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Function of the *N*-Acetyl-L-Histidine System in the Vertebrate Eye

Evidence in Support of a Role as a Molecular Water Pump

Morris H. Baslow

Nathan S. Kline Institute for Psychiatric Research, Center for Neurochemistry, Orangeburg, NY

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Abstract

N-acetyl-L-histidine (NAH) is a major constituent of poikilotherm brain, eye, heart, and muscle, but for which there is no known function. NAH is characterized by high tissue concentrations, a high tissue/extracellular fluid (ECF) gradient, and by a continuous selective and regulated efflux into ECF. In the eye, there is a complete compartmentalization of the synthetic and hydrolytic enzymes, with synthesis of NAH from AcCoA and L-histidine (His) occurring in the lens, and its hydrolysis to acetate and His restricted to surrounding ocular fluids. Using ^{14}C-isotopes, the cycling of NAH between lens and ocular fluids in a simple support medium consisting of NaCl (0.9%), Ca^{2+} (4 mEq/L) and D-glucose (5 m*M*) at pH 7.4 has previously been observed. In the present study, using the isolated lens of the goldfish eye, each of the components of that support medium has been individually varied in order to determine its effect on NAH release down its intercompartmental gradient. As a result of these and related studies, it is suggested that NAH may function as a metabolically recyclable gradient-driven molecular water pump. It is proposed that water influx or generation of metabolic water serves as the trigger mechanism to open a Ca-dependent gate for the release of NAH down its gradient, along with its associated water. Preliminary analyses suggest that in addition to its potential for multiple daily cycles, a strongly ionized hydrophilic molecule, such as NAH, may include a large power function as a result of its attraction to water, and it has been calculated that an aqua complex of each NAH molecule may have 33 dipole–dipole-associated water molecules as it passes into ECF. It is this unique combination of a capacity for multiple cycles per day, coupled with a large power function, that may allow for such an intracellular osmolyte to be present in relatively low concentration in comparison to total cellular osmolality, and yet to perform a large and important task with little expenditure of energy. With each NAH molecule recycled up to 10 times/d, and a power factor of 33, there could be 330 mmol of water transported/mmol of NAH each day. With typical NAH concentrations in brains of poikilothermic vertebrates of 5–10 mmol/kg, there is the potential for up to 3.3 mol (60 mL) of water to be removed each day/kg of brain, a value that represents about 8% of total brain water content. Dewatering of the released osmolyte would occur in two additional steps, consisting of its hydrolysis and the subsequent active uptake of its metabolites. It is also suggested that NAH is the archetype of several metabolically and structurally related cellular osmolytes found in both poikilotherms

and homeotherms, for which there is similarly no known function, and these may form a family of cycling hydrophilic osmolytes that serve as molecular water pumps in a variety of tissues. These include the basic His containing derivatives: NAH, carnosine, anserine, ophidine, and homocarnosine, and the acidic aspartate derivatives: *N*-acetyl-L-aspartate (NAA) and *N*-acetyl-L-aspartylglutamate (NAAG). In each of these cases, the high intracellular/extracellular osmolyte gradient appears to be maintained by combining a hydrophilic protein amino acid with a nonprotein moiety to block its use in other intracellular metabolic pathways, and by blocking catabolism of the derivative by maintaining its hydrolytic enzyme in an extracytosolic membrane or extracellular compartment. Unlike other known water-regulating mechanisms, the proposed cellular system is unique in that as a water pump, it can function as a water regulator independently of extracellular solute composition or osmolality. Finally, based on the hypothesis developed, the NAH system would represent the first cellular water pump to be identified.

Index Entries: *N*-acetylhistidine; *N*-acetylaspartate; homocarnosinosis; Canavan disease; carnosine; homocarnosine; water pump; osmolyte; osmoregulation; lens; brain.

Introduction

N-acetyl-L-histidine (NAH) and *N*-acetyl-L-aspartic acid (NAA) are major constituents of the vertebrate brain and eye with distinct phylogenetic distributions, but for which there is no known function. They are characterized by high tissue concentrations, high tissue/extracellular fluid (ECF) gradients, and a continuous regulated efflux into ECF. Evidence linking NAH and NAA metabolism has recently been reviewed, and the hypothesis developed that NAH and NAA complement each other and are metabolic analogs that are associated with an organism's osmotic environment, and are also involved in some aspect of membrane transport (Baslow, 1997). Previously, a postulated NAH pump mechanism at the surface of the fish lens has also been considered to be part of a universal phenomenon, which might also include other α-*N*-derivatives of histidine (His), such as the dipeptides carnosine (β-alanyl-L-histidine), anserine (1-methyl carnosine), ophidine (3-methyl carnosine), and homocarnosine (γ-aminobutyrl-L-histidine) as functional homologues (Baslow, 1967). Although NAA and NAH and related aspartate (Asp) and histidine (His) derivatives were discovered many decades ago, no physiological function for any of these compounds has yet been identified. In general, these substances are part of regulated intercompartmental biochemical cycles where they are synthesized within cells to the extent that they become important cellular osmolytes and exhibit high intra- or extracellular gradients. Next, these substances are released from cells in a regulated fashion, and in a different compartment, they are hydrolyzed. Finally, after hydrolysis, their components are taken up by cells and resynthesized into NAA, NAH, and their related derivatives once again. The importance of this cycling is made very clear in cases where the cycle has been disrupted. For example, a lack of aspartoacylase activity, which is necessary for the hydrolysis of NAA, as a result of Canavan disease, has profound neurological consequences. Canavan disease is a rare autosomal-recessive genetic disorder involving a gene on chromosome 17, which is required for the production of the acylase. In affected individuals, they are usually normal at birth, but after an early onset of the disease process, there is elevated brain NAA and *N*-acetyl-L-aspartylglutamate (NAAG), *N*-acetylaspartic aciduria, edema, demyelimation, and a spongy degeneration of the white matter of the brain, a syndrome that attests to the vital role of NAA biochemical cycling and metabolism in human brain (Baslow and Resnik, 1997). A similar human disease related to the metabolism and cycling of a His derivative is Homocarnosinosis. In this genetic disease, there is a deficiency of serum carnosinase, the enzyme that hydrolyzes brain synthesized homocarnosine, and the associated clinical symptoms are not unlike those of Canavan disease. This disorder is also characterized by normal birth, followed by an early onset of neurological symptoms with a variable phenotype that may consist of psychomotor retardation, hypotonia, tremor, myoclonic seizures, and

developmental delays (Perry et al., 1979; Lenney et al., 1983; Jackson et al., 1994). In addition to elevated tissue levels of homocarnosine, there is also a striking hypercarnosinuria. The neuromuscular aspects of this disease may be related to the distribution of carnosine, which is found in both brain and muscle. This disease also appears to be autosomal and recessive, with the locus for the gene that produces the missing enzyme on the distal long arm of chromosome 18 (Willi et al., 1997). From evidence provided by the analysis of the metabolic lesions that are associated with the biochemical aspartoacylase deficit in Canavan disease, it appears that the lack of enzyme activity is responsible for three metabolic outcomes. First, the intercompartmental NAA gradient is reduced 20- to 50-fold. Second, recycling of Asp from NAA is eliminated, and a near-field Asp deficit is induced. Finally, synthesis of NAA becomes dependent on far-field nutritional and *de novo* synthetic sources of Asp. Since homocarnosine and NAA share similar cycling characteristics, the metabolic outcomes in Homocarnosinosis would be expected to be similar. In this investigation, an attempt has been made to reproduce a key condition that prevails in both Canavan disease and Homocarnosinosis by isolating an organ that synthesizes several cycling osmolytes, and by eliminating the availability of the hydrolytic enzymes that form part of the intercompartmental cycle. In the eye, there is a complete compartmentalization of the synthetic (lens) and hydrolytic (ocular fluid) enzymes, and NAH undergoes a unique energy-driven lens-ocular fluid cycling (Baslow, 1967). Recently, NAA has also been found to be a universal constituent of the vertebrate lens (Baslow and Yamada, 1997a), and in the fish eye, both NAH and NAA are present simultaneously in many structures, including the choroid, ciliary body and iris, optic nerve, retina, and lens (Baslow and Yamada, 1997b). Based on these findings, the fish lens has been suggested as a model system for investigating the function of both NAH and NAA because of their presence in this relatively simple aneural and avascular organ, and the unique experimental opportunity that is provided by the compartmentalization of their synthetic and hydrolytic enzymes. Isolation of the lens disrupts the NAH and NAA cycles by eliminating their acylases, restricts formation of new NAH and NAA, and allows for experimental manipulation of the components of that cycle.

The lens is an organ that is particularly suited to these studies, since it behaves metabolically as if it were a single giant cell. The lens is made up of a single-cell type, which differentiates into epithelial and fiber cells as the lens grows. However, there are large gap junctions formed between cells that are maintained throughout the life of the lens through which the cells can pass metabolites and by virtue of which the lens is a syncytium of cells that easily and quickly share all solutes (Rae et al., 1996). Electrogenically, the lens maintains an electronegative interior owing to the positive internal–external potassium gradient that is established as if it were a single cell. Nourishment and removal of wastes and water for all cells of the lens are performed by the activities of a single layer of epithelial cells that form the outer surface of much of the lens, and whose apical membranes have pores, channels, and transporters that are needed for the movement of solutes between the lens interior and the ocular fluids, which bathe this avascular organ. Goldfish lens has been found to contain NAH and NAA at 3.30 and 2.03 μmol/g, respectively, representing about 4% of goldfish lens tissue osmolality. For other species, the concentration may be much higher, and whole tissue values for lens NAH of up to 21.73 μmol/g have been recorded. In the lens, NAH has been associated with the single layer of epithelial cells at its surface, just under the capsule. The identification of the lens-ocular fluid NAH cycle was originally made using ^{14}C-isotopes in a simple support medium consisting of NaCl (0.9%), Ca^{2+} (4 mEq/L), and D-glucose (5 m*M*) at pH 7.4. In this study, using the isolated lens of the goldfish eye, previous conditions have been reproduced, and each of the components of the support medium have been individually varied in order to investigate some of the factors that influence both the NAH tissue/ECF gradient, and the efflux of NAH into ECF.

Materials and Methods

Experimental Animals

For these studies, goldfish *Carassius auratus* were used. Fish weighed between 1 and 4 g, and lenses ranged between 1.5 and 4.1 mg. The fish were kept at 22–24°C in aerated fresh water under natural light conditions.

In Vitro Studies

Lenses were prepared from freshly dispatched fish by enucleation and then gently rolling on medium impregnated paper (Baslow, 1967). The isolation techniques used in this study have previously been shown to provide lenses that are electrogenically normal, maintain a positive potassium gradient for the duration of in vitro incubations, actively transport acetate and His into the lens, synthesize NAH, and selectively release synthesized NAH into the medium at a regulated rate (Baslow, 1967). Since a goal of the present study was to test the effect of external factors on the efflux of NAH observed previously, techniques similar to those used in the earlier work were employed. Where possible, lens pairs were used as controls for one another. Unless otherwise indicated, the standard bioassay was carried out in 1 mL of support medium containing NaCl, 0.9% (314 mosM/L); Ca^{2+}, 4 mEq/L (6 mosM/L); and D-glucose, 5 m*M* (5 mosM/L) at pH 7.4 and 22–24°C. The standard bioassay incubation time was 3 h. After enucleation and cleaning, lenses were subsequently transferred to parafilm for weighing, and then to media using a glass rod and the force of surface tension. Lenses used in the experiments, after isolation and weighing, were clear, nonsticky, and exhibited only point contact with the glass transfer rod. For dewatering and washing at the end of the incubation period, the lenses were recovered from the media and held in a platinum wire-loop cradle, so that lenses made no direct contact with the dry filter paper used to siphon fluids from the lens by capillary action. Subsequent transfer of the lens to parafilm for reweighing was made using a stainless-steel push rod. In some experiments, lenses were only weighed once, either at the beginning or at the end of the experimental procedure. Lenses used for further analyses were intact, round, and exhibited evidence of a positive internal pressure. After incubation, lenses were homogenized in water at 5 mg/mL and aliquots used for chemical and isotope analyses.

Measurement of Lens Viability

In these studies, ^{14}C-labeled amino acids were used to indicate lens viability. Alanine (Ala), His, and valine (Val) were used as markers. When used, each amino acid was added to the support medium at 0.1 m*M* (0.1 mosM/L) to which was then added 0.2 µCi of the same amino acid with a high ^{14}C specific activity. The specific activities for these amino acids were: L-Ala-UL^{14}C (Amersham), 155 mCi/m*M*; L-His-UL^{14}C (ICN Pharmaceuticals), 337 mCi/m*M*, and L-Val-L-^{14}C American Radiolabeled Chemicals, 55 mCi/m*M*.

Analyses of Radioactivity

Isotope counting was accomplished using aliquot samples of tissue homogenates or medium in 3 mL of Liquiscint (National Diagnostics) on a Packard 2200 Tricarb liquid scintillation counter, with time and counts adjusted for reproducibility of at least ±5%.

Analysis of NAH

Analysis of NAH was done after chromatographic separation using a butanol:acetic acid:water (4:1:5) system (Baslow, 1967). The NAH was visualized with Pauly reagent, and after elution with 1% butanol, the sample was analyzed at 510 n*M* using a Spectronic 21. The lower limit of this assay was 0.0001 µmol. For samples in the 0.0001–0.0020 µmol range, visual comparisons with known chromatographed standards were also made. Values obtained using these techniques provided data comparable to that obtained using an HPLC method (Baslow and Yamada, 1997a).

Analysis of Lens Clarity and Swelling

In addition to the degree of lens swelling obtained from pre and postincubation weights, and their ability to transport selected amino acids actively, lenses were also graded subjectively for clarity when placed in an intense source of light. For this analysis, and arbitrary scale of 1–6 was used, where 1 is clear and 6 is opaque. Since the clarity index (CI) used a noninvasive technique, the CI could be measured over shorter time periods during any given set of experimental conditions, and thus provide an independent set of progressive test data.

Results

Removal of Ca^{2+} from the Support Medium

As seen in Table 1, under the conditions of the standard bioassay, the amino acids used as mark-

Table 1
The Effect of External Calcium Ions on Amino Acid Uptake and Residual NAH in the Isolated Goldfish Lens

Lens[a] #	Calcium[b]	Amino acid[c]	CF[d]	Residual NAH,[e] μmol/g	Residual NAH as % of unincubated controls[f]
1	+	Ala	1.55	0.2	6.7
2	+	Ala	3.55	1.1	36.7
3	+	Ala	4.29	1.2	40.0
4	+	His	7.88	1.0	33.3
5	+	His	15.10	2.0	66.7
6	+	Val	6.97	2.4	80.0
7	+	Val	5.06	1.0	33.3
8	+	Val	7.85	0.6	20.0
9	+	Val	4.33	1.2	40.0
10	+	Val	6.56	1.6	53.3
Mean ± SD			6.31 ± 3.50[g]	1.2 ± 0.6[h]	41.0 ± 20.2
11	–	Ala	1.09	0.0	0.0
12	–	His	1.70	0.2	6.7
13	–	His	1.90	0.0	0.0
14	–	Val	1.16	0.0	0.0
15	–	Val	1.97	0.0	0.0
16	–	Val	1.57	0.0	0.0
Mean ± SD			1.51 ± 0.34[g]	0.03 ± 0.07[h]	1.1 ± 2.5

[a]Lens pairs: 1/11, 4/12, 5/13.
[b]Standard bioassay with (+) or without (–) Ca^{2+} at 4 mEq/L.
[c]Amino acids at 0.1 m*M* with 0.2 μCi of ^{14}C-high specific amino acid isotope tag.
[d]Concentration factor (CF) calculated as CPM/mL lens water/CPM per mL medium. Water content of lenses utilized was 53.5%.
[e]Lower limit of assay is 0.1 μmol/g.
[f]NAH content for unincubated control lenses # 47, 48 and pooled lenses 49–54 was 3.0 ± 0.0 μmol/g.
[g]Significant at $P = 0.0078$.
[h]Significant at $P = 0.0005$.

ers are taken up from the medium and concentrated about sixfold. In addition, during the 3-h incubation period, there is also a reduction of about half of the lens NAH. Removal of Ca^{2+} greatly diminishes amino acid uptake, and the concentration factor remains in a range somewhat above that of a diffusion equilibrium. There is also a marked effect on residual lens NAH, with NAH loss from the lens being almost complete in 3 h.

Removal of D-*Glucose from the Support Medium*

In Table 2, the effect of D-glucose on NAH loss is presented, and D-glucose appears to interact with lens NAH in that its presence in the incubation medium is associated with a 67% depletion of NAH. This D-glucose effect is probably also responsible for much of the NAH reduction of similar magnitude noted in lenses 1–10 in experimental data presented in Table 1, since the experimental conditions for these lenses are essentially the same as for lenses 17–24, except for the presence of the viability marker amino acids.

Modification or Removal of NaCl from the Support Medium

Decreasing the NaCl content of the support medium results in lens swelling owing to the

Table 2
The Effect of External D-Glucose on Residual NAH in the Isolated Goldfish Lens

Lens #[a]	D-Glucose[b]	Residual NAH (μmol/g)	Residual NAH as % of unincubated controls
17	+	0.2	6.7
18	+	0.2	6.7
19	+	1.0	33.3
20	+	3.0	100.0
21	+	1.0	33.3
22	+	1.4	46.7
23	+	1.0	33.3
24	+	0.1	3.3
Mean ± SD		1.01 ± 0.9	32.9 ± 29.5
25	–	2.9	96.7
26	–	2.2	73.3
27	–	2.3	76.7
28	–	3.6	120.0
29	–	3.0	100.0
30	–	2.5	83.3
31	–	1.4	46.7
Mean ± SD		2.6 ± 0.7	85.2 ± 21.6

[a]Lens pairs: 17/25, 18/26, 19/31.
[b]Standard bioassay with (+) or without (–) D-glucose at 5 m*M*.

influx of water during the incubation period. The relationship between lens swelling and osmolarity of the medium is shown in Table 3, where it is observed that the degree of lens swelling varies inversely with osmolarity. In addition, residual lens NAH tends to vary inversely with this water influx. In order to hold the swelling factor constant, the NAH level can be calculated as a function of the degree of swelling. For example, if a lens doubled in weight, the NAH content on a μmol/g basis would be reduced by half, and that value would become 100% of the expected lens NAH content. In this way, expected lens NAH would always be 100% regardless of how much the lens swelled. Any deviation from the expected NAH content would indicate a net gain or loss of NAH and suggest that there is some association between NAH and swelling. Based on this analysis, it is observed in Fig. 1 that there is a striking relationship between loss of lens NAH and swelling. This relationship indicates a *Y* intercept owing to NAH loss during the incubation period, with no swelling, to be about 35%, and that lens swelling, induced by a variety of factors is minimal (about 5%) until NAH is about 60% depleted, after which swelling then increases dramatically and is associated with NAH depletion of 70–80%.

Relationship Between Lens Swelling and Clarity

Using a subjective assessment of lens clarity, it has been observed that lens clarity varies inversely with the degree of lens swelling. Using the scale of 1–6, with 1 representing a clear lens and 6 representing an opaque lens, a constructed graph indicates that lenses with clarity indices of 1, 2, 3, 4, 5, and 6 would exhibit swelling of 0, 15, 30, 45, 59, and 74%, respectively.

Discussion

The high concentration of NAH at the surface of the lens and the steep gradient that is normally maintained along with its rapid turnover and energy expended to do so, coupled with the elabo-

Table 3
The Relationship Between Lens Swelling and Residual NAH in the Isolated Goldfish Lens

Lens #	Salinity[a] %	Swelling[b] index, SI	Expected[c] NAH, μmol/g	Residual NAH, μmol/g	% of expected NAH
32	0.00	1.75	1.71	0.25	14.6
33	0.00	1.22	2.46	0.40	16.3
34	0.00	1.43	1.75	0.40	22.8
Mean ± SD		1.47 ± 0.22	1.97 ± 0.34	0.35 ± 0.07	17.9 ± 3.5
35	0.45	1.35	2.22	0.40	18.0
36	0.45	1.41	2.13	0.70	33.3
37	0.45	1.30	2.30	0.60	26.0
Mean ± SD		1.35 ± 0.04[c]	2.22 ± 0.07	0.57 ± 0.12	25.8 ± 6.3
38	0.90	1.23	2.43	0.00	0.0
39	0.90	1.19	2.53	1.20	47.4
40	0.90	1.19	2.53	0.70	27.7
41	0.90	1.04	2.89	1.20	41.5
42	0.90	1.00	3.00	1.50	50.0
43	0.90	1.05	2.86	1.80	62.9
44	0.90	1.10	2.73	0.70	25.6
Mean ± SD		1.11 ± 0.08[d]	2.71 ± 0.20	1.01 ± 0.55	36.4 ± 19.1
45	1.80	1.00	3.00	2.00	66.6
46	1.80	1.13	2.65	0.10	3.8
Mean ± SD		1.07 ± 0.22	2.83 ± 0.18	1.05 ± 0.95	35.2 ± 31.4

[a]Standard bioassay with modified salinity in samples 32–37, 45, 46.
[b]The swelling index is lens weight after incubation/lens weight before incubation.
[c]The expected NAH is derived by dividing 3.0 (unincubated control value for NAH) by the SI. This value represents the effect on NAH of dilution owing to swelling alone.
[d]Significant at $P = 0.0029$.

rate metabolic machinery involved in the NAH cycle, have long suggested that it plays an important role at the lens surface. The results of this study have revealed several new aspects of NAH metabolism that may help to resolve the role of this enigmatic amino acid and help to discover the answer to the riddle of why it is passed through the lens membrane, only to be rapidly hydrolyzed, its metabolites actively taken up, and the molecule resynthesized once again.

Relationship Between External Ca^{2+} and Maintenance of the High Tissue/ECF NAH Gradient

Although the high lens/ocular fluid NAH gradient had been previously observed, it was not known whether this gradient was owing to a passive or an active process. In these studies, where lenses were made dysfunctional by removal of extracellular Ca^{2+}, it has been observed that the lenses lose their NAH, which suggests that the high NAH tissue/ECF gradient is probably the result of an active process in which Ca^{2+} plays an important role. The absence of Ca^{2+} not only results in the rapid depletion of lens NAH, but is also associated with the inability of the lens to transport amino acids actively. Whether the Ca^{2+} is directly or indirectly involved with maintenance of an active gating process regulating NAH efflux remains to be elucidated. However, there is reasonable evidence that the Ca^{2+} effect may be direct and operate at the level of a lens NAH transporter (NAHT). A good possibility for the projected NAHT is the

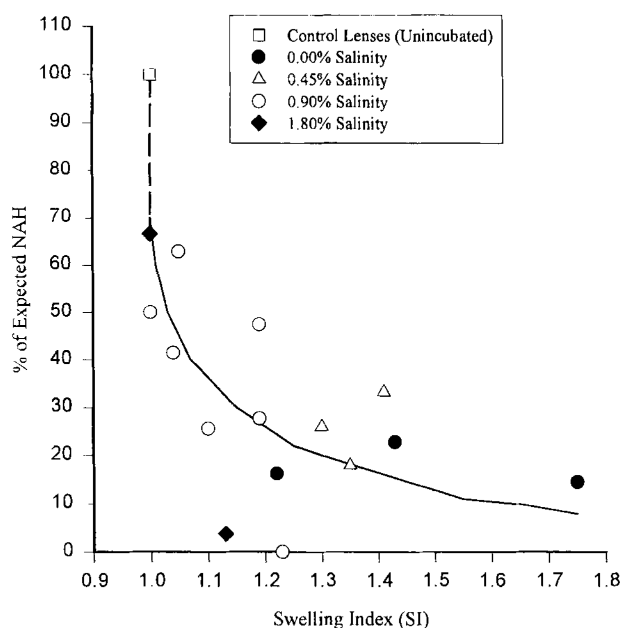

Fig. 1. Relationship between percent of expected lens NAH vs lens swelling in isolated goldfish lenses.

major intrinsic protein (MIP) of lens, with the ability to form gap junctions and other aqueous channels of large diameter (Unwin, 1989; Lea, 1996). In addition, Ca^{2+} is one of the ligands that regulates transport through these channels and where it operates, such that a decrease in cytosolic Ca^{2+}, as would be produced by elimination of extracellular Ca^{2+}, opens the channel. Although the function and regulation of MIP channels in the lens are as yet unknown, Lea (1996) comments that there is an expectation of finding a sensitively regulated water channel in the lens for the maintenance of its structural integrity. Rae and Levis (1984) measured currents from single ionic channels located on the apical membrane of lens epithelial cells and have identified a large channel varying in conductance from about 400 pS to 1.9 nS in different patches with no selectivity for small anions or cations. However, these authors speculate that such an unselective channel with a large conductance probably did not connect the cell's interior with its extracellular space because of the potential to dissipate the cell's ionic gradients rapidly. Nonetheless, perhaps such a large channel, with an affinity for and facilitating the diffusion of NAH, and with a Ca-ligand gate might also represent the sensitively regulated water channel postulated by Lea (1996). Thus, a reasonable interpretation of the results of this study, with regard to the efflux of NAH in response to removal of Ca^{2+} from the medium, is that the steep intercompartmental NAH gradient is normally maintained by a Ca-ligand gate controlling a water-filled NAHT channel at the apical membrane of lens epithelial cells, and that a reduction in cytosolic Ca^{2+} concentration, in response to removal of extracellular Ca, opens that channel.

Relationship Between External D-Glucose and NAH Efflux

D-Glucose appears to play a role in the reduction of lens NAH, and may be a major factor in the observed efflux of both NAH and NAA from lens and brain. Previously, Buniatian et al. (1965) have noted that glucose induced the efflux of NAA from rat brain slice preparations. D-Glucose usually enters cells down its gradient by a facilitated diffusion process through a family of related glucose transporters (GLUT), each with distinct physiological features and tissue distributions (Watanabe et al., 1994; Knott and Forrester, 1995), and the lens uses this energy supply for maintenance of osmotic balance and synthesis of metabolites (Zhang and Augusteyn, 1995). Of interest, it is also reported that some GLUT transporters may also act as water channels (Kumagai et al., 1994). Operating as a water channel, the GLUT transporters allow water to enter a cell along with D-glucose. Evidence that this water channel function is dependent on the actual passage of D-glucose is demonstrated by the fact that both water influx and D-glucose-facilitated transport are blocked by a variety of D-glucose-facilitated transport inhibitors as well as by L-glucose (Fischbarg et al., 1990). Glucose uptake has been reported to result in cell swelling in a variety of cells and tissues (Lang et al., 1998). In the lens, use of glucose for energy results in the production of ATP and in the formation of metabolites, which include CO_2 and water. Whereas the CO_2 readily migrates through the membrane lipids, down its gradient, the metabolic water that is produced must now be transported from intracellular to extracellular space, and counter to the continuous influx of water that results from that portion of the intracellular osmolytes that are present as nondiffusible macromolecules (Koch,

1994). Thus, a cell that must continuously remove intracellular water in order to reduce buildup of osmotic pressure faces the additional task of removing water that enters along with the movement of the glucose solute from extracellular to intracellular space, and with the conversion of that solute into additional intracellular solvent molecules produced by its metabolism. The observation in the present study that D-glucose in the extracellular medium induces the efflux of NAH may also explain the regulated efflux of NAH observed during the original studies of the NAH cycle in the eye. Moreover, based on this observation, the results suggest that there might be some relationship between NAH efflux and the need to remove both transported water and metabolic water produced by the oxidation of glucose.

Relationship Between Lens Swelling and NAH Efflux

In this study, for the first time, a relationship between water influx and NAH content of tissue has been observed. This relationship between loss of NAH and lens swelling, under a variety of osmotic conditions, is inverse, which suggests that water influx triggers the release of NAH and that NAH efflux may play some role in the movement of water out of the lens. Taylor et al. (1995), using a microdialysis technique in rats, noted a similar selective response of cerebral NAA to reduced osmolarity and suggested that NAA efflux might contribute to the protection of neurons against swelling. In a series of investigations of factors involved in cell swelling, Pasantes-Morales et al. (1994) used cultured cerebellar granule neurons, which are known to synthesize NAA (Urenjak et al., 1992), in studies of factors that affect the process of regulatory volume decrease mechanisms in cells exposed to reduced extracellular osmolarity. As a result of the studies, these authors concluded that it was clear that the mechanism underlying regulatory volume decrease in all cell types involves the activation of transmembrane permeability pathways leading to the efflux of intracellular osmolytes down their concentration gradients. Further, this efflux is through diffusion pores that are probably channels, and that this efflux can occur even in the absence of extracellular calcium. Finally, these authors suggested that the cellular water was removed by being carried by the gradient-driven exit of these intracellular osmolytes in the form of osmotically obligated water. Based on this premise, and although there may be other functions for NAH and the NAH cycle in various tissues, it would seem reasonable to assume that at least one of its functions as a cycling osmolyte is to transport water. Thus, the selective efflux of the osmolyte NAH down its gradient might serve as a way to carry cellular water, in the form of a specific quantity of water molecules for each NAH molecule that are associated as an obligated water shell. If this were the case, then the observation made in the present study that there was little lens swelling until lens NAH was sharply depleted has a logical explanation.

Proposed Function of the NAH Cycle in the Lens

Role as a Molecular Water Pump

Based on the results of many previous studies and on insights gained in the present study, a novel role for NAH in the lens is proposed. As a hypothesis, and in keeping with its known metabolic pathways, the compartmentalization of synthetic and hydrolytic enzymes, and its association with the epithelial cell layer at the lens surface, it is suggested that the NAH cycle may function as a pump for the elimination of both metabolic and other sources of tissue water. It is proposed that dedicated cells in the lens, at the lens–ocular fluid interface, synthesize the osmolyte NAH, and that the regulated release of NAH to ECF down its gradient, along with its associated water, would effectively result in the cotransport of water to ECF. In order to maintain the tissue–ECF osmotic balance, and for the cycle to continue, there must also be a rapid hydrolysis of the osmolyte by its specific acylase, accompanied by rapid uptake of the hydrolytic products. In carp, it has been calculated that the amount of NAH released from lens, 0.003 μmol/g/min (Baslow, 1967), could be hydrolyzed in 1.5 s, and in this study, the amount of His produced would be actively transported into the lens in 5.4 s. Energy would be expended in two places in the NAH cycle, for the active transport of the metabolic products of NAH back into the cell, and for the intracellular resynthesis of NAH. The lens of the eye, with its requirement to maintain a dehy-

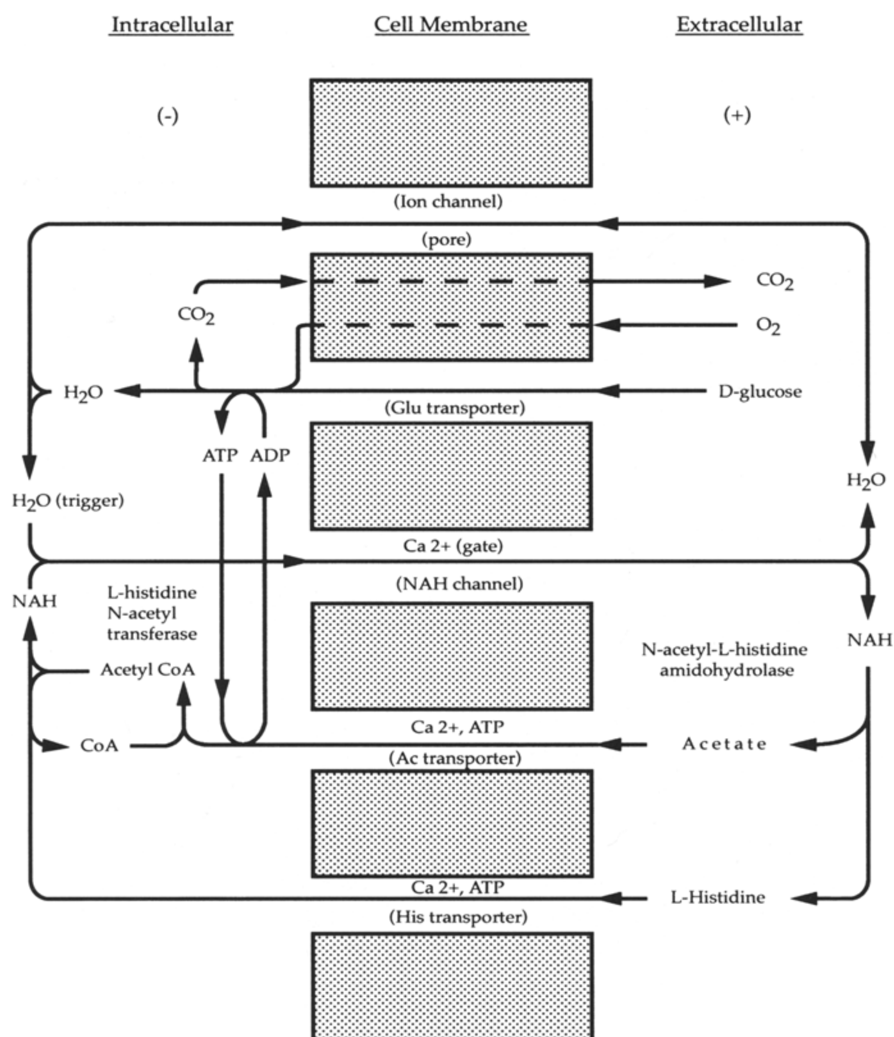

Fig. 2. Schematic representation of the NAH cycle in the lens of the eye, with associated trigger, gate, and energy source, operating as a gradient-driven molecular water pump.

drated state, and the retina and brain with their high energy demands and consequent high rate of production of metabolic water, would appear to be ideal candidates for such a water pump mechanism. This would also be especially important in light of the extreme sensitivity of the CNS to osmotic imbalance (Strange, 1992). A schematic representation of the proposed molecular water pump, which incorporates previous information, as well as the results of the present study is shown in Fig. 2. The basic components of the proposed pump mechanism are a suitable hydrophilic carrier molecule, a transporter with an appropriate trigger and gate, and a mechanism for dewatering and recycling the carrier molecule. The known NAH metabolic cycle clearly conforms to such a potential use. Obviously, a key component of this proposed system is the combined osmolyte–water transporter that is yet to be completely characterized. However, this kind of a combination osmolyte–water transport system, operating through a specific facilitated transporter for a similar-sized molecule as it travels down its gradient, has already been demonstrated for passage of the osmolyte, D-glucose across the plasma membrane by Fischbarg et al. (1990). The basic differences between the D-glucose–water transport, and the proposed NAH–water transport systems are in their direction of

flow, water influx with D-glucose, and water efflux with NAH, and the hydrophilic nature of NAH. The proposed function represents a synthesis based on many different experimental observations, inferences based on those observations, and on information derived from many similar and related biochemical studies. Thus, the hypothesis rests on a broad base, and although not all of the details are available, it does provide the basis for experimental verification. The hypothesis is also the least complicated solution that fits most of what is already known about NAH metabolism and correlates well with the results of a variety of other studies. When all of the factors are taken into consideration, the NAH system would appear to be well suited to function as a molecular water pump and to participate in the process of cellular osmoregulation.

Key Steps in the Proposed Molecular Water Pump Cycle

In considering the potential role of the lens-ocular fluid NAH cycle as that of an intercompartmental water pump, several aspects of that cycle required for its operation are brought into focus. These relate to questions of why NAH might be a selected carrier, and how the intercompartmental distribution of its synthetic and hydrolytic enzymes might be involved in creating an efficient water-regulating system. When analyzed in the context of the known biochemical cycle, it becomes apparent that components of the cyclical metabolism of NAH correspond to four key steps that would be required for the transport of water against a gradient. These four steps are illustrated in Fig. 3, and described below.

1. Derivative synthesis: The nature of a selected cycling osmolyte should be such that it has a strong attraction for water molecules, and that a high intracellular–extracellular gradient can be established and maintained, since it is this gradient that would drive the system. His is a strongly ionizable hydrophilic amino acid that would attract water molecules into one or more dipole–dipole aquacomplexed shells. However, His is also a protein amino acid and, in addition, participates in a number of other metabolic pathways as well. Therefore, it is unlikely that His could be raised to high enough intracellular concentrations to form the high intracompartmental gradient that is required to drive the system efficiently. The metabolic strategy that appears to have been followed to overcome this problem was to attach a nonprotein moiety to the His in order to block its utilization in any other intracellular metabolic pathways, and to block catabolism of the derivative by maintaining its hydrolytic enzyme in another compartment. In a recent review of cell volume regulatory mechanisms by Lang et al. (1998), such amino acid derivatives are referred to as "compatible osmolytes," in that they are molecules specifically designed to create osmolarity without compromising other cell functions. Thus, the purpose of acetylation step in the NAH cycle, as it relates to a potential water pump function, becomes clear.
2. Aquacomplexed water transport: NAH, being an His derivative, can be expected to attract polar water molecules. In addition, a known characteristic of the lens–ocular fluid NAH cycle is the regulated efflux of NAH into ocular fluid. Obviously, an NAH transporter is available that has a gate mechanism to allow both for a high intracellular concentration to be established and for a trigger mechanism to allow the regulated release of the NAH. This NAHT is yet to be characterized, but it is anticipated that it is a channel with Ca^{2+} as the gate ligand and that an increase in intracellular water is the trigger. Since several known Ca^{2+} ligand-gated channels respond by opening as intracellular Ca^{2+} concentration drops, it is reasonable to expect that an increase in intracellular water, which decreases intracellular osmolality and Ca^{2+} concentration, could serve as a homeostatic feedback trigger mechanism for the opening of a selective NAHT channel. As aquacomplexed NAH travels down its gradient into another compartment, intracellular water concentration would decrease, intracellular osmolality and Ca^{2+} concentration increase, and the elevated Ca^{2+} concentration could close the NAHT. The time frame for the opening and closing of the channel might be very rapid, which would allow for very tight control of intracellular osmolality. Lang et al. (1998), suggest that an intracellular mechanism serves cell volume regulation if it is modified by alteration of cell volume and this modification triggers the appropriate alter-

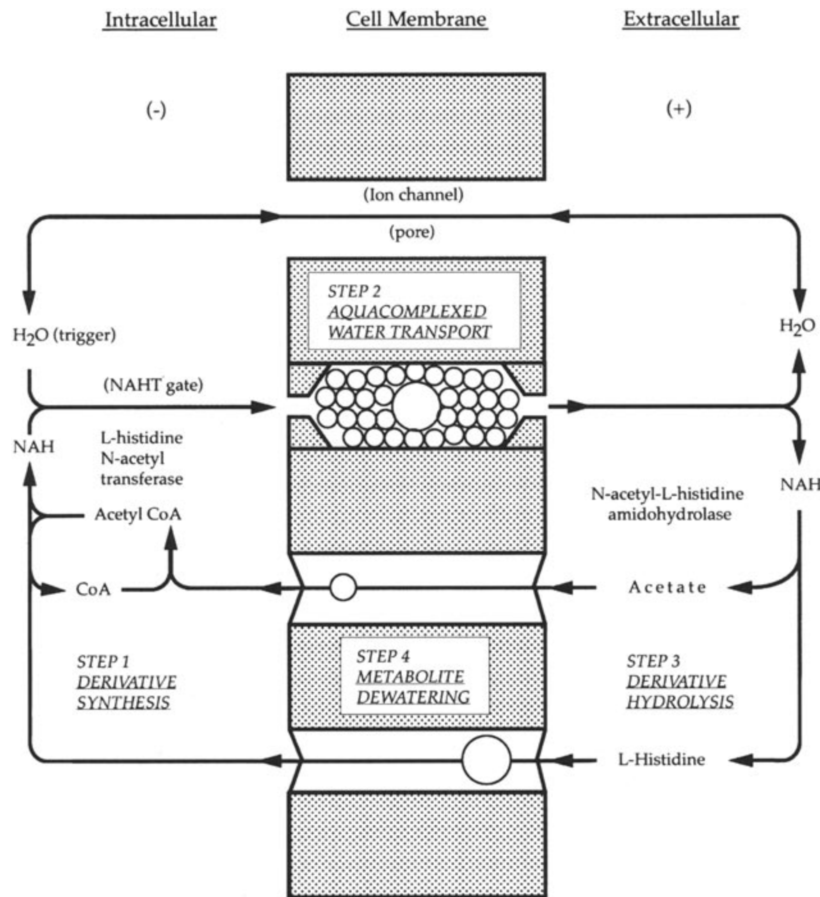

Fig. 3. Schematic representation of the NAH metabolic cycle and its participation in the four key steps that are required for NAH to operate as a molecular water pump.

ation of cell volume. They also indicate that Ca^{2+} may be involved in some aspects of regulatory volume decrease. NAH, being preformed, requires no additional energy and is therefore available for instantaneous use. It is also important to note that the proposed water pump, which is a carrier system, is NAH gradient-driven, and therefore, its transport is independent of all other osmolyte species or total osmolality of the receiving compartment.

3. Derivative hydrolysis: In the eye, the second compartment for NAH produced in the lens is the surrounding ocular fluid where NAH acylase is found. Since NAH is not actively taken up by the lens, if it were not hydrolyzed rapidly, the lens–ocular fluid gradient would drop, and the cycle and its function brought to a halt. Thus, the significance of the presence of high acylase activity in ocular fluid is that it hydrolyzes all NAH liberated from the lens, so that under normal conditions, there is almost no NAH in ocular fluid, and the intercompartmental gradient is maintained.
4. Metabolite dewatering: The final step in the known NAH cycle is that the hydrolytic products of NAH, His, and acetate, are rapidly transported into the lens for resynthesis of NAH. A question that arises is why the uptake of His, which is also an ionizable hydrophilic amino acid, would not bring water back into the cells of the lens. The answer is probably related to the different mechanisms of transport of NAH and His. Efflux of NAH is passive and gradient-driven, probably via a water-filled channel. However, its metabolites are actively transported back into the lens, certainly against a gradi-

ent for His, by means of much more water-restricting and energy-driven active transporters, where water molecules may be stripped from the transported substances (Sancho et al., 1995). The function of the hydrolytic enzyme therefore appears to be twofold: first, to hydrolyze the carrier molecule in order to maintain the high intercompartmental gradient, and second, to supply important metabolites that can be dewatered during their active transport into cells for their use in resynthesis of NAH. Although the identification of a cellular water pump has long been anticipated, none has ever been demonstrated in either multicellular or single-celled organisms (Heuser et al., 1993). However, Dupont (1983) offered a complex hypothesis in which it was suggested that Ca^{2+}-ATPase could have a dual role, in that it might serve as a water pump as well as an ion pump. This chemiosmotic hypothesis did contain some of the elements of the proposed NAH water pump mechanism. In a nonmetabolic cycling scheme, it was proposed that the carrier was a hexahydrated Ca^{2+}, transported through a channel, and the subsequent dewatering process was associated with its recycling in which the high-affinity binding of Ca^{2+} to a protein, a characteristic of active transport, resulted in its dehydration. Finally, the NAH and NAA systems in vertebrates have existed for at least several hundred million years and, in light of their potential for involvement in water regulation, it is of interest to note that the active site of human aspartoacylase is also represented in a bacterium (Hess, 1997), where it appears to have been conserved for several billion years.

An Estimate of the Power Function

As a molecular water pump, the higher the ratio of solvent-to-solute molecules, the more efficiently it will perform its function. An estimate of the power function (PF) or the numbers of osmotically obligated water molecules associated with each NAH molecule can be derived from the analyses of Hummer et al. (1996) on a small representative dodecapeptide (YFYNAKAGLCQT), which contained one charged amino acid (K), five polar residues (Y, Y, N, Q, T), a cysteine residue (C), and five nonpolar residues (F, A, A, G, L). These investigators calculated that there would be 2.04 water molecules/atom for united peptide atoms and hydrogen atoms forming part of polar groups. In NAH, there are 25 atoms with 9 H atoms not in polar groups. At a ratio of 2.04 water molecules/qualified peptide atom, one would expect there to be 33 water molecules associated with each molecule of NAH. Thus, the calculated PF for NAH is 33. A similar PF derived for the acidic NAA is 31. The functional significance of the rapid recycling of NAH becomes apparent when it is observed that there could be up to 10 cycles/d for each NAH molecule in the lens. Thus, from a relatively low standing molecular population, it would become possible to generate a substantial efflux of cellular water. For the goldfish lens containing 3 μmol of NAH/g and with 10 cycles/d and a PF of 33, it would be possible to remove 990 μmol (17.8 mg) of water/g of tissue each day, a value that is 1.8% of the weight of the tissue, and about 4% of the tissue water. With typical NAH concentrations in the brain of poikilothermic vertebrates of 5–10 mmol/kg, there is the potential for up to 3.3 mol (60 mL) of water to be removed each day/kg of brain. This value would represent about 6% of the weight of the tissue and about 8% brain tissue water. The energy utilized by such a system is low, and it has been calculated that the energy cost associated with NAA cycling in brain requires <1% of its daily energy budget (Baslow and Resnik, 1997). Key to the understanding of the system will be an analysis of the net efflux of water during each NAH cycle. However, with the large theoretical potential for water efflux, the experimental evidence that under conditions of increased water influx, tissue swelling is minimal until NAH is severely depleted, and the example provided by the GLUT water channel function, there is a strong likelihood that the NAH system will be found to constitute a highly effective molecular water pump.

Conclusions

Although the lens–ocular fluid NAH cycle in poikilotherms has been known for some time, understanding its functional significance has proven to be elusive. However, insights gained in the present study have brought into focus the poten-

tial for that cycle to be involved with the intercompartmental transport of water. The NAH system, by virtue of its association with the lens–ocular fluid interface and the cycling of NAH across this boundary, is well positioned to be a pump mechanism. Furthermore, the observed inverse relationship between residual lens NAH and lens swelling, under a variety of osmotic conditions, leads to the conclusion that this system might function as a water pump. Combining what is known about the chemistry and metabolism of NAH with the requirements to operate as a water pump, this could be accomplished by a four-step process in which the sequential steps include: intracellular synthesis of a hydrophilic carrier; facilitated transport of an aquacomplex of the carrier down its gradient; extracellular hydrolysis of the carrier and finally; dewatering of its metabolites during their active uptake. The steps in the process are generic and could apply to any other suitable hydrophilic carrier and its associated enzymes. Therefore, it is also suggested that NAH is the archetype of a class of metabolically and chemically related hydrophilic osmolytes that undergo similar intercompartmental cycles. These include the additional His derivatives; carnosine, anserine, ophidine, and homocarnosine, and the Asp derivatives, NAA and NAAG. In the human brain, four of the derivatives of His and Asp are simultaneously present and are found in a variety of cells, including both neurons and glia. Although their distribution within the brain varies, it is notable that in almost every part of the brain, these substances are paired, so that at least one Asp and one His derivative are represented. This leads to the intriguing notion that a balance between these derivatives is necessary for normal brain function, and to the concept that neuron function may require a biochemical dialog with, and participation in, some of the metabolic pathways of surrounding support cells. Based on the hypothesis that NAA and NAH and related compounds function as water regulators, the implications of their operational failure owing to inborn errors in their metabolism become clear. In Canavan disease, it is only the metabolism of NAA and NAAG that is affected, and in Homocarnosinosis, only carnosine and homocarnosine metabolism is affected. However, in each disease, the absence of their respective acylases results in a profound loss of brain function. If complete failure of one or more of these molecular cycles produces global neurological symptoms, it follows that regional or partial failures may also result in significant neurological disturbances that may be related to several brain disorders whose etiology is presently unknown. Tsai and Coyle (1995) have recently reviewed the often significant relationships between changes in brain NAA content and a variety of neuropathologies and neuropsychiatric disorders, but for which no underlining pathophysiological link has ever been established. The present analysis of the role of intercompartmental cycling osmolytes may provide such a link as well as a new horizon with which to evaluate further elements of CNS function. Finally, although some degree of uncertainty remains, based on the hypothesis, the NAH system would constitute the first cellular water pump to be identified, and NAH would be the first member of a new class of metabolically recyclable osmoregulators that could provide for a fourth mechanism, the pumping of water, by which cells can maintain osmotic homeostasis. The other three known dynamic mechanisms used by cells are not water pumps, and include secretion of macromolecules, facilitated diffusion of small organic osmolytes, and the use of ion pumps, all of which function by upregulating or downregulating extracellular solute composition and thereby creating sinks for the movement of water.

Note Added in Proof

While this paper was in press, an article was published (Meinild et al., 1998) that described a Na^+-glucose transporter (hSGLT1) as a molecular water pump involved in the active uptake of water. In that article, a mechanism very similar to that of the proposed archetypal NAH water pump was presented in which a specific number of water molecules (210) are cotransported uphill as a function of a gradient-driven $2Na^+$-glucose complex that is transported downhill via the cotransporter protein. This Na^+-glucose pump would operate as the obverse of the NAH efflux pump and function as a water influx pump. These apposed molecular water pumps may work in unison to achieve cellular water homeostasis.

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