in an LKB Ultrostainer (Rockville, MD) and examined in a Phillips EM 300 electron microscope at 80 kv.

RESULTS

The concentrations of taurine in the nervous system and the blood plasma of *Bufo boreas* are shown in Table I. The value given for toad brain represents primarily tissues from the cortex; the brain stem, including pons, medulla and cerebellar tissue flap were excluded from this measurement, as were the olfactory bulbs which are shown separately in Table I. Amphibian taurine concentrations in brain tissues are much lower than corresponding values for mammalian brain tissues (3,33–35), except in the olfactory bulbs and retina (Table I). In areas of the amphibian nervous system that contain little taurine, the concentration of glycerophosphoethanolamine is extremely high, much higher than in corresponding areas of mammalian brain (26, Baxter et al., unpublished data). Also the levels of taurine in the blood plasma of *Bufo boreas* is a fraction of that reported for mammalian blood plasma (36).

Table I. Taurine Concentrations in Tissues of Toad *Bufo Boreas*

Tissue	Taurine (μmol/gm wet wt.)	
Blood plasma	0.018*	± 0.006
Brain **	0.15 to 0.30***	± 0.08
Filum terminale	0.15	± 0.04
Olfactory bulbs	3.1	± 0.7
Retina	8.2	± 3.0

Mean ± S.D.
* μmol/ml plasma
** Brain tissue samples consisted of whole brain minus brain stem, pons, medulla, cerebellar flap and olfactory bulbs. The mid brain areas are comparatively small in toads, so that the bulk of each sample consisted of cortical tissues.
*** Seasonal variations in amino acid concentrations are shown for taurine by indicating the range.

After a single bolus injection of a hypoosmotic (25mOs) aqueous solution, plasma osmolality drops sharply within 15 minutes and then continues to decline gradually for another 3 hours (Figure 1a). A massive increase in taurine plasma concentration is initiated almost immediately with a 25-fold increase above normal levels recorded by 3 hours. When hypoosmolality in blood plasma is induced using a more gradual perfusion technique, a similar taurine tide in blood plasma is observed except that the rate at which taurine returns to normal appears more gradual (6). If plasma osmolality is maintained at very low levels for days, taurine concentration in plasma returns to normal levels (6). No systematic early change in the taurine concentration of "brain" tissue is observed (Figure 1a) and the hint of an increase after 4 or more hours becomes apparent only in animals maintained in a hypoosmotic state for several days (Baxter et al., unpublished data).

A single hyperosmotic injection of saline into the pp cavity elevates blood plasma osmolality almost immediately. An equilibrium is reached within 30 minutes. Osmolality declines gradually thereafter (Figure 1b). For

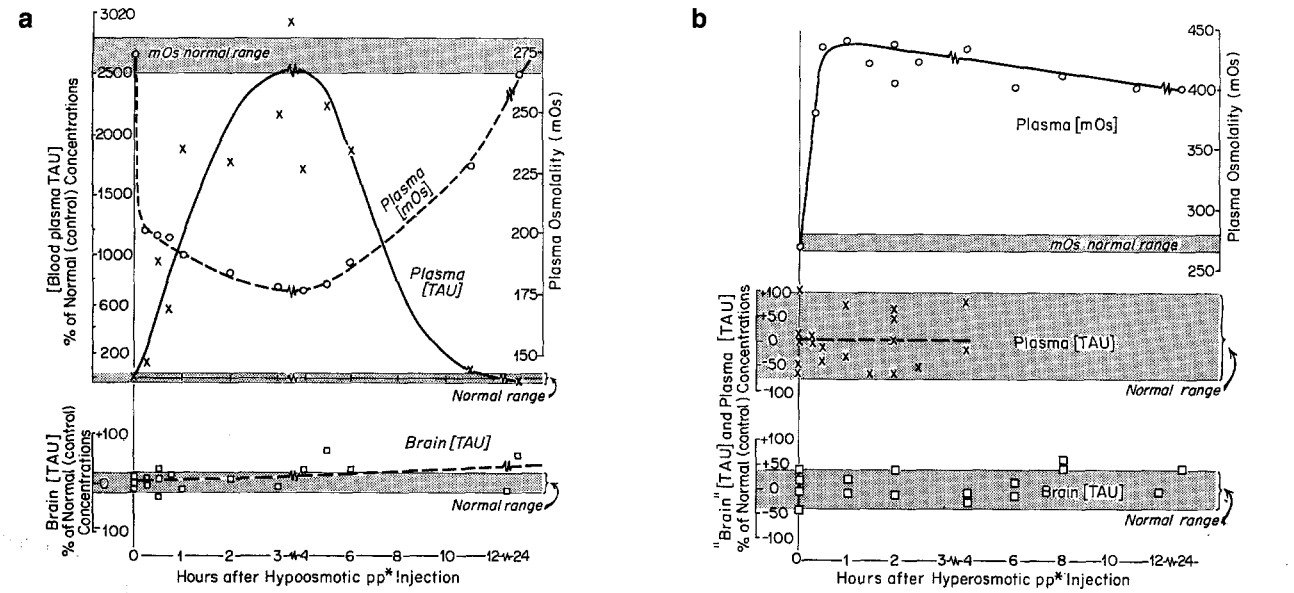

Fig. 1a. Correlation of Hypoosmolality in blood plasma with taurine concentrations** in plasma and brain tissues of *Bufo boreas*. 1b. Correlation of Hyperosomolality in blood plasma with taurine concentrations** in plasma and brain tissues of *Bufo boreas*. Footnotes for Figure 1a and 1b. *pp. = pleuroperitoneal cavity. Amphibians have no diaphragm so that an injection into the abdominal cavity becomes a pleuroperitoneal injection. **The normal (control) taurine concentrations are shown in Table 1 for both blood plasma and "brain" tissues.

the first 4 hours and some time thereafter taurine concentrations in "brain" and blood plasma remain within the normal range.

Plasma volume in the normal toad is around 7.5% of body weight during the summer months. Plasma volume in toads during winter months appears to be greater. Experiments performed with toads during the summer show that acclimation to hyperosmolality for 5 hours decreases plasma volume by 20% to 30%. In toads made hypoosmotic the plasma volume increased by more than 20% during a similar time period.

Initial and altered taurine concentrations are based upon measurements of taurine as μmoles/ml plasma (Table I and Figure 1a,b). This data, together with the measured changes in total plasma volume, show clearly that, instead of the apparent lack of change in total plasma taurine of hyperosmotic toads (Figure 1b), there is a net decrease in total plasma taurine. This decrease is paralleled by the apparent efflux of taurine from brain tissues as observed in the histochemical studies (Figures 2,3,4). The increased plasma volume in hypoosmotic toads shows that the net increase of taurine in their plasma is even greater than that indicated by the taurine tide (Figure 1a).

Changes in the water content of toad brain tissues appears to be minimal after exposure to altered plasma osmolalities for 1, 3, or 5 hours. However, in long-term experiments (2 to 10 days) the brains of toads maintained under hyperosmotic conditions showed a 4% decline in water content. Conversely amphibians maintained under hypoosmotic conditions for similar time periods exhibited a 9% increase in water content of brain tissues. The increase in total taurine concentration in brain tissue of hypoosmotic toads and decrease of total taurine concentration in brain tissues of hyperosmotic toads in these long-term experiments has been reported (6,37).

Immunohistochemical Results. Neurons of the toad brain do contain taurine under normal osmotic conditions, as determined by reactivity with a taurine antibody, although the staining is light or non-existent in some neurons in keeping with the relatively small taurine concentration in toad brain. This is illustrated for two different brain regions, frontal cortex and optic lobe, which have somewhat different neuronal populations (Figure 2 a,b; Figure 3 a,b). Hypoosmolar conditions result in greatly increased diffuse staining in the extracellular space and greatly reduced staining of neurons. This is illustrated in frontal cortex and optic lobe after 3 hours in hypoosmolar conditions (Figure 2 c,d; Figure 3 c,d); the staining after 1 hour in the same conditions is roughly intermediate (not shown).

Hyperosmolar conditions for 1 hour result in little change from control in frontal cortex, possibly with a little greater extracellular staining (Figure 2 e,f). The changes at this time in optic lobe are much greater and include deeper staining of many neurons and greater

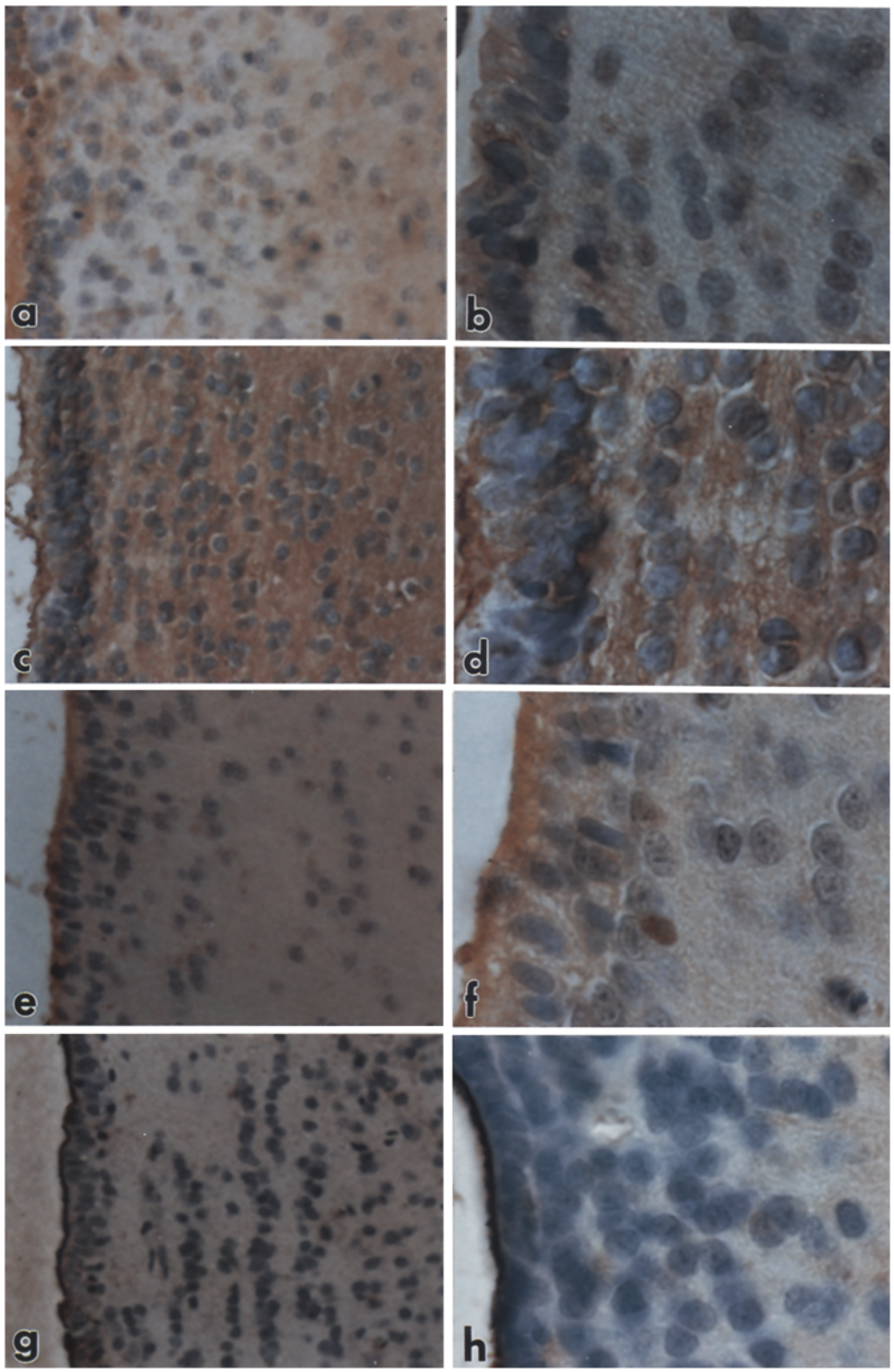

Fig. 2. Sections of frontal cortex stained with taurine antiserum and counterstained with hematoxylin. a,b from control animals; c,d from 3-hour hypoosmotic animals, e,f from 1-hour hyperosmotic animals; g,h from 3-hour hyperosmotic animals. a,c,e,g 230 X; b,d,f,h 570 X.

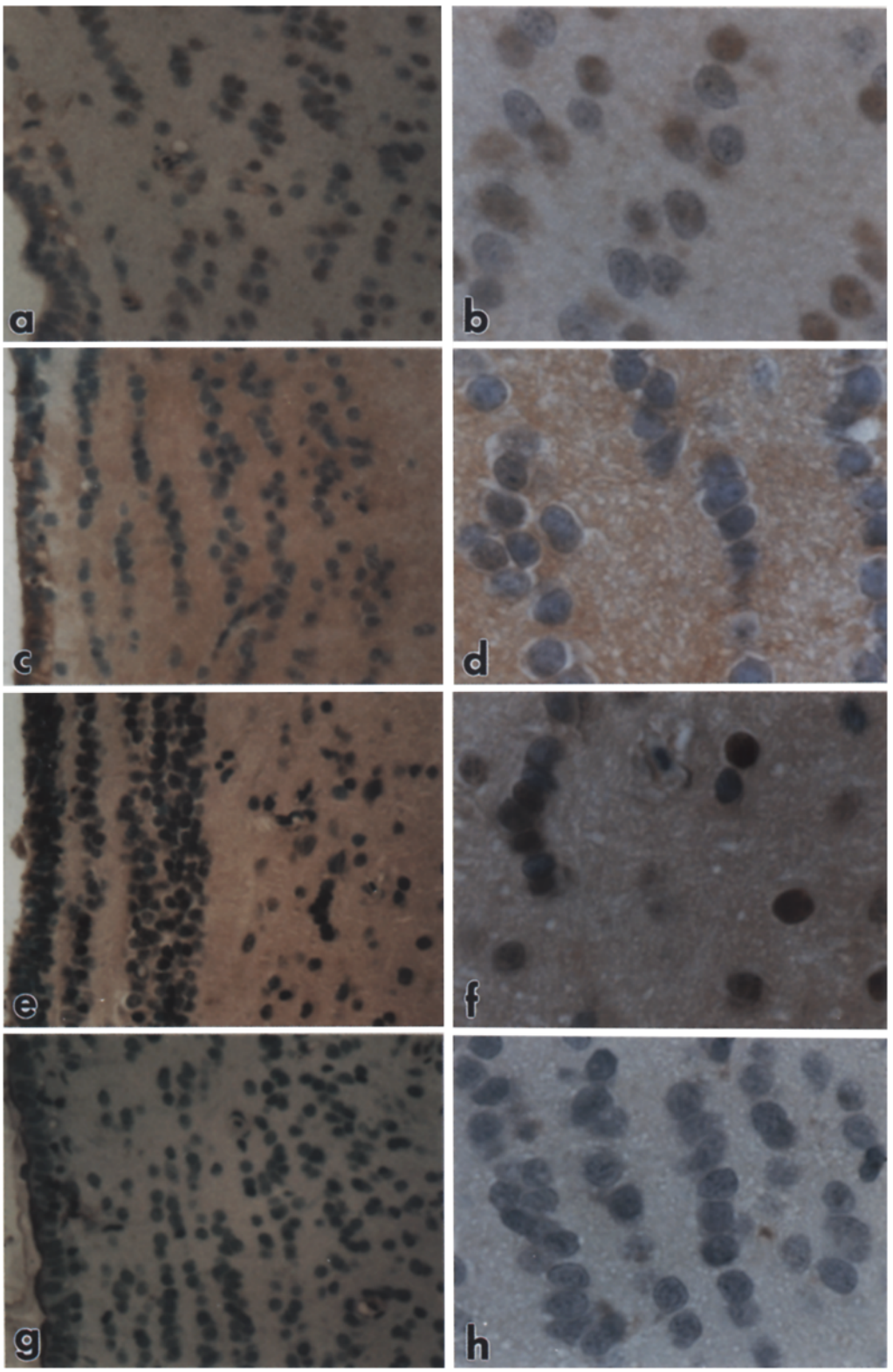

Fig. 3. Sections of optic lobe stained with taurine antiserum and counterstained with hematoxylin. a,b from control animals; c,d from 3-hour hypoosmotic animals; e,f from 1-hour hyperosmotic animals; g,h from 3-hour hyperosmotic animals. a,c,e,g 230 X; b,d,f,h 570 X.

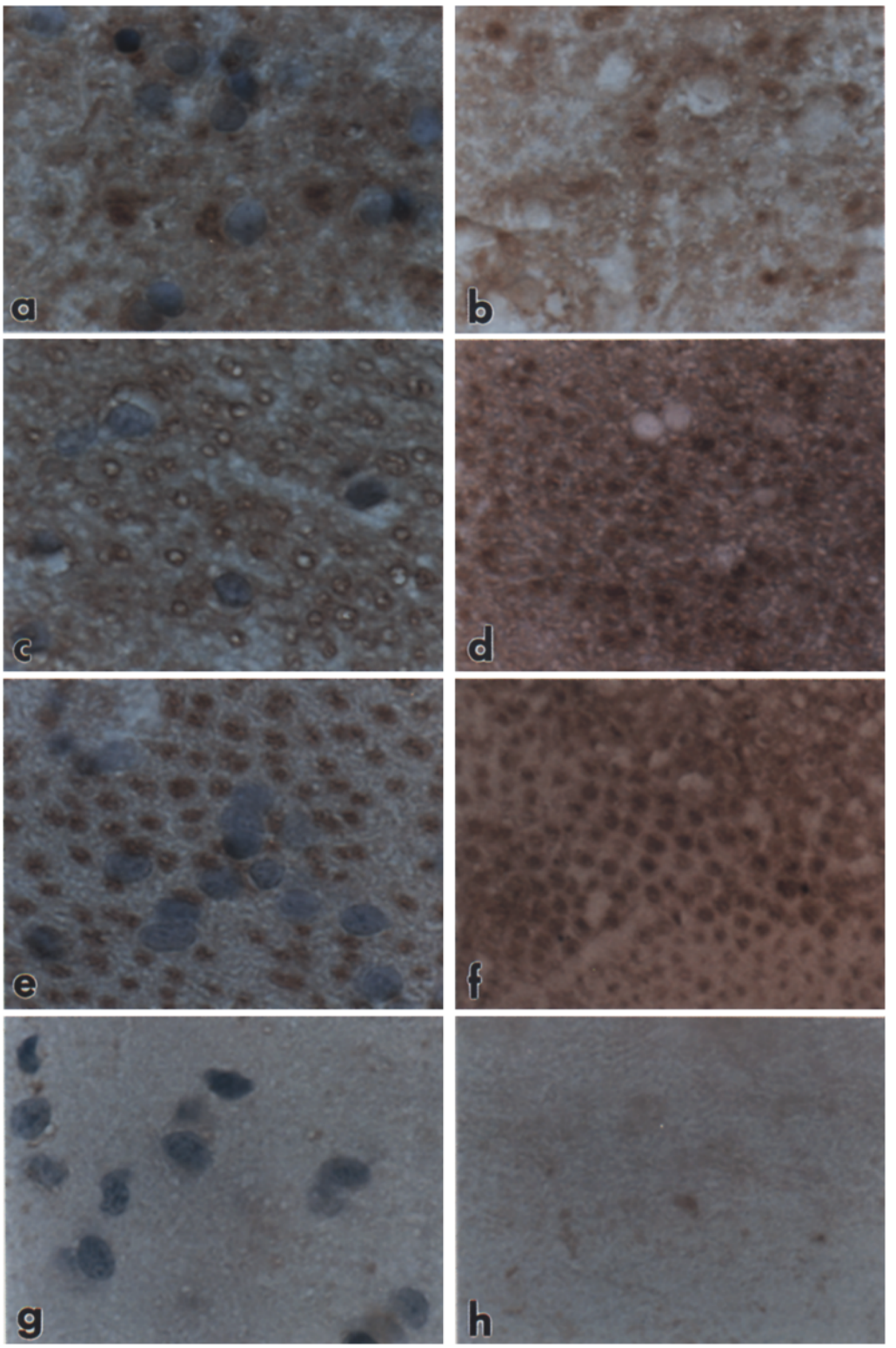

Fig. 4. Sections of thalamencephalon stained with taurine antiserum with and without counter-staining with hematoxylin. a,b from control animals; c,d from 1-hour hypoosmotic animals; e,f from 3-hour hypoosmotic animals; g,h from 3-hour hyperosmotic animals. 570 X.

staining of extracellular fluid (Figure 3 e,f). After 3 hours under hyperosmolar conditions, however, virtually no taurine staining is present (Figure 2 g,h; Figure 3 g,h). A third brain region, the thalamencephalon, followed the same general changes in staining under the different conditions described above for frontal cortex and optic lobe, and is illustrated to show the unique appearance of taurine staining in the extracellular space (Figure 4). In this region there are few neurons and very little taurine staining is apparent in neurons although there is considerable staining in the extracellular structures (Figure 4 a,b). Under hypoosmolar conditions, all taurine in neurons has disappeared, and appears to be concentrated in regular, vesicle-like structures in the extracellular space (Figure 4 c,d,e,f). This process is evident after 1-hour and appears complete by 3-hours. After 3-hours hyperosmolar conditions, virtually no taurine staining is present, even in these extracellular structures. Examination of this region at the ultrastructural level suggests that it contains bundles of nerve fibers which appear to have a great affinity for taurine, especially under hypoosmolar conditions (Figure 5).

DISCUSSION

The possible involvement of taurine in the regulation of water content and water balance in tissues of marine organisms and aquatic invertebrates has been evaluated repeatedly (see 38–40 for this literature). The majority, but not all of these studies reached affirmative conclusions. Only a few investigators reported data for tissues of the nervous system (39). Studies describing possible functions of taurine in tissues of mammalian species can be found summarized in three symposia on this subject (41–43). A striking correlation of water content and taurine concentrations in brain tissues was observed in a recent interspecies comparison with different rodents (5). The concentration of taurine in toad brain (Table I) further supports that correlation even outside the rodent order.

The plasma taurine tide (Figure 1a) elicited in toads acclimating to hypoosmotic conditions, can be elicited also in rats (44). The major difference between the two species is a much faster response time in the rat (37). Attempts to localize the tissue stores from which taurine is released into the plasma under hypoosmotic conditions have met with only partial success. Our (unpublished) studies show that platelet-bound taurine and the taurine from red blood cells are partial sources, a conclusion supported by other investigators (44). Many body tissues are rich in taurine and none have been excluded as possible donors. The many-fold increase of taurine in the plasma of hypoosmotic toads does not alter the taurine content of brain tissues, thereby excluding the areas studied as significant donors.

Taurine has been reported to stimulate the conversion of glucose to glycogen, presumably by its effect on insulin receptor binding mechanisms (45). In the toad, the synthesis and degradation of liver glycogen are dramatically affected by osmotic changes (46) and glycogen via glucose appears to be the most important source of carbon chains for the synthesis of tricarboxylic acid cycle-related, osmotically-active amino acids (47). Thus altered taurine levels might have an indirect osmoregulatory function. As yet this idea remains an unproven hypothesis.

Since the introduction of specific antibody techniques for the detection of individual amino acids within the matrix of brain tissues (19), several investigators have visualized the distribution of taurine in tissue compartments of the mammalian nervous system (see 32 for refs.). These reports indicate distinct localization of high taurine concentration in the cellular elements of tissues from various brain regions, particularly in neurons, dendrites and axons. The taurine concentration in similar regions of toad brain are relatively low, corresponding to the low density of neurons in these regions (Figures 2,3,4). Yet the results of the present study show quite clearly the presence of taurine (or taurine-like immunoreactivity) within neurons of the toad brains (Figure 2a,b, Figure 3a,b, Figure 4a,b). Under hypoosmotic conditions there is a distinct shift of taurine from intracellular locations to extracellular spaces. This phenomenon is seen in all three regions examined (Figure 2c,d, Figure 3c,d, Figure 4c,d,e,f) and has been observed also in in vitro studies where different criteria for taurine measurements were used (10,12,48,49). It represents a clearly demonstrated osmoregulatory function for taurine in tissues of the nervous system under hypoosmotic conditions. In the specialized thalamencephalon, taurine may be sequestered within axons (Figures 4 and 5).

To be an active osmolyte, taurine concentrations should increase in tissues under hyperosmotic conditions. Such results have been reported in brains of infant mice (7); in brain tissues of pregnant rats (8); and more recently in cultured astrocytes (11,50,51). This predicted result is evident after 1 hour in the optic lobe which shows increased staining of neurons and extracellular fluid (Figure 3e,f). The histological sections from brain tissues of toads kept under hyperosmotic conditions for 3 hours (Figure 2g,h Figure 3g,h Figure 4g,h), however, indicate a general depletion of taurine from the areas of cortex that have been studied. This surprising result is

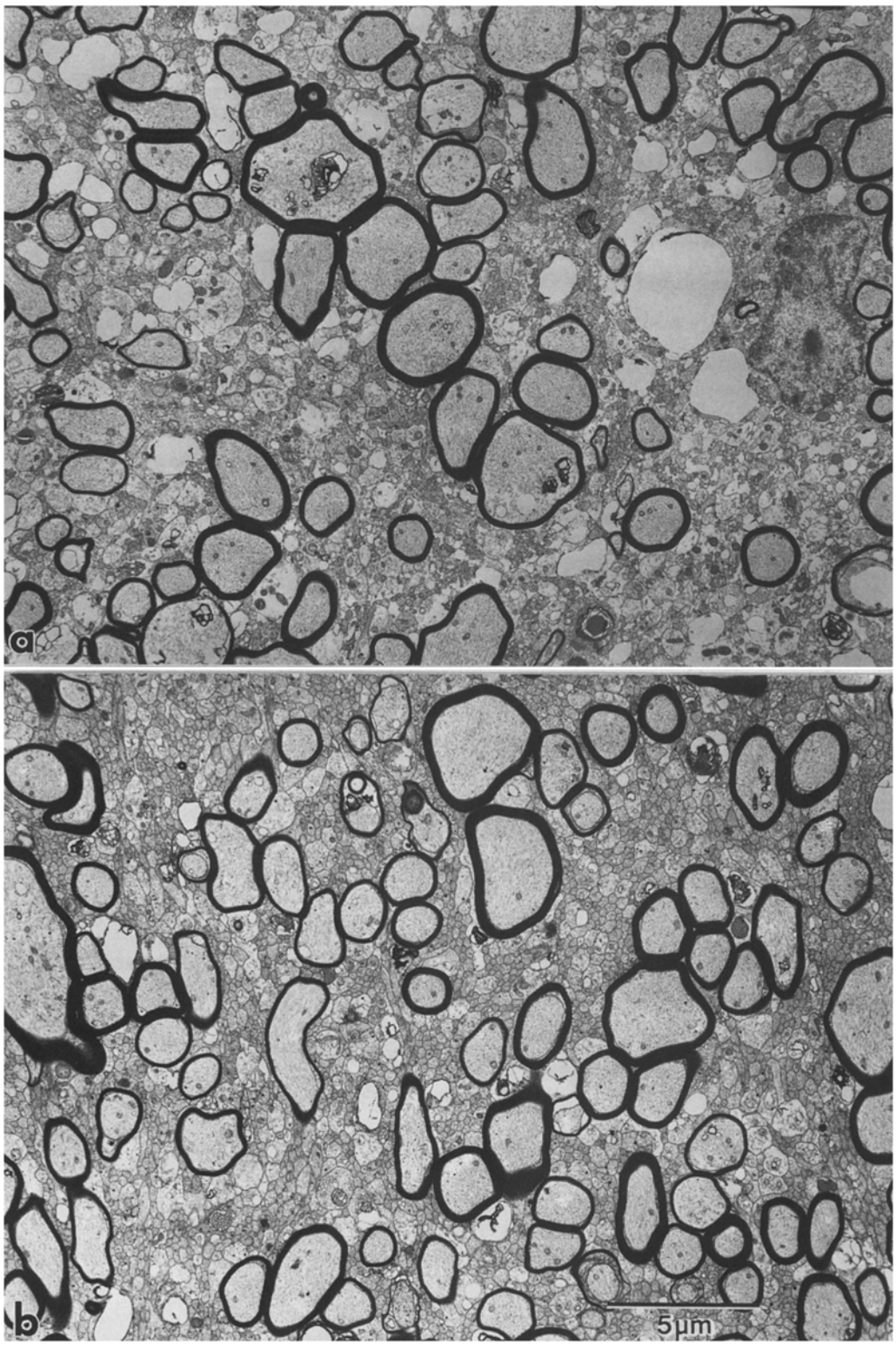

Fig. 5. Electron micrographs of thalamencephalon corresponding from regions shown in Fig. 4. a from control animal; b from 3-hour hypoosmotic animal.

consistent with the gradual depletion of taurine from brain tissues of toads maintained under hyperosmotic conditions for several days (6). Osmolality is just one of many variables that are known to affect the concentration and movement of taurine in and out of nervous tissues. (Ions, hormones, taurine gradients, transport mechanisms, the rate of osmotic induction, compartmental volume changes, the duration of an experiment, experimental conditions, cell types, brain areas, animal species, degree of maturation, nutritional states, etc.—all have been shown to influence taurine in specific nervous system tissues). The efflux of taurine from toad brain cortex under hyperosmotic conditions in vivo, may therefore represent the effect of one of these other variables.

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