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Amino Acids and Cell Volume Regulation

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Free amino acids play an important role in regulating cell volume in fishes. Four tissues/cells (skeletal muscle, RBC, brain, and myocardium) of the little skate, *Raja erinacea*, were selected for detailed study because of their special importance or unique advantage as experimental models. Three particular amino acids, β-alanine, taurine, and sarcosine play a predominant role in all four tissues. As in higher vertebrates, amino acid uptake in skate brain, heart, and RBC is mediated via a Na^+-dependent process.

Amino acids leave the skate brain rapidly in response to a sudden decrease in plasma osmolality and/or to a simultaneous drop in extracellular Na^+ concentration. However, although amino acids are important for volume regulation in normal brain cells, they do not appear to be likely candidates for the unidentified "idiogenic" osmolytes in mammalian brain cells.

The high concentration of taurine in skate myocardium is of special interest because of the special role of this amino acid in myocardial contractility. Thus, unlike β-alanine and sarcosine, taurine may play a dual role in regulating both cell volume and contractility of myocardial cells. The isolated skate atrium is well suited for *in vitro* studies of these two processes.

This discussion deals with the osmoregulatory responses involved in the maintenance of cell volume following an osmotic perturbation of the internal medium. The common skate (*Raja erinacea*) was chosen as a model for these *in vivo* and *in vitro* studies because of its ability to maintain functional viability while its body fluids are being markedly diluted following transfer of the fish from full strength to 50 percent sea water. The capacity of various cells to adjust to a diluted interstitium is of interest from the viewpoints both of natural history and of clinical medicine. The natural historian uses such knowledge in an attempt to understand the migrations of aquatic vertebrates between the sea and estuarial or fresh water. Such knowledge is of interest to the clinician because of its possible relationship to clinical situations such as the dialysis disequilibrium syndrome. The main finding arising from these studies on this elasmobranch is that free amino acids play a large role in regulating cell volume, and that transport of these solutes between cells and interstitium, in response to osmotic changes, is highly specific, carrier mediated and energy dependent.

OSMOREGULATION AND VERTEBRATE EVOLUTION

Paleontological and biological evidence indicates that the ostracoderm and placoderm forerunners of modern fishes lived in the sea exclusively throughout the Ordovician and into the late Silurian periods, but near the end of the Silurian many may have become euryhaline. Euryhaline fishes are those that have developed the capacity to live in both fresh and salt water. A majority of modern teleosts are now

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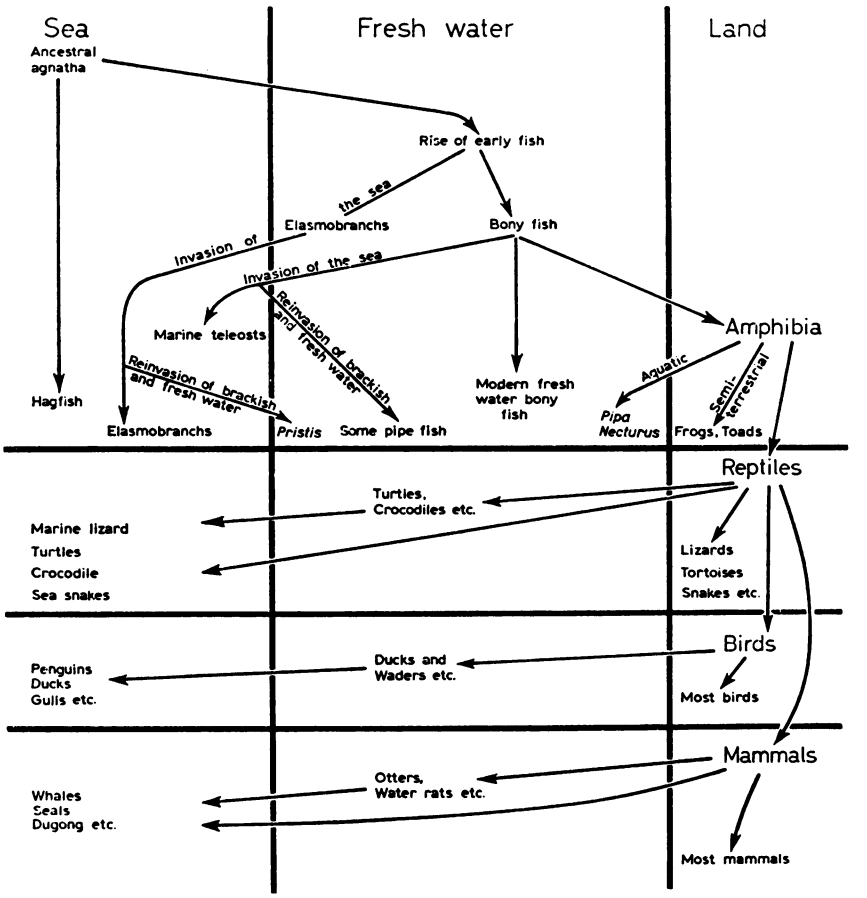


FIG. 1. Probable migrations between freshwater, sea, and land during the evolution of the vertebrates. With the exception of the cyclostome myxinoids or hagfishes, all modern fishes appear to have fresh water ancestors, and all marine vertebrates above the fishes are secondary invaders of the sea derived from terrestrial forerunners. From [3]; reproduced with permission.

marine dwellers, but it is generally agreed that they re-entered the seas at a relatively late date. The cartilaginous elasmobranchs may have evolved from remote ancestors that lived in inland waters but they appear to have been ocean dwellers since their first appearance in the Devonian [1,2]. The sole modern marine vertebrates that appear to have a continual record of sea dwelling since earliest times are the cyclostome myxinoids or hagfishes.

Subsequent challenges to osmoregulation in fishes resulted from the reinvasion of fresh water by several elasmobranchs and aglomerular teleosts. Of course, some of the teleosts that remained in fresh water have developed highly specialized salt and water transport devices that enable them to overcome the tremendous osmotic gradients encountered in reversing the journey from fresh water to the sea, and vice versa. Regulation of these devices will be discussed by subsequent authors, as will those devices which have evolved in response to the special need of terrestrial vertebrates to provide urinary concentrating processes which aid in conservation of water. These probable migrations between fresh water, sea, and land which seem to have occurred during the evolution of the vertebrates are illustrated in Fig. 1. The

higher vertebrates all arose from non-marine forms. Some representatives of each vertebrate class, with the exception of the amphibia, have returned to the sea. Some amphibians are semi-terrestrial but only the reptiles, birds, and mammals have achieved a truly arid-dwelling habitus. However, sizable representatives of each of these classes have successfully adapted to both marine and fresh water environments.

Such migrations from one environment to another cannot be managed unless the body fluid composition and concentration is controlled so as to render it compatible with the requirements of the animal's various cells and tissues. Fresh water vertebrates live in a hypotonic external environment and are threatened by an osmotic influx of water which is minimized by a relatively impermeable surface, as with many of the fishes and the aquatic reptiles and mammals. Excess water is lost by renal glomerular filtration, and subsequent ion loss is prevented by avid tubular retention. Active transport processes at the gill in fishes and in the skin of amphibians counteract ion loss. The water gradient between body fluids and external environment is reversed in most of the marine vertebrates, as the saline content of the sea, of course, is several-fold higher than plasma. Figure 2 is an outline of some of the specialized methods used by the various vertebrate classes to regulate salt and water balance.

A dynamic relationship prevails between external and internal osmolalities for most aquatic organisms, with the exceptions of *Myxine*, and the urea-retaining elasmobranchs and the lobe-finned fish *Latimeria*. Urea retention in elasmobranchs is due to both the relative impermeability of the gills and other body surfaces to the nitrogenous end-product and the reabsorption of over 90 percent of the compound from the glomerular filtrate. The nearly complete renal reabsorption of urea occurs by some process which is not entirely understood. *Myxine* has a salt concentration similar to that of the sea, whereas the elasmobranchs have about the same amount of salt as do the teleosts, but they synthesize urea more efficiently and retain it so efficiently that they are somewhat hyperosmotic to the sea. The rectal gland then removes excess sodium chloride, similar to the way salt is secreted by nasal glands in marine birds, and by orbital glands in lizards and turtles. Most euryhaline elasmobranchs are incomplete osmoconformers. Osmoconforming fishes are those whose body fluid osmolalities vary directly with the salinity of the environment. In contrast, osmoregulators have the homeostatic mechanisms necessary to maintain body fluid osmolalities constant in different salinities. Figure 3 shows the degree to which the euryhaline elasmobranch, *Pristis*, undergoes osmodilution during its non-marine phase. Blood osmolality of the totally non-marine elasmobranch, *Potamotrygon*, approaches that of fresh water teleosts. Most euryhaline teleosts, in contrast, are very effective osmoregulators. The estivating lungfish, *Protopterus*, converts ammonia production to urea during water deprivation which drives their blood osmolalities up while the relatively inert urea accumulates in body fluids and tissues to very high levels during the months or even years when these fish may be stranded in their cocoons out of water.

CELL VOLUME AND COMPOSITION CHANGES DURING ENVIRONMENTAL DILUTION IN THE LITTLE SKATE, *RAJA ERINACEA*

Although a great deal of work has been done on and much is known about the osmoregulation of the extracellular fluids of a wide variety of vertebrates, the processes involved in intracellular osmoregulation, i.e., cell volume regulation, have received relatively little attention until recent years. However, cell volume regulation in certain tissues such as brain and myocardium is critical for survival. There are two

	Liability to osmotic water loss	Liability to evaporative water loss	Blood conc. relative to medium conc.	Urine conc. relative to blood conc.
Elasmo-branchs	x	x	Isotonic	Isotonic
Teleosts	+	x	Hypotonic	Isotonic
Reptiles	x	+	Hypotonic	Isotonic
Mammals	x	+	Hypotonic	Hypertonic
Birds	x	+++	Hypotonic	Hypertonic

x = No liability
+ = Liability

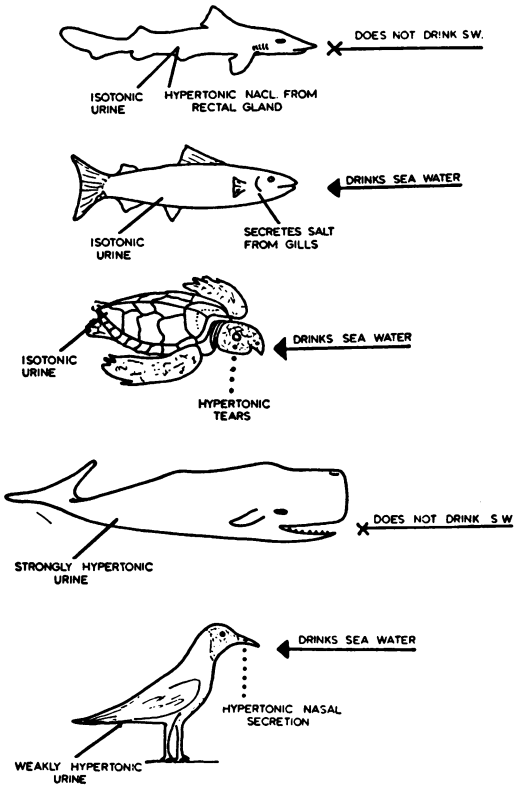
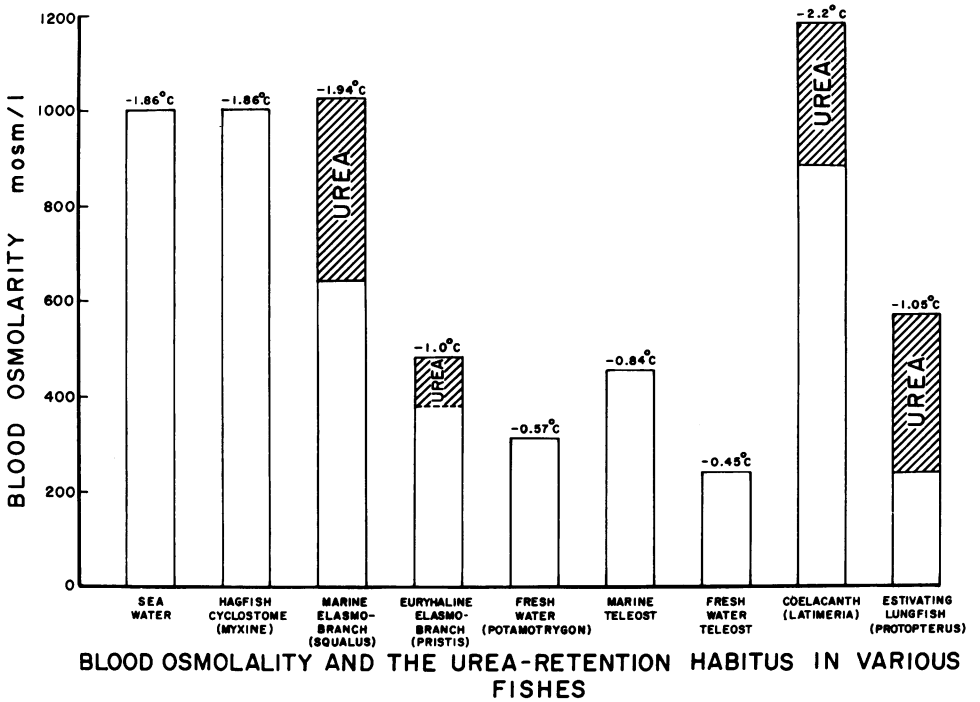


FIG. 2. Osmoregulatory devices used by various marine vertebrates to help control salt and water balance. From [3]; reproduced with permission.



BLOOD OSMOLALITY AND THE UREA-RETENTION HABITUS IN VARIOUS FISHES

FIG. 3. The approximate blood osmolal concentration in marine, estuarial, and fresh water fishes. Only *Myxine* has blood isosmotic with its external environment. The elasmobranchs and coelacanth urea-retaining fishes are typically hypertonic, even to their full-strength marine environments. Blood concentration, of course, is always hypertonic to medium in the fresh water fishes.

reasons for the relative paucity of studies on cell volume regulation in vertebrates. First, it is technically much more difficult to measure intracellular composition and volume compared to the relative ease with which these same measurements can be made on extracellular fluid (ECF). Second, it is difficult to produce changes in osmolality of the ECF of higher vertebrates sufficiently large to have a measurable effect on the composition and volume of the intracellular fluid (ICF) without killing the animal. It is for the second reason that we chose to study cell volume regulation in a lower vertebrate, the little skate, *Raja erinacea*, in which a wide range of ECF osmolality can be induced—as well as tolerated—by alteration of the salinity of the external environment.

The little skate is an incomplete osmoconformer; its ECF osmolality changes in relation to alterations in environmental salinity, but the osmotic pressure of the ECF remains above that of the medium. As shown in Fig. 4, gradual acclimation of little skates from full-strength to one-half strength seawater causes a significant drop in plasma concentrations of urea and NaCl (the two major solutes of skate ECF). However, ECF osmolality remains significantly above that of the diluted seawater both during the early and later stages of the acclimation program [5]. The marked fall in ECF osmolality is due to two factors: there is net influx of water into the fish which increases body weight about 10–15 percent [5]; and simultaneously the renal reabsorption of urea and NaCl from the glomerular filtrate falls from pre-dilution levels of 95 and 75 percent to 65 and 60 percent, respectively [5]. The net effect of these two factors is that the ECF osmolality of the diluted skates is 75 percent of that of skates kept in full-strength seawater (Table 1).

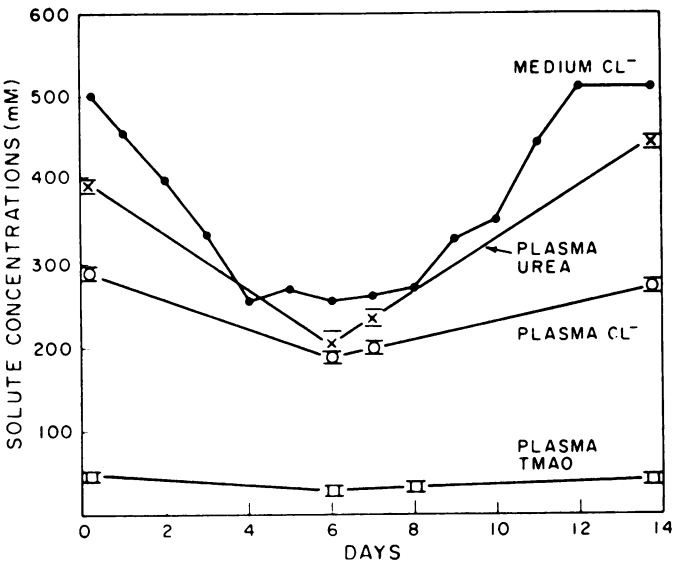


FIG. 4. Solute concentrations in medium and plasma of skates during dilution and reconcentration of seawater. Points with bars above and below represent means ± SE of 5–7 fish except for TMAO on day 14 which is average of 2 fish. TMAO = trimethylamine oxide. From [5]; reproduced with permission.

The influx of water into the skate leads to expansion of the extracellular fluid volume which is reflected in a marked increase (70 percent) in chloride space of skeletal muscle [6]. In contrast, cell volume appears to be closely regulated during environmental dilution. The cell water content of skeletal (wing) muscle averaged 70.5 g/100 g muscle for skates maintained in full-strength seawater and 71.4 g/100 g muscle for skates acclimated to half-strength seawater. This remarkably tight regulation of intracellular volume in the face of a marked (25 percent) dilution of the ECF was due in large part to the ability of the cells to unload osmotically active solute (Table 2) in response to the osmotic stress.

Role of Amino Acids in Cell Volume Regulation

Previous studies on a variety of osmotically tolerant invertebrates (see [7] for review) have shown that free amino acids play an important role in cell volume regulation during environmental dilution in these animals. For example, adaptation of the marine crab *Callinectes sapidus* to half-strength seawater is accompanied by a

TABLE I

Body Weights, Hematocrits, Extracellular Fluid Volumes of Muscle (Chloride Space), and Osmolar Concentrations of Plasma and Muscle in *R. erinacea* Before and After Dilution of Environmental Salinity

			Osmolality, mosmol/liter	
Seawater	Body Wt. kg	Cl Space, %	Plasma	Muscle
100%	.848	9.02	965	1,080
	±.03	±2.57	±18.1	±47
	(6)	(6)	(6)	(6)
50%	1.16	15.4	719	769
	±.10	±1.15	±13.6	±20.9
	(7)	(7)	(7)	(7)
P value*	>.05	<.01	<.001	<.001

Values are means ± SE. Number of fish per group are shown in parentheses.

*Group in 50% seawater compared to group maintained in full-strength seawater. From [6]; reproduced with permission.

TABLE 2

Solute Concentrations in Plasma and Within Erythrocytes and Muscle Cells of Skate (*Raja erinacea*) Maintained in Seawater and In Half-Strength Seawater

Sample	Seawater	K^+, meq/liter	Na^+ meq/liter	AA, mmol/liter	Urea, mmol/liter	TMAO, mmol/liter	Osmolality mosmol/liter
Plasma	100%	4.96 ± 0.38	299 ± 5.21	10.9 ± 0.49	361 ± 17.8	39.2 ± 2.7	965 ± 18
		(6)	(6)	(6)	(6)	(6)	(6)
Plasma	50%	4.25 ± 0.24	217 ± 5.05	12.2 ± 0.79	264 ± 11.9	30.2 ± 2.7	719 ± 14
		(7)	(7)	(7)	(7)	(7)	(7)
P value		NS	<.001	NS	<.01	<.05	<.001
Erythrocytes	100%	120.8 ± 4.44	50.7 ± 7.38	280 ± 6.18	413 ± 19.9	35.8 ± 1.5	
		(6)	(6)	(6)	(6)	(6)	
Erythrocytes	50%	135.7 ± 4.11	32.7 ± 2.12	150 ± 13.9	283 ± 15.4	29.3 ± 2.2	
		(7)	(7)	(7)	(7)	(7)	
P value		<.05	<.05	<.001	<.001	<.05	
Muscle cells	100%	161.8 ± 6.20	9.6 ± 2.58	214 ± 21.8	398 ± 18.2	63.9 ± 14.2	1,080 ± 47
		(6)	(6)	(6)	(6)	(6)	(6)
Muscle cells	50%	134.2 ± 1.90	4.1 ± 1.84	144 ± 19.8	264 ± 15.3	35.8 ± 9.4	769 ± 20
		(7)	(7)	(7)	(7)	(7)	(7)
P value		<.01	NS	<.05	<.001	NS	<.001

Values for solute concentrations are means ± SE. Number of fish per group indicated in parentheses. NS, not significant; i.e., probability >.05 indicates no significant difference between the means of solute concentrations for skates in 50% and those in full-strength seawater. From [6]; reproduced with permission.

decrease in body fluid osmolality from 1100 mOsmols/kg to about 850 and by a decrease in the total muscle cellular free amino acid concentration which approximately equals the fall in extracellular osmolality [8]. Amino acids have also been implicated in cell volume regulation in fishes such as the hagfish and osmotically tolerant teleosts. Muscle cells of the osmoconforming hagfish *Myxine glutinosa* contain high concentrations of free amino acids (approximately 200 mM). When the hagfish is adapted to diluted seawater, the concentrations of amino acids fall more than would be expected from the increase in water content and with a specificity that suggests the operation of one or more regulatory mechanisms [9,10].

Skeletal Muscle and Erythrocytes

Muscle was chosen for analysis because of its large contribution to total body mass. Erythrocytes, on the other hand, represent a cell population whose intracellular osmolyte composition can be directly determined unequivocally. As shown in Table 2, free amino acids comprise a significant fraction (20 percent–30 percent) of the total osmolality in tissues, such as erythrocytes and skeletal muscle, of skates in full-strength seawater. The remaining osmolality can be accounted for by sodium and potassium salts, urea, and trimethylamine oxide. During acclimation of the skates to half-strength seawater, cellular amino acid concentrations fall markedly: approximately 50 percent in erythrocytes and 33 percent in muscle cells. The percentage decrease in cellular amino acid concentration is approximately proportional to the decline in ECF osmolality in muscle but the decline observed in erythrocytes is greater than the drop in plasma osmolality. The different quantitative responses in muscle cells and erythrocytes may be related to differences in changes of inorganic electrolytes induced by environmental dilution in the two tissues. In erythrocytes K^+ (the major cation) concentration rises in diluted skates. In contrast K^+ concentration falls significantly in muscle cells during environmental dilution.

The data shown in Table 2 suggest an interesting scheme for cell volume regulation in the skate. The osmotic concentration of the body fluids in this elasmobranch is elevated by accumulation of high concentrations of urea—and to some extent trimethylamine oxide—in both intracellular and extracellular water. In contrast to the relatively low permeability of the external body surface and gills to urea, the plasma membranes of most other cells are freely permeable to urea. Thus it exerts a negligible osmotic effect across plasma membranes of cells such as erythrocytes and skeletal muscle. However, NaCl concentration, which is also elevated in the ECF of elasmobranchs compared to other vertebrates, does exert an osmotic effect between intracellular and extracellular fluids. The presence of such high concentrations of this osmotically active salt in the extracellular fluid must be matched by an equivalent amount of osmotically active solutes in the intracellular fluid to maintain cell volume. K^+ cannot be used in this role without disrupting the normal electrical forces operating to maintain potential differences across the cell membranes. Thus, amino acids are accumulated intracellularly to match, in part, the high concentration of NaCl in the ECF. During environmental dilution when plasma NaCl concentration falls 82 mmols/l from 299 to 217 (Table 2), muscle K^+ salts decrease 28 mmols/l and amino acids decline 70 mmols/l. The decline in cellular amino acid concentration helps to offset the increase in osmotic gradient between the ECF and ICF due to the fall in ECF NaCl and obviates the need for large changes in intracellular K^+. The latter aids in maintaining a normal potential difference across the cell membrane. The intracellular/extracellular K^+ ratio in muscle cells was 33 in skates kept in full-strength seawater and 32 in skates acclimated to half-strength seawater [6].

Previous studies on osmotically tolerant invertebrates and lower vertebrates indicated that specific amino acids such as taurine, glycine, and proline accumulated while these organisms were in full-strength seawater and that the concentrations of these amino acids were regulated in response to changes to environmental salinity [7]. Boyd et al. [11] found a similar condition in the little skate. As seen in Figs. 5 and 6, specific amino acids were retained by skate skeletal muscle and erythrocytes. Sarcosine and β-alanine comprised the major intracellular amino acids in skeletal muscle while taurine and β-alanine were predominant in erythrocytes. During acclimation to environmental dilution the cellular concentration of the major amino acids fell significantly. In contrast, the concentration of some of the other amino acids, present in much smaller amounts in muscle cells and erythrocytes, either did not change or actually showed a tendency to increase following dilution of the ECF.

It is interesting to speculate on why only certain amino acids have been selected to participate in cell volume regulation. In the little skate the three amino acids playing a predominant role are sarcosine, β-alanine, and taurine. One characteristic of these three amino acids is that they are relatively inert, metabolically speaking. For example, they are not found in protein so that relatively large alterations in their concentrations can be tolerated without disrupting cellular protein biosynthesis. Thus, these three amino acids may have been selected for the osmotic role they play because of their unique metabolic characteristics.

The mechanisms by which high cellular concentrations of specific amino acids are maintained in full-strength seawater and modulated during acclimation to environmental salinity are under investigation. Goldstein and Boyd [12] examined the β-alanine transport system of erythrocytes of the little skate. They found that both the uptake and efflux were Na^+ dependent in isolated erythrocytes; changes in Na^+ concentration corresponding to those observed in the ECF of diluted skates had significant and similar effects on both uptake and efflux. However, osmotic dilution of the incubation medium at constant Na^+ concentration accelerated β-alanine efflux

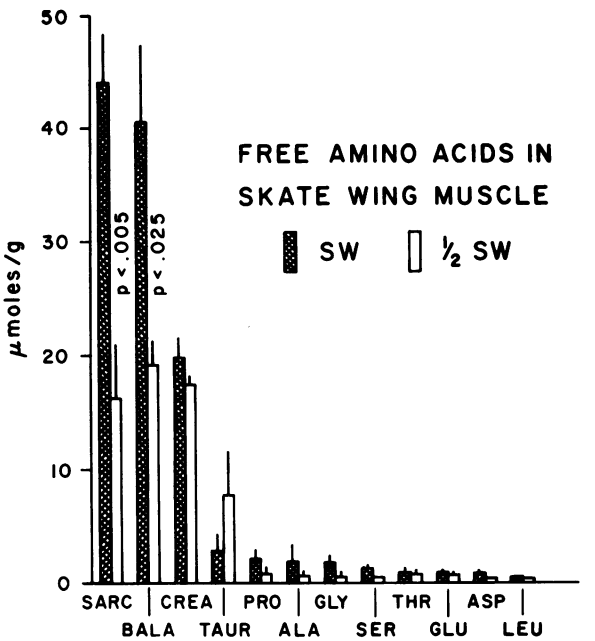


FIG. 5. Twelve most common free amino acids in skate wing muscle. ½ SW values significantly different from SW values are indicated by *p* values. From [11]; reproduced with permission.

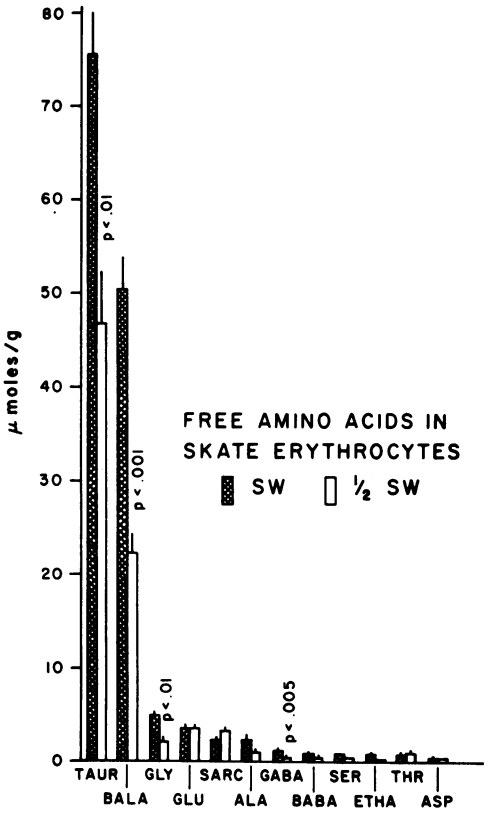


FIG. 6. Twelve most common free amino acids in skate erythrocytes. From [11]; reproduced with permission.

with no effect on uptake. Finally, they found that the β-alanine uptake system was markedly depressed in erythrocytes of skates that had been acclimated to half-strength seawater for one week. It appears, therefore, that there are a variety of factors involved in the regulation of β-alanine transport by skate erythrocytes and that alterations in more than one of these factors is responsible for the modulation in concentration of the amino acid during environmental dilution.

The remaining two tissues studied, brain and heart, were selected because of their critical importance for the survival of the animal. It is reasonable to expect that the functions of these two organs would be particularly sensitive to perturbations of osmolality and cell volume.

Brain

Sudden alterations in plasma osmolality may momentarily perturb the normal cellular osmotic equilibrium that exists between the intracellular and extracellular fluids and cause either tissue swelling or shrinking. Brain tissue is particularly susceptible and cerebral edema or sharp rises in cerebrospinal fluid pressure may occur during a rapid fall in osmolality of brain ECF. Such decreases in ECF osmolality are seen during treatment of diabetic ketoacidosis with insulin where a rapid fall in glucose and ketone bodies occurs, and during rapid dialysis of uremic patients in whom ECF urea concentrations are lowered markedly in a relatively short period of time. The brain swelling that occurs during these treatments has been attributed to the presence of unknown ("idiogenic") osmolytes in brain cells that exchange relatively slowly with the ECF [13].

Although several osmotically active solutes in brain have been ruled out as responsible for the brain swelling observed in these patients, the idea that intracellular amino acids may be the causative agents has not been tested. There are technical difficulties in measuring free amino acids in the mammalian brain because of their naturally low level and the problem of contamination by proteolysis during preparation of the sample for analysis. Thus, we used the little skate, in which cerebral amino acids are naturally markedly elevated, and which are readily amenable to experimentally induced rapid dilution of the ECF osmolality. The experiments were designed to test the hypothesis that free amino acids of brain cells remain elevated during rapid dilution of ECF and contribute to brain swelling under these conditions.

To simulate the sharp drop in ECF osmolality seen in clinical conditions such as rapid hemodialysis, skates were transferred from full-strength directly into half-strength seawater. As shown in Table 3, plasma osmolality fell by 10 percent within 4 hours, 30 percent within 24 hours, and almost 40 percent within 48 hours of dilution of the external medium. Brain osmolality, which is determined by the concentration of osmolytes within the cells, did not fall as rapidly since brain water content rose progressively within the 48-hour period studied. The concentration of free amino acids, the major constituents of the ninhydrin positive substances (NPS) in the brain of the little skate [14], fell somewhat faster than the osmolality of the ECF. Their concentrations decreased 25 percent within 4 hours, 35 percent within 24 hours, and approximately 50 percent within 48 hours. Therefore, retention of free amino acids cannot account for the osmotic disequilibrium observed between the intracellular and extracellular fluids of brain. If one can extrapolate from these experiments done on skates to the clinical conditions producing brain swelling, then free amino acids do not appear to be good candidates for the role of the "idiogenic" osmolytes responsible for the osmotic disequilibrium syndrome seen in patients. However, amino acids probably do play an important role in regulating cell volume in the normal brain [14].

The mechanism leading to the rapid release of free amino acids from the brain of skates following dilution of the ECF was investigated utilizing slices of brain incubated *in vitro*. As shown in Table 4, slices of whole brain incubated in Forster's elasmobranch saline solution [16] remained physiologically intact, judged by minimal losses of K^+ and NPS as well as maintenance of normal water content, for one hour

TABLE 3
Water and Ninhydrin Positive Substances in Brain and Osmolality in Plasma of *Raja erinacea* Following Rapid Acclimation to 50% Seawater

	% H_2O Brain	NPS (mM) Brain	Osmolality Plasma (mosmol/l)
Control (9)	78 ± 2	173 ± 20	957 ± 47
4 hr (6)	80 ± 1**	129 ± 20**	869 ± 22*
24 hr (6)	82 ± 4	114 ± 6*	684 ± 52*
48 hr (6)	87 ± 2*	90 ± 11*	595 ± 30*
72 hr (2)	82	83	571

Values in parentheses indicate the number of fish per group.
*$p < 0.001$; **$p < 0.01$; values are means ± S.D.

TABLE 4
Water Content, Ninhydrin Positive Substances and K^+ Concentration Changes in Brain Slices of *Raja erinacea*, Incubated *in vitro*

Medium	% Water	ΔNPS (%)	ΔK^+ (%)
Control (8)	77 ± 3	(-) 11 ± 4	(-) 18 ± 27
LiCl* (14)	90 ± 7***	(-) 35 ± 7***	(-) 58 ± 23***
Ouabain 10^{-4} M (12)	93 ± 5***	(-) 46 ± 5***	(-) 76 ± 18***
Dilute medium** (10)	90 ± 6***	(-) 35 ± 10****	(-) 37 ± 15****

Numbers of experiments per group shown in parentheses.
*LiCl was substituted for NaCl in the incubation medium.
**Dilute medium was prepared by decreasing NaCl from 300 to 200 mM and urea from 360 to 280 mM.
***$p < 0.001$.
****$p < 0.05$.

when incubated at 15°C. Since environmental dilution leads to a fall in osmolality of the ECF, we examined the effects of this perturbation on brain NPS concentrations *in vitro*. As shown in Table 4, dilution of the incubation medium produced a 24 percent net decrease in brain slice NPS content. A similar decrease was observed when brain slices were incubated in Na^+-free saline solution or in the presence of ouabain. Thus, environmental dilution appears to lead to increased efflux of amino acids from brain cells, in part at least, by reducing the concentration of Na^+ in the EDF. However, an effect of osmolality per se cannot be excluded.

Heart

Osmoregulatory studies on the skate heart focused on measuring the effects of osmodilution on the separate volumes of fluid compartments in atrial and ventricular myocardium, and in determining to what degree free amino acids and other osmotically active solutes participate in regulating cell volumes in both chambers when challenged by a diluted interstitium, *in vivo* and *in vitro*.

In a gradual environmental dilution procedure which reduced plasma osmolality by 33 percent, similar reductions in terms of mosm/kg wet wt could be observed in atrium and ventricle of *R. erinacea* (Table 5). Percentage water increased simulta-

TABLE 5
Osmolality and Water Content of Atrium, Ventricle, and Plasma Following Gradual Environmental Dilution

	Osmolality (mosm/l)			Water Content (% wet wt)	
Seawater	Atrium	Ventricle	Plasma	Atrium	Ventricle
100%	1091 ± 97 (6)	1052 ± 50 (9)	1041 ± 23 (7)	86 ± 1 (5)	83 ± 1 (7)
50%	715 ± 81 (3)	685 ± 58 (3)	701 ± 13 (4)	90 ± 2 (5)	86 ± 1 (9)
	$p < .05$	$p < .001$	$p < .001$	$p < .01$	$p < .01$

Values are means ± standard error. Number of fish per group shown in parentheses.

neously in atrium and ventricle. The response of this tissue to a lowered plasma osmolality is in agreement with earlier studies cited on skeletal muscle [6] and brain [14] of the skate. Table 6 shows the loss of some ninhydrin positive substances (NPS) by the atrium and ventricle following osmodilution under *in vivo* conditions. Note that these values are for total wet weight of tissue, whereas most of the NPS measured are intracellular.

To continue the study of cell volume regulatory responses in the skate atrium and ventricle, it was necessary to develop in *in vitro* preparation such that the experimental conditions could be more easily controlled. In early experiments where we attempted to use inulin distribution for the estimation of ECF volume and thereby the intracellular concentrations of NPS, Na^+, and K^+, ventricular strips became increasingly permeable to inulin after 2 hr. Accordingly there was a significant increase in its distribution due to the trauma resulting from cutting the strips, and perhaps also due to inner fibers that became increasingly permeable to inulin because of hypoxic conditions at the core of these relatively thick sections. The latter explanation is supported by further evidence that Na^+ leakage into ventricular myocardial cells and K^+ loss occurs as early as 2 hr after incubation. Armstrong et al. [17] reported significant changes in membrane permeability resulting in an altered distribution of total tissue water and electrolytes between intracellular and extracellular compartments following incubation of frog ventricular strips. Brown et al. [18] also have shown histologically that degeneration of rat atrial and ventricular strips occurs during *in vitro* incubation studies.

In contrast, the "hemiatrium" preparation appears more viable than the ventricle slice preparation. No NPS are released from hemiatria following a two-hour control incubation, and the transmural distribution of Na^+ and K^+ remains fairly constant.

TABLE 6

Ninhydrin Positive Substances (NPS), Na^+, and K^+ Concentrations (mmols/kg wet weight) *in vivo* and *in vitro*, Before and After Dilution, in Atrium and Ventricle

	NPS	Na^+	K^+
		in vivo	
Control atrium	103 ± 9	135 ± 6	59 ± 4
	(10)	(9)	(10)
Dilute atrium	56 ± 8	94 ± 8	49 ± 3
	(7)	(8)	(9)
	$p < .01$	$p < .001$	NS
Control ventricle	118 ± 10	116 ± 4	66 ± 5
	(14)	(11)	(13)
Dilute ventricle	79 ± 5	73 ± 4	54 ± 2
	(14)	(12)	(12)
	$p < .01$	$p < .01$	$p < .05$
		in vitro	
Control atrium	112 ± 10	125 ± 8	64 ± 4
	(12)	(7)	(7)
Dilute atrium	59 ± 4	101 ± 4	49 ± 1
	(7)	(10)	(11)
	$p < .001$	$p < .05$	$p < .001$
Control ventricle	91 ± 10	133 ± 3	47 ± 2
	(11)	(8)	(8)
Dilute ventricle	77 ± 7	133 ± 12	42 ± 1
	(9)	(9)	(10)
	NS	NS	$p < .05$

Values are means ± standard error. Number of fish per group shown in parentheses.

The permeability characteristics of the atrial preparation appear to remain chemically intact and they continue to beat spontaneously throughout the incubation period. The spongy structure and lack of a coronary circulation in the atrium facilitate diffusion of nutrients and gases from the incubation medium into the tissue. In addition, a minimal amount of cutting is required in contrast with the procedure used in preparation of ventricular strips.

Table 6 shows the loss of NPS under *in vitro* conditions in atrium following osmodilution. These values are for total wet weight of tissue. Intracellular concentrations of NPS calculated following the method of Forster and Goldstein [6] are shown in Table 7. Extracellular space measurements for use in determination of intracellular concentrations were made using distribution values of radiolabelled inulin following a two hour incubation *in vitro* [16]. ECS measurements were 39 ± 1 percent and 37 ± 1 percent for control and dilute atrium and 27 ± 2 percent and 22 ± 3 percent for control and dilute ventricle, respectively. These measurements were considerably greater than those reported for atrium and ventricle of the ray, *Dasyatis akajei*, which were 23.7 ± 1 percent and 15 ± 1 percent, respectively [19]. Our measurements are in close agreement with the extracellular space estimate of 26–29 percent made by Fenstermacher et al. [20] for the ventricle of the dogfish, *Squalus acanthias*, *in vivo*. Other reported values for ECS in cardiac tissue include 25 percent for frog heart slices *in vitro* [21] and 38 percent for isolated perfused rabbit heart [22]. Poole-Wilson and Cameron [23] report ECS measurements of 19.3 ± 4 percent, 21.7 ± 0.7 percent, and 29.5 ± 1.7 percent for left ventricle, right ventricle, and atria, respectively, in *in vivo* measurements on the rabbit. Danielson [24] also demonstrated that ECS measured *in vivo* was greater in the atrium than the ventricle of the toad. Extracellular space estimates derived from *in vitro* measurements have been shown to result in higher values than those derived from *in vivo* measurements. In addition, an increase in ECS values with increasing time of incubation, which has been attributed to increased hydration during incubation, have been reported by several groups [16,17,25,26].

In cardiac muscle of *R. erinacea*, atrial cells, in contrast to ventricle, regulate cell volume in a dilute environment by releasing significant quantities of NPS. Boyd et al.

TABLE 7
Intracellular Concentrations of Ninhydrin Positive Substances (NPS), Na^+, and K^+ in Atrium and Ventricle *in vitro* incubation

	NPS (mmols/l)	Na^+ (mmols/l)	K^+ (mmols/l)
Control atrium	208 ± 19	55 ± 9	120 ± 9
(*in vivo*)	(10)	(9)	(10)
Control atrium	216 ± 20	28 ± 5	131 ± 9
(*in vitro*)	(11)	(7)	(7)
Dilute atrium	110 ± 7	38 ± 8	97 ± 3
(*in vitro*)	(7)	(9)	(11)
	$p < .001$	NS	$p < .01$
Control ventricle	204 ± 15	74 ± 19	116 ± 8
(*in vivo*)	(14)	(11)	(13)
Control ventricle	155 ± 19	103 ± 6	81 ± 4
(*in vitro*)	(11)	(8)	(8)
Dilute ventricle	121 ± 11	106 ± 7	68 ± 3
(*in vitro*)	(9)	(7)	(10)
	NS	NS	$p < .05$

Values are means ± standard error. Number of fish per group are shown in parentheses.

[11] found that free amino acids (predominantly taurine) do not contribute significantly to intracellular osmoregulatory response to a diluted environment in cardiac muscle of the little skate. Ventricular tissue only was used in that study. As in the flounder ventricular muscle [27] there is a positive correlation between the decrease in intracellular K^+ and total osmolality decrease. Total atrial osmolarity of 1091 ± 97 mosm/l in the little skate can be closely accounted for by NPS, Na^+, K^+, and their covering anions. To this should be added approximately 75 mmols/l for trimethylamine oxide and approximately 300 mmols/l for urea. Despite the high Na^+ concentrations expressed per unit wet weight, intracellular Na^+ levels are considerably lower than those reported for the ray, *Dasyatis akajei* [19]. Intracellular Na^+ and K^+ concentrations reported for *R. erinacea* agree closely with the findings of Vislie and Fugelli [27] in the teleost ventricle, while NPS concentration changes are in agreement with their conclusion that amino acids (mainly taurine) play a major role in cell volume regulation in response to an osmotic stress.

In recent work we have been able to determine the response of individual free amino acids to osmotic perturbation in the heart of *R. erinacea.* Total intracellular amino acid concentrations are 168 ± 12 mmols/l and 170 ± 6 mmols/l in control atrium and ventricle and fall to 135 ± 16 mmols/l and 153 ± 4 mmols/l, respectively, following gradual acclimation to a dilute environment [28]. An amino acid profile analysis shows that the major constituent of the free amino acid pool is taurine which comprises 82 percent of the total free amino acids under control and dilute conditions [28].

Taurine in Intracellular Osmoregulation

Simpson et al. [29] were among the first to suggest that taurine played a substantial role in intracellular osmoregulation in invertebrates. Since that time many studies have been done on euryhaline invertebrate osmoregulation, and it is now widely accepted that amino acids play an important role in this process [29,30,31, 32,33,34,35,36,37]. In Mollusca, amino acids, and taurine are considered to be the generally occurring intracellular osmotic effectors [33,38]. Regulation of intracellular amino acid concentration has been shown in Crustacea in response to osmotic stress. This regulation is achieved via control of transport of amino acids across the cellular membrane and also by control of the cellular level of amino acid metabolism [7,36]. Pierce et al. [32] have demonstrated modification of amino acid transport in molluscan hearts. Hypoosmotic stress was shown to result in an increased efflux of NPS from isolated *Modiolus modiolus* hearts.

Recent studies in teleost fishes have implicated free amino acids (NPS) in intracellular osmoregulation in skeletal muscle. Lange and Fugelli [39] demonstrated decreases in total NPS in skeletal muscle of *Pleuronectes flesus* and *Gasterosteus aculeatus* following adaptation from seawater to fresh water. Higgins and Colley [40] and Ahokas and Duerr [41] showed increases in NPS following adaptation of the fresh water teleosts *Anguilla anguilla* and *Fundulus diaphanus* to SW and 30 percent SW, respectively. Few studies have been done, however, which give a detailed analysis of the individual amino acids responsible for maintenance of an intracellular osmotic balance. Ahokas and Sorg [42] were the first to show accumulation of taurine in a fresh water teleost in response to a hyperosmotic stress. Laserre and Gilles [43] demonstrated a decrease in muscle free amino acids of *Crenimugil labrosus* and *Paralichthyes lethostigma* following transfer from 200 percent SW to fresh water. Taurine was responsible for 45 percent of the total free amino acid decrease in the former and 54 percent in the latter. Colley et al. [44] demonstrated

sequential adaptation in NPS concentration in parietal muscle of *Agonus cataphractus* in response to sequential decreases in salinity. Taurine comprised approximately 90 percent of the intracellular amino acid pool and was responsible for 96 percent of the decrease in NPS following adaptation from 850 mosm to 200 mosm SW.

As mentioned previously, the major amino acid in atrium and ventricle of the skate is taurine, with concentrations of 139 ± 10 mmols/l found in the former and 137 ± 4 mmols/l in the latter under control conditions. These values are the highest reported in the heart of any species. Taurine, the sulfonate analogue of β-alanine, is also the most common amino acid in skate red blood cell [12] and brain [14] although its concentration is much less than that of sarcosine and β-alanine in skeletal muscle. Taurine is present in vertebrate heart in high concentration, often comprising up to 50 percent of the free intracellular amino acid pool. Within the skate heart, taurine makes up 82 percent of the amino acid pool and maintains a tenfold gradient between the intracellular concentrations found in atrial or ventricular tissue, and that found in plasma. Regulation of intracellular concentration is response to an osmotic stress has been demonstrated. In recent metabolic studies on cardiac muscle of *R. erinacea*, no synthesis or oxidation of taurine has been demonstrated [45]. Thus regulation of intracellular levels of taurine must take place via control of the transport process.

Characterization of Taurine Transport in the Heart

Figure 7 describes the uptake of ^{14}C taurine under control conditions. Following an initial period of equilibration, a linear uptake can be seen. The initial equilibrium values correspond well with concentrations found in extracellular fluid (.03–.039

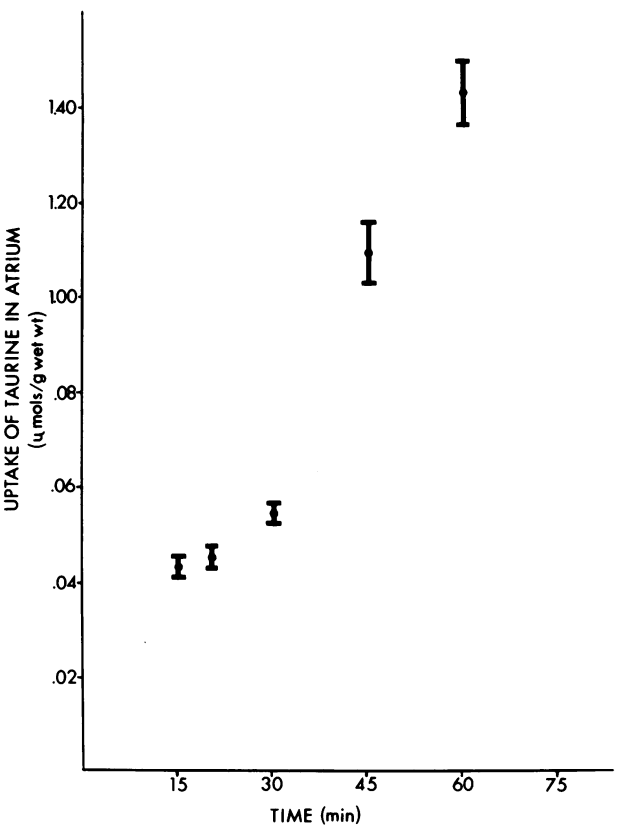


FIG. 7. Time course of taurine uptake by skate atrium *in vitro*.

μmol/g wet weight). The initial uptake of labeled taurine into the cell may be masked by that present in the extracellular fluid. A similar study of taurine uptake done by Grosso et al. [46] on isolated fetal mouse hearts in culture, demonstrated uptake of 6 μmols/g wet weight/hour. This rate is approximately fourfold that seen in the skate atrium. Those results were obtained at 37°C, whereas the skate studies were performed at 20°C. Awapara and Berg [47] were able to demonstrate linear uptake of taurine in rat heart slices for at least three hours. Specific transport systems for taurine have been demonstrated in a number of experimental systems including the perfused rat heart [48], rat heart and kidney slices [47], fetal mouse heart [46], Erlich tumor ascites cells [49], rat brain slices [50,51], rat brain synaptosomes [52], and human platelets [53].

The dependence of taurine transport upon sodium concentration was demonstrated in the skate "hemiatrium" preparation using the stepwise replacement of sodium chloride with choline chloride. Reduction of Na from 280 mM to 140 mM to 70 mM to 35 mM to 0 mM resulted in the following decreases in uptake: 30 percent, 50 percent, 56 percent, and 76 percent. Sodium dependence has previously been demonstrated for *in vitro* taurine transport into skate red blood cells [12] and brain [14] as well as in many mammalian systems [47,51,52,54]. Structurally related compounds tested for their ability to inhibit taurine uptake into the skate atrium *in vitro* are shown in Table 8. β-alanine was an effective inhibitor of taurine uptake. Uptake was reduced by 42 percent in the presence of 0.5 mM β-alanine (fivefold taurine concentration). α amino isobutyric acid and γ-aminoisbutyric acid had no significant effect on taurine uptake. This finding is in agreement with other studies done which demonstrate the existence of a transport system which is specific for the β-amino acids, and distinct from the α amino acid transport systems [49,50,52,54].

The taurine transport system in the skate atrium is also sensitive to the metabolic inhibitor Na azide and to the cardiotonic steroid ouabain in concentrations that inhibit the dephosphorylation of Na^+-K^+ ATPase located on the outside face of the cell membrane. Ouabain has been shown to decrease uptake by 50 percent in human

TABLE 8
Taurine Uptake by Skate Atrium, Expressed as Percentage of Control, as Affected by Various Agents Selected to Characterize the Transport System

Treatment	Percentage decrease in uptake of taurine	
Na dependence		
0 mM Na	-76.22 ± 3.71%	(4)**
35 mM Na	-56.27 ± 10.3%	(6)**
70 mM Na	-49.71 ± 7.76%	(6)**
140 mM Na	-30.35 ± 12.48%	(6)**
280 mM Na	0	
Competitive inhibition		
β-Alanine (10^{-5}M)	-42.40 ± 6.50%	(11)*
γ-Aminobutyric acid (10^{-5}M)	NS	(9)
α-Aminoisobutyric acid (10^{-5}M)	NS	(7)
Metabolic dependence		
Na azide (10^{-2}M)	-58.74 ± 1.27%	(3)*
Na^+-K^+ ATPase inhibition		
Ouabain (10^{-4}M)	-44.76 ± 2.89%	(4)*

Value are means ± standard deviation. Number of fish per group is shown in parentheses. *$p < 0.001$, **$p < 0.01$, NS = not significantly different from control.

platelets [53] and 45 percent in rat brain slices [51]. Stimulation of taurine uptake by ouabain has been shown in fetal mouse heart [46]; however, a suitable explanation for this stimulation has yet to be postulated.

SUMMARY

The problem of osmoregulation is an ancient one amongst the vertebrates and involves homeostatic control of both extracellular and intracellular body fluids. Although wide variation in the degree of control of extracellular fluids is observed in different vertebrates, it appears that cell volume is tightly regulated. Amino acids play a role in regulating cell volume in vertebrates, although their importance varies in different species. In the skate three amino acids, taurine, β-alanine, and sarcosine, are of major importance in regulating cell volume in several tissues, but each tissue has its own particular combination of the three playing that role. The levels of these amino acids are regulated, at least in part, by specific Na^+-dependent active transport processes that are responsive to changes in osmolality and composition of the extracellular fluid.

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