Cellular magnesium acquisition: an anomaly in embryonic cation homeostasis*

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Key words: magnesium, cation transporters, effect of fluoride on metabolism, early embryo homeostasis, yolk ion partition, ionic competition, sodium, potassium, calcium, teleost embryos, *Fundulus heteroclitus,* analytic methodology

* Early work in this report was performed at the Marine Biological Laboratory under AEC Grant No. 1343

Abstract

The intracellular dominance of magnesium ion makes clinical assessment difficult despite the critical role of Mg^{++} in many key functions of cells and enzymes. There is general consensus that serum Mg^{++} levels are not representative of the growing number of conditions for which magnesium is known to be important. There is no consensus method or sample source for testing for clinical purposes. High intracellular Mg^{++} in vertebrate embryos results in part from interactions of cations which influence cell membrane transport systems. These are functionally competent from the earliest stages, at least transiently held over from the unfertilized ovum. Kinetic studies with radiotracer cations, osmolar variations, media lacking one or more of the four biological cations, Na^{+}, Mg^{++}, K^{+}, and Ca^{++}, and metabolic poison 0.05 mEq/L NaF, demonstrated: (1) all four cations influence the behavior of the others, and (2) energy is required for uptake and efflux on different time scales, some against gradient. Na^{+} uptake is energy dependent against an efflux gradient. The rate of K^{+} loss is equal with or without fluoride, suggesting a lack of an energy requirement at these stages. Ca^{++} efflux took twice as long in the presence of fluoride, likely due in part to intracellular binding. Mg^{++} is anomalous in that early teleost vertebrate embryos have an intracellular content exceeding the surrounding sea water, an isolated unaffected yolk compartment, and a clear requirement for energy for both uptake and efflux. The physiological, pathological, and therapeutic roles of magnesium are poorly understood. This will change: (1) when ^{28}Mg is once again generally available at a reasonable cost for both basic research and clinical assessment, and (2) when serum or plasma levels are determined simultaneously with intracellular values, preferably as part of complete four cation profiles. Atomic absorption spectrophotometry, energy-dispersive x-ray analysis, and inductively coupled

plasma emission spectroscopy on sublingual mucosal and peripheral blood samples are potential methods of value for coordinated assessments.

Introduction

The four biological alkali cations, sodium, potassium, calcium, and magnesium, participate at the molecular level for homeostasis and in electrolyte pathophysiology [Elisaf, et al., 1997; Halperin and Kamel, 1998; Hill, 1990]. The first three are commonly determined together in blood chemistry profiles, but for reasons to be developed, magnesium is not generally included [Kost, 1993], often relegating it to the status of a special test [Crook, et al., 1994; Elin, 1988; Elin, 1991-92; Lim and Jacob, 1972; London, 1991].

The distribution of cations in the body directly influences the significance of plasma determinations (**Table 1**). To give a few examples, sodium, predominantly extracellular (ratio circa 10:1), often reflects water balance [Narins, et al., 1982], and potassium, even more predominantly intracellular (ratio circa 1:30), rises following physical injury to muscle and after hemolysis [Halperin and Kamel, 1998]. Similarly, changes in calcium balance (extracellular by a factor of 2-4 times, approximately 50% ionized and 50% bound to albumin or anions) are often due to bone disorders or parathyroid disturbance [Strewler, 2000]. In these instances peripheral blood values are useful in both the diagnostic and treatment phases of clinical care. The situation is different for magnesium. Magnesium is the least well known of the biological cations [Chamnongpol and Groisman, 2002; Lunin, et al., 2006; Shi, et al., 2002] in part because of greater difficulty in analysis, but some progress has been made recently isolating and describing magnesium transporters [Bui, et al., 1999; Kucharski, et al., 2000; Lunin, et al., 2006; Maguire, 2006].

This advance is illustrated in the context of renal tubular Ca^{++} reabsorption during thiazide-induced hypocalcemia and hypomagnesia [Nijenhuis, et al., 2005]. One effect is down regulation of Mg^{++} channels of the M subfamily in mice [Nijenhuis, et al., 2005].

Another aspect is a relationship to regulatory substances such as calcitriol (1,25-dihydroxyvitamin D_3) [Hoenderop, et al., 2001].

There is good evidence for genetic disorders of renal sodium-glucose and sodium-phosphate transporters, and the Na-Cl and Na-K-Cl cotransporters, specifically Bartter syndrome [Scheinman, et al., 1999; Simon and Lifton, 1996] and Gitelman syndrome, a variant of Bartter syndrome with hypomagnesemia and hypocalcuria [Gitelman, et al., 1966]. The renal mechanisms for magnesium include a lumenal Mg^{++} channel and a basolateral sodium-magnesium exchanger [Quamme, 1997].

Moreover, potassium depletion inhibits the reabsorption of Mg^{++} in the distal convoluted tubule [Quamme, 1997].

Plasma levels of magnesium are poor surrogates for disorders for which magnesium is considered to be part of pathogenesis [Cieslinski, et al., 1999; Elin and Hosseini, 1985]. This is mainly due to the intracellular location of most of the magnesium [Elin and Hosseini, 1985; Reinhart, et al., 1988; Reinhart, et al., 1990]. There has to be marked depletion of intracellular magnesium before hypomagnesemia is reflected in peripheral blood samples [Resnick, et al., 1993]. Compartmentalization is extreme, with total serum magnesium ranging from 0.3% [Elin, 1988] and 1.0% [Elin and Hosseini, 1985] up to a distribution ratio of 7% [Anderson, 1998], differences attributable to the greater intracellular content and the methods of determination.

Some of this uncertainty is due to an absence of consensus on the conditions and the most accurate test medium for magnesium: buffy coat leukocytes, erythrocytes, serum, or

sublingual epithelium (**Table 2**) [Elin, et al., 1990; Elin and Hosseini, 1985; Elin and Johnson, 1982; Haigney, et al., 1995; Haigney et al., 1998; Haung, et al., 1988; London, 1991; Maguire and Cowan, 2002; Martin and McGregor, 1986; Montes, et al., 1989; Nixon, et al., 1986; Reinhart, 1988; Ryzen, et al., 1986; Ryzen and Rude, 1990; Sjogren, et al., 1987; Urdal and Landmark, 1989].

Atomic absorption spectrophotometry identifies and quantifies magnesium [Huang, et al., 1988; Reinhart, et al., 1990]. Also applicable are energy-dispersive x-ray analysis [Haigney, et al., 1995] and inductively coupled plasma emission spectroscopy [Nixon, et al., 1986].

Moreover, the standard reference range for plasma magnesium is narrow [Eichhorn, et al., 1993], without agreement as to the optimum values for interpretative purposes. This has been reported in reference works as 1.5-2.5 mEq/L, or 0.75-1.25 mM/L)[Taylor, 1988] and 1.3-2.1 mEq/L, or 0.7-1.1 mM/L respectively [Henry, 1984, p. 1435].

Narrow homeostatic zones are also characteristic of potassium (3.5-5.0 mEq/L) and total (4.6-5.5 mEq/L; 2.3-2.8 mM/L) and ionized calcium (2.0-2.4 mEq/L; 1.0-1.2 mM/L) [Henry, 1984].

During the past 15 years or so, various reports have claimed certain clinical disorders are related to the magnesium economy of the body. A few examples are: mitral valve prolapse [Lichodziejewska, et al., 1997], low magnesium as a factor in insulin resistance [Fox, et al., 2001; Paolisso and Ravussin, 1995], low red blood cell magnesium in chronic fatigue syndrome [Cox, et al., 1991], as an important factor in electrocardiographic conduction during early myocardial ischemia [Reamer, et al., 1996], and in hypertension [Paolisso and Barbagallo, 1997; Touys, et al., 1989]. A relationship between all four cations in adult hypertension was explored by Touyz, et al. [1989].

Mg^{++} correlated more closely than Na^{+}, K^{+}, or Ca^{++} with measured blood pressure. This study demonstrates clearly the value of simultaneous four cation determination.

Similarly, changes in somatic magnesium economy have not been well studied in pregnancy and during fetal and neonatal development [Hallak and Cotton, 1993; Hillman, et al., 1977; Shaw, et al., 1990], critical time periods given the common use of intravenous magnesium sulfate as an antihypertensive agent in pregnancy, especially during labor and delivery [Mason, et al., 1996; McGuinness, et al., 1980; Mittendorf, et al., 2001; Stigson and Kjellmer, 1997].

Magnesium is a cofactor in various vital enzyme compelled metabolic reactions [Fox, et al, 2001; Qin, et al., 2006], in both cytoplasmic and mitochondrial compartments [Bertram, et al., 2006], and other cell functions [Grubbs and Maguire, 1987; Kelepouris, et al., 1993], many of which occur during early embryogenesis [Bregestovski, et al., 1992; Medina and Bregestovski, 1988; Nishikimi, et al, 2000; Rutenberg, et al., 2002].

Given this diversity of activity, two pertinent questions arise: **(1)** how is magnesium acquired *per se* from the medium of supply, and **(2)** is there a prefatory reserve of magnesium for rapid cell proliferation during blastogenesis, cell migration, differentiation, and organogenesis, when these aspects of the embryo are no longer in direct contact with initial sources? Later, the situation is compounded by the onset and maturation of the circulation.

Developmental physiology has not well addressed how and when these circumstances occur. The approach here is indirect, using a post fertilization teleost vertebrate model since comparable mammalian material is usually difficult to obtain.

The modern study of water and electrolyte metabolism arguably began with the seminal review of Manery [1954]. This monumental assessment referenced 629 citations

beginning with Berzelius in 1840. His reference list is a virtual catalogue of the leading physiologists and cell biologists of the first half of the twentieth century. The basis for asserting this review as the beginning of the modern era is that much of the critical work demonstrating cellular energy requirements for movement of ions into cells, from the 1930s into the early 1950s, was well covered by Manery [1954]:

> "...the simple view of two water compartments separated by a membrane of limited distensibility, freely permeable to water and to a few ions, is no longer tenable. Ions are known to move across membranes at varying rates although a relatively constant composition is maintained in cells. As long as water can move quickly [Edelman, 1952; Thompson, 1953] it will do so in response to osmotic pressure gradients, whether they be across the cell membrane or at different levels within the cell. Osmotic equilibrium is probably rarely attained but always approached. A steady state, both in respect to water and to ions, undoubtedly exists *depending on energy for its maintenance* [emphasis added] (p. 342)."

A fundamental question has to be answered before the kinetics of ion transport mechanisms (ion channels or transporters and energy conditions, *inter alia*) can be invoked, or integrated into an understanding the dynamics between the intracellular (cytosolic), interstitial, and intravascular compartments.

The question is, whether, at the molecular level, the four biological alkali metal cations have a physicochemical relationship based on atomic attributes, mediated by concentration through stochastic or modulated competitive means, or are they maintained and regulated entirely by transport and receptor or site binding reactions and other cellular devices?

Many studies, both clinical [Resnick, 1992; Tveskov, et al., 1994] and experimental [Carrier and Evans, 1976; Karpen, et al., 1993; Komatsu, et al., 1996; Laver, 1992; Marynick and Schaefer, 1975] have endeavored to establish direct relationships between

various subsets of the four cations, tasks made difficult by multicellular and multicompartmental aspects of the organism(s) involved and the specific activities of certain ions in different dynamical biological systems within the intact organism [Carrier and Evans, 1976; Parpart and Green, 1952]. Major examples include: **(a)** potassium and sodium exchange in nerve impulse transmission, **(b)** magnesium as active cofactor for many enzymes and protein synthesis [Wacker, 1969], and **(c)** calcium waves as the essential intracellular signal transducer [Frausto da Silva and Williams, 1994]. There are chemical differences between sodium, magnesium, potassium, and calcium but they share one vital attribute: *they have a positive charge*, i.e, they are electron acceptors within a competitive gradient range which may be significant outside of the role of express ion transport mechanisms [Frausto da Silva and Williams, 1944]. In fact, as a post cript to the quote from Manery provided above, the following from Frausto da Silva and Williams suggests a nearly complete conceptual evolution of cation exchange at the cell boundary since 1954:

> "We have seen that the uptake of metal ions M is almost invariably energized: consequently, it is not a true equilibrium process. However, if we assume a steady state in certain steps, we can treat the uptake problem as one in which the element M distributes between two very different phases containing, say, L_3 (a ligand) and L_2 separately and between which only M itself can equilibrate. We call this ‘equilibrium uptake' since the process of transport is presumed not to interfere. Selected uptake can then be dealt with through analysis of binding constants of M in two different compartments: the outer aqueous phase and the inner aqueous phase. The uptake of M will depend only on the stability of ML_2 relatively to ML_3 and no kinetic considerations or irreversible energized steps will be invoked in this approximation. (Energy is in fact put into the system just because L_2 and L_3 are synthesized in a controlled way and kept separate) (p. 33)."

This comprehensive construct has two presumptions ("...if we assume a steady state in certain steps..." *and* "...transport is presumed not to interfere..."). It is an

approximation based on a inner two compartment model. This may be oversimplified. The biological reality might be a more continuous rephasing across cytosol to reach action sites dependent on particular actions, in addition to the multiple steps ("a bucket brigade [Sun and Mauzerall, 1996]") in configuration and changes in energy levels during transit through ion channels [Eisenman and Horn, 1983; Eyring, 1963]. There is a distinction between cytosolic and mitochondrial compartments [Bertram, et al., 2006] Neither Manery [1954] nor Frausto da Silva and Williams [1994] examine prospects for either stochastic access to or modulated competition for receptor sites at the outer cell boundary or interactions dependent on the molecular attributes of the cations *per se*.

The distinction, if there is one, can be expressed as either *passive entry into* or *active uptake by* the exterior aspect of the channel as the first step in transmembrane passage. Similarly, the net effect or intracellular accumulation would be dependent on the means and efficiency of processes by which cations are expelled from or enabled to exit the cell [Levitt, 1984].

This report concerns the direct, and likely a modulated and competitive, relationship between all four cations by which to clarify the role of magnesium in pathophysiologic states and biological systems. The evidence obtained by the work supports the interpretation that osmotic aspects of the environment, changes in water content, have influence on the net balance of cations. Also reported are experiments with fluoride blocking metabolic bioenergetic mechanisms which affect the uptake, efflux, and distribution of the cations to the yolk and the embryo proper.

The experimental approach uses teleost embryos early in development and possibly before full maturation of specific ectodermal mechanisms to promote internal homeostasis [Moody, et al, 1991]. This will be developed in the discussion.

Materials and methods

Comparative analysis was carried out in four stages: **[1]** preparation of the biologic material, **[2]** their exposure to natural and artificial sea water media, the latter composed of solutions of the chlorides of the four cations in various but systematic subsets involving one, two, or three cations, with added radiotracer amounts of sodium, potassium, and calcium, and osmotic balanced solutions using sucrose as substitute for the absent cation(s), the *isosmotic* series, **[3]** a three part series, *heterosmotic*, with Marine Biological Laboratory (MBL) supply source sea water ($SW_{1.0}$), plus *(a)* half molar sea water ($SW_{0.5}$), and *(b)* a near twice molar sea water ($SW_{1.92}$), enriched by the chlorides of each of the four cations, across the entire set of unitary, binary, ternary, and quaternary solutions, without osmotic rebalancing, again with equivalent amounts of radiotracer sodium, potassium, and calcium, and **[4]** examining the relative passage or net take up of cations by radiotracer methods for sodium, potassium, and calcium, and photochemical means for magnesium, since a practical radiotracer isotope for Mg^{++} was unavailable at the time, and photochemical methods for total calcium.

Selected media were studied with sodium fluoride at 0.0125- 0.1 mEq/L to block the energy chain at pyruvate enolase [Allmann and Kleiner, 1980; Borei, 1945; Green and Parpart, 1950] and at other sites of the glycolytic energy cycle [Bartholmes, et al., 1987]. Paired sequential runs involved timed efflux of previously equilibrated tracers into media lacking that cation, followed by restitution. This subset partitioned uptake differences when yolk sac and embryonic body were independently tested. Yolk was removed from the embryos by insulin syringes fitted with beveled 27 gauge needles; yolk was immediately placed in separate planchets for beta counting or in microtubes for magnesium assessment.

Partition values were based on precise measurements of the XYZ dimensions of the three segments of 50 embryos proper (head, trunk, and tail) and the yolk sac (data not shown).

The experimental model

The experimental system was based on dechorionated embryos of *Fundulus heteroclitus* L., commonly known as the killifish, at Oppenheimer stages 22/23-26, mostly stages 23-24 [1937]. The killifish is a useful nearly ideal seasonal biological model. It is an ocean littoral or estuarine teleost, capable **(1)** of survival in widely varying salinities, **(2)** available in satisfactory numbers during a three month spawning season (late May to late August on the northeast Atlantic coast of North America), and **(3)** for which embryonic maturation and differentiation occurs within a transparent protective shell (*chorion*) along a series of well defined, easily verified stages [Armstrong and Child, 1965; Oppenheimer, 1937].

Indeed, *Fundulus* has been thoroughly studied from the perspective of cation movement under a wide range of conditions and over many years [Armstrong, 1928; Griffith, 1974; Guggino, 1980a; Guggino, 1980b; Jacob and Taylor, 1983; Laurent, et al., 2006; Loeb, 1914; Loeb, 1916; Maples and Bain, 2004; ; Marshall, et al., 1999; Potts and Evans, 1967; Scott, et al., 2004; Scott, et al., 2005; Shanklin, 1953; Shanklin, 1954a; Shanklin and Armstrong, 1954; Shanklin, 1956; Wood and Marshall, 1994]. A principal adaptation to salinity changes in post hatch fry and adult *Fundulus* is the chloride excretory cell in the gills [Copeland, 1947a,1947b,1947c,1948, 1950; Hossler, et al., 1985; Laurent, et al., 2006; Katoh, et al., 2003; Wood and Marshall, 1994]. The fish were obtained during the spawning period, early June through late August, by the Marine Resources Department of the MBL, Woods Hole, Massachusetts, and stripped eggs were

fertilized by stripped milt from males on a precise schedule. By this means the age of the embryos was known to within a half hour. By direct observation, from the circulating sea water at summer temperatures, chorionated fertilized eggs, typically 2.0 mm diameter, achieved Oppenheimer stages 22 and 23 between 96 and 120 hours post fertilization [Armstrong and Child, 1965; Oppenheimer, 1937]. At these stages embryonic development results in an ample subchorionic space, easily permitting surgical removal of the chorion (dechorionation) by a modified Nicholas method using iridectomy scissors under a binocular dissecting microscope at magnifications of 15-30X [Nicholas, 1927]. They were then maintained for 24 hours in sea water prior to entrance into each experimental series. The delay was used to eliminate embryos injured by the procedure. Minimal effort easily fertilized 500 or eggs per session. At peak efficiency up to 100 eggs per hour could be dechorionated, allowing for reasonable sample sizes for multiple simultaneous comparative runs. The surface of the *Fundulus* embryo at these stages is complete, without openings of any kind. The mouth, gills, and cloacal structures appear later during embryogenesis. The test scenario presents an intact surface ectoderm of *Fundulus*, roughly a complex cylinder mounted on a nearly spherical yolk sac to the various media. This simplified mesomorphic structure avoids the problems of compartmentalization of blood flow and differential organ allocations found in post embryonic organisms.

What these experiments could not do was to study directly the processes of embryonic maturation of ion transport mechanisms at the outer media:surface tissue interface or for internal cell to cell exchange (see discussion).

Sample size

Preliminary testing indicated the sensitivity of methods was such that only two to

five embryos were needed per planchet during radiation emission counting. The minimum sample size was set at 10 to average out any minor potential variations in embryonic mass and body surface area.

Preparation of artificial and partial sea waters

The cation content of Woods Hole sea water, drawn by a deep pump intake on the south side of the MBL, from Vinyard Sound, a part of Nantucket Sound, was determined contemporaneously with this work [Shanklin, 1954b]. **Table 3** displays the sea water analysis, a subsurface littoral oceanic medium. The source conditions are emphasized, since the sodium, potassium, and calcium concentrations differ somewhat from contemporaneous reports, mostly based on chlorinity ratios in pelagic samples [Culkin and Cox, 1966; Greenhalgh, et al., 1966; Lyman and Fleming, 1940; Morris and Riley, 1966; Riley and Tongudai, 1967], and the principal prior historical values [Dittmar, 1884]. A minor difference is the units of report: g/kg (all other reports) or mEq/L [Shanklin, 1954]. The major difference was the direct determination of all four cations, especially sodium by flame photometry [Shanklin, 1954], rather than by using joint precipitation of sodium and magnesium followed by allocations determined mostly by subtraction or secondary separations [Culkin and Cox, 1966; Greenhalgh, et al., 1967]. Only Culkin and Cox emphasized local variations in surface and subsurface waters. The 1954 results, determined at the outset of this work, were used to construct artificial sea waters as noted, including the isosmotic series, with sucrose for osmotic balance. Important for this report were consistency of the media preparation and the continuous observations *in situ* that *Fundulus* embryos thrived during exposure to these media and displayed normal sequences of development followed by hatching when not sampled for beta emissions or chemical testing.

<u>Radiotracer and photochemical determination of cations</u>

The radiotracer materials: ^{22}Na, ^{24}Na, ^{42}K, and ^{45}Ca, were obtained by the MBL from the Brookhaven National Laboratory, Upton, New York, overnighted by air freight to the nearest airport in Hyannis, Massachusetts. Other physicochemical means for testing standard solutions were also employed.

Different measures were required to determine the levels of cation transfer across the embryonic ectoderm. The majority were by ß-emission (electron loss) detected in the Geiger- Mueller radiation unit at MBL over a test range of 0.750- 1.050 kilovolts; all work reported here used the same tube (#3392 set at scalar 5) repeatedly calibrated against a ^{60}Co standard over a range of 60-90 counts per second [Loevinger and Berman, 1951]. This confirmed the strengths of the solutions provided and confirmed half lives as follows (shorter term isotopes): ^{24}Na, 14.8 hours, ^{42}K, 12.4 hours, and ^{45}Ca, 162.7 days. Minor corrections for thickness of specimen (self absorption) and tube dead space were made. The increase in number of embryos per sample to ten each was instrumental in keeping the variations observed on sample size and position in planchets for three determinations each to 2.4% for Na^+, 5.6% for K^+, and 4.7% for Ca^{++}. By contrast, photochemical measurements for Mg^{++} had a maximum variation of 1.1%, attributed to more complete extraction from embryos. There is the possibility in planchets for contamination or inadvertent removal of portions of embryos. The work reported here makes use of mean values only since the objectives were relative placement in a data matrix and proof of uptake, not statistical precision, followed in any case, from careful technique.

The regulations in effect at the time limited maximum permissible exposure to 6.25 mr/hr/8 hour day. This limit was never even closely reached in the approximately six months it took for that

phase of the work to be completed, three months each of two successive summer seasons of material availability. All radiation work required wearing daily detection badges and other standard precautions, especially when opening the containers from the Brookhaven facility.

Table 4 indicates the spectrum of radioactive forms of these cations. The nonavailability then of a useful radioactive isotope for magnesium, ^{28}Mg, was addressed by the use of photochemical methods. Three methods were examined. The indirect method of Simonsen, et al [1947] is based on a molybdivanate color matrix for phosphate following the precipitation of magnesium-phosphate complexes. One more direct method, by Ruigh [1929], uses para-nitrobenzeneazoresorcinol to form a lake, and another is a modified titan yellow assay [Garner, 1946; Orange and Rhein, 1951]. These three methods were found, by direct comparison, to be fully equivalent; in general, the titan yellow method was used more often, due to its relative simplicity. Isotope ^{28}Mg became available circa 1960 [Aikawa, 1960; Aikawa and Burns, 1960] and has been used sporadically [Hmiel, et al, 1986; Rojas and Taylor, 1975; Snavely, et al, 1989]. It is not readily available for studies on magnesium exchange between physiological compartments [Shaw, et al., 1990] currently because of a change in government policy in the 1980s resulting in an extremely high cost of the isotope [Maguire and Cowan, 2002].

It was considered *ab initio* that some calcium was bound and not readily exchanged during radiotracer uptake. Accordingly, total calcium was determined photochemically. The principal method employed was an adaptation of Kibrick, et al. [1952], using a chelating agent, ethylene diamine tetracetate, with murexide as indicator. Tetracetate as a greater affinity for calcium and the color of murexide is restored during titration.

In all instances in this work the experimental samples for chemical titration were compared regularly to stock test solutions of known concentration.

Time course of experimental runs

The basic work involving the heterosmotic and isosmotic runs was on samples maintained in the various media for ten hours. This interval was based on a prolonged test of equilibration of radiosodium ^{22}Na [**Figure 1**]. As the dominant cation of sea water and for many of the other media studied, this was considered *a priori* the best standard for a fixed end point. Use of ^{22}Na avoided a need to recalculate the raw data for degradation of signal over this time frame which would have been required had ^{24}Na been employed. Runs using fluoride in various concentrations were followed by 2.25 hour comparisons. Once the basic effects were found for this interval, efflux and uptake patterns were determined for each cation. Efflux was measured into media lacking the cation after uptake equilibrium had been reached; other sets depleted in the same manner were then studied during reuptake.

Flame photometry

A Toner-Pitts flame photometer with an internal lithium standard and a Beckmann spectrophotometer with a flame photometric attachment were used for total sodium and potassium determinations.

Results

The results are presented in two categories: **[1]** principal findings, and **[2]** special subsets, the results of fluoride and partitioning between embryo proper and the yolk sac.

Principal findings

These are presented in consolidated form in **Tables 5** and **6**. The *heterosmotic* series is Table 5. This arranges the media in descending order of molarity, with the ternary mixture without calcium in second place, and unitary calcium last of the fifteen different treatments reported here. Table 6 is the *isosmotic* series, in the same order. The three

media in the heterosmotic series are listed in descending order, 1.92, 1.0, and 0.5 sea water equivalents. Since there are four cations, a total of 60 media are reported. Overall, the dominant intracellular cation in these embryos is magnesium.

Several extremely high embryonic values appear in the table for artificial $SW_{1.92}$: **[1]** *sodium*, 103 mEq/L in the ternary medium lacking potassium, **[2]** *magnesium*, 111.4 mEq/L in the ternary mixture without calcium, and 108.6 mEq/L in the ternary solution lacking sodium, and **[3]** *calcium*, 17.6 mEq/L in the ternary medium lacking potassium. The *potassium* value construed as a *breakout* result is 6.10 mEq/L in the $SW_{1.92}$ binary mixture Na-K. There was one other very high magnesium value >100 mEq/L, 106.3, in the $SW_{1.92}$ Mg-K binary mix.

Table 7 displays the internal slopes of each triad for all mixtures which result when $SW_{1.0}$ values are normalized to 1.000. This indicates the combined effect of cation mix and the inherent osmotic differences between solutions at $SW_{0.5}$, $SW_{1.0}$, and $SW_{1.92}$, a range ratio of 3.84. The *italicized* ratios are between the individual result in $SW_{1.92}$ divided by the companion value for $SW_{0.5}$.

Twelve ratios exceeded 3.84 [**Table 8**], one for sodium, none for magnesium, four for potassium, and seven for calcium. The latter indicates the likelihood of efficient control of calcium homeostasis but not overt enhancement, since only two raw calcium levels exceeded the environmental concentration [**Table 5**, ternary NaMgCa, $SW_{1.92}$ and $SW_{1.0}$], more easily understood as a negative potassium effect.

The rankings and considerations noted above are not pertinent to the isosmotic series. **Table 9** shows the direct comparison of *heterosmotic* results in $SW_{1.0}$ with *isosmotic* levels, indicative of the effect of osmotic forces on the net uptake of the several cations. An interesting pattern develops when the heterosmotic results are subtracted

from the isosmotic values, each cation occurring eight times in the overall sequence. A negative value is when the heterosmotic value exceeds the isosmotic. Thirteen of the twenty eight media (quaternary sea water is the same in both series) had negative differences, five for sodium, four for magnesium, one for potassium, and three for calcium. **Figure 2** shows the osmotic effects for sodium and magnesium, the cations which have **(1)** the highest concentrations in the various media, **(2)** the highest uptake levels, and **(3)** the largest effect on osmotic balance when absent. The changes for potassium and calcium are much smaller empirically but appear nontrivial compared to the more restricted general uptake activity for either [**Table 6**]. **Figure 2** emphasizes an almost parallel effect on uptake of Na^+ and Mg^{++} by heterosmotic changes with different total concentrations. There is a slight convergence of the osmotic effect with exactly linear plots. The sodium regression line is: $\Delta_{Na} = 38.086[SW_{Conc}]-30.98$ and magnesium: $\Delta_{Mg} = 46.288[SW_{Conc}]-59.17$. The difference in absolute value at $SW_{1.92}$ was 12.5 mEq/L, about half that in $SW_{0.5}$ (24.2 mEq/L), a measure of the joint effect of the change in concentration and the osmotic force. Extrapolation suggests intersection of these vectors in an artificial sea water near 3.4 times base line, hypertonic beyond survival for the embryo. The linearity of result establishes (a) good precision in measurement, and (b) a uniformity of the osmotic effect on the major cations over this range of environment.

Interestingly, the two most divergent results, for solutions containing both sodium and magnesium, were ternary NaMgCa and binary NaMg, clearly a calcium effect since there is little osmolar difference. The largest positive difference was sodium in binary NaK (35.7). The difference for magnesium in ternary NaMgCa (-18.6) was larger than in unitary Mg (-12.3). The absolute difference between sodium and magnesium in ternary

NaMgCa was 45.4 and the next largest was 32.8 in binary NaMg. None of these differences seem related to the actual relative water content (data not shown). The colligative salt value (SV), when sodium was present, ranged from 1068 to 1290 mOsm, and when sodium was absent, from 17.4 to 222.4 mOsm.

These ranges are considered here as high and low groups for Mg, K, and Ca. There was one zone in this matrix where correlation between differences in water content and cation uptake took on coordinated, nonstochastic attributes. This is the grouping of ternary MgKCa (no Na^+), and binaries MgK, MgCa, and KCa. The ratio of segmental slopes against net uptake was plotted against their sea water concentration **[C]**; the three point plot is linear: **-0.00227982 [C] + 0.0698784**, over the range of 138.90 to 175.94 mOsm (data not shown).

Table 9 has the comparison between heterosmotic and isosmotic series with differences between the net values (heterosmotic subtracted from the isosmotic) and the ratios (heterosmotic divided by isosmotic). The data can be expressed as internal regressions but the pattern is clear from this more limited treatment. For example, the value for heterosmotic sodium tends to rise, with differences ranging from -7.5 to -26.8; the more singular decrease is binary NaK, with the lowest numerical ratio, 0.451. Moreover, sodium and potassium are the only ions, when unitary, to show a rise in the absence of osmolar balance. The effect of osmolar change and lack of potassium is very striking in ternary NaMgCa (no K^+), with a 24-fold rise in calcium uptake to a level exceeding that of the external medium by approximately 19%. Unitary magnesium and calcium have lower values in heterosmotic media.

The spread and pattern of values is evidence for the relative specificity of each medium, with complex effects from ion concentration and the osmotic environment. The

relation of the latter to the activation of membrane ion channels is considered in the discussion.

Special subsets

Small amounts of sodium fluoride, 0.01-0.10 mEq/L were added **[1]** to various media at the start of radiotracer uptake to equilibrium, and **[2]** after equilibration, to media lacking each of the cations in turn so as to track the efflux from embryos. The initial run, using ^{45}Ca for 2.25 hours, yielded unexpected findings. ^{45}Ca uptake was very rapid, a biphasic response to rising amounts of fluoride. A Lineweaver-Burk plot was nearly linear and the raw data well fitted the regression slope: **45.7 [mEq/L Ca^{++}] + 1.90** (data not shown).

More interesting was the result for 0.10 mEq/L fluoride, a concentration equilibrium with the external medium, 5.8 mEq/L calcium. This led to the choice of 0.05 mEq/L NaF for the remainder of the fluoride experiments [**Table 10**]. These showed different results, depending on the cation.

Potassium is the most mobile cation under these conditions, falling to similar baselines equally rapidly with and without fluoride, suggesting K^+ efflux is not bound to energy from the glycolytic cycle or through the cytochrome system. Sodium and calcium effluxes diverged over time. Na^+ loss was more rapid when fluoride was employed (1.0 h) demonstrating the strong Na^+ mechanism for ion retention is fluoride inhibited and, presumptively, energy dependent. Since there was no Na^+ in the external medium at this point in time, and minimal amounts after some is lost, interference with sodium uptake mechanism(s) cannot be postulated. By contrast, calcium behavior was the reverse: efflux under the fluoride effect took twice as long, 6.0 versus 3.0 hours. This seems to indicate that calcium efflux requires metabolic energy. Another anomaly of flux is seen

with magnesium [**Figure 3**]. The yolk compartment is unaffected by fluoride despite a faster rate of cellular loss, 5.0 versus 10.0 hours from the embryo proper.

Yolk magnesium content was a constant 30 mEq/L despite changes in external media, prior depletion of the embryo proper, upon restitution, with or without NaF (data not shown). Yolk is a sequestered compartment, apparently engaging in Mg^{++} homeostasis only as the yolk is absorbed later during embryonic maturation.

Discussion

General considerations

The four principal biological cations are closely related in the periodic table of elements, occupying a tight 2x2 matrix box. Sodium and potassium are in group IA, the third and fourth rows respectively, and magnesium and calcium are in group IIA, again the third and fourth rows, directly adjacent to sodium and potassium. **Table 11** shows pertinent aspects of these elements. The most important are the ionic diameters [Campbell, 1970; Diebler, et al, 1969; Maguire and Cowan, 2002] from which spatial volumes can be calculated [**Table 11**]. Cations in cytosol and interstitial fluids present to cellular membranes in the hydrated form (solvation) but pass through channels as either a *naked* ion or one with the hydration shell greatly diminished [Chong and Hirata, 1997; Dill, et al, 2005]. The same is true of anions [Coe, 1997]. The artificial sea waters used in this work used chloride as the sole anion. It was determined, *a priori*, to neglect the possible influence of sulfate despite its measured concentration in sea water, 12.25-12.29% of total anion content [Bianchi, 2007], reported in earlier work as a sulfate/chlorinity ratio of 0.14000 ∀ 0.00023 [Morris and Riley, 1966]. Work on cation antagonisms preliminary to this report used an all chloride formulary [Armstrong, 1928].

The ratio of the hydrated:naked radius of potassium is very favorable in spatial

terms, despite its relatively greater radius *per se* (a third larger than calcium, twice that of magnesium). Tredgold suggested the effective radius of cations to be taken into account in these considerations is about 0.6 Å larger than the Pauling radius {1973].

Energy is required to remove the hydration shell [Chong and Hirata, 1997; Samoilov, 1957], possibly a monumental effort in the case of magnesium, the smallest of fully ionized cations (0.65 Å), with a hydrated radius (4.76 Å) twice that of potassium. 1.73 times sodium, and 1.61 times calcium. These differences translate into an enormous spherical volume ratio for magnesium, *392.72* [**Table 11**] which could be a basis for intracellular sequestration.

The relation of spatial volumes does not explain all aspects of cation behavior in aqueous solution. For example, citrate salts in water at room temperature have a different sequence of affinity: K^+, 167 g/dl, Na^+, 72-92.6 g/dl, Mg^{++}, 20 g/dl, and Ca^{++}, 0.85 g/dl [Weast, 1975-76]. This hierarchy may relate to metabolic activity, given the importance of the citric acid cycle, but in this study, ion movement seems largely independent of chemical binding, in part due to the rapidity of flux. Moreover, cation influx may be affected by ions already in the cell [Matsuda, et al., 1987].

Evidence from several special experimental circumstances demonstrates the blocking role of some cations on others. Arellano, Woodward, and Miledi [1995] showed, using oocytes from *Xenopus*, that inward Na^+ and K^+ conductance was not directly due to membrane changes but to the *removal* of divalent blocking.

Mutation of a critical aminoacid in a cyclic nucleotide-gated channel altered the selectivity sequence for bivalent ions [Park and MacKinnon, 1995]. Studies of this type confirm the role of cations is in part due to their molecular attributes. In addition, magnesium is a strong Lewis acid (a substance accepting a pair of electrons, forming a

covalent bond) [Maguire, 2006]. The exchange rate of solvent water around hydrated Mg^{++} is substantially slower (.10^5/sec) than for Na^+, K^+, or Ca^{2+} (.10$^{8.5}$-10^9/sec) [Diebler, et al, 1969].

Further exploration into the physical chemistry of magnesium in aqueous solutions is beyond the intent and database of this paper. Rojas and Taylor reported a mean resting Mg^{++} influx from sea water into perfused squid giant axons at 55 mM $MgCl_2$ as 0.124 ∀ 0.080 p-mole/cm²/sec [1975]. Baker and Crawford [1972] measured an average resting influx of Mg^{2+} of 0.62 mol/cm²/sec in intact squid axons and concluded from their experiments the direction of net Mg^{++} transport was determined by the magnitude and direction of the gradient for Na^+. Although electrolytic aspects of ion movements were not considered in the experimental design of these studies, comparisons between the heterosmotic and isosmotic series do bear on the relationship between voltage-sensitive and solvent-sensitive processes in ion channel gating.

Rayner, et al [1992] examined the effect of hyperosmolar media on activation and deactivation of sodium channels. Their evidence ruled out the linear sequential and parallel independent models in favor of a coupled parallel model which incorporates features of voltage-sensitive and solvent- sensitive mechanisms [Rayner, et al., 1992]. The data presented here is consistent with their hypothesis and the factor of stretch activation of ion channels [Kirber, et al., 1988; Morris and Sigurdson, 1989].

Cation synergisms and antagonisms were reported by Portelli [1982]. Excess K^+, Mg^{++}, and H^+, and lower Na^+ blocked both electrical and mechanical activity in the isolated frog heart preparation while these effects were eliminated by increases in Ca^{++}, Na^+, and OH^- [Portelli, 1982].

The role of osmotic forces

Zimmerberg, et al. [1990] pointed out the selective nature of channels means exclusion of various molecular species, which nevertheless *must* (emphasis supplied) act osmotically on the channel interior, i.e., the physical configuration of the channel. Their test cation was potassium and the medium was the potassium channel of the giant axon of the squid *Loligo pealei*. Hyperosmolar sucrose dampened and slowed K^+ currents with minor changes in the exponential tail during restitution but did not influence voltage dependence [Zimmerberg, et al, 1990]. They considered a "blocking" action which forced closure of a proportion of channels in a given field. They defined *blocking* as inhibition of current flow through an open channel, then noted it would be difficult to distinguish such blocking from *fast flickering* of an open channel to a closed state driven by osmotic stress [Zimmerberg, et al, 1990]. Their plot of limiting Gibbs potential against the conductivity ratio of external solutions showed a marked difference between isosmotic and heterosmotic media [their figure 12].

The effect of osmotic stress on the surface of ectodermal cells in the experimental model shown here is clarified by the *narrow channel* concept of Levitt [1984]. He used the word *narrow* to mean a cross sectional diameter of the channel so small the conveyed ions or molecules could not pass each other during transit [Levitt, 1984]. Moreover, per Levitt, this has to incorporate the solvation or hydration shell of water about the cation. Na^+ is a smaller ion and has a tighter hydration shell than K^+. Sodium ions have about nine water molecules each with little change over an activity range; potassium differs with about seven water molecules at low concentrations but only five at high activity [Levitt, 1984]. His equations, based on this construct, matched those of measured experience better than the other mechanisms he discussed. In the experimental work described here, K^+ moved both in and out more quickly, these size differentials

notwithstanding. A mix of forces, thermodynamic, and physical or kinetic, has influence on K^+ channel selectivity [Grabe, et al., 2006]. The role of osmotic forces in several subsets of cation mix in these experiments is explored further in *progressions in magnesium sets* and *progressions in potassium-calcium sets*.

Magnesium and the ATP energy cycle

Evidence for relatively slower reaction times for magnesium is shown in **Figure 3**. Both efflux and uptake were slower, efflux about ten hours and uptake eight hours, compared to one hour for Na^+ and K^+ and three to six hours for Ca^{++}.

Maguire recently asked, "...why is ATP involved? [2006]" .The high energy phosphate bonds of substrate ATP are the energy end product of the glycolytic cycle and other enzymatic processes. Maguire stated:

> "There is no obvious reason why ATP is required for Mg^{2+} influx. The electrochemical gradient across the bacterial membranes is highly negative inside. This should provide far more potential energy than is necessary to drive Mg^{2+} influx at even micromolar extracellular Mg^{2+} concentrations. The normal role of P-type ATPases and a requirement for ATP is to mediate the efflux of a cation *against* its electrochemical gradient. MgtA and MgtB clearly do not mediate such a process. Indeed, it is often assumed that for ions such as Mg^{2+}, there must exist an efflux activity to prevent accumulation of the ion driven by the constant electrochemical gradient."

Fluoride inhibits ATP production as noted above. **Figure 3** shows that fluoride blocks nearly all magnesium uptake and hastens Mg^{++} efflux from the embryonic axis of *Fundulus*. Thus, an energy source is considered to be active in both directions. The efflux is not that of exponential diffusion but resembles controlled egress limited by carrier capacity. By contrast, fluoride releases Mg^{++} efflux from this mechanism and, as seen in Figure 3, the rapid fall to 5 hours could be exponential, lacking an intermediate time point. This will be discussed further under the section on *clinical considerations*.

The effect of fluoride on magnesium

Fluoride ion is a well known inhibitor of the glycolytic energy chain at pyruvate enolase [Allmann and Kleiner, 1980; Borei, 1945; Green and Parpart, 1950] and other sites of the glycolytic energy cycle [Bartholmes, et al., 1987]. Its use in these experiments was to determine the role of cell energy in a comparative sense. **Table 10** shows three unbalanced rates of efflux with and without fluoride. Fluoride appears to have no effect on potassium efflux but hastened sodium loss. This can be explained if homeostatic sodium balance is mainly the result of regulated uptake. Calcium, a bivalent ion, displayed slower efflux after fluoride treatment, suggestive of an energy dependent active process, the homeostatic effect of which is to maintain cellular Ca^{++} content. The anomalous result here is that magnesium requires an extremely long period for uptake from the outside concentration compared to the others and Mg^{++}, a bivalent ion, has the opposite pattern during efflux (**Figure 3, Table 10**). The result for calcium may be due in part to intracellular binding by proteins like calsequestrin which has been identified in eggs of the North Atlantic sea urchin (*Strongylocentrotus droebechchiensis*) [Lebeche and Kaminer, 1992] or ERcalcistorin/protein-disulfide isomerase [Lucero, et al., 1998]. Calsequestrin has been characterized with an apparent molecular weight of 54-58,000 daltons [Oberdorf, et al., 1988], distinguishing it from the calbindins [Fullmer and Wasserman, 1981; Gill and Christakos, 1993]. So far, no parallel evidence exists by which to characterize a *magsequestrin* although, despite the dissimilar behavior of Ca^{++} under the influence of fluoride, such a protein may well exist.

Progressions in magnesium sets

While casual examination of the extent of numerical data in **Tables 5-7** might suggest extreme complexity, even chaos, underlying the whole matrix, there are actually

well defined progressive series within when examined as to magnesium. To standardize discussion, the following descriptive comments will be from the perspective of the isosmotic series as shown in **Table 9**. *First*, there is a 19.9% decline (ratio = 0.801) for unitary magnesium in the heterosmotic state. Only the result for binary MgCa is nearly equivalent. Of those remaining, one showed inhibition, NaMgCa, (38.0%) and four showed facilitation: **(1)** ternary MgKCa, 12.5%; and **(2)** the triad of binaries, MgK, 20.8%; NaMg, 43.3%, with their companion ternary, NaMgK, 4.3%. The most consistent facilitation of Mg^{++} uptake is the absence of calcium. The lesser difference in ternary NaMgK has to be considered a further effect from interaction between sodium and potassium. *Second*, in ternary media, when both Mg^{++} and K^{+} are present, the result is facilitation of Mg^{++} and inhibition of K^{+}, although three effects out of four are small.

Progressions in potassium-calcium sets

The unitary value fell by 57.2% for potassium and fell substantially for calcium (81.3%) in their respective heterosmotic media. K^{+} and Ca^{++} were inhibited in the KCa binary (K^{+}, 74.9% and Ca^{++}, 71.0%). These changes implicate important interference with calcium and potassium transport by changes in the osmotic environment, possibly mediated through stretch-activated ion channels [Kirber, et al, 1988; Medina and Bregestovski, 1988; Morris and Sigurdson, 1989], as well as mutual K^{+}-Ca^{++} inhibition. The result, a large increase in Ca^{++}, from the absence of potassium in ternary NaMgCa, is strong evidence for the inhibition of calcium by potassium. The highest values for net calcium uptake are this circumstance: at $SW_{1.0}$, 6.9 mEq/L, an increase with respect to sea water, heterosmotic unitary calcium, and isosmotic calcium (718%, 1,605%, and 300% respectively), extraordinary values by any consideration. By contrast, there is little difference in the effect of calcium on sodium and vice versa in binary NaCa and in

heterosmotic $SW_{1.0}$, but the values of both are facilitated above the isosmotic state (sodium, +24.4%; calcium, +222.2%). This is further evidence for an effect of change in the osmotic environment on calcium balance. Intracellular binding of calcium would modulate this effect.

Progressions in sodium-potassium sets

In the other direction, the binary NaK shows inhibition with the heterosmotic effect, a 54.95% decrease in Na^+ and a 34.75% decrease for K^+ in $SW_{1.00}$.

Joint effects of osmotic change and the presence or absence of calcium on sodium and potassium are shown in **Table 12**. With ternary NaKCa as the baseline, binary NaK reflected different ratios at the three levels, $SW_{1.92}$, $SW_{1.0}$, and $SW_{0.5}$, and considerable increase in potassium uptake upon release from calcium inhibition, in inverse order with increasing total osmolarity. The opposite effect is seen for sodium under the same conditions. A roughly mirror image results when plotted together on a normalized Y axis (data not shown), suggestive of Na^+-K^+ reciprocal coordination. Other cations in the isosmotic series are mildly inhibitory toward their unitary values: **Ca>Na>K,** with the resulting ratio for potassium (1.097) almost identical to **Mg** (1.079), in sea water.

Ion channels in eggs and oocytes

Transformation of ova/oocytes through eggs, blastocysts, and gastrulas to embryos is rapid in many marine organisms, based intuitively on structures, proteins, and other substances *already on hand* in the maternal tissue. A case in point is mobilization of Ca^{++}, Mg^{++}, and phosphorus from the shell and yolk of embryonic alligators (*Alligator mississippiensis*), an oviparous reptile [Packard and Packard, 1989].

Calcium release channels in the cell cortex occur in <20% of immature oocytes of *Strongylocentrotus purpuratus* (Pacific sea urchin) [McPherson, et al., 1992]. Teleost

fertilization generates a calcium wave and membrane permeability for water increases from 4 to 10 days post fertilization (beyond the test range reported here) with a short term delay from Ca^{++} and indefinite delay from high Na^{+} content [Alderdice, 1988]. Cation channels have been found in frog oocytes (*Rana esculenta*) [Taglietti and Toselli, 1988], indicating a well conserved cell component. A general presence of ion channels in early embryos has been established [Moody, et al., 1991; Novak, et al., 2006].

Moreover, calsequestrin-like proteins [Henson, et al, 1989] and stretch-activated ion channels [Medina and Bregestovski, 1988] are present and active during fertilization and early post fertilization blastogenesis with voltage-dependent ion currents during meiosis and first mitosis in the ascidian *Boltenia villosa* [Coombs, et al, 1992]. There is evidence, however, that maturation involves and may require suppression [Okagaki, et al., 2001] of voltage-gated currents; this has been shown to occur in frog oocytes (*Rana esculenta*) [Taglietti, et al, 1984].

Calcium signal waves are operative during fertilization [Whitaker, 2006], implying prior competence of the cellular system. Ion channels have been found in early embryos of the chick and *Xenopus laevis* [Rutenberg, et al, 2002]. There are other aspects of this which seem pertinent here.

First, the chemistry of fluoride in these circumstances seems more complex than is usually considered. Phosphate combined with glucose has been shown to inhibit two cell development in the rat [Nishikimi, et al, 2000], and is further inhibited by the addition of sodium fluoride to egg culture media. The medium contained 1.19 mM $MgSO_4$ which may well have led to a $MgFPO_4$ complex formation similar to that which blocks enolase *in vitro* [Borei, 1945]. *Second*, the eggs of *Xenopus laevis* contain sufficient nucleoside triphosphate to make about 2500 nuclei, indicating a limit to the vital momentum within

the egg at fertilization [Woodland and Pestell, 1972]. An earlier paper put the limit of this momentum to occur in the blastula stage [Gurdon and Woodland, 1969]. *Third*, there is a relationship to the cell cycle which is reflected in oscillation of the resting potential, at least for potassium conductance, in the fresh water loach (*Misgurnus fossilis*) [Bregestovski, et al, 1992]. These critical periods are so early they have to be considered as determined by the status of the oocyte and egg membranes, both maternal products [Moody, 1995].

What is implied by these considerations is there has to be a transition period initially outside of transcription from post fertilization DNA, possibly activated largely if not wholly by the tensile forces of cell division, activating stretch-dependent ion channels while gathering sufficient magnesium and calcium "*reserves*" for the next stages of embryogenesis. During this time frame some adaptation to mechanisms encoded in the first several generations of cell proliferation and differentiation would have to follow. The activity of these early cells is directed toward mitosis which is dependent on an initial presence of microtubules. These are present in unfertilized Pacific sea urchin eggs [Pfeffer, et al, 1976] and in *Xenopus laevis* [Gard and Kirschner, 1987; Heidemann, et al, 1985]. Microtubule protein synthesis has been identified in early development [Raff, et al, 1975] and specifically during oogenesis and early embryogenesis in *Xenopus* [Pestell, 1975].

More recently, it has been shown that ß-catenin-mediated signaling, required for organizer formation in zebrafish, is controlled by residual maternal factors [Kelly, et al, 2000].

Clinical implications of early cation homeostasis

By the time placental vertebrates establish a fetal:maternal boundary, the initial

competencies have largely dispersed with concurrent environmental sources required for nutrition and maintenance. The passage of magnesium into the fetus across the placenta becomes requisite. Passage of ^{24}Mg in an *in situ* perfused rat placenta was determined by atomic absorption spectrophotometry [Shaw, et al, 1990]; this found evidence for at least partial active transport of Mg^{++}. The maternal serum levels of ^{28}Mg following intravenous infusion in the rabbit showed an apparent exponential decay asymptotic after about 8-10 hours [Aikawa and Bruns, 1960]. Fetal uptake was roughly biphasic with an early peak at about 6 hours, a decline for several hours, and then a slow rise to a maximum at 24 hours at termination of the experiments [Aikawa and Bruns, 1960]. The secondary rise correlated with a similar secondary decline in placental content, the result of dispersion within the fetus. More detailed analysis of the magnesium efflux curve, using the semilogarithmic method for equation fitting in nonlinear data [Schloerb, et al., 1950; van Liew, 1962] revealed a third order function indicative of a limiting intermediate compartment, the placenta (calculations not shown). This, in a more restricted manner, may well have been the what is shown in Figure 3, with the ectodermal layer as the rate limiting intermediate compartment for rapid efflux into the magnesium free solution.

A slightly more detailed distribution in the rat was reported by Hallak and Cotton [1993]. Surge infusion of $^{24}MgSO_4$ (270 mg/kq) without and with further reinfusion was studied photometrically. In the acute experiments maternal blood Mg^{++} rose from 2.0 ∀ 0.2 to 12.9 ∀ 1.0 mg/dL. Lesser increases were seen for amniotic fluid (+17.9%) and fetal blood (+14.3%). Fetal values were always somewhat higher. They also reported accumulation of magnesium in forebrain and hindbrain, more than the control values of about 38 and 41 mg/dL/g (sic) respectively.

The mean plasma Mg^{++} in fetal sheep was 1.08 ∀ 0.03 mM/L, nearly 14% higher than concurrent maternal values (0.95 ∀ 0.02 mM/L) [Barri, et al, 1990]. They infused 1.0mM/L $MgCl_2$/minute for 30 minutes. There were well correlated increases in both, maternal values rising 150-200% and fetal values by 17%. Barri, et al [1990] explored in sheep the role of parathyroid hormone in fetal Mg^{++}/Ca^{++} balance: **(1)** maternal thyroparathyroidectomy plus thyroid hormone replacement and infusions for constant maternal serum Ca^{++}, and **(2)** fetal thyroparathyroidectomy. In the first set of conditions maternal Mg^{++} fell nearly 50% in 24 hours but then slowly recovered to 76% of baseline by 7 days. Fetal Mg^{++} fell more slowly, a gradual exponential curve, over the entire week [Barri, et al, 1990]. Surgical removal of fetal parathyroid and thyroid had a complex effect on fetal Ca^{++} and Mg^{++} levels. Plasma Mg^{++} fell 30% by 48 hours, an undulating further decline to 11 days, with a slight rise by 18 days. The Ca^{++} level fell >50% by 72 hours, rebounded to 66.7% at six days, and then fell again with an effective plateau from 8-18 days approximating 47% of the baseline [Barri, et al, 1990], data indicative of some regulation of Mg^{++} and Ca^{++} homeostasis in the fetus by both maternal and fetal parathyroid glands.

These few studies are attentive to cation balance and control only in mature vertebrates, fetal and maternal. They provide little direct information relevant to cation acquisition and control before and after fertilization. Confirmation of the Mg^{++} levels of fetal brain reported by Hallak and Cotton [1993] would support a hypothesis of early embryonic intracellular sequestration, without countering the evident role of maternal cell membrane and cortex microstructures in the first stages of embryogenesis.

Summary and conclusions

The data presented: **(1)** establish the functional competency of early embryonic

teleost ectoderm to undertake homeostatic cation exchange, **(2)** establish that mechanisms are active from the earliest periods of embryo formation, **(3)** confirm that mechanisms for cationic transport require energy, apparently for both uptake and efflux, in some but not necessarily all situations (magnesium uptake and efflux both require an energy source by different mechanisms of action), **(4)** demonstrate that all of these cations influence the net levels of each of the other three, and **(5)** that osmotic pressure and water content of the external medium affects the net uptake and equilibrium of all four cations.

Cell membrane cation transporters contribute to distribution and concentration of Na^{+}, Mg^{++}, K^{+}, and Ca^{++} in early vertebrate embryos, but do not explain the differences in pattern of cation acquisition. The uptake of ^{22}Na reached intraembryonic equilibrium at ten hours which then served as the exposure time for all subsequent work. The metabolic blocking agent, sodium fluoride, has different effects on the energy related transport processes of the four biological cations. Variations in the water content of sea water (SW), at net effective concentrations of 0.5, 1.0, and 1.92 SW, compared with isosmolar corrections by addition of sucrose, elicited evidence of effects of osmotensile forces on cation movements. Magnesium behavior *in vivo* in this embryo model differed from the other cations but is directed at maintaining a high intracellular presence of this vital element. Some differences may be due to molecular attributes such as size and ion hydration ratios; others are best seen as specific dynamic competencies of post gastrular cells. The database reported here may support calculation of express difference equations of interactive behavior but such are considered to be beyond the scope of this largely observational report. The results strongly confirm the hypothesis of continuing maternal cellular contribution to early stages of vertebrate embryogenesis. Cation activity and interactions have clinical import, most particularly as the magnesium economy of the

body becomes more accessible by regularized clinical testing, *first*, to confirm whether magnesium levels are indirect or direct markers for certain disorders, and *second*, as possible means of following therapeutic use of intermolecular aspects of cation homeostasis. Unanswered is the genetic question of differentiation and maturation: are early embryonic cation channels and control mechanisms, of whatever type or power, merely transitional for later replacement in maturational processes, perhaps roughly equivalent to the vectors of maturation of hemoglobins Gower 1, Gower 2, and Portland [Clegg and Gagnon, 1981]? Further progress in understanding the role and actions of magnesium in biological and human physiological and pathological situations will require making ^{28}Mg much more easily available clinically and for basic cellular research.

Acknowledgements

Especial thanks are due four persons assisting in obtaining the numerous articles and reports accessed during preparation of this paper, many of which are cited in the references. Marion E. Freeland, Gainesville, Florida, made frequent use of the University of Florida Medical Center Library. Her counterparts, for extensive access to important papers, often the older literature, through interlibrary loan to and from the extensive collection of the MBL/WHOI Library at Marine Biological Laboratory, Woods Hole, Massachusetts, were Jennifer Walton, Matthew Person, and Colleen Hurter.