MAGNESIUM AND ITS TRANSPORT IN TILAPIA AND CARP: A STUDY BASED ON NUCLEAR METHODS

JOAN A. VAN DER VELDEN

Interfacultair Reactor Instituut van de Technische Universiteit Delft
ISBN 90-73861-01-2
NUGI 821
Trefw.: magnesiumhuishouding ; vissen ; onderzoek
MAGNESIUM AND ITS TRANSPORT IN TILAPIA AND CARP: A STUDY BASED ON NUCLEAR METHODS

Proefschrift

ter verkrijging van de graad van doctor aan de Technische Universiteit Delft, op gezag van de Rector Magnificus, prof. drs. P.A. Schenck, in het openbaar te verdedigen ten overstaan van een commissie aangewezen door het College van Dekanen op donderdag 25 april 1991 te 16.00 uur

door

Joannes Antonie van der Velden

geboren te Geldrop
doctorandus in de biologie
Dit proefschrift is goedgekeurd door de promotoren
prof. dr. S.E. Wendelaar Bonga
en
prof. dr. ir. J.J.M. de Goeij.

Ir. Z.I. Kolar en dr. G. Flik hebben als begeleiders in hoge mate
bijgedragen aan het totstandkomen van het proefschrift.
Het aan dit proefschrift ten grondslag liggende onderzoek werd grotendeels uitgevoerd bij de onderzoeksgroep Radio-isotoooptoepassingen van de afdeling Radiochemie van het Interfacultair Reactor Instituut van de Technische Universiteit Delft, Mekelweg 15, 2629 JB Delft; en bij de vakgroep Experimentele Dierkunde van de Faculteit der Natuurwetenschappen van de Katholieke Universiteit Nijmegen, Toernooiveld 25, 6525 ED Nijmegen.
CONTENTS

1: General introduction 1

2: Nucleonics
2.1: Introduction 7
2.2: Instrumental neutron activation analysis 9
2.3: Magnesium-27 12
2.4: Magnesium-28 15

3: Magnesium distribution
3.1: Introduction 27
3.2: Distribution in carp 28
3.3: Distribution in freshwater tilapia 31

4: Magnesium transport
4.1: Introduction 43
4.2: Tracer kinetical approach of transport in the water-fish system 44
4.3: Transport between carp and water 48
4.4: Transport across the intestine of tilapia 57
4.5: Transport across the basolateral plasma membrane of enterocytes 66

5: Effects of low-magnesium diets
5.1: Introduction 79
5.2: Effects on growth rate and elemental tissue concentrations of tilapia 81
5.3: Effects on the ion regulation of tilapia 91
5.4: Effects on growth rate and ion regulation of carp 100
5.5: Effects on the intake of magnesium in tilapia 109

6: Effects of low-magnesium ambient water
6.1: Introduction 115
6.2: Effects on early life stages of carp 116

7: General discussion 125

References 131
Summary 142
Samenvatting 145
Abbreviations 149
Symbols and compartment related subscripts 150
Dissertation related publications and contributions to international meetings 153
Dankwoord 155
Curriculum vitae 156
CHAPTER 1

GENERAL INTRODUCTION

AIM OF THE PRESENT STUDY

For vertebrate life forms magnesium is an essential element. It plays a pivotal, yet only partially understood, role in cellular physiology. Mg$^{2+}$ is intracellularly the most abundant divalent cation. It catalyses or activates hundreds of enzymes and plays a central role in the transfer, storage and utilization of energy and in the metabolism of carbohydrates, lipids and proteins. The biological significance of magnesium has been reviewed by Ebel & Günther [Gün80] and Aikawa [Aik81]. It has been suggested that magnesium may be a second messenger coordinating sustained responses of the cell (in contrast to the trigger function of the second messenger calcium) to changes in the extracellular fluid [Rub75; Rub77; Alv87].

The aim of the present study is to elucidate some aspects of the magnesium metabolism of fish. Therefore, the importance of magnesium transport routes between water and fish and between food and fish will be examined and the magnitude of the magnesium transport of these routes will be assessed. Attention will be paid to the dependence of fish on magnesium in external sources and on possible shifts in magnesium distribution, in magnesium transport and in ion regulation under different experimental conditions. Furthermore this study will deal with mechanisms of magnesium transport across intestinal epithelium.

MAGNESIUM PROPERTIES AND ITS OCCURRENCE IN THE BIOSPHERE

Magnesium, with an average abundance of 2.3%, ranks as the seventh element in the chemical composition of the earth's crust. Magnesium is present in all types of geological deposits (magmatic rocks, sediments, metamorphic rocks) and is in the earth's crust mostly present in silicates. Magnesium-silicates contain simple MgSiO$_4$ (olivine)
mixtures (with Ca, Al and Fe) like pyroxene and asbestos, and complex hydrated forms (with Na, K, Li, P etc.) like amphiboles, micas, chlorites and clay minerals. Magmatic magnesium-silicates are mostly formed at high temperature and high pressure and are instable at the earth’s surface. This leads to the formation of weathering products such as clay minerals and to leaching of magnesium into water [Kra67].

On ocean floors magnesium ranks as the fifth element with an abundance of around 4.5%. Here magnesium is mostly present in the form of MgCO₃ (magnesite). MgCO₃ is often mixed with CaCO₃ (calcite), and this complex is then called dolomite [Kra67].

The atomic mass of magnesium with the natural isotopic composition is 24.305. Magnesium has three stable isotopes, namely ²⁴Mg (abundance 78.99%), ²⁵Mg (abundance 10.00%) and ²⁶Mg (abundance 11.01%). The ionic radius of magnesium is 66 pm; this is relatively small compared with calcium (99 pm), sodium (97 pm) or potassium (133 pm) [Wea88]. The small size and relative large charge (+2) make Mg²⁺ to a 'hard metal' ion. Hard metal ions are not easily polarized, but are good polarisers of other molecules [Ver78]. Because of this great polarising ability, Mg²⁺ has a large hydration energy which makes most salts easily soluble. Magnesium salts of e.g. chloride, sulphate, nitrate and acetate dissolve well in water. The large hydration size of magnesium results in difficulties when passing through narrow water-filled channels; this explains the low permeability to magnesium of some biomembranes and epithelia [Fla84].

MAGNESIUM METABOLISM IN FISH

General

Studies dealing with magnesium in fish are scarce. They are mainly concerned with certain aspects of magnesium metabolism such as the magnesium distribution among fish tissues, dietary magnesium requirement, effects of high magnesium concentrations in the ambient water on the physiology and magnesium transport through different renal tubules.

With respect to magnesium, there is an essential difference in magnesium
handling between freshwater and seawater fish. The magnesium concentration in seawater is about 55 mmol.L\(^{-1}\), which is much higher than that of the blood plasma of fish (about 1 mmol.L\(^{-1}\), with about 50% in the ionic form). For maintenance of their magnesium balance, seawater fish may limit the entry of magnesium as well as promote magnesium excretion. On the other hand, in freshwater the magnesium concentration in general does not exceed 1 mmol.L\(^{-1}\). Because the blood magnesium level in freshwater fish is maintained at levels as found in marine fish, freshwater fish must compensate diffusional and excretory losses of magnesium by an active uptake of this element from the environment. Potential magnesium sources are the food and the ambient water, with gut and gills as possible entrance ports. This study deals exclusively with freshwater fish.

Magnesium sources

Dietary magnesium requirements and effect of a low-magnesium diet in fish have been studied by a number of workers. For optimum growth a magnesium requirement around 20 mmol.kg\(^{-1}\) diet has been reported for most fish species (see § 5.1). Effects of low-magnesium feeding are: a reduced growth rate [Ogi76; Cow77; Ogi78; Kno81; Gat82; Kno83; Shi88; Dab89; She89], a higher mortality [Ogi76; Gat82; Shi88], a lower magnesium concentration in blood plasma [Cow77; Ogi78; Gat82; Kno83], muscle and bone [Ogi78; Kno83; Shi88], a higher calcium concentration in muscle and bone [Ogi78; Kno83; Shi88] and a higher sodium concentration in muscle and bone [Cow77; Shi88].

In principle the ambient water can be an almost unlimited magnesium source for fish. Studies on the requirement of ambient magnesium in freshwater fish are not available. A few studies on the magnesium uptake (equals intake minus loss) from the water have been conducted [Hőb84]. These studies, however do not give information on the rates of the unidirectional transport routes between fish and water.

Magnesium handling

Three organs are specifically concerned with the exchange of magnesium between fish and the environment: the gut, the gills and the kidney.

Experiments using a magnesium deficient diet led to the conclusion that dietary magnesium is essential for the growth of carp [Ogi76] and thus that the gut is essential for the intake of magnesium from the environment. Solvent drag was suggested as a
possible mechanism for net magnesium transport through eel intestine [Nak86]. Studies on the mechanisms of magnesium intake in the gut are lacking.

In freshwater fish the gills are an important route for intake of ions from the environment, e.g. for sodium, chloride and calcium [Ber68; Gir80; Per88]. Evidence for branchial magnesium intake from the water is limited. Höbe et al. [Höb84] reported a branchial net magnesium intake of $2 \pm 13 \, \mu\text{mol.kg}^{-1}.\text{h}^{-1}$ for white suckers. This uptake was calculated from changes in the magnesium concentration of the external water in fish fitted with an urinary catheter to exclude contaminations by renal effluent. Frenzel and Pfeffer [Fre82] concluded that rainbow trout absorb significant amounts of magnesium from the water, as growth related accumulation of this element exceeded by far the amount magnesium fed. For tilapia it was reported that a high magnesium concentration in the water led to hypocalcemia and reduced osmotic water permeability of the gills [Wen83].

A major function of the kidney of freshwater teleosts is electrolyte reabsorption and production of large volumes of dilute urine. Sodium and chloride are almost completely reabsorbed in the renal tubules. Measurements of the magnesium concentrations in urine of freshwater teleosts suggest that also magnesium is reabsorbed against its concentration gradient [Hic69; Höb83]. For white suckers a renal magnesium excretion rate of $0.1 \pm 0.8 \, \mu\text{mol.kg}^{-1}.\text{h}^{-1}$ was reported [Höb84]. Intraperitoneal infusion of magnesium in freshwater rainbow trout led to a stimulation of renal excretion of magnesium; the majority of the infused load was excreted renally [Oik85] and not via intestinal or branchial routes.

THE EXPERIMENTAL ANIMALS

The studies described in this thesis were performed with two freshwater fish viz. tilapia and carp. Both fish belong to the class of Teleostei (bony fish).

The cichlid tilapia Oreochromis mossambicus (Peters), further called tilapia in this thesis, is a euryhaline species. It originates from Africa, but is now, being widely bred as a consumption fish, found throughout the tropical and subtropical latitudes. Except for a low water temperature, the tilapia is well known to tolerate and to adapt to numerous
strenuous environmental conditions such as high salinity, low pH, low oxygen tension and high ammonia levels [Che82]. The tilapia is a so-called "advanced teleost"; phylogenetically it is a recent species.

The common carp *Cyprinus carpio* L., further called carp in this thesis is a stenohaline freshwater teleost. The carp originates probably from the watershed of the Donau and from central Asia. Carp tolerate handling as well as poor water qualities (e.g. low oxygen tension). The common carp was one of the first domesticated fish. In China, this species has been cultured for at least 2400 years [Suz79].

These fish species were chosen on the basis of four major considerations. First, both species are tolerant on handling and can be easily kept and bred under laboratory conditions. Second, the physiology of both fish species has been thoroughly studied, especially with respect to endocrines and the hydromineral homeostasis. This may be useful in the interpretation of the data obtained in the present study. Thirdly, both species are commercially important. On the fourth place, tilapia and carp differ in several aspects (e.g. stenohaline against euryhaline; different endocrinological responses to high ambient calcium) which can be useful for comparative physiological studies.

Our first aim was to extend our general knowledge of the magnesium metabolism in fish. Transport epithelia of teleosts have successfully been used as models for transport of ions such as calcium, sodium, chloride and cadmium [Fli85b; Gro88; Ver87]. Therefore, another rationale in our studies on magnesium transport phenomena was to use these well established models of freshwater teleosts for the studies on magnesium transport in general. The gills of freshwater fish represent a model for a tight epithelium, the intestine represents a leaky epithelium.

**RADIOISOTOPES OF MAGNESIUM**

For metabolic studies of magnesium, insight in the transport rates of magnesium is important. The main approach to the determination of transport rates in general is the use of the radiotracer method. The known radioisotopes of magnesium are $^{20}$Mg ($t_{1/2}$ = 0.6 s), $^{21}$Mg ($t_{1/2}$ = 0.123 s), $^{22}$Mg ($t_{1/2}$ = 0.13 s), $^{23}$Mg ($t_{1/2}$ = 11.3 s), $^{27}$Mg ($t_{1/2}$ = 9.46 min), $^{28}$Mg ($t_{1/2}$ = 21.0 h), $^{29}$Mg ($t_{1/2}$ = 1.4 s) and $^{30}$Mg ($t_{1/2}$ = 1.2 s) [Led78]. The fact
that these radioisotopes of magnesium have relatively short half-lives, are scarcely commercially available and are often produced with a low activity and/or specific activity, have strongly limited their use. This is a major reason for the limited knowledge of the magnesium metabolism as compared to that of sodium, potassium or calcium. Dirks [Dir83] called Mg$^{2+}$ the forgotten cation.

Only two radioisotopes of magnesium have a half-life sufficiently long for biological applications, involving processes with a time scale of minutes or hours, namely $^{27}$Mg and $^{28}$Mg. Both radioisotopes can be produced in a nuclear reactor, although the specific activity to be obtained is somewhat limited. However, despite the limited specific activity, both radioisotopes have the property for usage as radiotracer in biological applications. These two radionuclides can be conveniently measured by their emission of $\beta^-$-particles and $\gamma$-rays.

OUTLINE OF THE PRESENT STUDY

The experiments described in this thesis deal with the production of radiotracers for magnesium and the magnesium metabolism of freshwater tilapia and carp. In chapter 2 the technique of instrumental neutron activation analysis, and the production and properties of two radioisotopes of magnesium are described. In chapter 3 the magnesium distribution and the magnesium inventory of carp and tilapia is described, mainly by the use of instrumental neutron activation analysis. Chapter 4 deals with magnesium transport (in vivo and in vitro) between the water and fish and food and fish. In chapter 5 effects of a low-magnesium diet on tissue mineral concentrations, on the ion regulation and on magnesium transport of fish is described. Chapter 6 deals with the effects of low ambient magnesium on early life stages of fish.
CHAPTER 2

NUCLEONICS

In this chapter the instrumental neutron activation analysis is described and the production of $^{27}\text{Mg}$ and $^{28}\text{Mg}$, their nuclear properties and the method of their quantification are treated.

§ 2.1

INTRODUCTION

For the measurements of elemental concentrations of magnesium, calcium and sodium several analytical techniques are available such as atomic absorption spectrometry, induced coupled plasma atomic emission spectrometry, spectrophotometry, complexometric titration, neutron activation analysis etc., all with their characteristic advantages and disadvantages. Neutron activation analysis was applied to measure magnesium, calcium and sodium concentrations in different tissues of fish. The technique used (instrumental neutron activation analysis: INAA) allows a simultaneous determination of these three elements in tissue samples without prior dissolution of the samples [Bod90b].

An important objective of this thesis was to obtain information on the transport of magnesium as occurring between water and fish as well as within the fish. The radiotracer method provides the means for the determination of the rate of this transport. There are two radioisotopes of magnesium to be utilized as radiotracer for magnesium namely $^{27}\text{Mg}$ and $^{28}\text{Mg}$, which are known for about 57 [Fer34] and 38 [She53] years, respectively. In the very first publication on $^{28}\text{Mg}$, Sheline & Johnson [She53] wrote: "Mg$^{28}$ has a half-life more than 130 times that of the longest-lived magnesium activity previously known, Mg$^{27}$ ($t_{\frac{1}{2}} = 9.6$ min). For this reason Mg$^{28}$ should find considerable use as a tracer, particularly in the study of photosynthesis. Its use in plant physiology, soil science, and certain parts of biochemistry is immediately evident. Preliminary experiments using $^{28}\text{Mg}$ as a tracer in plants have been encouraging". $^{28}\text{Mg}$
as $^{28}\text{Mg}^{2+}$ has ever since successfully been used in life sciences as radiotracer for the magnesium ion. In spite of the great importance of magnesium for living organisms the number of studies involving $^{28}\text{Mg}$ remained limited. This is probably due to the scarcity of $^{28}\text{Mg}$ caused by the difficulties in producing and distributing it. $^{28}\text{Mg}$ can be produced by nuclear reactions by charged particles directly [Mor67; Noz75; Pro76; Lun79] or indirectly via thermal neutron induced charged particles [Iwe53; Mel57; Sta63; Sta64; Rud65; Tör65; Mir70; Mal73; Rob74], by fast neutrons [Stü77; Kot77] or by high energy photons [Yag75; Pol89]. All these techniques require a further radiochemical separations.

$^{27}\text{Mg}$, although conveniently obtainable by a thermal neutron induced nuclear reaction, had until now not found applications as radiotracer in life sciences.

A set up for the production of $^{27}\text{Mg}$ and $^{28}\text{Mg}$, using the nuclear reactor of the IRI (Interfaculty Reactor Institute), was worked out [§ 2.3; § 2.4]. It should be noted that in two experiments described in this thesis [§ 4.3 and § 4.4] also $^{28}\text{Mg}$ obtained via photonuclear production [Pol89] was used. As the literature values of the half-life of $^{28}\text{Mg}$ show a significant diversity, the half-life of $^{28}\text{Mg}$ was determined [§ 2.4].

Radiotracer experiments are designed to provide information about the system. The data obtained from the experiments typically consist of values of tracer and tracee concentrations at various locations in the system at various time. Some systems may be regarded as being composed of a finite number of regions or phases called compartments, separated by boundaries, and having a uniform specific activity within each region. "Boundary" is used here in a general sense to represent the dividing line, real or conceptual between two compartments on the basis of physical, chemical, or biological properties [Bro68]. A common objective of a radiotracer study is to construct a compartmental model which represents the system in structure and/or behaviour on the basis of the available radiotracer data. This tracer kinetic approach, called compartmental analysis [Shi72], is used throughout this study. A further elaboration of the compartmental analysis pertaining to magnesium in the water-fish system is given in § 4.2 and § 4.3.
§ 2.2

INSTRUMENTAL NEUTRON ACTIVATION ANALYSIS

ABSTRACT
A fast instrumental neutron activation analysis technique was adopted for the
determination of magnesium, calcium and sodium concentrations in lyophilized fish
tissues. The reliability of the technique was established by measuring the magnesium and
calcium concentrations in Orchard Leaves and the sodium concentration in Kale,
reference materials with certified and recommended concentrations, respectively. The
ratios between the measured (average) values and the reference values were 1.02, 1.00
and 1.00 for magnesium, calcium and sodium, respectively, indicating that there were no
substantial systematic errors in the analytical technique used.

INTRODUCTION

Instrumental neutron activation analysis (INAA) comprises three consecutive steps. (i) Irradiation of a weighed sample for a predetermned period of time with
thermal neutrons in a nuclear reactor. This should result in the formation of measurable
quantities of one or more radionuclides from the element(s) to be determined. In most
cases this occurs due to a so-called (n,γ) nuclear reaction. Usually a standard containing
a known amount of the element(s) of interest or a known amount of zinc [Gir65] is
irradiated as well. (ii) Measurement of the activity of the obtained radionuclide(s) in the
sample and in the standard(s). Most of the radionuclides induced by the thermal neutron
irradiation emit γ-rays. The γ-ray spectroscopy using high-resolution semiconductor
detectors is the method of choice for their identification and quantification. (iii)
Calculation of the concentrations of the element(s) of interest.

The all-over reliability of the method should be assessed by regularly processing
reference materials (preferably with certified concentrations) resembling in composition
the sample material as much as possible.

The determination of the elements of interest namely magnesium, calcium and sodium is based on the nuclear reactions: \(^{26}\text{Mg}(n,\gamma)^{27}\text{Mg}\), \(^{48}\text{Ca}(n,\gamma)^{49}\text{Ca}\) and \(^{23}\text{Na}(n,\gamma)^{24}\text{Na}\), respectively. This results in \(\gamma\)-ray emitting radionuclides with half-lives of 9.46 min, 8.72 min and 15.02 h for magnesium, calcium and sodium, respectively. Due to the great number of samples to be measured a fully automated, instrumental method with a processing time of about 5 min per sample & zinc standard pair appeared to be most adequate for biological samples involved in this work [Bod90a].

MATERIALS AND METHODS

The reference materials used where "Standard Reference Material 1571 Orchard Leaves" (National Institute of Standards and Technology, Washington, USA) containing (0.62 ± 0.02)% magnesium and (2.09 ± 0.03)% calcium [Gla87] and "BOWEN's Kale" containing 2366 mg.kg\(^{-1}\) ± 12% sodium [Mur85]. The mentioned errors for magnesium and calcium refer to the standard deviations for a single determination and for sodium to the standard deviation of accepted laboratory means.

Typically 100 mg reference material and 50 mg zinc were encapsuled in two separate polyethylene vials. These were placed in another polyethylene vial ("rabbit") suitable for pneumatic transport to and from the reactor core. Each rabbit was irradiated for 15 to 20 s in the IRI nuclear reactor, where it was exposed to a thermal neutron flux of 1.3\times10^{17} \text{m}^{-2}\cdot\text{s}^{-1}. After irradiation the sample and zinc containing vials were manually removed from the rabbit and their activity consecutively measured with a \(\gamma\)-ray spectrometer consisting of a lead-shielded High-Purity Germanium detector (diameter: 40.5 mm, length: 51.4 mm; Ortec), a spectroscopy amplifier (model 673, Ortec) and a multichannel analyser (TN 1710; Tracer Northern) including a 200 MHz analog to digital converter. The samples to be measured can be placed on different calibrated distances from the detector. Corrections were applied for geometry effects related to filling height of the vial. Variations in dead-time during measurements were compensated for, by a dead-time stabilizer [Bru74]. The acquired data were transferred to a computer (PDP 11/44, Digital Equipment Cooperation) via a CAMAC interface. The identification and
the element assignment of γ-rays followed by peak quantifications based on the single comparator method using zinc as comparator element [Gir65] were carried out by a dedicated computer program [Kor73]. The actual concentrations of magnesium, calcium and sodium were calculated from the data pertaining to 0.844 & 1.015 MeV, 3.084 MeV and 1.368 & 2.754 MeV γ-ray peaks, respectively. Possible contributions to the \(^{27}\text{Mg}\) activity due to the reaction \(^{27}\text{Al}(n,p)^{27}\text{Mg}\) [Bow64] were found to be negligible.

RESULTS AND DISCUSSION

The mean values and their standard deviations of 8 separate determinations of magnesium and calcium concentrations in Orchard Leaves were 259 ± 21 mmol.kg\(^{-1}\) which is equivalent to (0.63 ± 0.05)% and 524 ± 50 mmol.kg\(^{-1}\) which is equivalent to (2.1 ± 0.2)%, respectively, while the certified values were (0.62 ± 0.02)% and (2.09 ± 0.03)%, respectively. The mean value and its standard deviation of 19 separate determinations of sodium concentrations in BOWEN's Kale was 103 ± 3 mmol.kg\(^{-1}\) which is equivalent to 2377 ± 71 mg.kg\(^{-1}\); while the recommended concentration is 2366 ± 284 mg.kg\(^{-1}\). The ratio between the measured (average) values and the reference values were 1.02, 1.00 and 1.00 for magnesium, calcium and sodium, respectively. This indicates the absence of any substantial systematic errors in the applied INAA procedure and proves its suitability for biological samples.
§ 2.3

MAGNESIUM-27

ABSTRACT
A method for the preparation of \(^{27}\text{Mg}\), a radionuclide with a half-life of 9.46 min, was set up. It is based on the thermal neutron irradiation of magnesium acetate enriched on \(^{26}\text{Mg}\). No contaminating radionuclides could be detected in the \(^{27}\text{Mg}\) preparations.

INTRODUCTION

In reviews on magnesium transport in biological systems, only \(^{28}\text{Mg}\) is given as a possible radiotracer for magnesium [e.g. Gru89]. However, for the study of fast magnesium transport processes (e.g. on cellular or subcellular level) \(^{27}\text{Mg}\), with a half-life of 9.46 min and emitting conveniently measurable radiation, can also serve as radiotracer for magnesium. A decay scheme of \(^{27}\text{Mg}\) is given in Figure 2.1. This section deals with the production of \(^{27}\text{Mg}\) by thermal neutron irradiation of a magnesium containing target according to the reaction \(^{26}\text{Mg}(n,\gamma)^{27}\text{Mg}\). Magnesium of natural isotopic composition contains 11.01% \(^{26}\text{Mg}\). The use of magnesium enriched on \(^{26}\text{Mg}\) increases the yield viz. the specific activity of \(^{27}\text{Mg}\).

MATERIALS AND METHODS

Aqueous solutions of magnesium acetate, with concentrations ranging from 60 to 140 mmol.l\(^{-1}\) obtained by dissolution of MgO, enriched to 97.1% \(^{26}\text{Mg}\) (Technabexport), in 100 \(\mu\text{mol.l}^{-1}\) acetic acid were used as target material. Typically 50 to 100 \(\mu\text{l}\) of this solution was sealed in a polyethylene vial and irradiated in the IRI nuclear reactor with a thermal neutron flux of 4.0·10\(^{16}\) m\(^{-2}\).s\(^{-1}\) for 10 min.

The radionuclidic analysis pertaining to the \(\gamma\)-rays and \(\beta^-\)-particles emitting...
radionuclides was carried out by γ-ray spectroscopy and liquid scintillation counting of \( \beta^- \)-particles. A 61 cm\(^3\) Ge(Li) detector (APY45A/N, Philips) with associated electronics, and a liquid scintillation counter (tri-carb 4000, Packard) adjusted to measure \( \beta^- \)-particles of 0.02 to 1.80 MeV were used for this purpose.

RESULTS AND DISCUSSION

The specific activity of \(^{27}\text{Mg}\) as calculated from the irradiation data was 46 GBq.mol\(^{-1}\) at the end of irradiation. Examination the γ-ray spectra revealed no other peaks than those belonging to \(^{27}\text{Mg}\). A plot of logarithms of the liquid scintillation
Plot of the logarithm of the counting rate versus time data obtained by liquid scintillation counting of a solution containing 28 kBq $^{27}$Mg (calculated value). The straight line represents the line of best fit through the data points (correlation coefficient $r = -0.99992$). Its slope is $-5.2978 \cdot 10^{-4} \pm 1.33 \cdot 10^{-6} \text{ s}^{-1}$ and the corresponding half-life value 568 ± 1 s or 9.47 ± 0.02 min.

counting data pertaining to the $\beta^-$-particle energy range of 0.02 to 1.80 MeV versus time is shown in Figure 2.2. A straight line was fitted through these data points (spanning about four half-lives of $^{27}$Mg) and the obtained slope value was calculated into the half-life value. The resulting figure of 9.47 ± 0.02 min was in good agreement with the literature value of 9.46 min indicating no relevant contamination of 0.02 to 1.80 MeV $\beta^-$-particles emitting radionuclides with half-lives differing from 9.46 min.
§ 2.4

MAGNESIUM-28

ABSTRACT
A novel method for the radiochemical separation of $^{28}\text{Mg}$ from reactor-neutron irradiated Li-Mg alloy was developed. It is based on the use of the inorganic ion-exchanger, polyantimonic acid, as a removing agent for both $^{24}\text{Na}$ and $^{56}\text{Mn}$. The final preparations were free of all $\gamma$-rays emitting contaminants but contained some tritium. The redetermination of the half-life of $^{28}\text{Mg}$ resulted in $20.915 \pm 0.009$ h.

INTRODUCTION

This section deals with the production of $^{28}\text{Mg}$ via the thermal neutron route based on two consecutive nuclear reactions proceeding in a $^6\text{Li}$- and $^{26}\text{Mg}$-containing target (Li-Mg alloy) exposed to thermal neutrons in a nuclear reactor: $^6\text{Li}(n,t)^4\text{He}$ and $^{26}\text{Mg}(t,p)^{28}\text{Mg}$. The $^{28}\text{Mg}$ produced in this way is not free from other radionuclides. Excluding relatively short-lived nuclides, where decay is virtually complete within one h after the end of irradiation, the following contaminants will remain [Rob74]: (i) tritium from $^6\text{Li}(n,\alpha)^3\text{H}$ reaction, (ii) $^{18}\text{F}$ from the $^{16}\text{O}(t,n)^{18}\text{F}$ reaction with oxygen present as impurity in the alloy and/or in the oxide film on the alloy foil, (iii) $^{24}\text{Na}$ from the $^{25}\text{Mg}(t,\alpha)^{24}\text{Na}$ and $^{24}\text{Mg}(n,p)^{24}\text{Na}$ and from $(n,\gamma)$-activation of any sodium impurities and (iv) contaminants resulting from the $(n,\gamma)$-activation of other possible impurities in the target material (for example $^{56}\text{Mn}$ from manganese). Various radiochemical separations of $^{28}\text{Mg}$ from irradiated Li-Mg alloys are known. The separation procedures typically comprise (i) dissolution of the irradiated Li-Mg alloy, (ii) precipitation of (some) impurities with $\text{H}_2\text{S}$ [Iwe53; Mel57], (iii) single or repeated precipitation of $\text{Mg(OH)}_2$ [Iwe53; Mel57; Sta63; Sta64; Tör65], (iv) dissolution of $\text{Mg(OH)}_2$ and removal of some contaminants with alumina and an anion exchange resin [Sta63; Sta64; Tör65].
and finally (v) recovery of $^{28}\text{Mg}$ from the eluate by repeated evaporation to dryness [Sta63; Sta64]. Although some of these steps have been omitted occasionally, the hitherto published separations are still rather complex and time-consuming.

The objective of this investigation was to develop a more compact radiochemical separation of $^{28}\text{Mg}$ from thermal neutron irradiated Li-Mg alloy that yields preparations free of all $\gamma$-rays emitting radionuclides but $^{28}\text{Mg}$ and its short-lived decay product $^{28}\text{Al}$ (half-life = 2.246 min). The central novelty of the proposed separation lies in the use of polyantimonionic acid (an inorganic ion-exchanger) a removing agent for both $^{24}\text{Na}$ [Gir68] and some other contaminants (for example $^{56}\text{Mn}$).

The final product, $^{28}\text{Mg}^{2+}$, was intended for the use as radiotracer for magnesium in metabolic studies (in fish) which occasionally may involve lengthy experiments and thus significant decay corrections. As the half-life of $^{28}\text{Mg}$ is still open to some doubt (see Table 2.1) a redetermination of its half-life becomes imperative.

MATERIALS AND METHODS

Production of $^{28}\text{Mg}^{2+}$

A 70 : 30 (weight)% Li-Mg alloy made up of elements of natural isotopic composition was used as the target material. The respective atomic abundances of the relevant isotopes being 7.5 (weight)% $^{6}\text{Li}$ and 11.01 (weight)% $^{26}\text{Mg}$. Typically a 20 - 60 mg weighing strip of alloy foil (thickness ≤ 1 mm) was cleaned in ethyl acetate, placed in a quartz capsule which was first evacuated, then filled with helium to prevent alloy components reacting with air components and to facilitate the dissipation of heat resulting mainly from the exoergic $^{6}\text{Li}(n,\alpha)^{4}\text{He}$ reaction and finally sealed using a $\text{H}_2$-$\text{O}_2$ burner [Sta64].

The capsule was put in an aluminium irradiation container and then irradiated in a nuclear reactor with a thermal neutron flux of $1.3\cdot10^{17}$ m\(^{-2}\).s\(^{-1}\) during 36 h and subsequently "cooled" for 4 h to allow for a complete decay of $^{28}\text{Al}$ formed in the co-irradiated capsule-container. Next the quartz capsule was cut open and the alloy strip transferred to an open, short (70 mm) but wide (35 mm in diameter) test-tube like vessel provided with a magnetic stirring bar. The vessel was constantly flushed with helium to
prevent flashing during the subsequent dissolution step and it was immersed in an ice-bath to prevent overheating and flashing during the dissolution step.

![Diagram of radiochemical separation scheme](image)

Fig. 2.3.
Radiochemical separation scheme for neutron irradiated Li-Mg alloys to MgCl₂ containing $^{28}$Mg.

Radiochemical separation

A preliminary $\gamma$-spectrometric analysis with a Ge(Li)-detector based $\gamma$-ray spectrometer of the same alloy irradiated for a shorter period of time (2 h in thermal neutron flux of $4.5 \times 10^{16}$ m$^{-2}$s$^{-1}$ followed by 5.6 h "cooling") revealed no other $\gamma$-rays emitting contaminants but $^{18}$F, $^{24}$Na and $^{56}$Mn. Hence, the primary aim of the separation procedure to be developed was the removal of these radionuclides.

The alloy strips were dissolved by slow addition of 1 - 1.5 ml 8 mol.l$^{-1}$ aqueous solution of HNO$_3$. Following dissolution, the vessel was immersed into an oil-bath, preheated to 120 °C and the solution was evaporated to dryness under constant stirring with a magnetic stirrer. The relatively high temperature facilitates the volatilization of last traces of water and acid. The vessel was then taken out of the oil-bath and the dry residue dissolved in 2.5 - 3 ml 0.1 mol.l$^{-1}$ KH phthalate, HCl buffer of pH = 3.5 (the buffer contains 50 ml 0.1 mol.l$^{-1}$ KH phthalate and 8.2 ml 0.1 mol.l$^{-1}$ HCl, diluted to 100 ml [Per74]). The solution obtained was poured into a glass column, inner diameter 9 mm, filled with 1.25 g (dry weight) polyantimonic acid ion exchanger Polyan HT 0.5 - 0.1 mm (Nimbuchem SA) previously washed five times with pH = 3.5 buffer and preconditioned
in the column with the same buffer. Additional 3 ml buffer were used to wash the (dissolution) vessel and the resulting solution was poured into the column as well. Both effluents were collected in a 15 ml centrifuge tube. Under constant stirring with a glass stirring rod about 4 ml 2 mol l\(^{-1}\) aqueous NaOH was added to the collected effluents. The end pH value should be higher than 12 in order to achieve a complete precipitation of Mg(OH)\(_2\). The precipitate was separated from the liquid by 10 min centrifugation at 1750 g. After discarding the supernatant, the Mg(OH)\(_2\) was dissolved in 1 ml 8 mol l\(^{-1}\) aqueous HCl and the solution was poured into a clean (dissolution) vessel provided with a magnetic stirring bar. The vessel was immersed into an oil-bath preheated to 120 °C and the solution was evaporated to dryness under constant stirring. The vessel was taken out of the oil-bath and the residue dissolved in 2 ml demineralized water. A scheme of this radiochemical separation is given in Fig. 2.3.

Product characterization

\(^{28}\text{Mg}\) decays to \(^{28}\text{Al}\) by emitting \(\beta^+\)-particles of \(E_{\text{max}} = 0.418\) (95\%) and 0.459 (5\%) MeV and \(\gamma\)-rays of 0.0306 (95\%), 0.4006 (36\%), 0.9417 (36\%), 1.3422 (54\%), 1.3728 (5\%) and 1.5894 (5\%) MeV. \(^{28}\text{Al}\) decays to stable \(^{28}\text{Si}\) by emitting \(\beta^-\)-particles of \(E_{\text{max}} = 2.878\) MeV (100\%) and \(\gamma\)-rays of 1.77870 MeV (100\%) [Led78]. A decay scheme of \(^{28}\text{Mg}\) and \(^{28}\text{Al}\) is given in Figure 2.4.

Both the radionuclidic analysis pertaining to the \(\gamma\)-rays emitting radionuclides and the absolute activity determination of the final \(^{28}\text{Mg}\)-containing solution have been carried out by \(\gamma\)-ray spectroscopy using an 80.1 cm\(^3\) Ge(Li) detector (Ortec) and associated electronics in conjunction with a computer (PDP 11/73, Digital Equipment Cooperation). A certified mixed radionuclide \(\gamma\)-ray reference source (Amersham) was used for both the energy and the absolute activity calibrations.

The irradiations with a time integrated neutron flux of \(1.7 \times 10^{22}\) m\(^{-2}\) yield 0.28 GBq \(^3\text{H}\) per mg \(^6\text{Li}\) (calculated value). The half-life of \(^3\text{H}\) is 12.346 year and it emits \(\beta^+\)-particles of \(E_{\text{max}} = 0.018\) MeV [Led78]. The quantity of tritium in the final solution was determined by liquid scintillation counting (Tri-Carb 4000 Series, Packard) of \(\beta^-\)radiation in a solution aliquot to which a proper volume of scintillation liquid was added. Such a counting was carried out at least 9 days after the irradiation (to allow for a complete decay of \(^{28}\text{Mg}\)).
The magnesium concentration in the final solution was determined in an aliquot of it by spectrophotometric titration with an EDTA solution using an automatic titration system (E 636 Titroprocessor including a E 616 Photometer, Metrohm). Prior to a titration the solution was made alkaline with ammonium and an "Indikator-Puffertablette" (Art. 8430, Merck) was added to the sample to be titrated. The chemical yield was calculated by relating the magnesium quantity as determined for the final solution with that in the target. Similarly, the specific activity of $^{28}\text{Mg}$-preparations
(expressed as Bq mol\(^{-1}\) at the end of the irradiation) was calculated by dividing \(^{28}\text{Mg}\)-concentration in the final solution by the magnesium concentration in this solution.

Determination of the half-life of \(^{28}\text{Mg}\)

The half-life determinations were made using two different methods for measuring the decrease of the intensity of the emitted radiation, namely \(\gamma\)-ray spectroscopy and Cerenkov counting for \(\gamma\)-rays and \(\beta^-\)-particles respectively.

The system used to measure \(\gamma\)-rays was composed of a 61 cm\(^3\) Ge(Li) detector (APY45A/N, Philips) and associated electronics in connection with a computer (PDP 11/44, Digital Equipment Cooperation). A 50 Hz reference-pulser (Model 1407, Canberra) was coupled to the preamplifier to provide signals to be utilized for the determination of counting losses (due to pile-up rejection, dead-time etc.). Namely, the ratio of the number \(p\) of pulser signals stored to the number \(G\) of pulser signals generated is identical to the ratio of the recorded number \(n\) of nuclear events to the corresponding number \(N\) of nuclear events occurring in the detector [BoI70]. Thus \(N = G(n/p)\). The \(\gamma\)-ray spectra were measured on samples withdrawn from the final solution and initially containing about 9 kBq \(^{28}\text{Mg}\). The samples were counted for 2000 - 4000 s and their decay was followed during 45 - 120 h and involving 25 to 80 spectra. Computer analysis of the spectra resulted in peak area values (n) for all recorded peaks. The data pertaining to 0.401 and 1.779 MeV \(\gamma\)-rays were corrected for counting losses and plotted as \(\ln N(t)\) versus time (t). The method of least squares was used to calculate the slopes of the obtained straight lines and the standard deviations of the slope data. Finally these data were transcribed into the corresponding half-life values and their standard deviations.

The counting of Cerenkov radiation resulting mainly from the 2.878 MeV \(\beta^-\)-particles of \(^{28}\text{Al}\) (the presence of tritium does not contribute to the Cerenkov counting) was carried out by a liquid scintillation counter (see above). The counting samples (10 ml) containing about 250 Bq \(^{28}\text{Mg}\) per sample were prepared by adding water to the aliquots of the final solution. The samples were counted for 600 - 900 s during 25 - 60 h (100 - 150 data points) and the half-life values calculated as described above for \(\gamma\)-rays except for the correction for counting losses which was not necessary.
RESULTS AND DISCUSSION

![Graph showing energy vs. counts per channel][1]

Fig. 2.5.
The γ-ray spectrum of the final solution measured with a Ge(Li) detector 8 h after the end of irradiation. The peaks numbered 1, 4, 6, 7 and 8 represent the respective 0.4006, 0.9417, 1.3422, 1.3728 and 1.5894 MeV γ-rays of $^{28}\text{Mg}$ [Led78]. The peak number 9 represents the 1.77870 MeV γ-ray of $^{28}\text{Al}$ (decay product of $^{28}\text{Mg}$) [Led78]. Peak numbered 2 represents the 0.511 MeV annihilation radiation (due to the pair formation). The remaining peaks, numbered 3 and 5 are the respective two-escape (0.757 = 1.77870 - 1.022 MeV) and one-escape peaks (1.268 = 1.77870 - 0.511 MeV) of the 1.77870 MeV γ-ray of $^{28}\text{Al}$.

It takes 1.5 to 2 h to separate $^{28}\text{Mg}$ from the irradiated Li-Mg alloy. The average
chemical yield ± its standard deviation was 87 ± 13% for 9 separations. The γ-ray spectra of the final preparations (Fig. 2.5) showed no contamination with either $^{18}$F, $^{24}$Na and $^{56}$Mn or any other γ-rays emitting radionuclide. It is worth-while to mention that, with regard to the retention of $^{56}$Mn by Polyam HT ion exchanger, the pH = 3.5 is the optimal one. At lower and higher pH's the removal of manganese is not complete. The average specific activity ± its standard deviation of six $^{28}$Mg batches was 220 ± 50 MBq.mol$^{-1}$ a value coherent with a literature value of 240 MBq.mol$^{-1}$ [Sta58].

In the final solution resulting from a 60 mg alloy target a tritium activity in the order of 50 kBq was measured. Note that irradiation of 60 mg alloy leads to the formation of about 9 MBq tritium.

The half-life values obtained with 4 different preparations and by using Ge(Li) detector ranged 20.93 to 21.08 h and 20.79 to 21.06 h for the 0.401 and the 1.779 MeV peaks respectively. The standard deviation of a single measurement was 0.01 to 0.02 h and 0.02 to 0.06 h for the first and the last data set. The respective average values and their standard deviations were 21.00 ± 0.06 and 20.89 ± 0.12 h and the overall average was 20.94 ± 0.10 h (relative standard deviation 0.48 %). The Cerenkov counting based half-life values (Fig. 2.6) obtained with two different preparations ranged 20.908 to 20.921 h with respective standard deviations 0.007 and 0.010 h. The average value is 20.915 ± 0.009 h (relative standard deviation 0.043%). Because of the minute relative standard deviation in the case of the Cerenkov counting the last value i.e. 20.915 ± 0.009 h is considered to be the half-life of $^{28}$Mg. As illustrated in Table 2.1 this value appears to be in good agreement with probably the last (in 1974) actually measured half-life value [Rot74] and with some of the later "compiled" values of unstated provenance.

A determination of the half-life of $^{24}$Na using the Cerenkov counting method resulted in a value of 15.029 ± 0.004 h, a value almost identical with a precise literature value of 15.030 ± 0.003 h [Eme72]. This confirms the adequacy of the present measurement of the half-life of $^{28}$Mg.

SUPPLEMENT

Some radiotracer experiments on magnesium transport require $^{28}$Mg$^{2+}$ of a
Fig. 2.6.
Plot of the natural logarithm of the counting rate versus time data obtained by liquid scintillation counting of a solution containing about 250 Bq $^{28}$Mg (estimated value). The straight line represents the line of best fit through the data points (correlation coefficient $r = -0.99998$). Its slope is $-0.033153 \text{ h}^{-1}$ and the corresponding half-life value 20.908 h.

Specific activity higher than the specific activity resulting from thermal neutron irradiation of Li-Mg alloys made up of elements of natural isotopic composition. The corresponding atomic abundances of the relevant isotopes are 7.5% $^6$Li and 11.01% $^{26}$Mg. In order to achieve a specific activity higher than the previously obtained 220 ± 50 MBq.mol$^{-1}$, the following approaches can be considered: (i) irradiation of the alloy in a higher neutron flux, (ii) irradiation of the alloy for a longer time period, (iii) enhancement of the Li:Mg ratio in the alloy or (iv) application of an alloy enriched on
Table 2.1
Chronological overview of $^{28}$Mg half-life data.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Year</th>
<th>Type*</th>
<th>Half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[She53]</td>
<td>1953</td>
<td>P</td>
<td>21.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>21.3 ± 0.2</td>
</tr>
<tr>
<td>[Iwe53]</td>
<td>1953</td>
<td>P</td>
<td>21.85 ± 0.32</td>
</tr>
<tr>
<td>[Wap53]</td>
<td>1953</td>
<td>P</td>
<td>21.4 ± 0.6</td>
</tr>
<tr>
<td>[Mar53]</td>
<td>1953</td>
<td>P</td>
<td>20.8 ± 0.5</td>
</tr>
<tr>
<td>[Sta58]</td>
<td>1958</td>
<td>P</td>
<td>21.3</td>
</tr>
<tr>
<td>[Wei63]</td>
<td>1963</td>
<td>P</td>
<td>20.88 ± 0.06</td>
</tr>
<tr>
<td>[Mor67]</td>
<td>1967</td>
<td>P</td>
<td>21.3 ± 0.2</td>
</tr>
<tr>
<td>[Rot74]</td>
<td>1974</td>
<td>P</td>
<td>20.93 ± 0.04</td>
</tr>
<tr>
<td>[Mar76]</td>
<td>1976</td>
<td>S</td>
<td>20.91 ± 0.03</td>
</tr>
<tr>
<td>[Led78]</td>
<td>1978</td>
<td>S</td>
<td>21.0</td>
</tr>
<tr>
<td>[Erd79]</td>
<td>1979</td>
<td>S</td>
<td>21.3</td>
</tr>
<tr>
<td>[Int83]</td>
<td>1983</td>
<td>S</td>
<td>20.91</td>
</tr>
<tr>
<td>[Tul87]</td>
<td>1987</td>
<td>S</td>
<td>20.90 ± 0.03</td>
</tr>
<tr>
<td>[Waa88]</td>
<td>1988</td>
<td>S</td>
<td>21.0</td>
</tr>
<tr>
<td>Present work</td>
<td>1990</td>
<td>P</td>
<td>20.915 ± 0.009</td>
</tr>
</tbody>
</table>

*P: Primary publication (original data), S: Secondary publication (data compilation).

the pertinent isotopes, $^6$Li and/or $^{26}$Mg. Option (i) is not possible in the IRI reactor. Option (ii) can only give a limited increase of the specific activity (e.g. an irradiation for 72 h instead of 36 h results in a 30% increase). Option (iii) will possibly result in an enhancement of the specific activity, but a reduction of the total activity produced. Option (iv) seem to be the most favourable if feasible. Although the use of an alloy containing $^6$Li and $^{26}$Mg only will yield the highest specific activity, the practical reasons led us to the choice of an alloy consisting of lithium, highly enriched on $^6$Li, and magnesium of natural isotopic composition. Namely, $^{26}$Mg was much more expensive than $^6$Li and it was not readily available in metallic form.

To obtain $^{28}$Mg of the mentioned higher specific activity, about 0.6 mm thick strips weighing about 60 mg, of a 75 : 25 % Li-Mg alloy with lithium enriched on $^6$Li to 94.84 % and magnesium of natural isotopic composition (Oak Ridge National Laboratory) were used. These strips were irradiated in the thermal neutron flux of $1.3 \times 10^{17}$ m$^{-2}$.s$^{-1}$ during 36 h and "cooled" for 4 h. The radiochemical impurities, namely
$^3\text{H}$, $^{24}\text{Na}$ and $^{56}\text{Mn}$ found in the target after irradiation were removed by a radiochemical separation analogous to the one described above but extended with a tritium recovery procedure. The irradiated alloy contained some $^{198}\text{Au}$ as well. An anion-exchange resin ($2\times 8$ Cl$^-$ form, Dowex) was used to remove this interfering radionuclide from the solution obtained by dissolution of the irradiated alloy in hydrochloric acid, evaporation to dryness and redissolution of the residue in the pH = 3.5 buffer.

The $\gamma$-spectrometric analysis (Ge(Li) detector) of the final product solution revealed no $\gamma$-rays emitting radionuclidic contaminants. However, the solution contained about 1 kBq tritium. The processing time for the irradiated alloy was 3 h at most. Specific activities of $^{28}\text{Mg}$ of about $1.3 \pm 0.3$ GBq mol$^{-1}$ were achieved, thus exceeding those obtained with the isotopically non-enriched alloy by a factor of 6 ± 2. Possible explanations for the enhancement of the specific activity by only a factor six, as the $^6\text{Li}$ abundance was increased by a factor of about 13, may be: inhomogeneity in the composition of the alloy, presence of oxygen and/or nitrogen on the surface of the alloy and the thermal neutron flux depression within the alloy strip. Particularly the latter is expected to be of predominant influence due to the combined effect of the very high thermal neutron capture cross-section of $^6\text{Li}$ and the thickness of the alloy foil. A tenfold increase of the specific activity was found by Stang et al. [Sta58] when using a 0.254 mm thick foil of an 75 : 25 % Li-Mg alloy in which lithium of natural isotopic composition was substituted for 96% $^6\text{Li}$. 
CHAPTER 3

MAGNESIUM DISTRIBUTION

This chapter describes research on magnesium distribution and magnesium inventory of carp and freshwater tilapia by means of instrumental neutron activation analysis. A comparison with data from the literature on magnesium concentrations in various tissues of freshwater teleosts is presented.

§ 3.1

INTRODUCTION

The transport of magnesium in transport epithelia such as gut, kidney and gills, which are involved in the magnesium metabolism of fish, is still poorly understood. For an understanding of magnesium metabolism (and homeostasis) in fish it is essential that information is available on the quantities of magnesium in different parts of the fish. Only a few studies have evaluated the magnesium distribution in freshwater teleosts. Essentially no studies on the total magnesium inventory of fish were available. The following studies on carp and tilapia report on the magnesium concentrations in tissues and on the total magnesium inventory of these fish. A short literature review of the magnesium concentrations in blood plasma and tissues of freshwater teleosts is given in § 3.3.
§ 3.2

MAGNESIUM DISTRIBUTION IN CARP

ABSTRACT
Neutron activation analysis and colorimetric methods were used to measure the magnesium distribution in non-growing carp weighing between 40 and 80 g. The magnesium concentration in the soft tissues was around 53 mmol per kg dry weight and in the hard (bony) tissues around 85 mmol per kg dry weight. About 53% of the magnesium was confined to soft tissues. The overall magnesium concentration in carp amounts to 13 μmol·g⁻¹.

INTRODUCTION

For studies on magnesium transport by compartmental analysis from fish to water and *vice versa*, information on the magnesium distribution within the fish is essential. At least the slowly exchanging and fast exchanging magnesium pools should be distinguished. For this reason the tissues of carp were divided in bony or hard tissues and in soft tissues. The underlying idea was that magnesium exchange is supposed to be slow in bony material and more rapid in soft tissues. The magnesium concentrations in both tissues were determined by instrumental neutron activation analysis [§ 2.1].

MATERIALS AND METHODS

Fish
Carp weighing from 42 to 83 g were obtained from laboratory stock. Fish (up to 10) were held in 120 l glass aquaria filled with 80 l artificial freshwater (initially) containing per litre 0.5 mmol NaCl, 0.06 mmol KCl, 0.2 mmol MgSO₄ and 0.2 mmol CaCl₂. The pH of water was maintained at 7.5 by means of an end-point titration system.
(ETS822, Radiometer) adding a sodium hydroxide solution. The water was kept at 24°C and filtered both by recirculation through a nylon-wool thermofilter (2113, Eheim) and continuously aerated. Twice a week one third of the aquarium content was replaced with freshly prepared water. The photoperiod was 12 h. The fish received daily six rations of dry fish food (Tetra) containing about 31 mmol.kg⁻¹ magnesium. The quantity of food was adjusted to match the maintenance level [Hui76].

Sampling

The quantities of magnesium in hard tissues, soft tissues and thus in the whole carp were assessed according to the following procedure. The fish were anaesthetized by placing them in 0.4 mmol.l⁻¹ solution of "MS-222" (Sigma) for 2 to 5 minutes, weighed and blood samples (mixed arterial and venous blood) of up to 2 ml were taken by puncture of the caudal vessels behind the anal fin using heparinized syringe fitted with a 23-Gauge needle. The fish were then killed by spinal transection. The blood was separated into cells and plasma by centrifugation at 9000 g for 3 minutes. A portion of the plasma was ultrafiltered using micro-collodion bags (SM 13202, Sartorius. Each carp was divided into hard (bones and scales) and soft (muscle and organs) tissue. The tissues were weighed, lyophilized and weighed again to assess the water content.

The magnesium concentrations in blood plasma and in its ultrafiltrate were determined colorimetrically (Diagnostic kit 595, Sigma) whereas the magnesium concentrations in the lyophilized tissues of carp were determined by instrumental neutron activation analysis [§ 2.2].

RESULTS AND DISCUSSION

The magnesium concentration in blood plasma of carp, Cₚ, amounts to 0.96 ± 0.06 mmol.l⁻¹ (mean value of 12 fish ± standard deviation). This result is close to 0.93 ± 0.05; 1.15 ± 0.16 and 0.94 ± 0.12 mmol.l⁻¹, reported for carp by Hunn [Hun72], Houston [Hou85] and Jensen [Jen90], respectively. The magnesium concentration in plasma ultrafiltrate was 0.61 ± 0.01 mmol.l⁻¹ (mean value of 4 fish ± standard deviation).
Table 3.1.
Magnesium distribution in carp. $W_f$ is the weight of the fish, $dW_h$ and $dW_s$ are the weights of respectively dry hard and dry soft tissue (all in g); $dC_h$, $dC_s$ and $C_f$ ($= Q_f/W_f$) are the magnesium concentrations in respectively dry hard tissue, dry soft tissue and (wet) fish (all in $\mu$mol.g$^{-1}$); $Q_h$ ($= dW_h \cdot dC_h$), $Q_s$ ($= dW_s \cdot dC_s$) and $Q_f$ ($= Q_h + Q_s$) are the magnesium quantities in respectively hard tissue, soft tissue and whole fish (all in $\mu$mol).

<table>
<thead>
<tr>
<th>$W_f$</th>
<th>$dW_h$</th>
<th>$dC_h$</th>
<th>$Q_h$</th>
<th>$dW_s$</th>
<th>$dC_s$</th>
<th>$Q_s$</th>
<th>$Q_f$</th>
<th>$Q_s/Q_f$</th>
<th>$C_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>42.3</td>
<td>3.23</td>
<td>87.7</td>
<td>284</td>
<td>5.39</td>
<td>49.1</td>
<td>265</td>
<td>549</td>
<td>0.48</td>
<td>13.0</td>
</tr>
<tr>
<td>62.2</td>
<td>4.24</td>
<td>77.1</td>
<td>327</td>
<td>8.02</td>
<td>47.8</td>
<td>383</td>
<td>710</td>
<td>0.54</td>
<td>11.4</td>
</tr>
<tr>
<td>72.4</td>
<td>5.64</td>
<td>81.6</td>
<td>460</td>
<td>9.33</td>
<td>57.8</td>
<td>539</td>
<td>999</td>
<td>0.54</td>
<td>13.8</td>
</tr>
<tr>
<td>83.1</td>
<td>5.63</td>
<td>92.9</td>
<td>523</td>
<td>11.13</td>
<td>57.9</td>
<td>644</td>
<td>1167</td>
<td>0.55</td>
<td>14.0</td>
</tr>
<tr>
<td>Mean Value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.53</td>
<td>13.0</td>
</tr>
<tr>
<td>Standard deviation of the mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The amounts of dry hard and dry soft tissues (including blood) per fish as well as the magnesium concentrations in these tissues are given in Table 3.1. The quantities of magnesium in hard and soft tissue were calculated by multiplying the concentrations with the respective tissue weights. The sum of these quantities represents the quantity of magnesium in whole fish. The results in Table 3.1 show that for the carp, weighing 42.3 to 83.1 g, about 53% of the fish magnesium is confined to soft tissue and that the quantity of magnesium in these fish is approximately proportional to their weights (13 $\mu$mol.g$^{-1}$). Tacon et al. [Tac84] found in carp (mean weight 55 g) a somewhat higher value of 17.3 $\mu$mol.g$^{-1}$.

A more extended study on the magnesium distribution in tilapia is described in § 3.3. In that paragraph, also a comparison of literature data on magnesium concentrations in plasma and tissues of freshwater teleosts is given.
§ 3.3

MAGNESIUM DISTRIBUTION IN FRESHWATER TILAPIA.

ABSTRACT
The magnesium and calcium quantities in several tissues of tilapia were determined by neutron activation analysis or colorimetric methods. Magnesium concentrations on dry weight basis ranged from 18 mmol kg\(^{-1}\) (blood plasma) to 140 mmol kg\(^{-1}\) (skeletal bone). Calcium concentrations ranged from 13 mmol kg\(^{-1}\) (soft tissue) to 4400 mmol kg\(^{-1}\) (dermal bone). In tilapia, the size of the body magnesium pool as a fraction of the total body weight changes, and care should be taken to extrapolate relations between the whole body magnesium quantity and body weight.

INTRODUCTION

Except for some blood plasma values [Wen83], no reports on tissue magnesium concentrations of freshwater tilapia are known to us. For insight in the magnesium metabolism of tilapia the distribution of magnesium and the magnesium inventory of the tilapia is of importance. As the magnesium concentration in tissues tends to be inversely related to the calcium concentrations [Geo75; Ise84], also the calcium concentration was determined. Furthermore a comparison with literature data on magnesium concentrations in blood plasma and tissues of freshwater teleosts is made.

MATERIALS AND METHODS

Fish

Male tilapia ranging in body weight from 25 to 52 g were obtained from laboratory stock. The fish were kept in fresh water which contained 0.2 mmol l\(^{-1}\) MgSO\(_4\),
0.2 mmol.l⁻¹ CaCl₂, 0.5 mmol.l⁻¹ NaCl and 0.06 mmol.l⁻¹ KCl. The water was maintained at pH 7.5 through the addition of Tris-HCl buffer (2.5 mmol.l⁻¹). The water was constantly aerated and kept at 28°C. The light regime was 12 h light and 12 h dark. The fish received six rations of tropical fish food (Tetra) per day by means of an automated food dispenser.

Sampling

The fish were anaesthetized with 0.4 mmol.l⁻¹ "MS-222" (Sigma) and weighed. Mixed arterial and venous blood was collected by puncture of the caudal vessels behind the anal fin using an ammonium heparin-rinsed tuberculin syringe fitted with a 23-Gauge needle. The blood was separated into plasma and cells by centrifugation at 9000 g for 3 min. Part of the blood plasma was ultrafiltered using micro-collodion bags (SM 13202; Sartorius). Samples were stored at -20°C. The scales, the testis, the intestinal tract including the stomach, the kidneys, the gills and the brain were collected separately; the branchial epithelium was scraped from the gill arches. After 1 minute of pressure cooking [Fle74] muscle was collected and the bone was divided into dermal, skeletal and head-region scalar bone. The remaining tissues were designated as "rest soft tissue". All fractionated material was weighed, lyophilized (except blood plasma) and weighed again.

Analytical methods

The determination of magnesium and calcium in the tissues was performed by instrumental neutron activation analysis (INAA) [§ 2.2]. The magnesium concentrations in the blood plasma and ultrafiltered blood plasma were determined colorimetrically with a magnesium kit (diagnostic kit no 595; Sigma) based on measurement of a coloured complex of magnesium and calmagite at 520 nm. The calcium concentrations in blood plasma and ultrafiltered blood were also colorimetrically determined with a calcium kit (diagnostic kit no 587; Sigma).

Statistics

Data are presented as mean values ± standard deviation (SD). The data were statistically evaluated by the Mann-Whitney U-test (one-tailed). Linear regression analysis was based on the least squares method; in a number of cases after natural
logarithmic transformation of the data. Statistical significance was accepted at the 1% level.

RESULTS

Size of the major parts of the body

The size of the three major parts of the body (scales, bone and soft tissue) correlate with the total dry weight of the fish \((5.7 < \text{d} W_f < 12.8 \text{ g})\) as follows:

\[
\text{d} W_c = 0.275 (\text{d} W_f)^{0.455} \quad [n=6] (1)
\]

\[
\text{d} W_b = 0.592 (\text{d} W_f)^{0.891} \quad [n=6] (2)
\]

\[
\text{d} W_s = 0.282 (\text{d} W_f)^{1.21} \quad [n=6] (3)
\]

where \(\text{d} W_f\) = total dry weight of the fish (g), \(\text{d} W_c\) = dry weight of the scales (g), \(\text{d} W_b\) = dry weight of the total bone (g) and \(\text{d} W_s\) = dry weight of the total soft tissues (g). The correlation coefficients \((r)\) for the linear regression of \(\ln \text{d} W_c\), \(\ln \text{d} W_b\) and \(\ln \text{d} W_s\) values on \(\ln \text{d} W_f\) values were 0.976, 0.994 and 0.997 respectively. Since the sizes of the body parts depends on the total body weight in a particular manner (cf. the exponents in equations 1, 2 and 3), the contribution of each body part to the total fish is a nonlinear function of the weight of the fish.

The mean body water content of the experimental fish was determined to be 74.7 ± 2.5%.

Magnesium and calcium concentration in the tissues

The concentration of magnesium and calcium in the various tissues showed no correlation with the body dry weight of the fish. For this reason, the data obtained for all fish analyzed were pooled per element and per tissue or body part. The average concentrations of magnesium and calcium are presented in Table 3.2. The magnesium
Table 3.2.
Magnesium and calcium concentrations (± standard deviation) in various tissues of freshwater tilapia. The number of observations are in brackets; \( dW \) stands for dry weight (g).

<table>
<thead>
<tr>
<th>tissue</th>
<th>concentration (mmol.kg(^{-1}) ( dW ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>magnesium</td>
</tr>
<tr>
<td>muscle</td>
<td>61 ± 8 (5)</td>
</tr>
<tr>
<td>kidney</td>
<td>44 ± 9 (5)</td>
</tr>
<tr>
<td>liver</td>
<td>42 ± 3 (3)</td>
</tr>
<tr>
<td>brain</td>
<td>27 ± 7 (5)</td>
</tr>
<tr>
<td>testis</td>
<td>37 ± 12 (6)</td>
</tr>
<tr>
<td>gills</td>
<td>57 ± 10 (5)</td>
</tr>
<tr>
<td>gallbladder</td>
<td>29 ± 2 (3)</td>
</tr>
<tr>
<td>packed cells</td>
<td>28 ± 5 (4)</td>
</tr>
<tr>
<td>intestinal tract</td>
<td>45 ± 15 (6)</td>
</tr>
<tr>
<td>rest soft tissue</td>
<td>60 ± 11 (4)</td>
</tr>
<tr>
<td><strong>Total soft tissue</strong></td>
<td><strong>60</strong></td>
</tr>
<tr>
<td>dermal bone</td>
<td>138 ± 36 (6)</td>
</tr>
<tr>
<td>skeletal bone</td>
<td>140 ± 19 (6)</td>
</tr>
<tr>
<td>scalar bone</td>
<td>66 ± 11 (6)</td>
</tr>
<tr>
<td><strong>Total bone</strong></td>
<td><strong>122</strong></td>
</tr>
<tr>
<td>scales</td>
<td>115 ± 18 (6)</td>
</tr>
<tr>
<td><strong>Total scales</strong></td>
<td><strong>115</strong></td>
</tr>
</tbody>
</table>

\(*\) Literature data of Flik et al. [Fli86a].

Concentration in blood plasma and ultrafiltered blood plasma (n=6) amounted to 1.12 ± 0.14 mmol.l\(^{-1}\) (which is equivalent to 18 mmol.kg\(^{-1}\) dry material) and 0.51 ± 0.11 mmol.l\(^{-1}\) respectively. The calcium concentration in blood plasma and ultrafiltered blood plasma (n=6) was 3.31 ± 0.04 and 1.86 ± 0.11 mmol.l\(^{-1}\) respectively.

Magnesium and calcium inventory

In three fish weighing about 8 g, magnesium and calcium inventories were calculated as the product of the mean concentration and the weight of the tissues. The inventories of magnesium and calcium are given in Table 3.3 and in Table 3.4, respectively. The calcium concentration in some soft tissues samples was below the INAA
Table 3.3. Magnesium inventory of tilapia with a dry weight of circa 8 g. Mean weight of tissues of freshwater tilapia (n=3) and the magnesium quantities of these tissues.

<table>
<thead>
<tr>
<th>tissue</th>
<th>weight (g)</th>
<th>quantity (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>muscle</td>
<td>2.420</td>
<td>0.148</td>
</tr>
<tr>
<td>kidney</td>
<td>0.013</td>
<td>0.57·10^{-3}</td>
</tr>
<tr>
<td>liver</td>
<td>0.052</td>
<td>2.18·10^{-3}</td>
</tr>
<tr>
<td>brain</td>
<td>0.018</td>
<td>0.49·10^{-3}</td>
</tr>
<tr>
<td>testis</td>
<td>0.021</td>
<td>0.78·10^{-3}</td>
</tr>
<tr>
<td>gills</td>
<td>0.028</td>
<td>1.65·10^{-3}</td>
</tr>
<tr>
<td>gallbladder</td>
<td>0.021</td>
<td>0.61·10^{-3}</td>
</tr>
<tr>
<td>packed cells</td>
<td>0.034</td>
<td>0.95·10^{-3}</td>
</tr>
<tr>
<td>intestinal tract</td>
<td>0.157</td>
<td>7.07·10^{-3}</td>
</tr>
<tr>
<td>rest soft tissue</td>
<td>0.671</td>
<td>4.03·10^{-2}</td>
</tr>
<tr>
<td><strong>Total soft tissue</strong></td>
<td><strong>3.462</strong></td>
<td><strong>0.203</strong></td>
</tr>
<tr>
<td>dermal bone</td>
<td>1.508</td>
<td>0.208</td>
</tr>
<tr>
<td>skeletal bone</td>
<td>1.561</td>
<td>0.219</td>
</tr>
<tr>
<td>scalar bone</td>
<td>0.714</td>
<td>0.047</td>
</tr>
<tr>
<td><strong>Total bone</strong></td>
<td><strong>3.785</strong></td>
<td><strong>0.474</strong></td>
</tr>
<tr>
<td>scales</td>
<td>0.712</td>
<td>0.082</td>
</tr>
<tr>
<td><strong>Total scales</strong></td>
<td><strong>0.712</strong></td>
<td><strong>0.082</strong></td>
</tr>
<tr>
<td><strong>Total body</strong></td>
<td><strong>7.959</strong></td>
<td><strong>0.759</strong></td>
</tr>
</tbody>
</table>

detection limit; for this reason a value determined in earlier experiments [Fli86a] using the thymol-blue method [Gin72] has been used.

For a 8 gram tilapia the distribution of magnesium and calcium is as follows: Magnesium is present predominantly in the bone (62%) and the soft tissues (27%); a significant fraction (11%) is located in the scales. The picture for calcium is different. Although the largest fraction (82%) is also present in bone; the scales account for a substantial part (18%), and only a small fraction (less than 1%) of the calcium is located in the soft tissues.

Figure 3.1 shows the quantities of magnesium in the scales (Q_s), total soft tissues (Q_d) and total bone (Q_b) as a function of body weight. The quantities of the three body parts are calculated as the product of the calculated weight (according to equation 1,2
Table 3.4.
Calcium inventory of tilapia with a dry weight of circa 8 g.
Mean weight of tissues of freshwater tilapia (n=3) and the calcium quantities of these tissues.

<table>
<thead>
<tr>
<th>tissue</th>
<th>weight (g)</th>
<th>quantity (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>soft tissue</td>
<td>3.462</td>
<td>0.05*</td>
</tr>
<tr>
<td>bone</td>
<td>3.785</td>
<td>13.78</td>
</tr>
<tr>
<td>scale</td>
<td>0.712</td>
<td>2.95</td>
</tr>
<tr>
<td>Total body</td>
<td>7.959</td>
<td>16.78</td>
</tr>
</tbody>
</table>

*) Calculated using literature data of Flik et al. [Fli86a].

and 3) and the mean magnesium concentration in these body parts.

Whole body magnesium inventory

The quantity of magnesium in the whole fish, calculated from the whole body magnesium quantities of the experimental fish, can be described as follows:

\[ Q_f = 0.1163(dW_f)^{0.896} \quad [n=6] \]

in which \( Q_f \) = total magnesium quantity of the fish (mmol). The correlation coefficient (r) for the linear regression of \( \ln Q_f \) values on \( \ln dW_f \) values was 0.961. The relationship between \( Q_f \) and \( dW_f \) is shown in Figure 3.1.

As can be seen from equation 4, the magnesium inventory is not linearly related to the fish weight; thus the overall magnesium concentration is a function of the fish weight. For instance, the average magnesium concentration calculated by means of equation 4 for a 6 g and a 12 g dry weight tilapia is 97 mmol.kg\(^{-1}\) and 90 mmol.kg\(^{-1}\), respectively.
Fig 3.1.
Quantities of magnesium in the scales, soft tissues, total bone and whole body as function of dry weight of tilapia. The magnesium quantities (mmol) in the whole fish, scales, total soft tissue and total bone is described by $Q_f = 0.1163(d\ W_f)^{0.896}$, $Q_c = 0.275(d\ W_f)^{0.455}.10^{-3}.115$, $Q_s = 0.272(d\ W_f)^{1.23}.10^{-3}.60$, and $Q_b = 0.595(d\ W_f)^{0.885}.10^{-3}.122$, respectively. The symbols indicate the actually measured inventories of the six experimental fish.

DISCUSSION

Magnesium concentrations in the tissues

There are substantial differences in magnesium concentration between the various
tissues. The white muscle of tilapia forms the major part of the total soft tissue magnesium pool. The muscle has a significantly higher magnesium concentration than some smaller body parts from the total soft tissue pool (plasma, packed cells, testis, brain, liver and kidney). The higher magnesium concentration in the muscle samples is in line with their higher Mg-ATP dependent myosin quantities. The magnesium concentration in the muscle may reflect the intracellular concentration. The value may be an underestimation, since no correction for extracellular space was applied. Assuming 7.54% extracellular space as reported for tilapia [Ass79], we come to a magnesium concentration of 66 mmol per kg dry weight for intracellular magnesium. Magnesium can be stored intracellularly in organelles such as mitochondria [Ebe80].

Similar to calcium, magnesium in blood plasma exists in three fractions: (i) free (ionic), (ii) complexed with citrate, hydrogencarbonate or phosphate, and (iii) protein-bound, non-ultrafiltrable magnesium. The former two fractions reflect the ultrafiltrable magnesium [Spe81]. The free or ionic magnesium is thought to represent the physiologically important fraction [Alv87].

The ultrafiltrable and the total magnesium concentrations in blood plasma are low, compared to the concentrations present in other tissues. However the concentration of free Mg$^{2+}$ in plasma is of the same order as in another fish and in terrestrial vertebrates. In rainbow trout (Oncorhynchus mykiss, formerly Salmo gairdneri) a free magnesium concentration of 0.57 mmol.l$^{-1}$ is found [Bjö85]. In horse plasma the free magnesium is 0.5 mmol.l$^{-1}$ [Mag87]. Speich et al. [Spe81] found an ultrafiltrable magnesium concentration of 0.593 mmol.l$^{-1}$ in human blood plasma, where 0.544 mmol.l$^{-1}$ is ionic and 0.049 mmol.l$^{-1}$ complexed. Gunn & Burns [Gun87] report an ultrafiltrable magnesium concentration in human blood plasma of 0.575 mmol.l$^{-1}$.

The magnesium concentrations of the total bone and scales are somewhat higher than in the soft tissues. Notably, scalar bone shows a significantly lower magnesium concentration than dermal and skeletal bone. It has been suggested that in higher vertebrates most of the skeletal magnesium is adsorbed to the mineral phase on the surfaces of apatite crystals [Ebe80; Ana81]. In mammals the incorporation of Mg$^{2+}$ ions into the calcium phosphate component of bone increases the stability. The magnesium containing component is called whitlockite [Dri82]. For fish, little is known about the chemical form of magnesium and its incorporation into bone.
Comparison with literature data

Literature data on magnesium concentrations in tissues of freshwater teleosts are scarce and fragmentary. The muscle of tilapia has a magnesium concentration of 61 mmol per kg dry material which is equivalent to 14 mmol.kg\(^{-1}\) wet material. For perch (*Perca fluviatilis*) a wet weight muscle magnesium concentration of 15 mmol.kg\(^{-1}\) has been reported [Lut72a] and for rainbow trout 12 and 13 mmol.kg\(^{-1}\) [Kno81; Kno83].

The magnesium concentration in the kidney and liver of tilapia has a magnesium concentration of 44 and 41 mmol per kg dry weight, respectively, which is equivalent to 7 and 8 mmol per kg wet material, respectively. These values are similar to those reported for the kidney in rainbow trout, namely 6 and 7 mmol.kg\(^{-1}\) wet weight [Cow77; Kno81; Kno83], and for the liver in rainbow trout 7 mmol.kg\(^{-1}\) wet weight [Kno81] and in perch 14 mmol.kg\(^{-1}\) wet weight [Lut72a]. In the kidney of carp a magnesium concentration of 41 mmol.kg\(^{-1}\) dry weight was reported [Dab90].

Houston [Hou85] reported magnesium concentrations in the range of 5.4 to 10.4 mmol.l\(^{-1}\) cell water for packed cells in four species of teleosts. In tilapia a concentration equivalent to 9.6 mmol.l\(^{-1}\) cell water was found.

The magnesium concentrations in blood plasma in freshwater teleosts from this study and from the literature are presented in Table 3.5. The magnesium concentrations for plasma found in this study are in the same range as those reported in the literature for freshwater teleosts. Since the fish compared were kept under different conditions, it appears that external conditions such as water temperature and magnesium concentration do not substantially influence plasma magnesium concentration. The blood plasma magnesium concentration seems also independent of the total body weight. Nanba *et al.* [Nan87] reported that in the carp the magnesium concentration in the blood plasma remains constant throughout the year.

The data determined for total bone magnesium concentration are in good agreement with data of Lutz [Lut72b], who found a concentration of 124 mmol.kg\(^{-1}\) dry material in the total bone of the perch.

On the basis of the above data, we conclude that there is only little variation between the magnesium concentrations in particular tissues of various freshwater teleosts, and this suggests a magnesium homeostatic mechanism in freshwater teleosts.
Table 3.5.
Data compilation of magnesium concentrations in plasma of freshwater teleosts.

<table>
<thead>
<tr>
<th>species</th>
<th>body weight (g)</th>
<th>n</th>
<th>temp (°C)</th>
<th>[Mg] (mmol.l⁻¹)</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anguilla anguilla</em></td>
<td>250-1000</td>
<td>8</td>
<td>12</td>
<td>2.07</td>
<td>[Che69]</td>
</tr>
<tr>
<td><em>Anguilla rostrata</em></td>
<td>300</td>
<td>7</td>
<td>12</td>
<td>1.41 ± 0.09</td>
<td>[Han89]</td>
</tr>
<tr>
<td><em>Carrasius auratus</em></td>
<td>30 ± 19</td>
<td>67</td>
<td>25</td>
<td>1.25</td>
<td>[But69]</td>
</tr>
<tr>
<td><em>Cyprinus carpio</em></td>
<td>106 ± 96</td>
<td>10</td>
<td>16</td>
<td>0.98 ± 0.05</td>
<td>[Hun72]</td>
</tr>
<tr>
<td></td>
<td>1100 ± 300</td>
<td>14</td>
<td>16</td>
<td>1.15 ± 0.16</td>
<td>[Hou85]</td>
</tr>
<tr>
<td></td>
<td>42-83</td>
<td>6</td>
<td>15</td>
<td>0.94 ± 0.12</td>
<td>[Nan87]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>24</td>
<td>0.96 ± 0.06</td>
<td>[§ 3.2]</td>
</tr>
<tr>
<td><em>Esox lucius</em></td>
<td>350-2000</td>
<td>10</td>
<td>10</td>
<td>0.12</td>
<td>[Wal87]</td>
</tr>
<tr>
<td><em>Mugil cephalus</em></td>
<td>240-1410</td>
<td>34</td>
<td>0.06</td>
<td>0.77</td>
<td>[Oik78]</td>
</tr>
<tr>
<td><em>Onchorhynchus kisutch</em></td>
<td>10-18</td>
<td>8</td>
<td>20</td>
<td>0.69</td>
<td>[Nor83]</td>
</tr>
<tr>
<td><em>mykiss</em></td>
<td>21</td>
<td>6-10</td>
<td>0.15</td>
<td>0.33</td>
<td>[Zba85]</td>
</tr>
<tr>
<td>62 ± 2</td>
<td>15</td>
<td>8-10</td>
<td>0.15</td>
<td>0.76</td>
<td>[Bjö85]</td>
</tr>
<tr>
<td>157 ± 47</td>
<td>70</td>
<td>10</td>
<td>0.15</td>
<td>0.61 ± 0.14</td>
<td>[Hou85]</td>
</tr>
<tr>
<td>140-190</td>
<td>8</td>
<td>11.5</td>
<td>0.15</td>
<td>0.66</td>
<td>[Oik85]</td>
</tr>
<tr>
<td><em>tshawytscha</em></td>
<td>66</td>
<td>18</td>
<td>0.05</td>
<td>0.99 ± 0.3</td>
<td>[Sno71]</td>
</tr>
<tr>
<td><em>Oreochromis mossambicus</em></td>
<td>20</td>
<td>10</td>
<td>25</td>
<td>0.2</td>
<td>[Wen83]</td>
</tr>
<tr>
<td><em>Perca fluviatilis</em></td>
<td>25-52</td>
<td>6</td>
<td>28</td>
<td>0.2</td>
<td>1.2</td>
</tr>
<tr>
<td><em>Salvelinus fontinalis</em></td>
<td>420 ± 155</td>
<td>24</td>
<td>14</td>
<td>0.76 ± 0.06</td>
<td>[Hou85]</td>
</tr>
</tbody>
</table>

40
Magnesium regulation

The small differences in magnesium concentration between the tissues of various teleosts suggest the presence of a magnesium homeostatic mechanism in teleosts. When fish indeed show magnesium homeostasis, questions arise concerning intake routes (gills, gut), bio-availability of magnesium, internal storage and regulatory mechanisms.

Whereas the gut has been well established as an intake route for magnesium [Chapter 1], Shearer [She89] provided indirect evidence (i.e. from diet experiments) that rainbow trout can take up magnesium directly from the water. Thus it is possible that the external water is an important magnesium source for fish. For calcium, fish bone contains the larger part of the whole body pool. Bone calcium can be mobilized in times of increased need for Ca^{2+} [Fle74; Ura85], although fish normally depend on the external calcium for homeostasis and growth, as has been shown for tilapia [Fli86a]. Interesting questions that require further investigation are whether fish, in analogy to Ca^{2+}, extract their Mg^{2+} required for their growth and homeostasis from the external water via the gills and whether the bone can act as a magnesium reservoir. In higher vertebrates, the bone pool can act as a magnesium buffer [Rog65; Ana81].

The studies reported in the following chapters were aimed at the delineation of magnesium transport routes in freshwater teleosts and how these routes contribute to homeostasis.
CHAPTER 4

MAGNESIUM TRANSPORT

In this chapter the transport of magnesium between fish and water and within the fish is described. The studies are based on the use of $^{27}\text{Mg}^{2+}$ and $^{28}\text{Mg}^{2+}$ as radiotracer for magnesium ions.

§ 4.1

INTRODUCTION

As mentioned earlier, data on magnesium transport in fish are scarce in the literature. Uptake of magnesium has been measured or assessed in some species [Höb84; She89]. However, in these studies no differentiation was made between the two components of uptake, i.e. intake and loss. To understand the magnesium metabolism and to recognize disturbances in the magnesium transport, it is essential that both components (intake and loss) are measured. We used the radioisotope $^{28}\text{Mg}$ to study the transport of magnesium between fish and water in vivo. Furthermore, $^{28}\text{Mg}$ was used to determine magnesium transport across the intestinal epithelium in vitro. Finally $^{27}\text{Mg}$ was used to demonstrate magnesium transport over basolateral plasma membranes of enterocytes, using inside-out oriented vesicles made thereof.
§ 4.2

TRACER KINETIC APPROACH OF TRANSPORT IN THE WATER-FISH SYSTEM

ABSTRACT
A radiotracer method based on compartmental analysis was applied to study the transport of magnesium between fish and water.

INTRODUCTION

The flow of elements between fish and water and vice versa using radiotracers has been subject of many investigations. However most tracer kinetical approaches are vague or based on two-compartmental systems [Mae56; Pot67; Kir70]. In a two-compartment system, the pertinent element studied in the fish is assumed to be present in one homogeneous compartment. Obviously this is not true. Therefore, a novel kinetical approach was followed in which the assumption of a two-compartmental system is not required.

RADIOTRACER METHOD AND COMPARTMENTAL ANALYSIS

With regard to magnesium, a water-fish system may be considered as a closed multi-compartment system with a number of intercompartmental connections (magnesium flows). One of the magnesium compartments represents magnesium dissolved in water. The other magnesium compartments are confined to the fish and represent magnesium in blood plasma, muscle, scales, bone and other tissues. To determine the outflow of a tracee from a compartment of such a multicompartment system, a tracer should be added to this compartment and subsequently the time curve for the tracer quantity in this compartment measured [Shi72]. At zero time the tracer moves out of this compartment only and no tracer is returning from any other
compartment, since none is present there. The instantaneous (down)slope of the curve for tracer quantity in the compartment to which tracer initially had been added, normalized to the tracer quantity in this compartment at zero time, is - except for the sign - equal to the rate constant of outflow of tracee. Thus for time $t = 0$,

$$-\frac{d[q(t)/q(0)]}{dt} = k$$  \hspace{1cm} (1)

where $q(t)$ and $q(0)$ are the tracer quantities (in Bq) at time $t$ (in s) and $t = 0$, respectively, and $k$ is the rate constant (in s$^{-1}$), defined as

$$k = \frac{F}{Q}$$  \hspace{1cm} (2)

where $F$ is the (out)flow of tracee (in mol.s$^{-1}$) and $Q$ is the traceee quantity (in mol) in the compartment into which the tracer was added at $t = 0$. Combining equations (1) and (2) one obtains:

$$F = -Q \frac{d[q(t)/q(0)]}{dt}$$  \hspace{1cm} (3)

or the outflow is equal to the product of the tracee quantity and the absolute value of the instantaneous (down)slope at $t = 0$.

Note that no assumption has been made with regard to the number of compartments and their connections. In this respect, the present tracer kinetic approach to ion transport in fish-water systems differs from that used by others [e.g. Mae56; Pot67; Kir70].

The outflow of magnesium from the water compartment and thus the flow of magnesium to fish from water $F_{fw}$ can in principle be calculated from the magnesium quantity in water $Q_w$ and the slope at $t = 0$ of time curves for the tracer quantity in water $q_w(t)$ normalized to the tracer quantity in water at $t = 0$, $q_w(0)$, using equation (3). However, in practice it may be difficult to estimate the initial (down)slope of the $q_w(t)/q_w(0)$ curve due, for example, to the poor alignment of data points or inadequate steepness of the curve. In such a case one can try to measure the tracer quantity in alive
fish \( q_f(t) \) and in water at \( t = 0 \), \( q_w(0) \). \( F_{fw} \) can then in principle be calculated from \( Q_w \) and the (up)slope of the \( q_f(t)/q_w(0) \) curves at \( t = 0 \) using equation (3) but omitting the negative sign. However, when the measurement of \( q_f(t) \) is not feasible and when only the quantity of tracer in the fish at the end of an experiment \( q_f(t_e) \) and the tracer quantity in water at \( t = 0 \) can be determined, one has to use the approximation:

\[
F_{fw} = Q_w[q_f(t_e)/q_w(0)]/t_e
\]  (4)

where \( t_e \) stands for the duration of the experiment. The underlying assumption for equation (4) is that \( q_f(t_e)/t_e \) is equal to \( d[q_f(t)/q_w(0)]/dt \) at \( t = 0 \).

The outflow of magnesium from blood plasma and thus - by assuming that there are no other outflows than from plasma to water - the flow of magnesium across the integument to water from fish, \( F_{wp} \), can in principle be calculated from the magnesium quantity in the plasma, \( Q_p \), and the (down)slope of the time curve for the tracer quantity in plasma, \( q_p(t) \), normalized to the tracer quantity in plasma at \( t = 0 \), \( q_p(0) \), using equation (3). However, none of these quantities are easily assessable. However, the tracer quantity in water, \( q_w(t) \), and the specific activity of magnesium in plasma at the end of the experiment, \( S_p(t_e) \) (in Bq.mol\(^{-1}\)), may well be determined. \( S_p(t_e) \) is defined as \( q_p(t_e)/Q_p \) and equal to \( \ast C_p(t_e)/C_p(t_e) \) where \( \ast C_p(t_e) \) is the tracer concentration in plasma (in Bq.l\(^{-1}\)) and \( C_p(t_e) \) is the magnesium concentration in plasma (in mol.l\(^{-1}\)) both at \( t = t_e \). By rearranging equation (3) and assuming that at zero time \( -d[q_p(t)/q_p(0)]/dt = d[q_w(t)/q_w(0)]/dt \) one obtains:

\[
F_{wp} = [Q_p/q_p(0)]dq_w(t)/dt
\]  (5)

or

\[
F_{wp} = [1/S_p(0)]dq_w(t)/dt
\]  (6)

where \( S_p(0) \) is the specific activity of magnesium and \( dq_w(t)/dt \) is the (up)slope of the time curve for tracer quantity in water, both at \( t = 0 \). In practice it may be difficult to determine \( S_p(0) \) in fish. However, for a limited time period \( S_p(t) \) is approximately
constant and consequently \( S_p(t_0) \) may be used instead of \( S_p(0) \). This yields an approximation:

\[
F_{wp} = \frac{[1/S_p(t_0)]dq_w(t)}{dt}
\]  

(7)

A determination of \( F_{wp} \) starts by introducing a certain quantity of tracer into the fish via intraperitoneal injection. To condition the fish for the actual experiments, the fish have to be kept in a large volume of water for an extended period of time. This procedure results in a partial transfer of tracer to water from fish and simultaneously in a distribution of tracer among the magnesium compartments of the fish, resulting in a period of a more or less steady specific activity in the plasma. An actual experiment starts (\( t = 0 \)) by placing a thus conditioned fish into a small volume of water.
§ 4.3

TRANSPORT BETWEEN CARP AND WATER

ABSTRACT
A radiotracer method was used to measure the magnesium flows to fish from water and vice versa in non-growing carp weighing less than 100 g. The trend of both "flow to fish from water" (F_{fw} in nmol.h\(^{-1}\)) and "flow to water from plasma" (F_{wp} in nmol.h\(^{-1}\)) versus fish weight (W_f in g) may be represented by the power functions F_{fw} = 0.16(W_f)^{1.48} and F_{wp} = 1.23(W_f)^{1.43}. Since F_{fw} < F_{wp} the carp have to gain magnesium from sources other than the water, namely from the food. In non-growing carp at least 80% of magnesium inflow originates from food.

INTRODUCTION

This investigation deals with the magnesium transport between carp and water. It was designed to establish the relative importance of the integument (gills and body surface) for magnesium intake by measuring the unidirectional flows of magnesium between fish and water. To our knowledge no such measurements have been reported in the literature so far.

MATERIALS AND METHODS

Fish
Carp weighing from 34.0 to 92.3 g were obtained from laboratory stock. Up to 10 fish were held in 120 l glass aquaria filled with 80 l artificial freshwater (initially) containing per litre 0.5 mmol NaCl, 0.06 mmol KCl, 0.2 mmol MgSO\(_4\) and 0.2 mmol CaCl\(_2\). The pH of water was maintained at 7.5 by means of an end-point titration system (ETS822, Radiometer) adding a sodium hydroxide solution. The water was kept at 24°C.
and filtered by recirculation through a nylon-wool thermofilter (2113, Eheim) and constantly aerated. Twice a week one third of the aquarium content was replaced with freshly prepared water. The photoperiod was 12 h light and 12 h dark. Daily the fish received six rations of dry fish food (Tetra) containing about 31 mmol.kg⁻¹ magnesium. The quantity of food was adjusted to match the maintenance level [Hui76]. During tracer exposure, the fish were not fed.

Radiotracer

²⁸Mg²⁺ was used as radiotracer for Mg²⁺. It was produced either by irradiation of Li-Mg alloys with thermal neutrons in the IRI nuclear reactor [§ 2.4] or by irradiation of PCl₃ with high-energy bremsstrahlung obtained from the NIKHEF 500 MeV electron linear accelerator [Pol89]. In both cases the irradiation was followed by separation of magnesium from irradiated material leading to aqueous solutions of MgCl₂ containing ²⁸Mg²⁺ and its decay product ²⁸Al³⁺. The specific activities of these preparations calculated from the activities as determined with Ge(Li) detectors just before the experiments and magnesium concentrations as determined by spectrophotometric titration with EDTA or by Atomic Absorption Spectrometry, were about 0.2 and 26 GBq.mol⁻¹ for the reactor and the accelerator production mode, respectively.

Mg²⁺ flow to fish from water

To determine Fᵣw a fish was weighed and placed into an all-glass vessel with 2 l artificial freshwater in which MgSO₄ was replaced by MgCl₂ containing 80 kBq ²⁸Mg. The water was constantly aerated with pre-humidified air and kept at 24°C. By means of a peristaltic pump the water was recirculated (mean residence time of water in the vessel was 520 s) through a glass spiral round about a cylindrical (diameter: 76 mm, length: 76 mm) NaI(Tl)-scintillation detector (Type 12S, Harshaw Chemie) equipped with a multichannel analyzer (Series 35, Canberra). During the experiments the spectra of the γ-radiation emitted by both ²⁸Mg and ²⁸Al present in water were measured in subsequent intervals (counting time per interval 1000 or 5000 s) and stored in a personal computer. The areas of the 0.401, 0.942 and 1.342 MeV peaks - all belonging to ²⁸Mg only - were determined for all data points and then corrected for decay.

49
The experiments lasted 21.5 up to 25.4 h (= t_e) in all but two cases (for the fish weighing 92.3 and 54.2 g t_e was 5 and 14.5 h, respectively) and were stopped by adding "MS-222" (Sigma) to the water (final concentration 0.4 mmol.l⁻¹). After 2 to 5 min the fish were taken out of the radioactive water, rinsed with artificial freshwater, blotted with filter paper and killed by spinal transection. Finally the fish were minced and aliquots were put into one or more 20 ml counting vials. Each vial contained about 20 ml minced fish. The amount of minced fish per vial was determined by weighing the vials before and after filling. The γ-ray spectra of samples of minced fish were measured in a well-type (diameter: 76 mm, length: 76 mm) NaI(Tl)-scintillation detector (Type 125W12, Harshaw Chemie) connected to the before-mentioned counting equipment. To allow for the decay of ²⁸Al present in the samples at the moment of sampling and for the establishment of a ²⁸Mg-²⁸Al equilibrium all samples were measured at least 30 min after sampling. The counting time was 500 or 1000 s. The 20 ml water samples taken at the beginning of the experiment were measured in a similar way. All the pulses pertaining to 0.1 - 2.0 MeV region were taken into account. Their number was corrected for background and decay of ²⁸Mg and the counting rates were calculated.

During the experiments no measurable decrease of radiotracer concentration in the water recirculating around the detector was observed. Consequently, F_{fw} (in mol.h⁻¹) had to be calculated using equation (4) in which:

\[ Q_w = V_w C_w \]  

(8)

where \( V_w \) is the volume of water (in l) and \( C_w \) is the magnesium concentration in water (in mol.l⁻¹);

\[ \frac{q_f(t_e)}{q_w(0)} = \frac{R_f(t_e) W_f}{R_w V_w} \]  

(9)

where \( R_f \) is the (mean) counting rate per unit weight of minced fish (in counts.s⁻¹.g⁻¹) measured as 20 ml sample(s), \( W_f \) is the weight of the fish (in g) and \( R_w \) is the counting rate per unit volume of water (in counts.s⁻¹.l⁻¹) measured as 20 ml sample; \( t_e \) is expressed in h.
Mg$^{2+}$ flow to water from plasma

To determine $F_{wp}$, the fish were weighed and injected intraperitoneally with 0.1 - 0.2 ml saline (0.9% NaCl) with 2 - 4 μmol MgCl$_2$ and approximately 80 kBq $^{28}$Mg. After the injection the fish were put into a 50 l all-glass aquarium with 30 l constantly aerated, artificial freshwater which was kept at 24°C. After 9 - 14 h the fish were taken out, rinsed with artificial freshwater for 2 min and the experiment was started by placing a fish into an all-glass vessel with 1.7 l artificial freshwater which was constantly aerated with pre-humidified air and kept at 24°C. Water samples of 20 ml were taken 1, 5, 10, 15, 30, 45, 60 min and then every 30 min thereafter. To each of these samples Al$^{3+}$ (as AlCl$_3$ dissolved in dilute sulphuric acid) was added to serve as hold-back carrier for $^{28}$Al$^{3+}$ and thus prevent its adsorption onto the inner wall of the counting vial. The experiments, during which the fish were not fed, lasted for 5 h and were stopped by transferring the fish into 2 l artificial freshwater containing 0.4 mmol.l$^{-1}$ "MS 222" for 2 to 5 min. A blood sample (mixed arterial and venous blood) of up to 2 ml was taken by puncture of the caudal vessels behind the anal fin using heparinized syringe. The fish were then killed by spinal transection. The blood was separated into cells and plasma by centrifugation at 9000 g for 3 min. The magnesium concentrations in the blood plasma and in its ultrafiltrate were determined colorimetrically (Diagnostic kit 595, Sigma). The fish was minced and put into counting vials. See above for details on these procedures. Counting samples containing 0.250 - 0.863 ml blood plasma and water adding up to 20 ml were prepared.

The $^{28}$Mg activity in liquid samples was determined by Cerenkov counting of β$^-$-particles of (mainly) $^{28}$Al by means of a Liquid Scintillation Counter (Tri-carb 300c, Packard Instruments). The samples were measured at least 30 min after sampling to allow for the establishment of a $^{28}$Mg-$^{28}$Al equilibrium. The counting data were corrected for background and decay of $^{28}$Mg. The tracer quantities in water for all sampling times were calculated by taking into account the amount of tracer removed from water by subsequent sampling:

\[
q_w(i) = \{R_w(i)[V_w \cdot (i-1)v_w] + \sum_{i=1}^{n} R_w(i-1)v_w\} / \epsilon
\]  \hspace{1cm} (10)

51
where \( i \) is the sequential sample number, \( R_w(i) \) is the counting rate of \( i \)-th water sample (in \( \text{counts.s}^{-1}.\text{l}^{-1} \)), \( v_w \) is the sample volume (in l) and \( \epsilon \) is the counting efficiency (in \( \text{counts.s}^{-1}.\text{Bq}^{-1} \)). In the following calculations the counting efficiency \( \epsilon \) cancels out and therefore knowledge of its actual value is not required. Note that for \( i = 1 \ t = 1 \ \text{min}, \ i = 2 \ t = 5 \ \text{min} \ \text{etc.} \) Hence for \( i = 1, \ R_w(i-1) = R_w(0) = 0. \)

\( F_{wp} \) (in \( \text{mol.h}^{-1} \)) was calculated using equation (7) in which:

\[
S_p(t_e) = \frac{[R_p(t_e)/\epsilon]}{C_p}
\]  

(11)

where \( R_p(t_e) \) is the counting rate of plasma (in \( \text{counts.s}^{-1}.\text{l}^{-1} \)) pertaining to \( t_e = 5 \ \text{h} \) and \( C_p \) the magnesium concentration in plasma (in \( \text{mol.l}^{-1} \)) a mean value (determined in separate measurements as described above) for all fish instead of \( C_p(t_e) \) values for each individual fish was used (this because all the plasma was needed for the preparation of the counting samples).

The (up)slope at \( t = 0 \) of the \( q_w(t) \) versus time (in \( \text{h} \)) curve (in \( \text{counts.s}^{-1}.\text{e}^{-1}.\text{h}^{-1} \)), required for equation (7), was determined by fitting a straight line through data points for \( t \leq 1 \ \text{h} \) calculated by equation (10). This was carried out by linear regression analysis. Fig. 4.1 shows a typical example of such a procedure.

RESULTS AND DISCUSSION

\( \text{Mg}^{2+} \) flow to fish from water

The \( F_{fw} \) values, obtained for fish weighing 36.7 to 92.3 g, are shown in Fig. 4.2 as function of the fish weight. Linear regression of \( \ln F_{fw} \) values on \( \ln W_f \) values yielded a power function for which the equation is:

\[
F_{fw} = a(W_f)^b
\]  

(12)

where \( F_{fw} \) is expressed in \( \text{nmol.h}^{-1} \), \( a = 0.16 \) and \( b = 1.48 \) (Fig. 4.2). The correlation coefficient (r) for the above-mentioned linear regression was 0.880.

Magnesium dissolved in water may enter the fish through the integument (gills
Fig. 4.1.
The tracer quantity in the water (in counts s\(^{-1}\) \(\cdot\) \(\epsilon\)^{-1}) versus time (t in h) curve as obtained in a "flow to water from plasma" experiment with a 46.2 g carp injected with tracer 11 h before \(t = 0\). The slope of the curve at \(t = 0\) was calculated by fitting a straight line through data points for \(t \leq 1\) h.

and body surface) and via the gut by drinking. The \(F_{fw}\) values as obtained in this study may comprise both of these routes. The drinking related part of \(F_{fw}\) may be estimated by using a drinking rate value of 51 \(\mu\)l.h\(^{-1}\). (100 g\(^{-1}\)), as obtained for Carassius auratus [Mot69]. This results in 5 nmol.h\(^{-1}\) magnesium for a 50 g carp which is only about 10\% of \(F_{fw}\).
Mg$^{2+}$ flow to water from plasma

The $F_{wp}$ values, obtained for fish weighing 34.0 to 77.6 g, are shown in Fig. 4.2 as function of the fish weight. Linear regression of $\ln F_{wp}$ values on $\ln W_f$ values showed that the trend of the plotted data points may be represented by a power function for which the equation is analogous to equation (12) but with $a = 1.16$ and $b = 1.51$ (Fig. 4.2). Here $r$ was 0.886.

The magnesium may leave the fish through the integument and by urinary and faecal excretion. The $F_{wp}$ values as obtained in this study represent the integumental route, viz. the flow of magnesium along the route: plasma $\rightarrow$ gills and body surface $\rightarrow$ water, only. However, this statement is subject to the condition that during the very first hour of the experiment the urinary and faecal excretion of $^{28}$Mg$^{2+}$ does not significantly contribute to the tracer quantity in water.

As the specific activity of plasma was determined 5 h - thus not really shortly - after the start of an experiment, the question may arise whether the use of $S_p(t_c = 5 \text{ h})$ instead of $S_p(0)$ does not lead to a significant overestimation of $F_{wp}$ due to the possible loss of tracer from plasma and consequently: $S_p(t_c)/S_p(0) < 1$. One may estimate this ratio by supposing a uniform specific activity of magnesium throughout the extracellular fluid and consequently equal specific activities in its two components, namely plasma and interstitial fluid ($S_i$) i.e. $S_p(t) = S_i(t)$ for $0 \leq t \leq t_c$. The tracer balance for extracellular fluid and water results in the relation:

$$S_p(t_c)/S_p(0) = 1/[1 + q_w(t_c)/S_p(t_c)[Q_p + Q_i]]$$ \hspace{1cm} (13)

where $Q_i$ is the magnesium quantity in interstitial fluid (in mol). The tracer quantity in water at the end of the experiment $q_w(t_c)$ was calculated by means of equation (10) (for $t_c = 5 \text{ h}$). Hence $Q_p = V_p C_p$ where $V_p$ is the volume of plasma in carp (in l) and $C_p$ is the magnesium concentration in plasma (in mol.l$^{-1}$) [§ 3.2], and $Q_i = V_i C_i$ where $V_i$ is the volume of the interstitial fluid in carp (in l) and $C_i$ is the magnesium concentration in the interstitial fluid (in mol.l$^{-1}$). According to Thorson [Tho61] in carp $V_p = 1.8 \times 10^{-5} W_f$ and $V_i = 13.7 \times 10^{-5} W_f$. For $C_i$ the magnesium concentration in plasma ultrafiltrate [§ 3.2] was used. The foregoing estimation procedure yields $S_p(t_c)/S_p(0)$ ranging from 0.69 to 0.86 or equal to 0.78 $\pm$ 0.05 (mean value for 7 fish $\pm$ standard
Fig. 4.2. Magnesium flow to fish from water, $F_{fw}$ (squares + lower curve) and to water from plasma, $F_{wp}$, both versus fish weight ($W_f$ in g). Given are the primary (circles + upper curve) as well as the for $S_p$ decrease corrected $F_{wp}$ values (triangles + middle curve). The curves represent the corresponding best-fit power function as given in the text.

deviation). Apparently the specific activity of magnesium in plasma of carp changes during the 5 h lasting experiments. Although the decrease of specific activity in plasma remains limited to 31% or less, the preceding $F_{wp}$ values were corrected by multiplying each of them with the corresponding $S_p(t_c)/S_p(0)$ ratio (Fig. 4.2). Linear regression of (new) $\ln F_{wp}$ values on $\ln W_f$ values resulted in a power function analogous to equation (13) with $a = 1.23$ and $b = 1.43$ (Fig. 4.2, middle curve). For this regression $r = 0.924$. 

55
Magnesium balance

Growing fish constantly incorporate magnesium in their body. The uptake or net flow of magnesium into the fish, $F_{\text{net}}$, is the resultant of two oppositely directed flows: $F_{\text{in}}$ being the inflow of magnesium to fish from both water ($F_{\text{fw}}$) and food ($F_{\text{ff}}$) and $F_{\text{out}}$ being the outflow of magnesium to water from fish along both the integumental ($F_{\text{wp}}$) and excretory routes ($F_{\text{ef}}$).

The present study deals with fish fed at maintenance level thus with non-growing fish. The whole body magnesium quantity in these fish is expected to remain constant before and during the experiment. Obviously $F_{\text{net}} = 0$ which means that $F_{\text{in}} = F_{\text{out}}$ or:

$$F_{\text{fw}} + F_{\text{ff}} = F_{\text{wp}} + F_{\text{ef}}$$

(14)

The curves in Fig. 4.2 show that for the carp under consideration $F_{\text{fw}} < F_{\text{wp}}$ for all $W_{f}$ values. For both the fish weighing 40 and 80 g $F_{\text{fw}}/F_{\text{wp}}$ (corrected) = 0.16 and thus it may be regarded as independent on $W_{f}$. By rearrangement of equation (14) and using the preceding $F_{\text{fw}}/F_{\text{wp}}$ value one obtains:

$$F_{\text{ff}} = 5.3F_{\text{fw}} + F_{\text{ef}}$$

(15)

Evidently $F_{\text{ff}} >> F_{\text{fw}}$ and amounts to at least 80% of the magnesium inflow into the carp. Consequently $F_{\text{ff}}$ represents the most important route for magnesium inflow to these non-growing fish. Ogino & Chiou [Ogi76] came to a similar conclusion, namely "that carp cannot absorb sufficient amount of magnesium from the rearing water to meet their requirement". Note that the conclusion of these authors was based on measurements of the magnesium content of growing fish put on diets containing different amounts of magnesium and not on direct measurements of unidirectional flows. The above-mentioned status and our own results indicate that, in contrast to calcium [Fl98a], a significant fraction of the magnesium inflow in carp is effected along another route than the integumental one: apparently the intestinal route. This route is dealt with in the next section.
§ 4.4

TRANSPORT ACROSS THE INTESTINE OF TILAPIA

ABSTRACT

Bidirectional magnesium fluxes measured in vitro across stripped intestinal epithelium of freshwater tilapia revealed a net flux of magnesium from mucosa to serosa, indicating that an active step is involved in the transepithelial transport. Replacement of sodium in the saline by NMDG decreased the flux from mucosa to serosa, as well as the flux in the opposite direction and abolished the net flux. The flux from mucosa to serosa was inhibited by the addition of ouabain. From an estimation of the magnesium concentration in the enterocytes, we conclude that an uphill transport step may be located in the basolateral membrane. The presence of a sodium dependent exchange mechanism for magnesium extrusion in the basolateral membrane of fish intestinal epithelium is postulated.

INTRODUCTION

Studies on Mg\(^{2+}\) transport across membranes and membrane systems have been reviewed extensively by Ebel & Günther [Ebe80], Flatman [Fla84] and Günther [Gün87]. Measurements of the Mg\(^{2+}\) flow through the integument of carp have given evidence that the major intake of magnesium in fish is not from the water via the gills, but from the food via the intestinal tract [§ 4.2]. These observations are in line with the results of Ogino & Chiou [Ogi76], who showed that dietary magnesium is essential for growth in carp. Also, young Oreochromis niloticus depend on dietary magnesium for growth [Dab89].

Little is known about the mechanisms of Mg\(^{2+}\) transport in fish intestine. Nakamura & Hirano [Nak86] suggest solvent drag as a mechanism for net Mg\(^{2+}\)
transport in eel. For mammals, the reports on this topic are equivocal. In the small intestine of the rat, diffusion [Ros62a; Ald70; O'D73] as well as solvent drag [Beh74] were reported to underlie magnesium absorption, although the possibility of an active component was not excluded. For the guinea pig intestine, active transport of magnesium was demonstrated [Ros62b; Par87]. Parallel to our studies, Karbach & Rummel [Kar90] reported that the mucosa to serosa and serosa to mucosa magnesium fluxes across rat ileum are magnesium concentration dependent. According to these authors the mucosa to serosa flux has a non-diffusive cellular component; the serosa to mucosa flux was diffusive and probably restricted to the paracellular pathway.

Proceeding from this state of knowledge we have analyzed Mg\(^{2+}\) fluxes over the intestinal epithelium *in vitro*, using stripped intestinal epithelium of the tilapia and \(^{28}\)Mg as a radiotracer.

**MATERIALS AND METHODS**

Fish

Tilapia weighing around 150 g, were obtained from laboratory stock and kept in Amsterdam municipal water with a magnesium concentration of about 0.25 mmol.l\(^{-1}\) and at a temperature of 28±2°C. The photoperiod was automatically controlled (12 h light: 12 h dark) and the fish were fed once daily with tropical fish food (Tetra) containing about 31 mmol magnesium per kg dry weight. Fish were sacrificed by spinal transection just behind the gills. The intestine was cut free just after the stomach, transferred to saline (see media) and flushed with the same solution. Next, the intestine was cut lengthwise and the mucosa stripped of its underlying muscular layers as described by Albus *et al.* [Alb79]. All experiments were performed at room temperature.

Media

The control saline contained (in mmol.l\(^{-1}\)): NaCl (117.5), KCl (5.7), NaHCO\(_3\) (25.0), NaH\(_2\)PO\(_4\) (1.2), CaCl\(_2\) (2.5), MgSO\(_4\) (1.0) and mannitol (28.0); the saline was oxygenated with a mixture of 95% O\(_2\) and 5% CO\(_2\). In some cases ouabain (0.1 mmol.l\(^{-1}\)) was added to the saline bathing the serosa. In some cases sodium was substituted by N-
methyl-D-glucamine adjusted to pH 7.4 with HCl (for NaCl), Choline-bicarbonate (for NaHCO₃) and KH₂PO₄ (for NaH₂PO₄). The extra addition of KH₂PO₄ was compensated for by using 4.5 instead of 5.7 mmol.l⁻¹ KCl.

Radiotracers

The ²⁸Mg was produced at the NIKHEF (Amsterdam) by photonuclear irradiation of PCl₃ [Pol89]. The specific activity was about 26 GBq.mol⁻¹.

The extracellular space of intestinal segments was determined using (hydroxy [¹⁴C]methyl)-inulin (Amersham) with a specific activity of 575 GBq.mol⁻¹.

Mg²⁺ flux determinations

Segments of approximately 1 cm² of stripped intestinal epithelium from the proximal 15 cm of the intestine were fixed in a holder leaving an exposed tissue area of 0.2 cm². This holder formed the partition between two half-chambers, denoted as "a" and "b". The set up has been described in detail by Groot et al. [Gro79]. The transport of Mg²⁺ was followed using ²⁸Mg²⁺ as radiotracer.

Prior to an experiment, both half-chambers were filled with 1.9 ml magnesium-free saline. At time zero 0.1 ml of a ²⁸Mg²⁺-containing MgSO₄ solution was added to half-chamber "a", and 0.1 ml of MgSO₄ solution was added to half-chamber "b". The resulting magnesium concentration in both half-chambers was 1 mmol.l⁻¹, as confirmed by atomic absorption spectrometry. The contents of both half-chambers were constantly stirred and gassed. After 1 min, 0.5 h and 1 h, 0.5 ml saline samples were taken from half-chamber "b". After each sampling, 0.5 ml of tracer-free 1 mmol.l⁻¹ magnesium-containing saline was added to the sampled half-chamber. At the end of the experiment (time 1.5 h) 0.5 ml saline samples were taken in triplicate from both half-chambers. The radioactivity of the samples was determined by liquid scintillation counting.

Three sets of experiments were carried out to determine the magnesium flux to serosa from mucosa and/or to mucosa from serosa in i) control saline, ii) sodium-free saline, and iii) saline with 0.1 mmol.l⁻¹ ouabain on the serosal side.
Extracellular space, magnesium, sodium, potassium and water content

To determine the extracellular space and the elemental and water content of the stripped intestinal epithelium under the three experimental conditions, 1 cm² segments of stripped epithelium were pre-incubated for 15 min in 40 ml of control or sodium-free saline. Subsequently the segments were transferred for 1 h to 40 ml of fresh (experimental) saline containing 110 nmol.l⁻¹ ¹⁴C-inulin. All salines were continuously stirred by a flow of humidified gas (95% CO₂ + 5% O₂). Upon completion the segments were removed, blotted on moist filter paper (no.1, Whatman), weighed in tared aluminium weighing boats (Heraeus), dried to constant weight at 70°C and weighed again. The dried tissue was extracted for at least 2 h in 0.1 N HNO₃ and the extract was divided into samples to determine the radioactivity (for extracellular space) and the magnesium, sodium and potassium content.

The extracellular space, defined as the inulin space, was calculated as the ratio of the ¹⁴C-radioactivity per g dry tissue and the ¹⁴C-radioactivity per ml medium. The intracellular space was calculated as the difference between tissue water content and extracellular space and was expressed in ml.g⁻¹ dry weight of epithelium.

Analytical methods

The concentrations of magnesium, sodium and potassium were determined by atomic absorption spectrophotometry (Perkin-Elmer 305).

The radioactivity of the samples was determined by liquid scintillation counting (Tricarb 2660, Packard) using a scintillation cocktail (Insta-gel II, Packard). The radioactivity of ²⁸Mg-containing samples was determined at least 30 min after sampling. From this time on, (i) all radioactivity (from ²⁸Mg or its daughter product ²⁸Al) stems from ²⁸Mg present during the experimental set and (ii) the decay ratio of ²⁸Mg and ²⁸Al is constant.

Statistics

Data are presented as mean values ± the standard deviation (SD). Only data points within a 95% confidence interval (i.e. within two SD's) were taken for the calculation of the mean. Data were analyzed statistically using Student’s t-test. Statistical significance was accepted at the 5% level.
Tracer kinetic approach

The data obtained in this study were subjected to compartmental analysis. The set up described here for the study on Mg$^{2+}$ transport (using $^{28}$Mg$^{2+}$ as radiotracer), should be considered to present a closed, non-steady-state three compartmental model. In non-steady-state multicompartmental models the formulas for direct calculation become very complex [Shi72]. Therefore, an approximation was made to a steady-state two compartmental model assuming that both the net quantity of magnesium transported and the quantity of magnesium associated with the epithelium are negligible compared with the quantity of magnesium in the saline of each half-chamber. This assumption has been verified for this particular set up (see Results).

Starting from equation (5) [§ 4.2] and from the fact that $J_{ba} = F_{ba} / A$, where $J_{ba}$ is the flux to compartment "b" from compartment "a" (in nmol.h$^{-1}$.cm$^{-2}$), $F_{ba}$ is the flow to compartment "b" from compartment "a" (in nmol.h$^{-1}$) and A is the exposed area for transport (in cm$^2$), one obtains:

$$J_{ba} = \frac{[Q_a/q_a(0)] dq_b(t)}{dt A} \tag{16}$$

where $Q_a$ is the magnesium content of the 2 ml of saline of half-chamber "a" (in nmol), $q_a(0)$ is the tracer quantity in half-chamber "a" at time zero (in Bq), $dq_b(t)/dt$ is the slope at time zero of the time curve for the tracer quantity in compartment "b".

Since the flux might have been disturbed short time after time zero because of the addition of magnesium, we always based the calculation of the magnesium flux on the 1 h and 1.5 h data points. This assumption was justified as the tracer appearance rate proved to be essentially constant in time over the total experimental period. Thus $dq_b(t)/dt$ at time zero can be described as $[q_b(t_2) - q_b(t_1)] / (t_2 - t_1)$, in which $t_1$ is 1 h and $t_2$ is 1.5 h. Assuming that the change in the total activity as well as the change in the specific activity is negligible over the experimental period, one obtains:

$$J_{ba} = \frac{[Q_a/q_a(t_2)][q_b(t_2) - q_b(t_1)]}{(t_2 - t_1) A} \tag{17}$$

As $q(t) = R(t) / \epsilon$, where $R(t)$ is the counting rate in the 2 ml saline (in counts.s$^{-1}$)
and $\epsilon$ is the counting efficiency (in counts.$s^{-1}.Bq^{-1}$), we can replace $q(t)$ in equation (17) by $R(t)/\epsilon$. In the resulting equation, the counting efficiency, $\epsilon$, cancels out and therefore knowledge of its actual value is not required. This results in the final equation:

$$J_{ba} = \frac{Q_a/R_a(t_2)[R_h(t_2)-R_h(t_1)]}{(t_2-t_1)} A \tag{18}$$

RESULTS

Magnesium flux

The data on the magnesium fluxes across the intestinal epithelium are shown in Table 4.1. In the control situation, the magnesium flux to serosa from mucosa is significantly higher than the flux to mucosa from serosa. A net flux of about 23 nmol.$h^{-1}.cm^{-2}$ was calculated. Replacement of Na$^+$ caused a drastic inhibition of the flux to serosa from mucosa as well as to mucosa from serosa. In the sodium-free saline there is no significant net Mg$^{2+}$ flux. Addition of ouabain to the serosal saline decreased the flux to serosa from mucosa significantly.

Table 4.1.
Magnesium fluxes ± standard deviation across stripped intestinal epithelium of freshwater tilapia under different experimental conditions. The number of observations are indicated between brackets.

<table>
<thead>
<tr>
<th>saline conditions</th>
<th>Mg$^{2+}$ flux (in nmol.$h^{-1}.cm^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>to serosa from mucosa</td>
</tr>
<tr>
<td>control</td>
<td>39 ± 19 (9)</td>
</tr>
<tr>
<td>sodium-free</td>
<td>6 ± 3 (7)</td>
</tr>
<tr>
<td>+ ouabain</td>
<td>16 ± 2 (3)</td>
</tr>
</tbody>
</table>

Extracellular space and tissue magnesium, sodium and potassium content

Under control conditions, the epithelial water content was 4.2 ± 0.6 ml.$g^{-1}$ dry weight stripped epithelium and the extracellular space was 3.4 ± 0.7 ml.$g^{-1}$; from these values we calculated a cellular water content of 0.8 ml per g of dry weight epithelium.
(n = 19). The epithelial magnesium, sodium and potassium concentrations (in mmol.kg\(^{-1}\) dry weight epithelium) were 27.9 ± 4.1, 390 ± 89 and 352 ± 27, respectively. The calculated cellular concentrations for magnesium, sodium and potassium (in mmol.l\(^{-1}\) cell water) were 7.9 ± 1.6 (n = 18), 87 ± 14 (n = 17) and 98 ± 14 (n = 16), respectively. Under sodium-free conditions, the cell water content decreased by 18%; in the presence of ouabain it increased by 17%. However, the magnesium concentration of the total epithelium was not significantly affected. Under sodium-free conditions the cellular magnesium concentration was 11.3 ± 3.4 (n = 18), in the presence of ouabain it was 6.4 ± 1.1 mmol.l\(^{-1}\) (n = 18). Under sodium-free conditions, the sodium concentration decreased by 69% and the potassium concentration was not significantly affected. In the presence of ouabain the sodium concentration increased by 91% and the potassium concentration decreased by 67%.

The dry weight of a 0.2 cm\(^2\) tissue sample of stripped epithelium, as used in the flux experiments, was 0.46 ± 0.14 mg (n = 23). The mean total magnesium content of 0.2 cm\(^2\) stripped epithelium under control conditions was calculated to be 13 ± 4 nmol.

DISCUSSION

In stripped intestinal epithelium of tilapia, a net magnesium transport occurs from mucosa to serosa. Since the magnesium concentrations in both half-chambers are equal and there is no electrical gradient over the intestinal epithelium, the net magnesium transport will result from net water transport (solvent drag), from an active transport mechanism, or from both.

Studies on water fluxes across stripped intestine of freshwater teleosts have been performed for the Japanese eel (Anguilla japonica) and demonstrated a net flux of 5.5 μl.h\(^{-1}\).cm\(^{-2}\) in the middle section of the intestine [And78]. The net water flux of non-stripped intestine was found to be considerably higher. In the same species of eel, Ando [And74] reported a net water flux of 16.5 μl.h\(^{-1}\).cm\(^{-2}\) in non-everted gut sacs in vitro (and thus in non-stripped epithelium). During an in vitro study with non-everted sacs of tilapia, Mainoya [Mai82] found a water flux across the anterior intestine of 0.44 ml.g\(^{-1}\).h\(^{-1}\).
Proceeding from an epithelial water content of 4.2 ml.g⁻¹ dry weight and a dry weight of 2.3 mg.cm⁻² (0.46 mg per 0.2 cm²) we calculate a wet weight of 12 mg.cm⁻² and hence we estimate a maximum net water flux for tilapia of 5.3 μl.h⁻¹.cm⁻² for a non-stripped epithelium. However, the value given by Mainoya [Mai82] was obtained in Ringer solution which contained glucose (this will increase the water transport via Na⁺/glucose co-transport), and thus the value in glucose-free saline, as used in our study, was probably lower. Nevertheless, even if we proceed from this very high water transport rate in our set-up, a net magnesium flux of 5.3 nmol.h⁻¹.cm⁻² at most may be predicted via solvent drag. This value is considerably lower than the actual net flux of magnesium observed in our stripped epithelium. Therefore, we predict that solvent drag is not the major mechanism for net magnesium transport in the tilapia intestine.

Starting from the known cell potential of -60 mV in tilapia enterocytes [Bak88] and the saline Mg²⁺ concentration of 1 mmol.l⁻¹ one may predict an equilibrium concentration in the cell of 100 mmol.l⁻¹ Mg²⁺. Our analysis of the tissues indicates a total concentration of about 8 mmol.l⁻¹ magnesium. Thus, the magnesium uptake across the brush-border membrane could be passive and driven by an electrochemical potential difference of at least 25 mV. It is generally accepted that most of the intracellular magnesium will be bound. For instance in oxygenated human red blood cells the free Mg²⁺ was 13% of the total magnesium [Ouw89]. Thus, the intracellular Mg²⁺ activity in the enterocyte too will be much lower than the total cellular concentration and, therefore, the inward driving force given above will be an underestimate. Conversely, transcellular transport requires energized extrusion at the basolateral membrane against an electrochemical potential difference of at least 30 mV. The data presented are very similar to the data of Karbach & Rummel [Kar90] for stripped rat ileum. These authors pointed to a total diffusive paracellular magnesium flux to mucosa from serosa and a cellular flux to serosa from mucosa that was at least partly non-diffusional.

The ratio of cellular potassium and sodium found in this study is low. As noted earlier by Groot [Gro81] acclimation of fish to water of higher temperature results in a higher total sodium concentration in the enterocytes. However, it should be stressed that the data given refer to total sodium and potassium concentrations and not to ion activities. The Na⁺/K⁺-ATPase of the tilapia enterocytes has a Kₘₙ(K⁺) of 9 mmol.l⁻¹ [Fli90] and this suggests strongly that the cellular Na⁺ will be much lower (viz. around
9 mmol.l⁻¹) than the total sodium concentration [Pre88]. As the cellular magnesium concentrations under the various experimental conditions differed only slightly, the measured fluxes can be compared. When Na⁺ in the saline is replaced by the inert cation NMDG⁺ [Pal89] both unidirectional fluxes are reduced and the net magnesium flux is abolished. Addition of ouabain to the serosal saline, to block the Na⁺/K⁺-ATPase activity, reduced the flux from mucosa to serosa. Therefore, we postulate the involvement of a Na⁺-dependent mechanism for active magnesium extrusion in the basolateral membrane of the enterocytes of freshwater tilapia. A possible mechanism is that of a Na⁺/Mg²⁺ exchanger analogous to the Na⁺/Ca²⁺ exchanger described for tilapia enterocytes [Fli90]. The presence of a Na⁺/Mg²⁺ exchange mechanism has recently been demonstrated in chicken erythrocytes [Gün85] and squid giant axons [DiP88]. Lüdi & Schatzmann [Lüd87], however, conclude that in human red blood cells magnesium extrusion is unlikely to depend on a Na⁺/Mg²⁺ exchange mechanism that depends on an inwardly directed Na⁺ gradient. They suggest an ATP-energized extrusion mechanism that depends on external Na⁺ for conformational translocation in the ionophoric part of the exchanger. Apparently, the mechanisms for magnesium transport differ among cell types and among species.
§ 4.5

TRANSPORT ACROSS THE BASOLATERAL PLASMA MEMBRANE
OF ENTEROCYTES

ABSTRACT
Vesicles were prepared from an enterocyte membrane fraction, enriched in basolateral plasma membranes. The membrane configuration of the preparation was 15% inside-out oriented vesicles, 30% right-side out oriented vesicles and 55% leaky membrane fragments. The radiotracer $^{27}\text{Mg}^{2+}$ was produced by thermal neutron irradiation of Mg(CH$_3$COO)$_2$ containing magnesium enriched in $^{26}\text{Mg}$ (97.1%). The specific activity was around 35 GBq.mol$^{-1}$. Intake of Mg$^{2+}$ in vesicles was stimulated by ATP, but not by ADP or the non-hydrolyzable ATP analogue ATP-$\gamma$-S. Ouabain did not affect this Mg$^{2+}$ transport, nor were Na$^+$ or K$^+$ required. The Mg$^{2+}$ transport process obeyed Michaelis-Menten kinetics ($K_{1/2}$ for Mg$^{2+}$ : 1.12 ± 0.35 mmol.l$^{-1}$, $V_{\text{max}}$ : 5.10 ± 0.53 nmol.min$^{-1}$.mg protein$^{-1}$). The affinity for Mg$^{2+}$ of the transporter is in line with an extrusion mechanism that is activated by cytosolic free Mg$^{2+}$ levels. We conclude that the basolateral plasma membrane of the fish enterocyte contains a Mg$^{2+}$ translocating ATPase. To our knowledge this is the first report providing direct evidence for an ATP-driven Mg$^{2+}$ transport process across plasma membranes of animal cells.

INTRODUCTION

In § 4.4 we demonstrated a net mucosa to serosa Mg$^{2+}$ flux in the proximal part of the tilapia intestine. This influx results from a transcellular movement of Mg$^{2+}$. Evidence is accruing from research on a variety of species including man [Mil79; Qua80; Ren85; Par87; Har90; Qua90; Kar90; § 4.4] that transcellular movement of Mg$^{2+}$ occurs in transporting epithelia. The picture that emerges from physiological research on the transcellular movement of Mg$^{2+}$ [Qua80] reminds strongly of the (more established) transport route described for the transcellular movement of Ca$^{2+}$: passive entry at the
apical membrane down the electrochemical gradient, cytosolic buffering and energized export at the basolateral plasma membrane. Although data on the free Mg\(^{2+}\) concentration in the cytosol are scarce, it is generally believed to be in the millimolar range and to be kept far below the equilibrium concentration [Alv87]. One may then calculate that the transmembrane electrochemical gradient allows passive entry of Mg\(^{2+}\) into the cell [e.g. § 4.4]. Indeed, Quamme & Dai [Qua90a] and Quamme & Rabkin [Qua90b] have demonstrated a unique, regulated and specific (e.g. Ca\(^{2+}\) insensitive) Mg\(^{2+}\) entry pathway in cardiac myocytes as well as in a renal cell line. Extrusion mechanisms for Mg\(^{2+}\) have been most extensively studied in erythrocyte membrane preparations and both cation (Na\(^{+}\)-gradient) dependent [Gün89a,b,c] and ATP dependent [Lüd87; Fre89] mechanisms have been postulated as mechanisms for cellular magnesium homeostasis. But also for magnesium transporting epithelia such mechanisms were postulated: a Na\(^{+}\)/Mg\(^{2+}\) exchange activity was implicated in the Mg\(^{2+}\) reabsorption mediated by the cells of the thick ascending limb of the loop of Henle [Qua80]. However, also in the Mg\(^{2+}\) secreting renal tubules of the seawater flounder a sodium dependent Mg\(^{2+}\) extrusion mechanism has been postulated [Ren85]. Thus, the presence and location of such an extrusion mechanism does not automatically predict the direction of net Mg\(^{2+}\) flow over the epithelium. The picture emerging is that extrusion mechanisms guarantee magnesium homeostasis of the cells that make up the Mg\(^{2+}\) transport epithelium, and that the electrophysiological conditions of the epithelium determine net flow of Mg\(^{2+}\). Important analogies appear to exist between Mg\(^{2+}\) extrusion processes in evertate tissues (e.g. squid axon [Wee76]) and vertebrate tissues and this wide occurrence of related mechanisms may indicate the relevance of Mg\(^{2+}\) extrusion for cellular magnesium homeostasis in general.

The mucosa-to-serosa transport of Mg\(^{2+}\) across tilapia intestine is for a major part transcellular and in situ Mg\(^{2+}\) extrusion appears Na\(^{+}\) dependent [§ 4.4]. These observations prompted us to assess Mg\(^{2+}\) transport over the basolateral plasma membrane, using resealed vesicles prepared from these membranes. We were unable to demonstrate Na\(^{+}\)-gradient driven Mg\(^{2+}\) transport in these vesicles, but we advance evidence for an ATP-driven Mg\(^{2+}\) pump.
chapter 4

MATERIALS AND METHODS

Fish

Male tilapia weighing around 200 g, were obtained from laboratory stock. The fish were kept in Delft municipal water with a magnesium concentration of about 0.34 mmol.l⁻¹ and at a temperature of 28°C. The light regime was 12 h light and 12 dark. The fish were fed a commercial food (Trouvit) six times per day using an automated food dispenser.

Statistics

Data are presented as mean values ± standard deviation, unless stated otherwise. Data were analyzed statistically using Student's t-test or one-way analysis of variance (Anova) where appropriate. Statistical significance was accepted when \( P < 0.025 \). Data on the kinetics of ATP-dependent Mg²⁺ transport by plasma membrane fragments were analysed by non-linear regression analysis [Lea87].

Isolation and characterization of basolateral plasma membranes

Basolateral plasma membranes were isolated as described in detail by Flik et al. [Fli90]. This procedure was used with some minor modifications. In short, fish were killed by spinal transection and the intestinal tract was rapidly removed from the fish. The proximal one third of the intestine was flushed with an ice-cold saline (150 mmol.l⁻¹ NaCl, 1 mmol.l⁻¹ HEPES/Tris pH 8.0, 0.1 mmol.l⁻¹ EDTA) and cut lengthwise. The mucosa was scraped off onto an ice-cooled glass plate. The scrapings were disrupted with a dounce homogenizer with a loosely fitting pestle (30 strokes) in a sucrose buffer (250 mmol.l⁻¹ sucrose, 10 mmol.l⁻¹ HEPES/Tris pH 8.0, 0.5 mmol.l⁻¹ DTT, 100 U.ml⁻¹ aprotinine). The suspension thus obtained was centrifuged for 25 min. at 1,540 g (L7-55 with rotor SW41, Beckman). The supernatant was centrifuged again for 35 min at 154,000 g. The top white fluffy part of the pellet was removed by careful whirling and resuspended (100 strokes in the same douncer device) in an isotonic sucrose buffer (250 mmol.l⁻¹ sucrose, 10 mmol.l⁻¹ HEPES/Tris pH 7.4, 100 U.ml⁻¹ aprotinine). The resuspended membranes were mixed with 1.25 volumes of 4.38 mol.l⁻¹ sucrose in 10 mmol.l⁻¹ HEPES/Tris pH 7.4. Aliquots of this suspension were carefully covered with
isotonic sucrose buffer and subsequently centrifuged isopycnically for 2 h at 154,000 g. The membranes appearing at the interface after centrifugation were collected with a tuberculin syringe, mixed with 300 mmol.l⁻¹ sucrose (or 150 mmol.l⁻¹ NaCl or KCl) in 20 mmol.l⁻¹ HEPES/Tris (pH 7.4) and centrifuged for 35 min at 154,000 g. The pellet was rinsed twice with the same buffer and vesiculation of the membranes was completed by resuspending them in 250 μl of this buffer by 25 passages through a 23-G needle. For the Mg²⁺ transport assays, the membranes were stored on ice without being frozen. Part of the membrane preparation was frozen to determine protein content, Na⁺/K⁺-ATPase activity and membrane orientation. Protein was determined conform Lowry et al. [Low51]. The protein content of membrane suspensions thus obtained was 17 ± 3 mg (n=8) "bovine serum albumin" equivalents. The Na⁺/K⁺-ATPase activity in the preparations was 143 ± 25 μmol P₅₀.h⁻¹.(mg protein)⁻¹ (n=6). The membrane configuration, determined as described recently [Fli90] was (15 ± 5)% inside-out oriented vesicles, (30 ± 8)% right-side-out oriented vesicles and (55 ± 7)% leaky membrane fragments (n=6). The membrane preparation was not enriched in apical membranes as judged from a purification factor (ratio of specific activity in the plasma membrane fraction and in the initial homogenate) of 0.56 ± 0.10 (n=6) for the apical membrane marker enzyme sucrase [Dah64]. The characteristics of the membrane preparation are very similar to values reported before for a comparable membrane preparation, in which Ca²⁺ extrusion mechanisms were defined [Fli90].

Radiotracer

The radiotracer ²⁷Mg²⁺ (t½ = 9.46 min) was used to study Mg²⁺ transport. The ²⁷Mg was produced by irradiation of Mg(CH₃COO)₂, containing magnesium enriched in ⁶⁷Mg to 97.1%, in a nuclear reactor [§ 2.3]. The specific activity at the start of the experiments was about 35 GBq.mol⁻¹. The ²⁷Mg activity was determined on β⁻-emission using a liquid scintillation counter (tri-carb 4000, Packard).

Vesicle Mg²⁺-uptake assays

Mg²⁺ transport into vesicles was determined using a rapid filtration technique, essentially as described by van Heeswijk et al. [Hee84]. The composition of the assay
medium was (final concentrations in mmol.l\(^{-1}\), at 37°C): sucrose (300) or NaCl (150) or KCl (150), Hepes/Tris (20, pH 7.4), Tris-ATP (0 or 3), HEEDTA (0.5), CDTA (0.5) and Mg\(^{2+}\) (up to 5.0). The (free) Mg\(^{2+}\) concentrations were varied by the addition of Mg(CH\(_3\)COO)\(_2\) to the assay medium and the actual free levels of Mg\(^{2+}\) were calculated as suggested by van Heeswijk et al. [Hee84] using the stability constants of the Mg\(^{2+}\) chelating ligands (ATP, ADP, HEEDTA and CDTA) given by Sillèn & Martell [Sil64]. The first and second protonations of the ligands were taken into account and the stability constants adjusted in accordance with the pH, temperature and ionic strength of the medium as suggested by Harrison & Bers [Har87]. In some experiments ATP was replaced by ADP or ATP-\(\gamma\)-S (Boehringer). The incubation temperature was 37°C. In some experiments ouabain (Boehringer) was dissolved in the assay medium to a final concentration of 1 mmol.l\(^{-1}\).

Five \(\mu\)l membrane suspension was pipetted to the wall of an Eppendorf tube containing 120 \(\mu\)l complete medium (at 37°C). The reaction was started by vortex mixing the contents of the tube. The reaction was stopped after 0 (blank) or 300 s by the addition of 1 ml icecold stop buffer containing 300 mmol.l\(^{-1}\) sucrose (or 150 mmol.l\(^{-1}\) NaCl or KCl), 20 mmol.l\(^{-1}\) HEPES/Tris pH 7.4 and 0.1 mmol.l\(^{-1}\) LaCl\(_3\)). For the blank determination the stop buffer was pipetted on top of the vesicle suspension pipetted against the wall. From the 'stopped reaction' 50 \(\mu\)l was taken to assess the \(^{27}\text{Mg}\) specific activity and 1 ml was filtered (RC 55; pore size 0.45 \(\mu\)m, Schleicher & Schuell) to collect the membranes. The filters were rinsed twice with the same buffer. The amount of membrane protein per filter was around 80 \(\mu\)g. The filters with retained \(^{27}\text{Mg}\) were placed in glass counting vials with 10 ml scintillation cocktail (flo-scint IV, Packard) and the radioactivity was determined. The vesicular Mg\(^{2+}\) intake was calculated from the difference in the \(^{27}\text{Mg}\) accumulated after 300 and 0 s. ATP-driven Mg\(^{2+}\) transport was defined as the difference in Mg\(^{2+}\) intake in the presence and absence of ATP. Pilot experiments on time dependency of magnesium intake in basolateral plasma membrane vesicles of tilapia enterocytes demonstrated that a 300 s period may yield an estimate for the initial velocity of the transport process (data not shown).

The half-life of \(^{27}\text{Mg}\) and the duration of most of the assays (300 s) allowed us to run 6 assays per batch of \(^{27}\text{Mg}\) produced. However, a steady loss of enzyme activity was observed over relatively short time periods (up to 60% decrease in \(V_{\text{max}}\) values on
the day of membrane isolation). Therefore we have normalized data obtained during the
day to the first values obtained on that day, in order to overcome the introduction of
large standard errors resulting from a loss of enzyme activity in the membrane
preparation. To this end each run of assays (dictated by the production of the $^{27}\text{Mg}$)
included a determination of $\text{Mg}^{2+}$ transport at 0.81 mmol.l$^{-1}$ $\text{Mg}^{2+}$.

RESULTS

Table 4.2.
$\text{Mg}^{2+}$ intake in enterocyte plasma membrane vesicles.

<table>
<thead>
<tr>
<th>conditions</th>
<th>-ATP</th>
<th>+ ATP</th>
<th>ATP-driven</th>
</tr>
</thead>
<tbody>
<tr>
<td>150Na$_i$/150K$_o$</td>
<td>0.16±0.11 (11)</td>
<td>0.32±0.30 (12)</td>
<td>0.16</td>
</tr>
<tr>
<td>150Na$_i$/150K$_o$ + monensin</td>
<td>0.14±0.10 (6)</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>150Na$_i$/150Na$_o$</td>
<td>0.15±0.09 (4)</td>
<td>0.79±0.09 (2)</td>
<td>0.64</td>
</tr>
<tr>
<td>150K$_i$/150K$_o$</td>
<td>0.08±0.04 (12)</td>
<td>0.29±0.05 (5)</td>
<td>0.21</td>
</tr>
<tr>
<td>150Na$_i$/150K$_o$ + 1·10$^{-6}$ Ca$^{2+}$</td>
<td>0.32±0.12 (5)</td>
<td>0.70±0.25 (7)</td>
<td>0.38</td>
</tr>
<tr>
<td>300sucrose$_i$/300sucrose$_o$</td>
<td>0.54±0.24 (22)*</td>
<td>1.89±0.78 (21)*</td>
<td>1.35</td>
</tr>
</tbody>
</table>

The $\text{Mg}^{2+}$ intake was determined at a calculated free $\text{Mg}^{2+}$ level of 0.81 mmol.l$^{-1}$ in
media with and without 3 mmol.l$^{-1}$ ATP. All values were corrected for '0' s values (see
Materials and Methods section). Data are expressed in nmol $\text{Mg}^{2+}$ taken in per min per
mg membrane protein. Mean values ± standard deviation are given; the number of
observations are indicated between brackets. Conditions: the subscripts $i$ and $o$ refer to
the media inside and outside of the vesicles, respectively (e.g. 150Na$_i$/150K$_o$ stands for
vesicles loaded with 150 mmol.l$^{-1}$ NaCl and transferred to medium containing 150
mmol.l$^{-1}$ KCl). n.d.: not determined; * significantly different from all other values in the
same column (one-way analysis of variance).

Assay conditions

Table 4.2 summarizes data on $\text{Mg}^{2+}$ intake under a variety of conditions. In all
instances, 3 mmol.l$^{-1}$ ATP enhanced the intake of $\text{Mg}^{2+}$. Vesicles kept in 300 mmol.l$^{-1}$
sucrose showed a higher $\text{Mg}^{2+}$ intake than vesicles prepared in ionic environments (150
mmol.l$^{-1}$ Na$^+$ or K$^+$). Moreover, the ATP-stimulated $\text{Mg}^{2+}$ intake (defined as the
difference in $\text{Mg}^{2+}$ intake in the presence and in the absence of 3 mmol.l$^{-1}$ ATP) was
the highest in vesicles in sucrose media. The presence of an outwardly directed \( \text{Na}^+ \) gradient did not stimulate the intake of \( \text{Mg}^{2+} \) into vesicles: no significant differences in \( \text{Mg}^{2+} \) intake were found when 150 mmol.l\(^{-1}\) \( \text{Na}^+ \) loaded vesicles were transferred to medium containing 150 mmol.l\(^{-1}\) \( \text{K}^+ \) or 150 mmol.l\(^{-1}\) \( \text{Na}^+ \). Moreover, addition of the \( \text{Na}^+ \)-ionophore monensin did not decrease the \( \text{Mg}^{2+} \) intake when \( \text{Na}^+ \) loaded vesicles were transferred to \( \text{K}^+ \) medium (in the absence of ATP). The presence of 1 \( \mu \text{mol.l}^{-1} \) \( \text{Ca}^{2+} \) in the medium did not affect the \( \text{Mg}^{2+} \) intake. Under these conditions a \( \text{Na}^+ \) driven \( \text{Ca}^{2+} \) uptake of around 10 nmol.min\(^{-1}\).(mg protein\(^{-1}\)) may be anticipated in this vesicle preparation [Fli90]. Proceeding from these findings we have performed the following experiments with vesicles in sucrose media.

Substrate specificity of \( \text{Mg}^{2+} \) transport

As shown in Fig. 4.3, in an ATP-free medium and at a calculated free \( \text{Mg}^{2+} \) concentration of 0.81 mmol.l\(^{-1}\), the \( \text{Mg}^{2+} \) intake was per mg protein was \( 0.87 \pm 0.32 \) nmol.min\(^{-1}\) (n=6). The addition of 3 mmol.l\(^{-1}\) ADP or ATP-\( \gamma \)-S did not affect this intake. However, the addition of 3 mmol.l\(^{-1}\) ATP stimulated the \( \text{Mg}^{2+} \) intake 2.2-fold to \( 1.89 \pm 0.78 \) nmol per min per mg protein. Ouabain (1 mmol.l\(^{-1}\)) did not affect the ATP-driven \( \text{Mg}^{2+} \) intake (1.51 \( \pm 0.67 \) nmol.min\(^{-1}\).(mg protein\(^{-1}\))).

Kinetics of ATP-dependent \( \text{Mg}^{2+} \) transport

Fig. 4.4 represents the free \( \text{Mg}^{2+} \) concentration ([\( \text{Mg}^{2+} \)] in mmol.l\(^{-1}\)) dependence of \( \text{Mg}^{2+} \) intake (v in nmol.min\(^{-1}\).(mg protein\(^{-1}\))). Fitting the data by non-linear regression analysis to the Michaelis-Menten equation, \( v = (V_{\text{max}}[\text{Mg}^{2+}])/(K_{\text{v}},+[\text{Mg}^{2+}]) \) yielded a half maximum activation concentration (\( K_{\text{v}} \)) for \( \text{Mg}^{2+} \) of 1.12 \( \pm 0.35 \) mmol.l\(^{-1}\) and a maximum velocity (\( V_{\text{max}} \)) of 5.10 \( \pm 0.53 \) nmol.min\(^{-1}\).(mg protein\(^{-1}\)) (n=5).

DISCUSSION

To our knowledge this is the first report providing direct evidence for an ATP-driven \( \text{Mg}^{2+} \) transport process across plasma membranes. The ATP-dependency of the \( \text{Mg}^{2+} \) intake, the Michaelis-Menten kinetics and therefore the evidence for a single
Fig. 4.3.
Substrate specificity of Mg\(^{2+}\) transport by basolateral plasma membrane fragments of tilapia enterocytes. Mg\(^{2+}\) transport was assayed at a concentration of 0.81 mmol.l\(^{-1}\) Mg\(^{2+}\) and at 3 mmol.l\(^{-1}\) of the respective substrates. ATP, but not ATP-\(\gamma\)-S or ADP stimulated Mg\(^{2+}\) intake significantly in basolateral plasma membrane vesicles.

Mg\(^{2+}\) transport site, and the \(K_{i/2}\) for Mg\(^{2+}\) of 1.12 mmol.l\(^{-1}\) lead us to conclude that this transporter may be an \(E_{1}E_{2}\)-type ATPase activated by cytosolic Mg\(^{2+}\).

In the absence of ATP significant intake of Mg\(^{2+}\) occurred (all values represent numbers obtained as the difference in Mg\(^{2+}\) intake after '0' and 300 s). At least some of this Mg\(^{2+}\) intake may reflect Mg\(^{2+}\) binding to the membranes: Bers et al. [Ber86b]
Fig. 4.4.
Kinetics of ATP-dependent Mg$^{2+}$ transport by basolateral plasma membrane fragments of tilapia enterocytes. Initial rates of Mg$^{2+}$ intake into vesicles of these membrane fragments at 3 mmol.l$^{-1}$ ATP were plotted as a function of the calculated free Mg$^{2+}$ concentration and the data were fitted by non-linear regression analysis according the Michaelis-Menten equation. Each point represents a mean value $\pm$ standard deviation for 6 to 21 observations. The half maximal activation concentration ($K_{m}$) $\pm$ standard deviation for the ATP driven Mg$^{2+}$ transport of the preparation is $1.12 \pm 0.35$ mmol.l$^{-1}$ and the maximum velocity ($V_{\max}$) $\pm$ standard deviation is $5.10 \pm 0.53$ nmol.min$^{-1}$.mg protein$^{-1}$.

have drawn a similar conclusion for the binding of Ca$^{2+}$ to cardiac sarcolemmal vesicles, that increased when strong ionic media (containing NaCl or KCl) were replaced by sucrose media. Assuming analogy for Ca$^{2+}$ and Mg$^{2+}$ binding sites with respect to competition by Na$^{+}$ or K$^{+}$ for binding to these sites, such competition could explain the
higher Mg$^{2+}$ intake observed in vesicles in sucrose media compared to NaCl or KCl media.

Mg$^{2+}$ transport in the tilapia epithelium is partly Na$^{+}$ dependent [§ 4.4]. Unexpectedly, therefore, no significant differences in Mg$^{2+}$ intake were observed when Na$^{+}$ loaded vesicles were transferred to K$^{+}$ medium and to Na$^{+}$ medium (the classical differential assay for Na$^{+}$ gradient driven counter-transport, e.g. for Na$^{+}$/Ca$^{2+}$ exchange [Flie90]). Also no differences were found between Mg$^{2+}$ intake in the presence of a Na$^{+}$ gradient and its absence as determined in K$^{+}$ loaded vesicles transferred to K$^{+}$ medium. The presence of DTT (added as an antioxidant) during the initial steps of the membrane isolation could have abolished any Na$^{+}$/Mg$^{2+}$ exchange activity in our membrane preparation, assuming an analogy between a presumed Na$^{+}$/Mg$^{2+}$ exchanger and the Na$^{+}$/Ca$^{2+}$ exchanger for this sulfhydryl reagent, that keeps the Na$^{+}$/Ca$^{2+}$ exchanger 'locked' [Par88]. However, also in membranes isolated in the absence of this reagent during isolation we could not demonstrate Na$^{+}$-driven Mg$^{2+}$ transport. Furthermore, one could argue that the 300 s duration of the assay was too long to properly assay a Na$^{+}$ gradient driven Mg$^{2+}$ intake. However, the Mg$^{2+}$ intake appeared linear in time at least up to 300 s.; moreover, proceeding from a rate of Mg$^{2+}$ intake of 0.16 nmol.min$^{-1}$.mg protein$^{-1}$ in the absence of ATP for Na$^{+}$ loaded vesicles transferred to K$^{+}$ medium (Table 4.2), a vesicular space of 3 µl.mg protein$^{-1}$ [Flie90] and 45% resealed vesicles, we calculate an intravesicular Mg$^{2+}$ concentration of $(0.16\cdot5)/(0.45\cdot3) = 0.59$ mmol.l$^{-1}$ after 5 min. Assuming that two Na$^{+}$ are exchanged for one Mg$^{2+}$ [Gün89c], it follows that this process would have hardly (only by 1.18 mmol.l$^{-1}$) influenced the dissipation of the initial Na$^{+}$ gradient of 150 mmol.l$^{-1}$.

Under all conditions tested, addition of ATP stimulated Mg$^{2+}$ intake in vesicles and the strongest in vesicles in sucrose media. At the moment we have no data to elucidate what causes the lower ATP-driven Mg$^{2+}$ transport in vesicles kept in high ionic strength media compared to vesicles kept in sucrose. Clearly, competition between Na$^{+}$ or K$^{+}$ and Mg$^{2+}$ for binding to a carrier could explain this phenomenon, but appropriate kinetical data are lacking. The fact that Mg$^{2+}$ intake is stimulated by ATP in vesicles kept in sucrose media (containing no detectable Na or K) strongly suggest that this transport is mediated by a carrier (the process is saturable) that does not require Na$^{+}$. 

75
or K⁺ for its action. As ATP-γ-S does not stimulate Mg²⁺ intake it appears not to be a kinase mediated protein phosphorylation, a mechanism that underlies the activation of Na⁺/Mg²⁺ exchange activity in squid axon [DiP88]. Our results support the suggestion drawn by Frenkel et al. [Fre89] that Mg²⁺ extrusion (whatever carrier is involved) in human erythrocytes requires ATP as an energy supply.

One could argue that the Mg²⁺ intake resulted (indirectly) from Na⁺/K⁺-ATPase activity that was shown to maintain a Na⁺ gradient sufficiently steep to drive Ca²⁺ transport in these vesicles [Fli90]. However, in our particular set up no Na⁺ or K⁺ was present to stimulate this ATPase. Moreover, no evidence for a Na⁺/Mg²⁺ exchange activity could be advanced by creating a Na⁺ gradient over the membrane vesicles (a gradient that does stimulate Ca²⁺ transport in these vesicles [Fli90]. Finally, the specific Na⁺/K⁺-ATPase inhibitor ouabain exerted no effect on the ATP-driven Mg²⁺ transport. Thus a Mg²⁺ translocating ATPase appears to exist in the plasma membrane of the fish enterocyte.

The affinity of the Mg²⁺ transporter for Mg²⁺ (1.12 ± 0.35 mmol.l⁻¹) is in good agreement with the notion that the Kᵥ₂ value of a particular ion that activates a carrier at a cytosolic side is always close to the resting cytosolic activity of that ion [Pre88]. The intracellular free Mg²⁺ levels reported in the literature cover a considerable range, but the higher values may have originated primarily from technical problems related to Mg²⁺ measurements [Alv87]: typical values are e.g. 0.50 - 1.29 mmol.l⁻¹ for *Helix aspersa* neurons [Alv84] and 0.80 - 1.69 mmol.l⁻¹ for frog skeletal muscle cells [Alv86]. The values of 3 - 4 mmol.l⁻¹ in frog, sheep and ferret striated muscle cells [Hes82; Lóp84] may have resulted from interferences of Na⁺ or K⁺ with the Mg²⁺ electrodes available at that time.

Assuming that the Kᵥ₂ of the enzyme indeed reflects the free Mg²⁺ in the cytosol [Pre88], this correlation strongly favours the presence of an intracellular Mg²⁺ site of activation. Conversely, it argues against a role for an ecto-ATPase activity [Ste87] in our assays (where the right-side-out oriented vesicles could contribute), as this kind of enzyme was shown to be inhibited by Mg²⁺ and may also depend on non-hydrolyzable ATP-analogues for its activity. Also, the Kᵥ₂ of the plasma membrane Mg²⁺ pump is in line with reported Kᵥ₂ values for intracellular magnesium stores: Diwan et al. [Dìw79] reported a Kᵥ₂ for Mg²⁺ uptake by rat liver mitochondria of 0.7 mmol.l⁻¹. Any
compartment that exports Mg\(^{2+}\) from the cytosol may be predicted to have a \(K_v\) in this concentration range.

Proceeding from the assumption that the ATP binding site of the Mg\(^{2+}\) transporter is directed towards the cytosol, the ATP-driven Mg\(^{2+}\)-transport occurs in the 15% inside-out oriented vesicles of the membrane preparation. The actual maximum velocity of the Mg\(^{2+}\) transport process is thus 6.7 times higher for a 100% inside-out oriented preparation (around 34 nmol.min\(^{-1}\). (mg protein\(^{-1}\)). This \(V_{\text{max}}\) for Mg\(^{2+}\) transport in these vesicles is roughly 15.6-fold higher than the ATP-driven Ca\(^{2+}\) transport and 2.5-fold higher than the Na\(^{+}\)-gradient driven Ca\(^{2+}\)-transport (13.6 nmol.min\(^{-1}\).mg\(^{-1}\)) reported for these membranes [Fli90]. Yet this value for \(V_{\text{max}}\) may be an underestimation, considering the loss of enzyme activity observed in our membrane preparations. However, comparing the net mucosa-to-serosa Ca\(^{2+}\)- and Mg\(^{2+}\)-fluxes reported for the tilapia intestine of 34 and 23 nmol.h\(^{-1}\).cm\(^{-2}\), respectively [Fli90; § 4.4], the Mg\(^{2+}\)-transport-ATPase appears to operate in situ below saturation, but may well be a driving force of physiological significance.

Future studies should focus on the relative importance of this Mg\(^{2+}\)-transport-ATPase for Mg\(^{2+}\) transport in vitro as well as in vivo, and its occurrence in other species. The unprecedented use of \(^{27}\)Mg\(^{2+}\) as radiotracer for Mg\(^{2+}\) for such studies should be considered by those who have access to reactor facilities.
CHAPTER 5

EFFECTS OF LOW-MAGNESIUM DIETS

In this chapter effects of a low-magnesium diet on growth rate, on the magnesium, calcium and sodium concentration in various tissues, on magnesium transport and on ion regulation are described.

§ 5.1

INTRODUCTION

Magnesium deficient feeding of freshwater teleosts often increased the mortality or leads to changes in ionic composition of the fish [Shi88; Dab89; She89]. Magnesium requirements of freshwater fish have been established by a number of researchers in experiments in which fish were fed with low-magnesium diets. For optimal growth of the rainbow trout (Oncorhynchus mykiss) magnesium requirements have been reported to be 10-20 mmol.kg$^{-1}$ diet (initial fish weight 16 and 35 g; [Kno81]), 25-29 mmol.kg$^{-1}$ diet (initial fish weight 0.9 g; [Ogi78]) and 54-58 mmol.kg$^{-1}$ diet (initial fish weight 12 g; [She89]). Magnesium requirements of around 20 mmol.kg$^{-1}$ diet have been reported for channel catfish (Ictalurus punctatus; initial fish weight 5 to 8 g; [Gat82]), for the carp (initial fish weight 2.8 g; [Ogi76]) and the guppy (Poecilia reticulata; initial fish weight 0.17 g; [Shi88]). For young Nile tilapia (Oreochromis niloticus) with an initial fish weight of 10 g, optimum growth occurred at 24 to 32 mmol.kg$^{-1}$ diet [Dab89].

From the above literature data on the magnesium requirement of freshwater fish, we have concluded that a control diet should contain about 30 mmol magnesium per kg. To study the physiological effects of low-magnesium diets, two low-magnesium diets (magnesium concentration 13 and 1 mmol.kg$^{-1}$) and a control diet were used. Further details on these diets are given in § 5.2.

In this chapter we report on the effects of low-magnesium diets on growth rate, on the magnesium, calcium and sodium concentrations in various tissues and on the magnesium intake from the water. Further, some pilot experiments have been performed
on the effects of low-dietary magnesium on the ion regulation of freshwater fish. Our studies concerned young (1-9 g) and adult (about 100 g or heavier) tilapia and adult carp (about 100 g or heavier). In the literature, data on low-magnesium feeding concern only young fish or small fish species (up to 50 g). The rationale for studying young and adult fish specimens was to assess possible differences in magnesium uptake strategies between young and adult fish. Finally, we compared the responses of two fish species, tilapia and carp. These species were selected because the tilapia is known as a fish showing strong adaptive responses and the carp as a fish showing tolerance rather than adaptation to strenuous conditions.
§ 5.2

EFFECTS ON GROWTH RATE AND ELEMENTAL TISSUE CONCENTRATIONS OF TILAPIA

ABSTRACT
The growth rate and magnesium, calcium and sodium concentration in scales, bone and muscle of freshwater tilapia, weighing about 150 g or heavier, were followed during low-magnesium feeding. No mortality was observed. The growth rate decreased in fish on low-magnesium diets, but no changes were observed in the magnesium concentration in the scales, bone or muscle. Neither there were changes observed in calcium or sodium concentrations in these tissues. We conclude that adult tilapia fed a low-magnesium diet manage, in contrast to other fish species, to maintain their magnesium balance and must obtain magnesium from the water.

INTRODUCTION

In fish species other than tilapia, a low-magnesium diet often led to a high mortality, a reduced growth rate and/or to changes in the elemental concentration of tissues [e.g. Shi88]. In this section we tested the effects of two low-magnesium diets on the growth rate and elemental tissue composition of the tilapia. This study will also allow us to evaluate the importance of dietary magnesium for this species. These parameters were determined in tilapia of about 150 g or heavier, fed on low-magnesium diets.

MATERIALS AND METHODS

Fish
Adult male tilapia were kept in all glass aquaria filled with 450 l Nijmegen tap
water. The water was filtered by recirculation (500 l.h\(^{-1}\)) through an 800 l biological filter system and continually refreshed with 300 l water per day. The magnesium, calcium and sodium concentrations of the tap water were 0.20 ± 0.03, 0.7 ± 0.1 and 0.6 ± 0.1 mmol.l\(^{-1}\), respectively, the pH was around 7.6 and the temperature was kept at 25°C. The automatically controlled light regime was 12 h light and 12 h dark. About 1 month prior to the start of the experiment, the fish were placed under these conditions and fed a control diet (see below).

Diets

The fish received six rations of food per day by means of an automated food dispenser. For food three diets were used, namely a control diet and two low-magnesium diets (Diet "A" and "B"). The composition of the control diet is shown in Table 1. In the experimental diets MgO and MgSO\(_4\).2H\(_2\)O were partly or completely replaced by glucose monohydrate. The total magnesium concentration as determined by atomic absorption spectrophotometry was 30 mmol.kg\(^{-1}\) in the control diet, 13 mmol.kg\(^{-1}\) in diet "A" and 1 mmol.kg\(^{-1}\) in diet "B". All diets were produced by Hope Farms.

Experimental design

To determine the growth rate, fish were weighed weekly on a balance (PM34 Delta Range, Mettler). The use of this balance allows weighing of free swimming fish, which minimize stress to netting. For data analysis, the individual weights of the fish were ranked per group. The growth data were equidistantly plotted in a three-dimensional figure, with group size, week number and body weight on the X-, Y- and Z-axis, respectively.

Throughout the experiment fish were randomly sampled for the determination of the magnesium, calcium and sodium concentration in the scales, bone and muscle. To this end fish were killed by spinal transection. From both sides of the fish, ten scales were collected at fixed spots rostrally near the side line. White muscle was collected from the dorsal musculature. Good care was taken not to include bony parts. After microwave-cooking, three vertebrae of the caudal spinal cord were collected and freed of adherent muscle. All fractionated tissue material was weighed, lyophilized and weighed again to obtain the tissue water content. The magnesium, calcium and sodium concentrations of
Table 5.1.
Composition of the control diet (data: Hope Farms, Woerden, The Netherlands).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% in diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>32.0</td>
</tr>
<tr>
<td>Gelatin</td>
<td>8.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>3.0</td>
</tr>
<tr>
<td>Fish oil</td>
<td>3.0</td>
</tr>
<tr>
<td>α-cellulose</td>
<td>6.0</td>
</tr>
<tr>
<td>Carboxymethyl cellulose</td>
<td>2.0</td>
</tr>
<tr>
<td>Glucose monohydrate</td>
<td>34.2</td>
</tr>
<tr>
<td>Vitamin/trace element mix</td>
<td>5.0</td>
</tr>
<tr>
<td>KH₂PO₄·2H₂O</td>
<td>1.2</td>
</tr>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>1.7</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1.7</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>1.7</td>
</tr>
<tr>
<td>Choline chloride 50 %</td>
<td>0.3</td>
</tr>
<tr>
<td>MgO</td>
<td>0.1</td>
</tr>
<tr>
<td>MgSO₄·2H₂O</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The vitamin and trace element mix supply per kilogram of diet: 10,000 IU vitamin A (added as retinyl acetate); 1000 IU cholecalciferol; 10 mg menadione sodium bisulfite; 200 mg dl-α tocopherol; 20 mg thiamine mononitrate; 20 mg riboflavin; 20 mg pyridoxine HCl; 75 mg niacin; 50 mg calcium dl-pantothenate; 0.02 mg cyanocobalamin; 0.1 mg biotin; 5 mg folic acid; 200 mg inositol; 100 mg Fe (as Fe₃O₄); 115 mg Mn (as MnO); 100 mg Zn (as ZnO); 7.6 mg Cu (as CuCO₃·Cu(OH)₂); 4.5 mg I (as Ca(IO₃)₂); 0.75 mg Co (as CoSO₄·H₂O); 3.3 mg Mo (as (NH₄)₄Mo₇O₂₄·4H₂O); 0.09 mg Se (as Na₂SeO₃). Glucose monohydrate was used as carrier of this mix.

scales, white muscle and skeletal bone were determined on the lyophilized samples by non-destructive instrumental neutron activation analysis. This method of analysis is described in § 2.2.

Experiment 1

A group of 41 fish (mean initial weight 158 ± 34 g) was fed the low-magnesium diet "A", and another group of 41 fish (mean initial weight 172 ± 39 g) the control diet. The fish were weighed weekly and fed daily 1.5% of the total wet weight of the group for 10 weeks, subsequently 1.25% for 2 weeks, and 1% thereafter. Fish fed on this regimen consumed all the food provided. The experiment lasted for 14 weeks.
Experiment 2

One group of 40 fish (mean initial weight 199 ± 41 g) was fed the low-magnesium diet "B", and another group of 41 fish (mean initial weight 213 ± 45 g) with the control diet. The fish were weighed weekly and fed daily 1.5% of the total wet weight of the group. The experiment lasted for 19 weeks.

Statistics

Data are presented as mean values ± standard deviation, unless stated otherwise. Data were statistically analysed using the Student's t-test. The normal distribution of the body weight data was assessed by the use of the Kolmogorov-Smirnov test [Web88]. The data proved to be normally distributed (data not shown). Therefore, the use of the Student's t-test is justified. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

No mortality, diseases or apparently abnormal behaviour was observed in the tilapia fed control or experimental diets.

In Experiment 1, the mean body weights after 14 weeks had increased from 158 ± 34 g (n=41) to 205 ± 45 g (n=19) and from 172 ± 39 g (n=41) to 288 ± 64 g (n=20) for the experimental and the control group, respectively. From these data we calculated a mean increase in body weight over a 14 weeks period of 30% and 67%, respectively. Three-dimensional plots of the weight profile of the tilapia populations are given in Figures 5.1A and 5.1B. In Experiment 2, the mean body weights after 19 weeks had increased from 199 ± 41 g (n=40) to 306 ± 77 g (n=19) and from 213 ± 45 g (n=41) to 383 ± 67 g (n=19) for the experimental and control group, respectively, equivalent to a mean increase in body weight over a 19 weeks period of 54% and 80%, respectively. Three-dimensional plots of the weight profile of the tilapia populations are given in Figures 5.1C and 5.1D. In Experiment 1 as well as in Experiment 2 the weights of the experimental and control fish at the start of the experiment did not differ significantly. Upon completion of the experiments the experimental groups showed a significantly lower mean body weight ($P < 0.002$) than the control groups.
Figure 5.1.
Effects of low-magnesium diets on the weight profile of tilapia populations. Three-dimensional plots of week number (X-axis) versus group size number (Y-axis) versus wet body weight (Z-axis). A) Experiment 1, control (30 mmol.kg⁻¹ diet); B) Experiment 1, diet "A" (13 mmol.kg⁻¹ diet); C) Experiment 2, control (30 mmol.kg⁻¹ diet); D) Experiment 2, diet "B" (1 mmol.kg⁻¹ diet).

Both in Experiment 1 and 2 the concentrations of magnesium, calcium and sodium of the scales, muscle and skeletal bone did not change during the experimental
period. The mean concentrations of the elements in these tissues are given in Table 5.2. Except for a slight and statistically significant ($P < 0.002$) increase in the muscle calcium concentration of tilapia fed diet "A", no differences between control and experimental animals were observed.

**Table 5.2.**
Mean tissue element concentrations ± standard deviation (in mmol.kg$^{-1}$ dry weight) as determined by instrumental neutron activation analysis. The number of observations are in brackets.

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>diet &quot;A&quot;</td>
<td>control</td>
<td>diet &quot;B&quot;</td>
</tr>
<tr>
<td>Mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>scales</td>
<td>106 ± 12 (22)</td>
<td>101 ± 17 (19)</td>
<td>102 ± 10 (27)</td>
<td>99 ± 13 (28)</td>
</tr>
<tr>
<td>muscle</td>
<td>61 ± 6 (19)</td>
<td>63 ± 4 (17)</td>
<td>65 ± 6 (27)</td>
<td>65 ± 5 (28)</td>
</tr>
<tr>
<td>bone</td>
<td>130 ± 16 (15)</td>
<td>123 ± 22 (17)</td>
<td>128 ± 13 (27)</td>
<td>126 ± 17 (32)</td>
</tr>
<tr>
<td>Ca</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>scales</td>
<td>4050±300 (21)</td>
<td>4000±300 (20)</td>
<td>3850±350 (27)</td>
<td>3800±450 (28)</td>
</tr>
<tr>
<td>muscle</td>
<td>12± 2 (14)</td>
<td>15± 2 (13)*</td>
<td>12± 3 (26)</td>
<td>12± 2 (25)</td>
</tr>
<tr>
<td>bone</td>
<td>4700±550 (15)</td>
<td>4650±400 (17)</td>
<td>4450±300 (27)</td>
<td>4450±400 (26)</td>
</tr>
<tr>
<td>Na</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>scales</td>
<td>232 ± 8 (13)</td>
<td>226 ± 11 (13)</td>
<td>238 ± 13 (27)</td>
<td>231 ± 14 (28)</td>
</tr>
<tr>
<td>muscle</td>
<td>62 ± 17 (10)</td>
<td>60 ± 10 (9)</td>
<td>55 ± 8 (26)</td>
<td>56 ± 8 (28)</td>
</tr>
<tr>
<td>bone</td>
<td>199 ± 14 (9)</td>
<td>208 ± 16 (10)</td>
<td>218 ± 17 (28)</td>
<td>213 ± 15 (31)</td>
</tr>
</tbody>
</table>

control diet : 30 mmol Mg per kg  
diet "A" : 13 mmol Mg per kg  
Diet "B" : 1 mmol Mg per kg  
*P < 0.002

The water content in the tissues did not differ between control and experimental animals. The mean water content of scales, muscle and skeletal bone of fish sampled in Experiment 1 were 36.1 ± 5.0% (n=29), 77.0 ± 1.2% (n=29) and 44.0 ± 3.9% (n=29), respectively; for Experiment 2 these values were 41.2 ± 3.9% (n=34), 75.7 ± 1.0% (n=34) and 41.3 ± 6.3% (n=34), respectively.
DISCUSSION

Adult freshwater tilapia survived low-magnesium diets for at least 19 weeks without noticeable effects on the magnesium concentrations in the scales, bone or muscle with controls. These results contrast with reports in the literature on other fish species. For instance, in rainbow trout (initial weight 21 to 35 g) the muscle magnesium concentration after about 19 weeks of feeding with a diet containing 1.5 mmol magnesium per kg was 15% lower than in fish on the control diet containing 24 mmol.kg\(^{-1}\) [Kno81; Kno83]. In guppy of about 0.2 g a decrease of 12% was observed after 15 weeks feeding with a diet containing 8 mmol magnesium per kg, instead of the control value of 37 mmol.kg\(^{-1}\) [Shi88]. For the bone compartment, the reported decreases in magnesium concentration in fish fed a low-magnesium diet were even larger. In carp of about 8 g fed a diet containing 3 mmol magnesium per kg for four weeks, bone magnesium was 40% lower than in the controls receiving diet with 41 mmol.kg\(^{-1}\) [Ogi76]. In channel catfish of about 8 g a decrease of 54% in 10 weeks was observed in the bone magnesium concentration of fish fed a diet containing 1.5 mmol magnesium per kg (the control diet contained 43 mmol.kg\(^{-1}\)) [Gat82]. In 1 g rainbow trout a decrease of 70% was observed after 6 weeks feeding with a diet containing 2 mmol magnesium per kg compared to 32 mmol.kg\(^{-1}\) in the control assay [Ogi78]. In rainbow trout with an initial weight of 21 g, Knox et al. [Kno83] observed a decrease of 12% in the bone magnesium concentration after 19 weeks feeding with a diet containing about 1 mmol magnesium per kg (the control food contained 24 mmol.kg\(^{-1}\)). A reduction of 60% in 15 weeks was observed in 0.2 g guppy on a diet containing 8 mmol magnesium per kg. In this experiment the control diet contained 37 mmol.kg\(^{-1}\) [Shi88]. The higher sodium concentrations in muscle and bone and the higher calcium concentrations in bone reported for fish fed low-magnesium diets [Cow77; Ogi78; Kno81; Kno83; Shi88] also conflict with the results in this study. We suggest that species differences in ion regulatory capacity must explain the impact of low-magnesium diets on tissue magnesium, calcium and sodium concentrations of these fish, as will be discussed below. However, it should be noted that all literature reports refer to experiments with younger fish or smaller fish species than the fish used in our experiments.
The values for magnesium and calcium reported in this study for muscle, bone and scales of the control fish are in line with earlier reports by us on tilapia fed a commercial standard fish food (containing around 31 mmol magnesium per kg) and kept under comparable water conditions [Fliss6a; § 3.3]. This indicates that the artificial control diet used in the present experiments is a food of full value.

The decreased growth rate of tilapia under low-magnesium feeding conditions observed in this study is in line with literature data on rainbow trout, carp, channel catfish, guppy and Nile tilapia [Ogi76; Cow77; Ogi78; Kno81; Kno83; Gat82; Shi88; Dab89; She89]. The absence of mortality in our study contrasts with studies on carp, channel catfish and guppy [Ogi76; Gat82; Shi88].

### Table 5.3.
Growth and magnesium accumulation in tilapia.

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>diet &quot;A&quot;</td>
<td>control</td>
<td>diet &quot;B&quot;</td>
</tr>
<tr>
<td>$W_f(0)$</td>
<td>172</td>
<td>158</td>
<td>213</td>
<td>199</td>
</tr>
<tr>
<td>$W_f(t_c)$</td>
<td>288</td>
<td>205</td>
<td>383</td>
<td>306</td>
</tr>
<tr>
<td>$\Delta Q_f$</td>
<td>2007</td>
<td>833</td>
<td>2864</td>
<td>1832</td>
</tr>
<tr>
<td>$\Delta t$</td>
<td>98</td>
<td>98</td>
<td>133</td>
<td>133</td>
</tr>
<tr>
<td>$\Delta Q_f/\Delta t$</td>
<td>20.5</td>
<td>8.5</td>
<td>21.5</td>
<td>13.8</td>
</tr>
<tr>
<td>Feeding</td>
<td>1.4</td>
<td>1.4</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>$[\text{Mg}]_{\text{food}}$</td>
<td>30</td>
<td>13</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>$Q_{\text{food}}$</td>
<td>96.6</td>
<td>33.0</td>
<td>134.1</td>
<td>3.8</td>
</tr>
</tbody>
</table>

$W_f(0)$ and $W_f(t_c)$ represent the mean wet body weight at the start and the end of the experiment (in g), respectively. $\Delta Q_f$ represents the difference between the calculated total magnesium inventory in the fish at the start and at the end of the experiment (in μmol) according to $Q_f = 116.3(0.253W)^{0.896}$ [§ 3.3]. $\Delta t$ represents the duration of the experiment (in day). Feeding represents the amount of diet per unit of body weight fish per day (in % day$^{-1}$). $[\text{Mg}]_{\text{food}}$ represents the magnesium concentration in the food (in mmol kg$^{-1}$). $Q_{\text{food}}$ represents the magnesium quantity in the food consumed per fish (in μmol day$^{-1}$).

We conclude that adult tilapia fed a low-magnesium diet still augment their total body magnesium pool, although not to the degree observed under control conditions. Fish fed diet "A" (13 mmol kg$^{-1}$) as well as diet "B" (1 mmol kg$^{-1}$) showed a decreased
growth rate. Therefore, we conclude that the dietary magnesium requirement for optimal growth is more than 13 mmol.kg\(^{-1}\) food. Apparently for survival and maintenance of magnesium homeostasis the requirement is equal to or less than 1 mmol magnesium per kg food. We conclude then that the magnesium concentration of the food can be a limiting factor for growth. This conclusion might suggest that tilapia are fully dependent for their magnesium supply from the food, and unable to extract magnesium from the water. However our data show that this conclusion is not correct. Diet "B" provides the fish with around 4 \(\mu\)mol magnesium per day (Table 5.3). From the increase in body weight and the total body magnesium concentration in these fish [§ 3.3] it can be calculated that the fish were in fact accumulating magnesium at a rate of 14 \(\mu\)mol per day (Table 5.3). Apparently these fish had access to another source of magnesium than the food, and this can only be the water. The fish may drink it or take it up with the food or absorb magnesium directly from the water via the integument, in particular the gills. For a 20 g tilapia kept under comparable water conditions a drinking rate of 28 \(\mu\)l.h\(^{-1}\) is reported [Fli85a]. A linear extrapolation of this value for a 250 g tilapia results in a magnesium intake via drinking of only 1.7 \(\mu\)mol.day\(^{-1}\). This indicates that the intestinal uptake of magnesium cannot account for the magnesium accumulation we determined in the fish put on the low-magnesium diets. This points to the gills as an additional route of magnesium intake from the water in tilapia. For tilapia direct measurements of magnesium intake from the water are not available. For carp however, we determined a magnesium intake from the water of 70 nmol.h\(^{-1}\) for a 60 g carp [§ 4.3]. Assuming a comparable intake for tilapia we calculate, by linear extrapolation of this value to a 250 g tilapia, a magnesium intake via the integument of 7 \(\mu\)mol.day\(^{-1}\). Thus the estimated total magnesium intake of fish fed diet "B" from food and water (drinking and integumental intake) is of the same order as the growth related magnesium increase in the fish. However it should be noted that these fish, in analogy to carp [§ 4.3], will also have a considerable loss of magnesium. Future studies should also address magnesium loss under low-magnesium feeding conditions. The question remaining is how tilapia exactly keep up with their magnesium balance under normal and under low-magnesium feeding conditions. In general, tilapia adapt relatively fast and well to changes in their environment such as a drop in ambient calcium concentration or water pH [Che82;
chapter 5

Wen84; Fli86a; Fli89b]. Possible adaptations to low-magnesium feeding may be (i) a more efficient absorption of magnesium in the intestinal tract, (ii) diminished magnesium loss via gills, body surfaces and kidney, (iii) higher intake of magnesium from the water via the integument or by drinking or (iv) any combination of these possibilities.

Further measurements of drinking rates, magnesium intake from the water, magnesium loss and Mg$^{2+}$ fluxes across the intestinal epithelium of tilapia fed control and low-magnesium diets may give information on the possible adaptation strategies of this fish to low-magnesium food. Some of these aspects will be studied and/or discussed later on in this thesis.
§ 5.3

EFFECTS ON THE ION REGULATION OF TILAPIA

ABSTRACT
Feeding tilapia a low-magnesium diet (1 mmol.kg⁻¹) decreases their growth rate but does not affect the blood ionic composition at least on the long term. Pituitary prolactin cell activity is enhanced as judged from the rate of synthesis of ³H-leucine labelled prolactin in vitro and from the ultrastructure of the prolactin cells. Reduction of the Na⁺-intake and proliferation of chloride cells, phenomena typical for enhanced prolactin cell activity, were observed. We conclude that the ion regulation of tilapia changed during low-magnesium feeding, even though no net effects on elemental composition of the body compartments were found.

INTRODUCTION

As reported earlier, in several studies in which freshwater teleosts were fed low-magnesium diets, the elemental composition in tissues of these fish had changed [Chapter 1; § 5.2]. This indicates that reduced dietary magnesium intake may disturb the regulation of ions other than magnesium. However, in adult freshwater tilapia fed on a diet containing only 1 mmol magnesium per kg, we could not find changes in the magnesium, calcium or sodium concentration of scales, muscle or bone [§ 5.2]. However, although the elemental tissue concentrations in low-magnesium fed tilapia had not changed, it is of course possible that changes occur in the regulation of these ions. A similar effect was found in tilapia exposed to acidified water: whereas the total body pool of sodium of these fish exposed to acidified water had not altered, we found changes in the intake and loss of sodium indicating that adaptation of the sodium regulation to the altered environmental conditions had taken place [Fli89b]. Thus for evaluation of the
effects of low-magnesium feeding on ion regulation, some parameters other than ion concentration and elemental composition of tissues have to be examined. Therefore a regulator and some regulator targets we studied in addition. An important regulator that dominates the water and ion homeostasis of the tilapia is the hormone prolactin. The main function of this pituitary hormone is considered the control of the permeability to water and ions of the integument, in particular the gill surface [Ber86a; Wen89a]. The hormone was further shown to enhance the calcium uptake capacity of the gills [Fli86b]. Reliable assays for determining prolactin levels in the blood are not available yet for the fish species used in this thesis. Therefore, prolactin cell activity was determined by biochemical estimations of prolactin synthesis rate and electron microscopical examinations. A target for prolactin are the chloride cells [Fos82; Ede84; Fli89a]. These cells contain the mechanisms for the active intake of sodium and calcium ions from the water [May87]. We estimated the chloride cell density in the inner opercular membrane; a sensitive parameter for determinations of changes in active transport of ions in general [Fos81]. Other parameters chosen, which are known to correlate with changes in prolactin activity, were the branchial sodium intake and blood plasma composition. The hematocrit values were determined because changes have been related to primary ion regulatory failure [Mil82].

MATERIAL AND METHODS

Fish

Male tilapia were obtained from laboratory stock. At the beginning of the experiments the mean body weight of the groups of fish used was about 200 g. The fish were kept in all glass aquaria with 450 l Nijmegen tapwater at a maximal density of 40 g fish per litre water. The total magnesium concentration in the water was 0.20 ± 0.03 mmol.l⁻¹ (n = 3), the water pH was around 7.6 and the temperature controlled at 25°C. The water was recirculated through a 800 l biological filter system and continually refreshed with a flow of 300 l tapwater per day. The automatically controlled light regime was 12 h light and 12 h dark.
Diets

The fish received food in dry pellet form at a dose of 1.5% of the estimated total wet body weight of the population per day, in six rations per day using an automated food dispenser. Control fish received a diet containing 30 mmol Mg per kg; experimental fish received a diet containing 1 mmol Mg per kg. Both these diets are described in § 5.2.

Blood parameters

Fish were anaesthetized with 0.4 mmol.l⁻¹ "MS222" (Sigma). Blood (mixed arterial and venous) was collected by puncture of the caudal vessels with a syringe fitted with a 21-G needle. Calcium-heparin (Radiometer) was used as an anticoagulant. Next the fish were killed by spinal transection. Blood was collected and hematocrit determined. Blood cells were separated from plasma by centrifugation (3 min, 9000 g) and the plasma stored on ice until further assay. Part of the plasma (200 μl) was ultrafiltered using Ultrafree-MC filter units (UFC3 TGC00, Millipore) with a 10 kilodalton (kDa) molecular cut-off. Blood and blood plasma analyses were carried out on the day of sampling.

The total magnesium concentration of plasma and ultrafiltered plasma was determined with a magnesium kit (diagnostic kit no. 595, Sigma). Blood Ca²⁺ was determined with an Ionic Calcium Analyzer (Radiometer) as described by Fogh-Anderson [Fog81]. Hematocrit was assessed after 10 min centrifugation of heparinized blood samples in 75 μl glass capillaries. Plasma osmolarity was assessed with a micro-osmometer (Roebling) using distilled water and a 300 mOsm.kg⁻¹ standard (Sigma) as reference.

Prolactin cell activity.

Immediately after the fish had been killed the pituitary gland was removed and transferred to Hanks' balanced salt solution (HBSS; H13387, Sigma). Next the rostral pars distalis (the so-called prolactin lobe which consists almost exclusively of prolactin cells [Flis89a]) was dissected. Its volume was determined using a binocular microscope equipped with an ocular micrometer. The rostral pars distalis was bisected. One part was fixed and processed for electron microscopy [Wen89b]. The other part was preincubated in HBSS at 25°C for 60 min and subsequently incubated for 4 h in 50 μl HBSS to which
3.3 GBq $[^3]$H-leucine (Amersham) had been added. Next the tissue was washed in HBSS (three times 1 min) and homogenized in 500 $\mu$l 0.05 mol.l$^{-1}$ acetic acid with a tight glass-to-glass Potter-type homogenizer device and centrifuged at 9000 g for 10 min; the supernatant was lyophilized and stored dry at -20°C until further assay. The newly-synthesized products released to the medium during the 4 h labelling period were collected by trichloro-acetic acid (10% final concentration) precipitation [Lac70]; 1 $\mu$g bovine serum albumin was added to improve recovery. After overnight incubation at 0°C the samples were centrifuged for 30 min at 9000 g and the pellet rinsed twice with diethylether and subsequently stored at -20°C until further assay.

The proteins in the rostral pars distalis extract and in the incubation medium samples were separated by SDS polyacrylamide (15%) slab gel electrophoresis (Mini Protean II, Biorad) following the protocol of Laemmli [Lac70]. After electrophoresis the gels were fixed in 40% methanol:10% HAc in water (30 min), 5% methanol:7% HAc in water (30 min) and 10% glutaraldehyde (British Drug Houses) in water (30 min) and subsequently rinsed in flowing tapwater for at least 1 h. For fluorography the fixed gels were impregnated with PPO [Bon74]. Before drying (slab dryer model 443, Biorad), the gels were dehydrated for 8 - 10 h in 50% methanol in water containing 3% glycerol. Preflashed X-ray film (XAR-5, Kodak) was used; exposure time was 24 - 72 h at -80°C. Fluorographs were scanned densitometrically with a transmission scanning densitometer (Model 1650, Biorad). An example of such a densitometric scan is shown in Fig. 5.2.

The molecular weight of the products were estimated by comparison with $^{14}$C-methylated protein molecular weight markers (Amersham). The radioactivity recovered from the 21 and 19 kDa areas in the homogenate plus that in the media was taken as measure for the synthesis rate of the prolactin cells.

Opercular chloride cell density

Freshly dissected opercula were incubated for 60 min in tapwater containing 20 $\mu$mol.l$^{-1}$ DASPEI (ICN Biomedicals), a vital stain for mitochondria. Next, the opercula were rinsed in tapwater and the density of fluorescent cells established at anatomically fixed spots as described by Foskett et al. [Fos81] Per fish 25 sites (with a total surface area of 5 mm$^2$) in a single operculum were scored. The opercular chloride cell density is expressed in the number of chloride cells per mm$^2$. 

94
effects of low-magnesium diets

Fig. 5.2.
Densitometric scan of fluorograph of products newly synthesized by the rostral pars distalis in vitro after SDS-polyacrylamide slab gel electrophoresis. The apparent atomic mass unit of protein markers is given on the X-axis. The lower scan reflects a sample of the control fed tilapia and the upper scan of tilapia fed a low magnesium diet, both after 19 weeks of feeding.

Sodium intake

The Na⁺ intake was calculated from the initial rate of ²⁴Na⁺ intake by the fish from the water [§ 4.2]. In short, fish was exposed to 0.4 MBq.l⁻¹ ²⁴Na (procedure of production described by Flik et al. [Fli89b]) in a total volume of 3 l. After 2 h the fish was rinsed with tapwater (twice for 1 min) and once with tapwater containing 35 mmol.l⁻¹
NaCl. Next the fish was killed, microwave cooked and homogenized with water (65% of the body wet weight). Quintuple samples of approximately 5 g, weighed to the nearest three decimals, were analyzed for $^{24}$Na $\gamma$-emission, using a NaI(Tl) $\gamma$-ray spectrometer (Ultrogamma II, LKB).

Statistics

Data are presented as mean values ± standard deviation. Data were statistically analysed using the Student's $t$-test. Significance was accepted for $P < 0.05$.

RESULTS

Prolactin cell activity

The diet had no effect on the prolactin cell volume as the relative size of the rostral pars distalis compared to the total pituitary gland had not changed (data not shown).

As judged from their ultrastructure, however, the prolactin cells of the experimental tilapia showed clear signs of stimulation (Fig. 5.3). In the experimental fish the rough endoplasmic reticulum was more abundant and frequently giant mitochondria were observed.

Also the prolactin cell activity as measured by the pituitary prolactin synthesis rate in vitro was enhanced in the tilapia fed a low-magnesium diet. The rate of prolactin synthesis after 8 weeks had increased by 20% ($n=5; P < 0.05$) by 70% and after 19 weeks ($n=5; P < 0.001$).

---

Fig. 5.3.
Electron micrographs of prolactin cells of tilapia; er, endoplasmic reticulum; G, Golgi area; 9090 x. Fig. 5.3.1: Untreated control (fish kept under standard laboratory conditions); Fig. 5.3.2: Control fish on experimental diet with normal magnesium content (magnesium: 30 mmol.kg$^{-1}$); Fig. 5.3.3 & 5.3.4: Fish on low-magnesium diet (magnesium: 1 mmol.kg$^{-1}$); compared to the controls the cells are enlarged and the endoplasmic reticulum (Fig. 5.3.3) and Golgi areas (Fig. 5.3.4) are more extensive.
Opercular chloride cell density

Tilapia fed a low-magnesium diet had significantly higher densities of chloride cells in the inner opercular epithelium compared with control fed tilapia, both after 8 weeks (223 ± 39 (n=7) versus 53 ± 14 (n=7); P < 0.001) and after 19 weeks (175 ± 28 (n=8) versus 56 ± 12 (n=8); P < 0.001) of experimentation.

Sodium intake

After 19 weeks of feeding the low magnesium diet the sodium intake had decreased by 35% from 8.5 ± 1.8 (n=5) in the controls to 5.5 ± 1.6 μmol.h⁻¹ per 100 g fish (n=5; P < 0.025) in the experimental tilapia.

Blood parameters

As shown in Table 5.4, no significant differences could be demonstrated in blood parameters between control and experimental fish.

Table 5.4.
Blood and blood plasma parameters of tilapia fed a control (magnesium: 30 mmol.kg⁻¹) and a low-magnesium (1 mmol.kg⁻¹) diet. Fish were sampled 8 and 9 weeks after the start of the experiment; no differences were found between control and experimental groups at either time and, therefore, the samples have been pooled. Mean values ± standard deviation are given with the number of observations in brackets.

<table>
<thead>
<tr>
<th>blood parameters</th>
<th>control diet</th>
<th>low-magnesium diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>hematocrit (%)</td>
<td>28.1 ± 3.5 (12)</td>
<td>27.7 ± 4.5 (13)</td>
</tr>
<tr>
<td>plasma osmolarity (mOsm.kg⁻¹)</td>
<td>329 ± 7 (12)</td>
<td>326 ± 7 (13)</td>
</tr>
<tr>
<td>blood Ca²⁺ (mmol.l⁻¹)</td>
<td>1.72 ± 0.03 (12)</td>
<td>1.69 ± 0.08 (13)</td>
</tr>
<tr>
<td>plasma Mg (mmol.l⁻¹)</td>
<td>1.50 ± 0.04 (5)</td>
<td>1.35 ± 0.28 (6)</td>
</tr>
<tr>
<td>plasma ultrafiltrable Mg (mmol.l⁻¹)</td>
<td>0.98 ± 0.07 (5)</td>
<td>0.90 ± 0.21 (6)</td>
</tr>
</tbody>
</table>

DISCUSSION

We did not find changes in hematocrit, blood calcium, plasma osmolarity and plasma magnesium levels in tilapia fed a low-magnesium diet. These blood parameters were chosen because they may reflect actions of regulators and/or actions of targets for
regulators with respect to ion regulation. These results are in line with the results presented in § 5.2 where the magnesium, calcium and sodium concentrations in various tissues had not changed in tilapia put on the same diet. However at the level of regulators and regulator targets of ion regulation we found significant effects of low-magnesium feeding. Therefore we may conclude that a low-magnesium diet effects the ion regulation in tilapia and that these blood parameters are not appropriate indicators for these subtle changes.

Apparently tilapia has the ability to maintain the elemental concentrations in blood and tissues within normal ranges under low-magnesium feeding conditions. The changes in prolactin cell activity, chloride cell density and sodium intake clearly indicate that this requires adjustments in regulatory and effector mechanisms for ion regulation. We conclude that tilapia is able to adapt successfully its ion regulation to low-magnesium food. Successful adaptations of tilapia have been reported earlier to e.g. low calcium stress [Fli86a] and to low water pH [Fli89b]. These adaptive capacities distinguish tilapia from many other freshwater fish species. With respect to low-magnesium food the adaptive capacity of tilapia is superior to that of species such as rainbow trout (Oncorhynchus mykiss), channel catfish (Ictalurus punctatus) and guppy (Poecilia reticulata) [Cow77; Gat82; Kno83; Shi88]. In contrast to tilapia, the latter species do not maintain the concentrations of magnesium, calcium and/or sodium in their tissues [Chap. 1] and/or in blood plasma when put on a low-magnesium diet [Cow77; Gat82; Kno83]. Also the carp is reported not to adapt to low-magnesium diets [Ogi76]. In a comparative study we performed similar experiments with carp [§ 5.4] as described here and in § 5.2 for tilapia.
§ 5.4

EFFECTS ON GROWTH RATE AND ION REGULATION OF CARP

ABSTRACT
Low-magnesium feeding of carp weighing 100 g or more for 17 weeks did not result in mortality. However, reductions were found with respect to the growth rate and the magnesium concentration in blood plasma and bone. Furthermore some parameters for changes in the ion regulation were examined. Low-magnesium fed carp showed a decreased branchial Na\(^+\)/K\(^+\)-ATPase activity, an increased chloride cell density, and changed sodium and magnesium concentrations in the bone. It is concluded that adult carp depend on magnesium in the food for maintenance of the magnesium balance. Low-magnesium fed carp are not able to adapt to these conditions within 17 weeks. Low-magnesium feeding also affects the regulation of ions other than magnesium.

INTRODUCTION

Diet experiments with freshwater tilapia (about 200 g) showed that tilapia can survive low-magnesium feeding and even keep on growing without depleting the magnesium pools of scales, bone or muscle [§ 5.2]. We concluded that adult tilapia has adaptational mechanisms to survive low-magnesium feeding. However, during this low-magnesium feeding of tilapia effects were found on the regulation of ions other than magnesium [§ 5.3]. The question arises whether an other teleost species, viz. the carp, has similar capability to cope with low-magnesium feeding conditions and thus can (i) survive, (ii) maintain its growth rate, (iii) maintain its magnesium balance and (iv) adjust its ion regulation. We followed the experimental design as reported for a comparable study on tilapia [§ 5.2; § 5.3] and selected parameters that yield information on growth rate, elemental tissue composition and on ion regulatory phenomena. The elemental composition was studied in bone and in blood plasma. Ion regulatory phenomena were examined by studying a regulator of the ion balance (prolactin cell activity), some
regulator targets (chloride cell density and branchial Na\(^+/K^+\)-ATPase) and some effects of regulation on the ionic composition of the blood plasma. Branchial Na\(^+/K^+\)-ATPase activity is considered the driving force for active Na\(^+\) uptake and H\(^+\) extrusion in the gills. This enzyme activity is concentrated in the chloride cells [May87]. The hematocrit was determined because it may reflect drastic changes in ion regulation [Mil82] and the health condition of the fish.

**MATERIALS AND METHODS**

**Fish**

Adult carp were kept in all glass aquaria with 450 l Nijmegen tap water. The magnesium concentration of the water was 0.20 ± 0.03 mmol.l\(^{-1}\), the pH was around 7.6 and the temperature was 25°C. The water was filtered by recirculation through an 800 l biological filter system and continually refreshed with 300 l water per day. The photoperiod was automatically controlled (12 h light: 12 h dark). The fish were fed daily 2% of the total wet weight of the group by means of an automated food dispenser and were weighed weekly on a balance (PM34 Delta Range, Mettler). No food was administered on the days the fish were weighed.

Two weeks before the start of the actual experiment, a group of fish was randomly divided into two groups of 36 fish weighing 88 ± 14 g (controls) and 90 ± 14 g (experimentals). During these two weeks both groups were fed the control diet.

The experiment was started by providing the experimental group (109 ± 22 g) a low-magnesium diet (magnesium: 1 mmol.kg\(^{-1}\)); the control group (103 ± 16 g) received an equal ration of control diet (magnesium: 30 mmol.kg\(^{-1}\)). Both diets are described in § 5.2. The experiment lasted 17 weeks. With intervals of 2 to 5 weeks 5 fish were sampled at random from the populations to determine several parameters. Mixed arterial and venous blood was collected by puncture of the caudal vessels using an ammonium heparin-rinsed tuberculin syringe. The fish were killed by transection of the spinal cord. Then the fish was microwave-cooked and three vertebrae of the caudal spinal cord were collected and freed of adherent muscle. The bone tissue was weighed, lyophilized and
weighed again.

At week 10 and week 17 an opercular membrane, the gills and the pituitary gland were removed from the 5 fish in each group.

Analytical methods

The blood was separated into plasma and cells by centrifugation at 9000 g for 3 min. Part of the blood plasma was ultrafiltered using filter-units (ultrafree-MC UFC3 TGC00, Millipore) with a 10 kilodalton (kDa) molecular cut-off. The magnesium and calcium concentrations of blood plasma and ultrafiltered blood plasma were determined with diagnostic kits (diagnostic kit no 595 and 587, Sigma). The total osmolarity of the blood plasma was determined with a micro-osmometer (Roebling) using distilled water and a 300 mOsm.kg\(^{-1}\) standard (Sigma). Hematocrit was assessed after 10 min centrifugation of heparinized blood samples in 75 μl glass capillaries. The soft tissue of the gills was scraped off with a glass slide and collected in an isotonic buffer containing 300 mmol.l\(^{-1}\) sucrose, 20 mmol.l\(^{-1}\) HEPES/Tris pH 7.4, 0.1 mmol.l\(^{-1}\) EDTA, 0.1 mmol.l\(^{-1}\) DTT (Sigma), 100 U.ml\(^{-1}\) aprotinin (Sigma) and 50 U.ml\(^{-1}\) sodium-heparin. The scrapings were homogenized in this buffer using a tight glass-to-glass Potter homogenizer. Protein was determined with a protein assay (Biorad) using bovine serum albumin as a reference protein. Substrate accessibility was optimized [Fli90] by treatment with the detergent saponin (20 μg.ml\(^{-1}\) membrane protein). The Na\(^{+}/K^{+}\)-ATPase activity was determined by the method of Bonting & Caravaggio [Bon63].

For an estimation of the rate of prolactin synthesis, the freshly dissected rostral pars distalis ("prolactin lobe") of the pituitary gland was preincubated for 30 minutes and subsequently incubated for 4 h in 50 μl medium containing 1 MBq [\(^{3}\)H]leucine (Amersham). The lobes and the proteins collected from the incubation media were subjected to SDS-gel electrophoresis [§ 5.3; Wen83]. Newly synthesized proteins were visualized by fluorography. The molecular weight of the proteins was estimated by comparison with \(^{14}\)C-methylated protein molecular weight markers (Amersham). The protein bands were quantified by densitometric scanning (model 1650, Biorad).

The density of chloride cells in the inner opercular membrane was determined by fluorescence microscopy of the opercula that had been incubated at room temperature in water containing 5 μmol.l\(^{-1}\) DASPEI (ICN Biomedicals) for 45 min as described in
The magnesium, calcium and sodium concentrations of the skeletal bone were determined by instrumental neutron activation analysis as described earlier [§ 2.2].

Statistics

Data are presented as mean values ± standard deviation of the mean. Data were statistically analysed by Student’s t-test. The body weights of the experimental and control group were statically tested for a normal distribution at the start and at the end of the experiment, using the Kolmogorov-Smirnov test [Web88]. Statistical significance was accepted at $P \leq 0.05$ unless indicated otherwise.

RESULTS

During the experiments no mortality was observed and all fish were apparently healthy. The blood hematocrit was not affected. Both groups were steadily growing from $103 \pm 16$ g (n=36) to $415 \pm 28$ g (n=11) and from $109 \pm 22$ g (n=36) to $356 \pm 60$ g (n=11) after 17 weeks for the control and experimental group, respectively. A mean decrease in the growth rate over this 17 week time period of 21% can be calculated for carp fed the low-magnesium diet. The body weights of the experimental and control group at the beginning and end of the experiment were normally distributed. The body weight ranges at the start of the experiment were comparable; the range in body weights at the end of the experiment was larger in the experimental group (262-444 g) than in the control group (355-450 g).

The magnesium concentration in vertebral bone of the control group did not change significantly during the experimental time period; the mean value was $99 \pm 19$ mmol.kg$^{-1}$ (n=25). However in the experimental group, this concentration had decreased to $66 \pm 7$ mmol.kg$^{-1}$ (n=5) after 2 weeks and further to $42 \pm 9$ mmol.kg$^{-1}$ (n=5) after 17 weeks. Both values are significantly different from their respective controls. The calcium concentrations in the bone of both the control and experimental group did not change in time and the mean values in time were $3800 \pm 250$ mmol.kg$^{-1}$ (n=25) and
3850 ± 350 mmol.kg⁻¹ (n=25), respectively. These calcium concentrations do not differ significantly. The sodium concentration of the bone after 2 weeks was not significantly different for control and experimental carp, viz. 188 ± 6 mmol.kg⁻¹ (n=5) and 198 ± 21 mmol.kg⁻¹ (n=5), respectively. However, after 17 weeks the sodium concentration had increased (P<0.02) in the low magnesium fed carp, viz. from 217 ± 8 mmol.kg⁻¹ (n=5) in the controls to 234 ± 9 mmol.kg⁻¹ (n=5) in the experimentals. The mean water content of the vertebrae of control and experimental carp was not significantly different viz. (52 ± 4)% (n=25) and (51 ± 3)% (n=25), respectively.

Table 5.5.
Mean values ± standard deviation of plasma concentrations (in mmol.l⁻¹), plasma osmolarity (in mOsm.kg⁻¹), hematocrit value (in % of the total blood volume), Na⁺/K⁺-ATPase activity of the original homogenate of carp branchial epithelium (in μmol P₄.h⁻¹.mg⁻¹ protein), prolactin cell activity (in arbitrary units) and chloride cell density (number.mm⁻²) on the opercular membrane after 10 and 17 weeks feeding of a low-magnesium diet (1 mmol.kg⁻¹) and a control diet (30 mmol.kg⁻¹). The number of all observations is 5.

<table>
<thead>
<tr>
<th></th>
<th>week 10</th>
<th>week 17</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>experimental</td>
</tr>
<tr>
<td>Plasma concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>0.78±0.10</td>
<td>0.33±0.19***</td>
</tr>
<tr>
<td>ultrafiltrable Mg</td>
<td>0.66±0.08</td>
<td>0.30±0.14***</td>
</tr>
<tr>
<td>Ca</td>
<td>2.44±0.09</td>
<td>2.30±0.15**</td>
</tr>
<tr>
<td>ultrafiltrable Ca</td>
<td>1.80±0.10</td>
<td>1.62±0.07**</td>
</tr>
<tr>
<td>Plasma osmolarity</td>
<td>260±4</td>
<td>263±4</td>
</tr>
<tr>
<td>Hematocrit percentage</td>
<td>23±3</td>
<td>25±1</td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase activity</td>
<td>23.3±4.1</td>
<td>8.2±4.3***</td>
</tr>
<tr>
<td>Prolactin cell activity</td>
<td>100±32</td>
<td>66±30</td>
</tr>
<tr>
<td>Chloride cell density</td>
<td>25±6</td>
<td>38±14</td>
</tr>
</tbody>
</table>

* P < 0.05
** P < 0.02
*** P < 0.01

The mean concentrations of the plasma and ultrafiltrable plasma magnesium and calcium, the osmolarity of the plasma, the blood hematocrit, the branchial Na⁺/K⁺-ATPase, the prolactin cell activity and the chloride cell density of control and
low-magnesium fed carp at week 10 and week 17 are given in Table 5.5. As shown in this table, plasma and ultrafiltrable plasma magnesium concentrations were significantly reduced. Plasma calcium was slightly reduced at week 17, and plasma ultrafiltrate calcium both at week 10 and 17. Plasma osmolarity, blood hematocrit and prolactin cell activity were not notably changed. In the electron microscope the ultrastructure of the prolactin cells of both groups, as examined after 17 weeks, was very similar (Fig. 5.4). Significant reduction was found in the specific Na\(^+\)/K\(^+\)-ATPase activity of the gills. The number of chloride cells was significantly increased after 17 weeks (Table 5.5).

Fig. 5.4.
Electron micrographs of prolactin cells of carp; 9700 x.
The cells show nuclei surrounded by small rings of cytoplasm containing many electron dense granules. The structure is similar in controls (Fig. 5.4.1) and in carp put on a low-magnesium diet for 17 weeks (Fig 5.4.2).
DISCUSSION

Following carp 17 weeks on a low-magnesium diet, we observed a decrease in the magnesium concentration of blood plasma and vertebral bone. Furthermore some signs of a disturbance of the ion regulation such as a decreased branchial Na⁺/K⁺-ATPase activity and an increased opercular chloride cell density were observed. The absence of mortality and of notable changes in hematocrit indicate that the health of the fish was not seriously affected by the low-magnesium diet. This is further indicated by the observation that the fish continued to grow. Thus, apparently carp are not fully dependent on a strict control of the plasma magnesium levels. They tolerated the low-magnesium levels in the blood and other tissues without notable effects on their general condition for at least four months.

The parameters of the control carp determined in this study are in good agreement with earlier reports on carp. We found a mean magnesium concentration in blood plasma and in plasma ultrafiltrate of 0.94 ± 0.18 mmol.l⁻¹ (n=15) and 0.73 ± 0.11 mmol.l⁻¹ (n=15), respectively. Previously we reported a magnesium concentration in blood plasma and in plasma ultrafiltrate of 0.96 ± 0.06 mmol.l⁻¹ and 0.61 ± 0.01 mmol.l⁻¹, respectively [§ 3.2]. Hunn [Hun72], Houston [Hou85] and Jensen [Jen90] found magnesium concentrations in carp blood plasma of 0.93 ± 0.05 mmol.l⁻¹, 1.15 ± 0.16 mmol.l⁻¹ 0.94 ± 0.12 mmol.l⁻¹, respectively. We found a blood plasma calcium concentration of 2.20 ± 0.44 mmol.l⁻¹ (n=15). Earlier reports give values of 1.99 ± 0.05 [Hun72] and between 2.6 - 3.3 mmol.l⁻¹ [Nan87]. The plasma osmolarity of 260 ± 4 mOsm.kg⁻¹ is close to that reported by Nanba et al. [Nan87], viz. around 250 mOsm.l⁻¹. We found a magnesium and calcium concentration in the vertebrae of 99 ± 19 mmol.kg⁻¹ (n=25) and 3820 ± 270 mmol.kg⁻¹ (n=25), respectively. Ogino & Chiou [Ogi76] found in carp, fed a diet with a comparable magnesium concentration, values of about 105 and 3120 mmol.kg⁻¹ for magnesium and calcium, respectively. From this comparison of the elemental composition of important magnesium and calcium pools in the fish, we conclude that our control diet does not disturb the magnesium or calcium balance of carp and may be considered as a sufficient diet.

The decrease in the magnesium concentration of the vertebrae we found in carp on a low-magnesium diet, is in agreement with the report of Ogino & Chiou [Ogi76] who
found in bone of small carp (around 8 g), fed a diet containing 3.3 mmol magnesium per kg, a decrease from 105 to 64 mmol.kg\(^{-1}\) after 4 weeks. Apparently, both carp of around 8 and 100 g mobilize magnesium from their bone compartment. A decrease in the magnesium concentration of bone was also observed in some other freshwater teleosts fed a low-magnesium diet such as rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*) and guppy (*Poecilia reticulata*) [Ogi78; Gat82; Kno83; Shi88]. An increase of the sodium concentration in bone was observed in low-magnesium fed rainbow trout and guppy [Kno83; Shi88].

Table 5.6.
Growth and magnesium accumulation in carp.

<table>
<thead>
<tr>
<th></th>
<th>control diet</th>
<th>low-magnesium diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>(W_f(0))</td>
<td>103</td>
<td>109</td>
</tr>
<tr>
<td>(W_f(t_c))</td>
<td>415</td>
<td>356</td>
</tr>
<tr>
<td>(\Delta Q_f)</td>
<td>4056</td>
<td></td>
</tr>
<tr>
<td>(\Delta t)</td>
<td>119</td>
<td>119</td>
</tr>
<tr>
<td>(\Delta Q_f/\Delta t)</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Feeding</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>([\text{Mg}]_{\text{food}})</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>(Q_{\text{food}})</td>
<td>155</td>
<td>5</td>
</tr>
</tbody>
</table>

\(W_f(0)\) and \(W_f(t_c)\) represent the mean wet body weight at the start and the end of the experiment (in g), respectively. \(\Delta Q_f\) represents the increase of the total magnesium inventory (in mmol) assuming a concentration of 13 \(\mu\text{mol.g}^{-1}\) [§ 3.2]. \(\Delta t\) represents the duration of the experiment (in day). Feeding represents the amount of diet per unit of body weight fish per day (in \%/day\(^{-1}\)). \([\text{Mg}]_{\text{food}}\) represents the magnesium concentration in the food (in mmol.kg\(^{-1}\)). \(Q_{\text{food}}\) represents the magnesium quantity in the food consumed per fish (in \(\mu\text{mol.day}^{-1}\)).

In Table 5.6 it is shown that under the control conditions the quantity of magnesium in the food for one fish is 155 \(\mu\text{mol.day}^{-1}\). For growth this fish needs 34 \(\mu\text{mol.day}^{-1}\), assuming an all-over magnesium concentration in the carp of 13 \(\mu\text{mol.g}^{-1}\) [§ 3.2]. However, under low-magnesium feeding conditions the mean quantity of magnesium in the food supply for one fish amounts to 5 \(\mu\text{mol.day}^{-1}\) only. Obviously this low-magnesium diet is not enough to supply the magnesium required for growth at a rate.
found in the control fish.

Because we found changes in the branchial Na\(^+\)/K\(^+\)-ATPase activity, chloride cell density and in the concentration of bone sodium and plasma calcium, we may conclude that low dietary magnesium also influences ions other than magnesium in carp. This was also concluded for tilapia [§ 5.3]. However, in several important respects the responses of carp to a low-magnesium diet are clearly different from that of tilapia studied under identical conditions [§ 5.2; § 5.3]. We observed that low-magnesium fed tilapia showed a small decrease in their growth rate, but in contrast to carp no changes were detected in the magnesium concentration of the bone or the blood plasma. Furthermore, in tilapia a significant increase of the prolactin cell activity was observed, whereas in carp prolactin cell activity had not changed. Tilapia show responses that characterize these fish as an effective adaptor to changes in environmental conditions. Conversely, carp react more as a tolerator than as an adaptor to low-magnesium feeding. Tolerance rather than adaptation has been reported earlier for carp exposed to other strenuous conditions such as anoxia [Thi89]. The differences in magnesium regulation between the two fish species make them suitable objects for further comparative studies on magnesium balance.
§ 5.5

EFFECTS ON THE INTAKE OF MAGNESIUM IN TILAPIA

ABSTRACT

Freshwater tilapia, weighing between 1 and 9 g, can survive low-magnesium feeding for at least 3 weeks. The magnesium intake from the water does not differ under control and low-magnesium dietary conditions. Thus, the reduced dietary magnesium is not compensated for by increased uptake from the water. Conversely, our data indicate that during low-magnesium feeding the fish economize in their magnesium by reducing magnesium losses by more than 90%.

INTRODUCTION

As we found for tilapia a net magnesium uptake over stripped intestine [§ 4.4] and a very low dietary magnesium requirement for survival [§ 5.2] we investigated whether the intake of magnesium from the water in low-magnesium fed tilapia could compensate for a severe reduction in the magnesium content of the food.

MATERIALS AND METHODS

Fish

Tilapia weighing from 1 to 9 g were obtained from laboratory stock. Fish (density not exceeding 1.5 g fish per litre) were held in 120 l glass aquaria filled with 80 l artificial freshwater (initially) containing per litre 0.3 mmol NaCl, 0.1 mmol Na₂SO₄, 0.06 mmol KCl, 0.2 mmol MgCl₂ and 0.2 mmol CaCl₂. The pH of the water was maintained at 6.5 by means of an end-point titration system (ETS822, Radiometer) by the addition of sodium hydroxide solution. The water was kept at 27°C and filtered by recirculation.
through a nylon-wool thermofilter (2113, Eheim) and constantly aerated. Twice weekly one third of the aquarium content was replaced with freshly prepared water. The photoperiod was 12 hours. The fish were fed on a control or a low-magnesium diet. Diet pellets (Hope Farms) of which the composition has been given in § 5.2 were crushed and dissolved in gelatine [Wol51; Ber68]. The resulting magnesium concentrations were about 25 and 1 mmol.kg\(^{-1}\) dry weight for the control diet and low-magnesium diet, respectively. The daily ration of diet was 5% dry weight of the fish wet weight. All fish were raised with the control diet but the experimental fish were fed on low-magnesium diet at least 3 weeks prior to the experiment.

Radiotracer

The radionuclide \(^{28}\text{Mg}\)\(^{2+}\) was used as tracer for \(\text{Mg}^{2+}\). It was produced by irradiation of Li-Mg alloys, containing Li enriched to 94.84% \(^6\text{Li}\) (Oak Ridge National Laboratory), with thermal neutrons in the IRI nuclear reactor, followed by a radiochemical separation of magnesium from the irradiated material leading to an aqueous solution of \(\text{MgCl}_2\) containing \(^{28}\text{Mg}\) and its decay product \(^{28}\text{Al}\). The specific activity of this preparation was about 1 GBq.mol\(^{-1}\) [§ 2.4].

\(\text{Mg}^{2+}\) intake from the water

To determine the magnesium intake from the water, a fish was weighed and placed into an all-glass vessel with 1 l artificial freshwater containing 0.2 mmol.l\(^{-1}\) \(\text{MgCl}_2\) including \(^{28}\text{Mg}\) with a radioactivity concentration of 200 kBq.l\(^{-1}\). The water was constantly aerated with pre-humidified air and kept at 27°C. At the start of the experiment 20 ml of the water was sampled in 20 ml glass counting vials for radioactivity determinations.

The experiments, during which the fish were not fed, lasted 0.5 to 20 hours. At the end of the experiment fish were rinsed for 1 min in one litre of fresh artificial water twice, anaesthetized in 0.4 mmol.l\(^{-1}\) “MS 222” (Sigma), killed by transection of the spinal cord, frozen in liquid nitrogen, and weighed. After 2 minutes the belly was cut open and the complete content was taken out. The belly content and the fish minus the belly content were placed in two 20 ml glass counting vials. To correct for the counting geometry, the fish was pressed against the bottom of the counting vial and the height of
the fish layer in the counting vial was measured. In analogy to the procedures followed for the determination of extra-intestinal Ca\(^{2+}\) intake [Pan80], the activity in the fish without belly content was used to calculate the extra-intestinal Mg\(^{2+}\)-intake or integumental Mg\(^{2+}\)-intake. The activity in the belly content was used to estimate for the drinking rate.

In order to allow for the decay of \(^{28}\)Al present in the samples at the moment of sampling and for the establishment of a \(^{28}\)Mg-\(^{28}\)Al equilibrium all samples were measured at least 30 min after sampling. Both water and fish samples were counted on γ-rays in a well-type (diameter: 76 mm, length: 76 mm) NaI(Tl)-scintillator (Type 12SW12, Harshaw Chemie) connected to a multichannel analyser (Econ II series, Tracor Northern). All γ-rays from 0.1 to 3 MeV were taken into account. The counting time was 1000 s. The counting rates were corrected for background, decay and counting geometry. The integumental magnesium intake or flow to fish from water, \(F_{fw}\) (in nmol.h\(^{-1}\)), was calculated using an equation analogous to equation (4) of § 4.2:

\[
F_{fw} = Q_w(0)[q(t)/q_w(0)]/t
\]

where \(Q_w(0)\) is the quantity of magnesium in the water at the beginning of the experiment (in nmol), \(q(t)\) is the tracer quantity in the fish at time \(t\) (in Bq), \(q_w(0)\) is the quantity of tracer in the water at \(t = 0\) (in Bq) and \(t\) is the time (in h). This equation is subject to the assumption that the absolute value of the slope of the \(q(t)/q_w(0)\) versus time curve at \(t = 0\) is equal to \([q(t)/q_w(0)]/t\) for \(t \leq 20\) h [§ 4.2, § 4.3]. In pilot experiments this assumption was justified for experimental times up to 20 h.

RESULTS

No mortality, diseases or apparently deviating behaviour were observed in tilapia fed on either the control or the experimental diet. Both groups showed significant growth.

The results on the integumental magnesium intake in fish, \(F_{fw}\) (in nmol.h\(^{-1}\)),
The integumental magnesium intake (\(F_{fw}\) in nmol.h\(^{-1}\)) versus fish weight (\(W_f\) in g) in control (squares) and low-magnesium (plus signs) fed freshwater tilapia. The lines delineate the power functions \(F_{fw} = 4.52(W_f)^{0.58}\) for normal fed tilapia (dotted line) and \(F_{fw} = 4.41(W_f)^{0.54}\) for low-magnesium fed tilapia (solid line). These were obtained by linear regression of \(\ln F_{fw}\) versus \(\ln W_f\).

\(\text{versus}\) fish weight, \(W_f\) (in g) are shown in Figure 5.5. Linear regression of \(\ln F_{fw}\) on \(\ln W_f\) showed that the trend of the plotted data points may be represented by a power function, \(F_{fw} = a(W_f)^b\). The best fitting power function for control-fed tilapia yielded for \(a: 4.5 \pm 1.3\) and for \(b: 0.58 \pm 0.08\) (\(n=33; r=0.802; P < 0.001\)) and for low-magnesium fed tilapia for \(a: 4.4 \pm 1.3\) and for \(b: 0.54 \pm 0.13\) (\(n=35; r=0.602; P < 0.001\)). There is no significant difference between the two power functions.
The $^{28}\text{Mg}$ associated with the belly content varied greatly. No significant relation was found between $^{28}\text{Mg}$ in the belly content and the fish weight or the duration of the experiment. The calculated average magnesium intake into the belly content was $16 \pm 26$ and $6 \pm 8$ nmol.h$^{-1}$ for control and low-magnesium fed tilapia, respectively.

DISCUSSION

Tilapia weighing 1 - 9 g can survive low-magnesium feeding for at least 3 weeks. We could clearly demonstrate intake of magnesium from the water. However this intake appears independent of the diet magnesium concentration. Thus, when the dietary magnesium content is reduced, the fish do not compensate for this reduction by increasing the magnesium intake from the ambient water.

The magnesium uptake is the resultant of the total magnesium intake (via integument, eating and drinking) and the total magnesium loss (via integument, urine and faeces). The magnesium intake of a 6.5 g tilapia (as an example) under control and low-magnesium feeding conditions is shown in Table 5.7. The total magnesium intake for a 6.5 g tilapia is maximally 368 and 32 nmol.h$^{-1}$ for control and low-magnesium fed fish, respectively.

Table 5.7.
Magnesium intake ($\pm$ standard deviation) in a 6.5 g tilapia fed on a control diet (magnesium concentration 25 mmol.kg$^{-1}$) or a low-magnesium diet (magnesium concentration 1 mmol.kg$^{-1}$).

<table>
<thead>
<tr>
<th>Route</th>
<th>Magnesium intake (in nmol.h$^{-1}$)</th>
<th>control diet</th>
<th>low-magnesium diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>integument drinking</td>
<td>13 $\pm$ 4</td>
<td>12 $\pm$ 5</td>
<td></td>
</tr>
<tr>
<td>eating *)</td>
<td>16 $\pm$ 26</td>
<td>6 $\pm$ 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>339</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>estimated total</td>
<td>368</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

*) Calculated assuming a complete absorption of magnesium from the food.
Whereas the fish do not compensate for low-magnesium feeding by increasing their magnesium intake from the water, they may economize on magnesium by reducing magnesium loss. Our data indeed provided evidence for this possibility. The growth related magnesium uptake of a 6.5 g tilapia under control conditions can be estimated proceeding from its growth rate (19.1% in 3 months [Fli89b]) and the total fish magnesium content (0.1163[0.253W]^{0.896} \text{ mmol [§ 3.3]}) . It is calculated then that tilapia has a magnesium uptake of around 14 nmol.h^{-1}. This means that for tilapia fed control diets the total magnesium loss amounts to about 368-14 = 354 nmol.h^{-1}. However the low-magnesium fed tilapia, assuming a magnesium intake of 32 nmol.h^{-1} (Table 5.7) and a normal growth rate, will lose magnesium at a rate of 32-14 = 18 nmol.h^{-1}. In fact the growth rate is reduced in fish on a low-magnesium diet [§ 5.2]. But even if we assume that no growth did occur in our experiments, the total magnesium loss will have been maximally 32 nmol.h^{-1}. These data imply that tilapia fed a low magnesium diet suppress their loss of magnesium by more than 90%. For further analysis of this adaptation mechanism, experiments have to be carried out to investigate the routes that are suppressed (integumental, intestinal or renal).
CHAPTER 6

EFFECTS OF LOW-MAGNESIUM AMBIENT WATER

In this chapter some effects of low-magnesium in the ambient water on early life stages of fish are described.

§ 6.1

INTRODUCTION

Of the two potential magnesium sources available for fish, the intake of magnesium via the food is of primary importance [§ 4.3]. In § 5.5 we showed that tilapia did not compensate for decreased dietary magnesium availability by increasing the intake of magnesium from the water. This does certainly not imply that the water magnesium is unimportant for fish under all conditions. Of special interest are the early life stages of fish, when they do not eat and are fully dependent on the yolk and for their food supply. In this chapter we report on the effect of reduced ambient water magnesium concentrations on embryo’s and larvae of carp.
§ 6.2

EFFECTS ON EARLY LIFE STAGES OF CARP

ABSTRACT
Carp eggs, fertilized in vitro, were allowed to develop in freshwater with magnesium concentrations varying from 0.001 to 0.10 mmol.l\(^{-1}\). Magnesium concentrations below 0.010 mmol.l\(^{-1}\) seriously impeded carp embryonic development: the incidence of deformed larvae and mortality steeply increased to 100% at water magnesium concentrations of 0.001 mmol.l\(^{-1}\). Thus, early life stages of carp require ambient magnesium for survival and successful development. The magnesium and calcium concentration of the developing eggs was dependent on the ambient magnesium concentration. The uptake of magnesium in eggs decreased and the uptake of calcium increased with decreasing ambient magnesium concentrations. However, the uptake of the sum of these divalent ions seems independent of ambient magnesium concentrations. This indicated a competition between magnesium and calcium for (passive) uptake into developing eggs.

INTRODUCTION
In this section we focus on the effects of low-magnesium levels of the ambient water on the development of carp eggs fertilized in vitro. Fish eggs contain significant amounts of magnesium and it is thought that a part of the magnesium in eggs is associated with the yolk [Hay46]. Therefore, the yolk may serve as a magnesium source for the developing embryo. However, the number of cells increase rapidly before hatching and it seems likely that developing fish eggs must extract magnesium from the ambient water to supply these cells with this essential element. This aspect is dealt with below. We used carp eggs to evaluate the dependence on ambient magnesium of early life stages of fish, that are not yet feeding.
MATERIALS AND METHODS

*In vitro* fertilization.

Carp weighing about 1500 g, were held at 23°C in Nijmegen tap water containing about 0.2 mmol.l⁻¹ magnesium. Carp gametes were obtained through hormonal induction of ovulation and spermiation by intramuscular injection of carp pituitary powder [Yas68; Cha76], suspended in 0.9% NaCl solution. Gametes of both sexes were stripped and mixed in glass petri dishes. Fertilization was induced by the addition of water (23°C). After 5 min of incubation, the eggs were rinsed and the dishes, each containing approximately 300 eggs, were placed in an experimental unit. Every experimental unit had five incubation chambers containing 4 l medium, in which the petri dishes with eggs were placed, and a reservoir containing another 20 l medium. The experimental medium was recirculated by pumps through this experimental unit. The water in the basin was kept at pH 7.8 (± 0.1) by controlled addition of 0.01 mol.l⁻¹ H₂SO₄ using pH-stat equipment. The water was thermostatted at 23°C and was irradiated with ultraviolet light before it reentered the basin, to inhibit development of fungi.

Experimental media

The experimental media contained 0.06 mmol.l⁻¹ KCl, 0.8 mmol.l⁻¹ CaCl₂, 3.5 mmol.l⁻¹ NaCl and 0.33 mmol⁻¹ NaHCO₃ in demineralized water. To manipulate the magnesium concentration of the media, graded amounts of MgSO₄ were added. Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) analysis (plasma 200, Instrumentation Laboratory) of the media thus obtained, yielded values for magnesium concentrations of 0.001, 0.004, 0.007, 0.010, 0.020 or 0.10 mmol.l⁻¹. All media were set at 0.1 mmol.l⁻¹ sulphate using Na₂SO₄. The water magnesium concentrations varied within 10% of the nominal concentrations during the incubation period, as determined afterwards (see below).

Experimental procedure

After the fertilization procedure, unfertilized eggs were removed and the number of fertilized eggs (around 1500) per experimental medium was assessed. Every 12 h until
the end of the experiment (up to 170 h after fertilization) dead and mouldy eggs and, later on, dead larvae were counted and removed. Fifty h after fertilization, the tail movement rate (beats per minute) was determined for 20 embryos in each experimental group. As soon as hatching was observed, the number of deformed larvae was assessed every 6 h until all larvae had hatched. The identification of deformed larvae was based on gross macroscopical appearance. Larvae were designated deformed when they were slightly crescent to almost corkscrew-shaped. The percentage deformation was expressed as the ratio of the number of deformed larvae and the total number of larvae hatched. About 10 h after hatching the number of pigmented cells on one side of the lateral abdominal skin of 20 larvae was determined using a binocular microscope. Furthermore attention was given to the frequency of the heart beat, the occurrence of embolism and the presence of tissue necrosis. Throughout the experiments 5 ml water samples were taken from the experimental units every 24 h to assess the magnesium concentration by ICP-AES analysis.

In a parallel experiment the mineral content of the eggs under the experimental conditions was determined; petri dishes with about 30 eggs were placed in each experimental medium. After 6, 24, 48, 63 and 76 h a dish was removed and 25 eggs were weighed, lyophilized and weighed again. To the dried eggs 300 μl concentrated HNO₃ was added. After 24 h, 1 ml demineralized water was added and mixed, and 1 ml of this solution was added to 3 ml demineralized water. The total magnesium, calcium and sodium concentrations were measured by ICP-AES.

Statistics

Data in the text are presented as mean values ± the standard deviation, unless otherwise stated. Data were analyzed statistically using Student’s t-test. Statistical significance was accepted at the 5% level.

RESULTS

The magnesium and calcium concentrations of the eggs after 6 h exposure to the experimental media had not changed significantly and were around 30 and 17 mmol.kg⁻¹
Figure 6.1.
The mean magnesium concentration (± standard error of the mean) of eggs (in mmol.kg⁻¹ dry weight) versus time after fertilization of the eggs (in h) during development in different ambient magnesium concentrations (viz. 0.004, 0.010 and 0.10 mmol.l⁻¹). The number of experiments is 3; the number of eggs measured per determination is 25.

dry weight, respectively. At 76 h after fertilization eggs exposed to 0.1 mmol.l⁻¹ had increased their magnesium concentration to 58 ± 3 mmol.kg⁻¹ and their calcium concentration to 34 ± 5 mmol.kg⁻¹ (n=3). A concentration-dependent inhibition of this magnesium uptake was observed with decreasing water magnesium levels (Fig. 6.1). At 0.10 mmol.l⁻¹ magnesium in the water, the egg magnesium concentration almost doubled
Figure 6.2.
The mean calcium concentration (± standard error of the mean) of eggs (in mmol.kg⁻¹ dry weight) versus time after fertilization of the eggs (in h) during development in different ambient magnesium concentrations (viz. 0.004, 0.010 and 0.10 mmol.l⁻¹). The number of experiments is 3; the number of eggs measured per determination is 25.

from around 30 to 58 mmol.kg⁻¹ between 6 and 76 h after fertilization. At 0.010 mmol.l⁻¹ magnesium in the water this increase was around 70% and at 0.004 mmol.l⁻¹ only around 25%. In contrast, calcium uptake increased with decreasing ambient magnesium levels (Fig. 6.2). The calcium concentration of the eggs doubled at 0.1 mmol.l⁻¹ and increased around fourfold at 0.004 mmol.l⁻¹ magnesium in the water. The sodium concentration in the eggs after 6 h exposure was 122 ± 10 mmol.kg⁻¹ (n=18) and had increased after 76 h exposure to 189 ± 37 mmol.kg⁻¹ (n=18). The sodium concentration of the eggs was
not affected by the experimental conditions. Also the water content of the eggs was not affected by the experimental media and was determined to (83 ± 4)% (n=43).

Table 6.1.
Mortality of embryo's and larvae and the relative number of deformed carp larvae exposed to different ambient magnesium levels from fertilization until 170 h after fertilization. The values represent the mean of 3 separate experiments ± standard deviation (for details see section materials and methods).

<table>
<thead>
<tr>
<th>[Mg\textsuperscript{2+}] (mmol.l\textsuperscript{-1})</th>
<th>Mortality (%)</th>
<th>Deformation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>100 ± 0</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>0.004</td>
<td>74 ± 45</td>
<td>62 ± 20</td>
</tr>
<tr>
<td>0.007</td>
<td>41 ± 52</td>
<td>8 ± 8</td>
</tr>
<tr>
<td>0.010</td>
<td>4 ± 7</td>
<td>19 ± 27</td>
</tr>
<tr>
<td>0.020</td>
<td>0 ± 0</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>0.100</td>
<td>0 ± 0</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

The mortality of embryos and larvae, and the incidence of deformed larvae under the experimental conditions are shown in Table 6.1. At a magnesium concentration of 0.1 mmol.l\textsuperscript{-1} in the water, no mortality and essentially no deformed larvae (1 ± 1)% were observed up to 170 h after fertilization. Both the mortality and relative number of deformed larvae increased with a decrease in ambient magnesium concentration, leading to a 100% mortality and (96 ± 3)% deformed larvae at a magnesium concentration of 0.001 mmol.l\textsuperscript{-1}.

The frequency of tail movements of embryos just before hatching and the pigmentation of larvae are shown in Table 6.2. Both these parameters show decreases when the egg and larval stages are exposed to water with a low magnesium concentration. Furthermore, we observed that exposure of larvae to water with a very low magnesium concentration (0.001 mmol.l\textsuperscript{-1}) had a very low heart rate, showed embolism and, upon light microscopical observation, necrosis of brain tissue and muscle (data not shown).
Table 6.2.
The frequency of tail movements just before hatching and the pigmentation (i.e. the number of observed pigmented cells on one side of the lateral abdominal skin) 10 h after hatching of carp, exposed to different ambient magnesium levels. The values represent the means of 60 observations ± standard deviation.

<table>
<thead>
<tr>
<th>[Mg^{2+}] (mmol.l^{-1})</th>
<th>Tail movements (beats per minute)</th>
<th>Pigmentation (relative density)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>2 ± 2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>0.004</td>
<td>3 ± 3</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>0.007</td>
<td>3 ± 4</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>0.010</td>
<td>8 ± 7</td>
<td>9 ± 7</td>
</tr>
<tr>
<td>0.020</td>
<td>10 ± 9</td>
<td>12 ± 8</td>
</tr>
<tr>
<td>0.100</td>
<td>24 ± 3</td>
<td>26 ± 7</td>
</tr>
</tbody>
</table>

DISCUSSION

Our results show that for the development of carp, low levels of magnesium in the ambient water (critical lower level around 0.01 mmol.l^{-1}) are essential. At magnesium levels below 0.01 mmol.l^{-1} deformation, tissue necrosis and death were observed in early life stages. As the eggs did not loose magnesium, the magnesium present in yolk and perivitelline fluid does not suffice for successful development of the egg. Our data show that in developing eggs the magnesium concentration increased. Consequently, there is uptake of magnesium from the water. Apparently, at water magnesium levels below 0.01 mmol.l^{-1} the accumulation of magnesium is hampered and the development retarded as indicated by several parameters (Fig. 6.1; Table 6.1 & 6.2). Thus, early life stages of carp require magnesium from the water for survival. The elemental concentrations we found for carp eggs (30, 17 and 122 mmol.kg^{-1} for magnesium, calcium and sodium, respectively) are close to values found by Chetty and Agarwal [Che84] for eggs of the same species. They reported magnesium, calcium and sodium concentrations in the eggs of about 35, 25 and 109 mmol.kg^{-1} dry weight, respectively.

The uptake of ions from the water may be driven by the perivitelline potential [Pet86], by ion-exchange [She87], directly by a transporting enzyme or by any
combination of these. Assuming that all magnesium and sodium in the water are in their ionic form and that the equilibrium potential between perivitelline fluid and ambient water for eggs of carp is comparable to that for eggs of freshwater Atlantic salmon (*Salmo salar*) under the conditions given [Pet86; Edd90], the equilibrium potential will be around -40 mV. Calculating the equilibrium magnesium concentration in the perivitelline fluid at an ambient magnesium concentration of 0.1 mmol.l\(^{-1}\) using the Nernst equation, we come to a value of 2.3 mmol.l\(^{-1}\). The magnesium concentration measured in whole eggs was 5.1 mmol.l\(^{-1}\). However, in analogy to e.g. cytosolic conditions, the free magnesium concentration in the egg is predicted to be much lower than the total magnesium concentration. We conclude then that at an ambient magnesium concentration of 0.1 mmol.l\(^{-1}\), magnesium transport driven by the electrochemical potential difference is possible. At an ambient magnesium concentration of 0.01 mmol.l\(^{-1}\), we calculate an equilibrium concentration of 0.23 mmol.l\(^{-1}\). Under these conditions magnesium transport, driven by the electrochemical potential difference, is unlikely. In the case of an ambient magnesium concentration of 0.004 mmol.l\(^{-1}\), the equilibrium concentration is 0.09 mmol.l\(^{-1}\) and potential driven magnesium transport will be negligible. The above reasoning may explain the data presented in Fig. 6.1, where we show that an ambient magnesium concentration of 0.004 mmol.l\(^{-1}\) inhibits magnesium uptake from the water almost completely.

A remarkable phenomenon observed was the stimulation of calcium uptake in eggs by decreasing ambient magnesium levels (Fig. 6.2). The decrease in magnesium uptake was almost compensated by the increase in calcium uptake. In other words, the increase in the sum of magnesium and calcium appeared to be independent of the magnesium content of the ambient water. This indicates a competition between calcium and magnesium for (passive) uptake into developing eggs. In higher vertebrates hypomagnesemia (as a result of magnesium deficiency) may result in hypercalcemia, in a decrease of tissue magnesium and an increase of tissue calcium concentrations [Geo75; Gev88]. Similarly, in adult tilapia a high magnesium concentration of the ambient water resulted in a hypermagnesemia and hypocalcemia [Wen83].

In addition to mortality and deformation, also a decreased heart beat rate, tail movement rate and pigmentation indicated the dependency on water magnesium of early
life stages of carp. It has been shown recently in our laboratory by evaluating the same parameters that a low water pH also hampers the development of early life stages of carp [Oye91]. A decrease in heart beat rate and pigmentation has also been reported for early life stages of freshwater rainbow trout (*Oncorhynchus mykiss*) exposed to a low pH or to a low ambient calcium concentration [Nel82]. Clearly, the ionic composition of the ambient water is of importance for the development of fertilized eggs. This holds true for the magnesium concentration as well as for the calcium and proton concentration of the water.

We conclude that ambient magnesium is required for successful embryonic and larval development of carp. The essential role of magnesium in many cellular physiological events may account for this magnesium dependency. Given the competition between calcium and magnesium, inhibitory effects of high calcium concentrations on embryonic development should be considered in future research.
CHAPTER 7

GENERAL DISCUSSION

NUCLEAR METHODS IN STUDIES ON MAGNESIUM PHYSIOLOGY

The studies described in this thesis show that the application of nuclear methods can significantly extend our understanding of magnesium physiology in vertebrates. Non-destructive instrumental neutron activation analysis is a handy and reliable technique to measure the magnesium concentration along with other physiologically important minerals such as sodium and calcium, in fish and probably in most vertebrate tissues while the tracer method provide the basis for the determination of relevant magnesium transport rates.

Despite the short half-lives and limiting specific activity obtainable by the production techniques used, the radioisotopes $^{27}\text{Mg}$ and $^{28}\text{Mg}$ proved to be adequate radiotracers for magnesium in the particular experimental designs chosen. The choice towards one of these radioisotopes to be applied as radiotracer depends on the set up of the experiment (such as length of the experiment and the specific activity required). The applications of $^{27}\text{Mg}^{2+}$ and $^{28}\text{Mg}^{2+}$ as radiotracer for $\text{Mg}^{2+}$, as reported in this thesis have yielded new insights in the magnesium physiology of fish, and we consider these radiotracers to be a useful tool for studies on magnesium physiology in general.

MAGNESIUM REGULATION IN FRESHWATER FISH

Embryonic carp have a strict dependency on ambient magnesium for their development. A low magnesium concentration (less than 0.01 mmol.l$^{-1}$) in the environmental water yielded a high mortality [§ 6.1]. Adult non-growing carp take in magnesium from the water, but this intake compensates only about 15% of the total loss of magnesium, and this means that adult carp under 'normal' conditions take in the majority of magnesium from their food [§ 4.3]. Feeding adult carp a low-magnesium diet
for 17 weeks, resulted in a diminished growth rate (by 21%) and decreased the magnesium concentration in blood plasma and bone drastically. However it did not result in an increased mortality. This indicates that adult carp can not maintain their magnesium balance under low-magnesium conditions. The strategy that carp use to survive low-magnesium diets appears to be that of a tolerance. The low-magnesium feeding did not effect the prolactin cell activity but it did effect the chloride cell density and branchial Na⁺/K⁺-ATPase activity, indicating that low dietary magnesium influenced the ionoregulatory mechanisms of these fish in general [§ 5.4].

Young tilapia (1-9 g) fed a low-magnesium diet did not change the branchial magnesium intake from the water [§ 5.5]. Adult tilapia seem to take in magnesium via the intestine since a net magnesium transport to serosa from mucosa was found [§ 4.4]. Adult tilapia (around 100 g or more) survived low-magnesium feeding for at least 19 weeks without any mortality or changes in the magnesium composition of blood plasma, muscle, bone or scales. As the magnesium intake from the low-magnesium diet does not suffice for the growth related magnesium uptake of the fish, these low-magnesium fed adult tilapia had to take in magnesium from the water [§ 5.2; § 5.3]. Just as in the case for carp, low-magnesium levels in the diet influenced the ionoregulation of tilapia in general. In these fish prolactin cell activity was enhanced, chloride cell density had increased and sodium intake had decreased. As tilapia appear to maintain a magnesium balance and show stimulation of the prolactin cell activity during low-magnesium feeding, we conclude that this species survives this low-magnesium feeding by adaptation [§ 5.2; § 5.3]. A question arising from the experiments with tilapia is whether the intake of magnesium from the low-magnesium diet and the intake from the water is sufficient for the growth related magnesium increase in these low-magnesium fed fish. Extrapolations of the intake per gram fish (as determined in § 5.5) to 200 g tilapia fed a diet containing 1 mmol magnesium per kg (as determined in § 5.2), resulted in a total intake (thus via water and food) which is in the same order as the growth related magnesium increase (as determined in § 5.2) and thus could explain our results. However, care should be taken in extrapolation of data on the magnesium intake. Furthermore it can not be excluded that there are differences in the strategy of surviving low-magnesium feeding between young and adult tilapia.

The differences in survival strategy and in maintenance of the magnesium balance
between carp and tilapia may be related to differences in the euryhalinity of these species and the endocrines involved.

Future studies must also address the contribution of magnesium loss to the magnesium balance of low-magnesium fed fish. As was discussed in chapter 5, regulation of magnesium loss may be an important other faculty for fish to cope with low-magnesium diets.

A MODEL FOR TRANSEPITHELIAL MAGNESIUM TRANSPORT IN FISH ENTEROCYTES

On the basis of the results presented in this thesis on magnesium transport across intestinal epithelium of freshwater tilapia and additional data from the literature, a tentative model for transepithelial magnesium transport is presented in Figure 7.1.

Magnesium transport across the intestinal epithelium may follow paracellular as well as transcellular routes. Paracellular transport is a commonly encountered phenomenon in transepithelial ion transport [Gro88]. Several authors [And74; And78; Mai82] reported on water fluxes across fish intestinal epithelia and concluded that these fluxes follow probably paracellular routes for the major part. Magnesium may be cotransported with this water; such cotransport is called "solvent drag". Solvent drag has been suggested as an important mechanism for net magnesium transport across the intestinal epithelium in eels [Nak86]. However, on the basis of reported water fluxes and of measurements of magnesium transport [§ 4.4] across intestinal epithelium we calculated that solvent drag can contribute to the total net magnesium movement for 25% at most. Thus 75% or more of the magnesium transported from mucosa to serosa follows a transcellular route.

Starting from the above conclusion that the major mucosa-to-serosa route of magnesium across the intestinal epithelium is transcellular, the question arises whether this transport is active, passive or both. We propose that magnesium enters the epithelial cells passively across the apical membrane and is transported subsequently by active mechanisms across the basolateral membrane. In tilapia enterocytes the transcellular
Fig 7.1.
Model for transepithelial magnesium transport in tilapia intestine. Paracellular transport of magnesium is possible from mucosa (M) to serosa (S) and vice versa, and the permeability to magnesium of the tight junctions (tj) at the mucosal side will determine the rate of transport. Magnesium may be (partly) transported via this route by "solvent drag". Transcellular transport of magnesium from mucosa to serosa involves two membrane transitions viz. over the apical membrane and over the basolateral plasma membrane. We propose that the entry of magnesium into the cell is passive and driven by an electrochemical potential (Ψ). Intracellular magnesium can be transported as free Mg$^{2+}$, complexed (e.g. to ATP or other ligands; MgL) or in a bound form (e.g. to proteins). Furthermore magnesium can intracellularly be sequestered in cytoplasmic organelles. The extrusion of magnesium across the basolateral plasma membrane is either mediated by a sodium-dependent transport mechanism, such as a Mg$^{2+}$/Na$^{+}$-exchanger driven by the sodium gradient over the basolateral membrane or by an ATP-dependent magnesium pump (Mg$^{2+}$-ATPase) or both.
membrane potential is -60 mV [Bak88] and the total magnesium concentration around 8 mmol.l⁻¹ cell water [§ 4.4]. Applying the Nernst equation and using the above-mentioned values and a temperature of 301 K, we calculate that passive inward transport may occur above an external magnesium activity as low as 0.08 mmol.l⁻¹. However, this value is an overestimation since the intracellular magnesium concentration used in the Nernst equation, refers to the total magnesium concentration. The free or active magnesium concentration that actually should be used is probably around 1 mmol.l⁻¹, the $K_M$ of the transport Mg²⁺-ATPase of the enterocyte [§ 4.5; Pre88]. Based on the above considerations it is predicted that magnesium transport across the apical membrane of fish enterocytes is passive.

Magnesium transport across intestinal epithelium proved to be partly sodium dependent [§ 4.4]. Replacement of Na⁺ by the inert cation NMDG⁺ resulted in a diminished magnesium flux from mucosa to serosa. Since the addition of the specific blocker of the Na⁺/K⁺-ATPase, ouabain, dissipated the Na⁺ gradient significantly and at the same time reduced the mucosa-serosa flux, the presence of a Na⁺/Mg²⁺ exchanger driven by the sodium gradient over the basolateral plasma membrane seems likely. Then for Mg²⁺-transport an analogy with the Ca²⁺-transport would exist, because a Na⁺-dependent Ca²⁺ transport in stripped intestinal epithelium as well as a Na⁺/Ca²⁺ exchanger has been demonstrated in tilapia enterocytes [Fli90]. On the basis of the above considerations we predicted a Na⁺-dependent Mg²⁺-exchange mechanism to be present in the basolateral membrane of fish enterocytes. Unfortunately, experiments designed to show Na⁺/Mg²⁺ exchange in basolateral plasma membrane vesicles in an assay system as described for Na⁺/Ca²⁺ exchange [Fli90] were unsuccessful for as yet unexplained reasons [§ 4.5].

Magnesium could also be transported across the basolateral plasma membrane via a Mg²⁺-ATPase as suggested by Lüdi & Schatzmann [Lüd87]. Our demonstration of magnesium transport across basolateral plasma membranes in sucrose medium provided direct evidence for an ATP-dependent Mg²⁺ extrusion mechanism [§ 4.5]. Neither ADP nor the non hydrolyzable ATP analogue, ATP-γ-S, promoted Mg²⁺ transport over the basolateral membrane indicating an ATP-specificity. Since no sodium or potassium was present in the experimental media and ouabain exerted no effects on this ATPase
activity, the transport mechanism appears unrelated to Na\(^+\)/K\(^+\)-ATPase. Therefore, the existence of a new and so far unique Mg\(^{2+}\)-transport-ATPase is postulated in the basolateral membrane of fish enterocytes.

No conclusions can be drawn on the relative importance of the two magnesium transport mechanisms postulated in the basolateral plasma membrane, because, as indicated above, data are still incomplete.

The intracellular transport of magnesium was not investigated in this study. Cellular magnesium is not distributed homogeneously: the magnesium concentration in various subcellular compartments differs considerably [Ebe80]. Magnesium can be divided in three fractions namely complexed, bound and free. A considerable amount of magnesium will be complexed (e.g. with ATP, which gives a stable complex [Ver78]) or bound to proteins. Free cellular magnesium forms only a minor part of the total cellular magnesium although it likely represents the physiologically most important fraction as "static" regulator of cell functions [Alv87]. In oxygenated human red blood cells the free Mg\(^{2+}\), as determined with NMR-techniques, was 13% of the total magnesium [Ouw89]. Intracellular transport of magnesium could take place in the free form, in the complexed form (e.g. transport of magnesium-ATP complexes to the basolateral plasma membrane, the location of many ATPase activities) or bound. In addition cellular organelles such as microsomes and mitochondria may act as stores of cellular magnesium [Geo75; Diw79; Ebe80]. Cytosolic magnesium can be sequestered as well as shuttled by these cytosolic organelles.
REFERENCES

Bode, P. (1990a) Instrumental neutron activation analysis in a routine way. J. Trace and
Microprobe Techniques 8: 139-154.
DiPolo, R. & L. Beaugé (1988) An ATP-dependent Na⁺/Mg²⁺ countertransport is the
only mechanism for Mg extrusion is squid axons. Biochim. Biophys. Acta 946: 424-428.


135


Peterson, R. H. & D.J. Martin-Robichaud (1986) Perivitelline and vitelline potentials in teleost eggs as influenced by ambient ionic strength, natal salinity, and electrode electrolyte; and the influence of these potentials on cadmium dynamics within the egg. Can. J. Fish. Aquat. Sci. 43: 1445-1450.


Probst, H.J., S.M. Qaim & R. Weinreich (1976) Excitation functions of high-energy α-


Speich, M., B. Bousquet & G. Nicolas (1981) Reference values for ionized, complexed,
Törkő, J. (1965) Erfahrungen zur herstellung von $^{28}$Mg am reaktor durch (t,p)-reaktion. Kernenergie 8: 219-220.
Zutphen.


SUMMARY

Magnesium is an essential element and plays a pivotal role in fish physiology. Despite its importance, the magnesium metabolism is still poorly understood especially when compared to that of sodium, potassium or calcium. The gap in our knowledge on magnesium metabolism is partially due to the short half-lives and the limited availability of radioisotopes of magnesium required as radiotracers in physiological studies. Only two radioisotopes of magnesium have a half-life sufficiently long for particular biological applications involving processes on a minute or hour time scale, viz. $^{27}$Mg ($t_{1/2} = 9.46$ min) and $^{28}$Mg ($t_{1/2} = 20.915$ h). Both radioisotopes can be obtained by thermal neutron irradiation of magnesium containing materials in a nuclear reactor, and were produced at the Hoger Onderwijs Reactor, Delft. The production of $^{27}$Mg was based on the nuclear reaction $^{26}$Mg(n,$\gamma$)$^{27}$Mg. Magnesium acetate with magnesium enriched on $^{26}$Mg to 97% was used as target material. Ten minutes irradiation in a neutron flux of $5.6\cdot10^{16}$ m$^{-2}\cdot$s$^{-1}$ yielded a product with specific activity of about 65 GBq.mol$^{-1}$. $^{28}$Mg was produced according to two consecutive nuclear reactions: $^6$Li(n,t)$^4$He and $^{26}$Mg(t,p)$^{28}$Mg. A thin strip of lithium-magnesium alloy was used as target material. Irradiation for 36 h in a neutron flux of $1.3\cdot10^{17}$ m$^{-2}\cdot$s$^{-1}$, yielded a specific activity of about 0.25 and 1 GBq.mol$^{-1}$ for an alloy of natural compositions and for an alloy enriched on $^6$Li to 95%, respectively. However $^{28}$Mg produced in this way is contaminated with other nuclides (e.g. $^3$H, $^{24}$Na, $^{56}$Mn, $^{198}$Au) and, therefore, a radiochemical separation of the target material was necessary. For this purpose a relative fast (about 1.5 to 3.0 h) radiochemical separation procedure was developed. The procedure yielded a product free of all $\gamma$-ray emitting contaminants. As the half-lives of $^{28}$Mg reported in the literature showed some diversity, also the half-life of $^{28}$Mg was redetermined to be 20.915 h. Beside the radiotracer method another nucleonic technique was used in the studies described in this thesis viz. instrumental neutron activation analysis. Using this technique lyophilized fish tissue samples were analysed for magnesium, calcium and sodium contents.

The study of magnesium metabolism in freshwater fish concerned the internal distribution, the requirement and dependency of fish for external magnesium sources. To
this end magnesium transport routes were assessed and the effects of low-magnesium diets and low-magnesium ambient water on fish were examined. Furthermore a tentative model for magnesium transport in fish enterocytes was composed. Two consumption fish, both belonging to the class of the teleosts, namely the tilapia (*Oreochromis mossambicus*) and the carp (*Cyprinus carpio*) were used in this thesis.

For an understanding of magnesium metabolism and homeostasis in fish it is essential to obtain information on the quantity of magnesium in different parts of the fish. Therefore, the magnesium concentration of different tissues of tilapia and carp was determined, and the total magnesium inventory of the fish assessed.

In carp, a radiotracer method was used to measure the magnesium flows to fish from water and to water from plasma. As the intake from the water was less than the total loss, carp have to obtain magnesium from other sources than the water. In non-growing carp, weighing around 75 g, at least 80% of the total magnesium intake originates from the food.

The mechanisms of magnesium uptake via the intestinal tract of tilapia were studied by two *in vitro* experiments. First the magnesium movements were determined across the intestinal epithelium, after this was separated from the underlying tissue layers. A net magnesium flux in the mucosa to serosa direction was found and evidence was provided that an active step is involved in the transepithelial transport. The equilibrium concentration of magnesium as calculated on the basis of literature data on the cell potential of tilapia enterocytes, was higher than found in actual determinations of the magnesium concentration in the enterocyte. This points to a passive intake of magnesium across the apical membrane and an energized magnesium extrusion mechanism at the basolateral plasma membrane of fish enterocytes. This transcellular transport, in particular the translocation across the basolateral membrane, seems to be at least partly sodium dependent. Proceeding from this experiment the Mg$^{2+}$ transport into inside-out oriented resealed vesicles of the basolateral plasma membrane was determined under various conditions. The magnesium intake in inside-out vesicles, representing the Mg$^{2+}$ extrusion over the basolateral plasma membrane *in vivo*, is at least partly effected by a Mg$^{2+}$-transport-ATPase. The $K_{v5}$ for Mg$^{2+}$ of this enzyme was around 1 mmol.l$^{-1}$. To our knowledge this is the first report of a magnesium-transporting
ATP-dependent enzyme.

Diet experiments were performed to determine whether tilapia and carp, both weighing around 150 g, normally depend on magnesium from their food and whether and how they were able to survive on a low-magnesium diet. Both species of fish survived a low-magnesium diet (1 mmol Mg per kg pellet diet) for at least 17 weeks. In tilapia, the low-magnesium diet induced a small decrease in the growth rate, but no changes in the magnesium concentrations of blood plasma, bone, muscle or scales. However in carp, the low-magnesium diet not only decreased the growth rate but also significantly decreased the magnesium concentrations of blood plasma and bone. In both fish species the low-magnesium diet influenced the ion regulation of the fish. However, also in this respect a difference was found between tilapia and carp: only in tilapia an increase in the prolactin cell activity of the pituitary gland was found. It is suggested that tilapia and carp have different mechanisms to survive low-magnesium feeding conditions, namely adaptation and toleration, respectively.

The integumental intake of magnesium from the water in tilapia, weighing, 1 to 9 g, did not differ whether these fish were fed a control or a low-magnesium diet. For survival, these low-magnesium fed tilapia have to minimize their loss of magnesium.

The dependency on magnesium in the ambient water was tested in early life stages of carp. Ambient water with low-magnesium concentrations (less than 0.01 mmol.1⁻¹) had drastic effects on carp embryos and larvae, such as a high mortality, skeletal deformation and tissue necrosis.

From the research described in this thesis, it is clear that nuclear techniques can contribute substantially to a better understanding of the magnesium metabolism of fish. On the basis of the results on magnesium transport across the intestinal epithelium, a model for transepithelial magnesium transport has been postulated.
SAMENVATTING

Magnesium en het transport ervan in tilapia en karper: een op radiotracer-methode en activeringsanalyse gebaseerde studie.

Magnesium is een essentieel element voor organismen en speelt een belangrijke rol in de fysiologie van vissen. Ondanks het belang van magnesium is over het metabolisme ervan nog relatief weinig bekend vergeleken met de kennis omtrent het metabolisme van natrium, kalium en calcium. Hieraan ligt vooral ten grondslag de korte halveringstijd en de beperkte verkrijgbaarheid van magnesiumradioisotopen, die gebruikt kunnen worden als tracer voor magnesium in fysiologische studies. Slechts twee radioisotopen van magnesium hebben een halveringstijd ($t_{1/2}$) die lang genoeg is voor het bestuderen van bepaalde biologische processen gedurende een tijdsinterval van een half uur of een paar dagen, namelijk $^{27}$Mg ($t_{1/2} = 9,46$ minuten) en $^{28}$Mg ($t_{1/2} = 20,915$ uur). Beide radioisotopen zijn met succes geproduceerd in de Hoger Onderwijs Reactor te Delft. Bij de produktie van $^{27}$Mg werd gebruik gemaakt van verrijkt (97%) $^{26}$Mg (als magnesium-acetaat) en de reactie $^{26}$Mg(n,γ)$^{27}$Mg. Bestraling gedurende tien minuten in een neutronenflux van $5,6 \times 10^{16}$ m$^{-2}$s$^{-1}$ leverde een specifieke activiteit op van 65 GBq.mol$^{-1}$. Bij de produktie van $^{28}$Mg werd gebruik gemaakt van een lithium-magnesium legering waarin, bij neutronenbestraling, de volgende twee-stapsreactie optreedt: $^{6}$Li(n,t)$^{4}$He; $^{26}$Mg(t,p)$^{28}$Mg. Bestraling gedurende 36 uur in een neutronenflux van $1,5 \times 10^{17}$ m$^{-2}$s$^{-1}$ leverde voor een legering van natuurlijke samenstelling en voor een legering van met (95%) $^{6}$Li verrijkt lithium respectievelijk een specifieke activiteit van 0,25 en 1 GBq.mol$^{-1}$ op. Na bestraling bevat de legering naast $^{28}$Mg, en de dochternuclide $^{28}$Al, ook verontreinigingen van andere nucliden (zoals $^{3}$H, $^{24}$Na, $^{56}$Mn en $^{198}$Au) en derhalve was een (radio)chemische scheiding van het eindprodukt noodzakelijk. Hiertoe werd een, ongeveer 1,5 - 3,0 uur durende, radiochemische scheiding ontwikkeld. Het eindprodukt na de scheiding was geheel vrij van γ-straling uitzendende radionucliden anders dan $^{28}$Mg en de dochternuclide $^{28}$Al. Gezien het belang van een nauwkeurig bepaalde halveringstijd en de verschillen tussen de gerapporteerde
halveringstijden van $^{28}\text{Mg}$ in de bestaande literatuur, werd deze opnieuw bepaald. Dit resulteerde in een halveringstijd van 20,915 uur. Naast de radiotracermethode is tijdens het onderzoek ook gebruik gemaakt van een andere nucleaire techniek namelijk instrumentele neutronenactiveringsanalyse. Deze techniek werd gebruikt voor het analyseren van gevriesdroogde weefsels van vissen met betrekking tot magnesium, calcium and natrium.

Het onderzoek naar de magnesiumhuishouding in vissen is uitgevoerd door het bepalen van de magnesiumverdeling in de vis en het bestuderen van magnesiumtransport tussen vis en water en door het darmepithel. Verder zijn de behoeften aan magnesium uit voedsel en water bepaald en zijn effecten van magnesium-arm voer op magnesiumverdeling, magnesiumtransport en ionienergietralie beschreven. Het onderzoek is gericht op de proefdiermodellen tilapia (Oreochromis mossambicus) en karper (Cyprinus carpio). Beide soorten zijn belangrijke consumptievissen en behoren tot de beenvissen.

Om een inzicht te krijgen in de magnesiumhuishouding is het allereerst essentieel om kennis te hebben over de verdeling van magnesium in de vis. Daartoe is de magnesiumconcentratie in bepaalde weefsels en organen bepaald. Een radiotracermethode werd gebruikt voor het meten van magnesiumtransport van water naar karper en van het bloedplasma van de karper naar het water. Daar de inname van magnesium vanuit het water in de karper kleiner is dan het totale verlies van de vis aan het water, moet de karper magnesium uit andere bronnen dan water halen. Hiervoor komt alleen het voer in aanmerking. Karpers van rond de 75 g, die niet groeiden (dit betekent dat de inname gelijk is aan het verlies) halen tenminste 80% van het benodigde magnesium uit het voer.

De opnamemechanismen van magnesium in de darm van de tilapia werd onderzocht door middel van in vitro experimenten. Eerst werd het transport van magnesium over het darmepithel, waarvan de spierlaag was verwijderd, in beide richtingen gemeten. Er bleek een netto-transport van magnesium te zijn van de mucosale kant naar de serosale kant. De evenwichtsconcentratie van magnesium in darmcellen, berekend uit literatuurgegevens over de celpotentiaal van darmcellen van tilapia, bleek hoger te zijn dan de gemeten magnesiumconcentratie. Dit wijst op een passief
magnesium transport over het apicale membraan van de darmcel en op een actieve, energie vergende, stap voor het magnesium transport over het basolaterale membraan van de darmcel. Dit epitheliaal transport over het basolaterale membraan lijkt natrium-afhankelijk te zijn. Uitgaande van het aktief transport over het basolaterale membraan, werd de magnesiumopname bekeken in gesloten blaasjes van het basolaterale plasmamembraan van disarmellen waarvan de cytosolische zijde naar buiten gericht was. De magnesiumopname in deze blaasjes, en dus in feite het transport van de darmcel naar plasma \textit{in vivo}, lijkt (althans deels) verzorgd door een Mg$^{2+}$-transporterend ATP-afhankelijk enzym. De $K_v$ van dit enzym ligt rond de 1 mmol.l$^{-1}$. Dit magnesium transporterend enzym is, voor zover ons bekend, wel voorspeld maar nog nooit eerder gevonden.

Om de vragen te kunnen beantwoorden of zoetwatervissen zoals tilapia en karper een magnesium-arm dieet kunnen overleven en wat de eventuele mechanismen voor het overleven zijn, werden dieetexperimenten uitgevoerd. Beide vissoorten, met een gewicht rond de 150 g of meer, vertoonden gedurende 17 weken geen sterfte als ze gevoerd werden met een magnesium-arm dieet (1 mmol Mg per kg pellet). De tilapia gevoerd met het magnesium-arm dieet liet een geringe daling in de groeisnelheid zien, maar er werden geen effecten op de magnesiumconcentratie in bloedplasma, spier, bot of schub gevonden. De karper, daarentegen, vertoonde naast een verminderde groeisnelheid ook een duidelijke daling van de magnesiumconcentratie van bloedplasma en bot. In beide vissoorten had een magnesium-arm dieet echter effect op de ionenregulatie. Ook in dat opzicht zien we verschillen tussen tilapia en karper. In tegenstelling tot de karper leidt een magnesium-arm dieet bij de tilapia tot een verhoging van de prolactine activiteit. De verwachting is dat tilapia en karper verschillende mechanismen gebruiken om een magnesium-arm dieet te overleven. Bij de tilapia lijkt dit adaptatie te zijn en bij de karper tolerantie.

De inname van magnesium uit het water, met uitzondering van die inname \textit{via} de darm (door drinken), werd gemeten in kleine (1-9 g) tilapia's. De tilapia neemt niet méér magnesium op uit het water wanneer deze gevoerd wordt met een magnesium-arm dieet, waaruit blijkt dat een magnesiumtekort in de vis niet kan worden gecompenseerd door de opname van magnesium uit het water te verhogen. Het in het water voorkomend
magnesium bleek wel van groot belang voor de vroege levensstadia van vissen. Lage magnesiumconcentraties in het water (lager dan 0,01 mmol.l\(^{-1}\)) veroorzaakten drastische effecten op de embryo's en larven van de karper zoals een hoge mortaliteit, misvorming en necrose.

Uit het in dit proefschrift beschreven onderzoek blijkt dat het gebruik van radionucliden van magnesium een belangrijke bijdrage kan leveren aan onderzoek naar de magnesiumhuishouding van vissen en waarschijnlijk ook van hogere vertebraten. Voor een vergelijkend fysiologisch onderzoek naar de magnesiumhuishouding bij vissen zijn de proefdiermodellen tilapia en karper geschikt gezien hun verschillende reacties op magnesium-arm dieet. Uit het verrichte onderzoek naar het magnesiumtransport in de darm en gebruik makende van literatuurgegevens wordt een model van magnesiumtransport door een darmcel van een vis naar voren gebracht.

De belangrijkste resultaten van het in dit proefschrift beschreven onderzoek kunnen als volgt worden samengevat:

-Diverse nucleaire technieken blijken geschikt te zijn voor de bestudering van de magnesiumhuishouding in dierlijke organismen zoals kon worden aangetoond voor zoetwatervissen.

-De hiertoe benodigde radioactieve tracers \(^{27}\text{Mg}^{2+}\) en \(^{28}\text{Mg}^{2+}\) kunnen middels een kernreactor worden geproduceerd.

-In de karper is de opname van magnesium uit het voedsel groter dan uit het water.

-In de basolaterale membranen van tilapiadarmcellen is een, tot nu toe onbekende, magnesiumtransporterende enzym-aktiviteit aangetoond.

-De strategie voor het overleven van een magnesium-arm dieet lijkt bij de tilapia en karper gebaseerd op respectievelijk adaptatie en tolerantie.

-Jonge tilapia's gevoed met een magnesium-arm dieet compenseren dit niet door meer magnesium op te nemen uit het water.

-De embryonale en larvale stadia van karpers zijn erg gevoelig voor lage magnesiumconcentraties in het water.
ABBREVIATIONS

ADP : adenosine 5'-diphosphate
ATP : adenosine 5'-triphosphate
ATP-γ-S : adenosine 5'-0-(3-thiotriphosphate)
calmagite : 1-[1-hydroxy-4-methyl-2-penylazo]-2-naphthol-4-sulphonic acid
CDTA : trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
DASPEI : 2-(dimethylaminostyryl)-1-ethylpyridiniumiodine
DTT : dithiothreitol
EDTA : ethylenediaminetetraacetic acid
HAc : acetic acid
HEEDTA : N-Hydroxyethylethylediaminetriacetic acid
HEPES : N-2-hydroxyethylpiperazin-N'-2-ethanesulphonic acid
"MS222" : 3-aminobenzoic acid ethyl ester. Methanesulphate salt
NMDG : N-methyl-D-glucamine
Ouabain : 4β,20[22]-cardenolide-1β,3β,5α,11α,14,19-hexol-3-[6-deoxy-α-L-mannopyranosyl]
PPO : 2-5-diphenyloxazole
SDS : sodium dodecyl sulphate
Tris : tris-hydroxymethylaminomethane
SYMBOLS AND COMPARTMENT RELATED SUBSCRIPTS

1) SYMBOLS

A : area (in cm$^2$)

C$_{a}(t)$ : concentration (e.g. of magnesium) at time t (in mol.l$^{-1}$)

*$_{C_{a}}$ : tracer concentration (in Bq.l$^{-1}$)

Da : atomic mass unit; the dalton is one twelfth of the mass of the nuclide $^{12}$C.

E$_{\text{max}}$ : maximal energy of the $\beta$-particles of a radioisotope (in MeV)

F$_{\text{ba}}$ : flow (e.g. of magnesium) to compartment b from compartment a (in mol.s$^{-1}$ or nmol.h$^{-1}$)

F$_{\text{in}}$ : flow (e.g. of magnesium) into the fish (in nmol.h$^{-1}$)

F$_{\text{net}}$ : net flow [F$_{\text{in}}$ - F$_{\text{out}}$] (in nmol.h$^{-1}$)

F$_{\text{out}}$ : flow (e.g. of magnesium) out of the fish (in nmol.h$^{-1}$)

g : relative centrifugal force

i : sequential sample number

J$_{\text{ba}}$ : flux (e.g. of magnesium) to compartment b from compartment a (in nmol.h$^{-1}$.cm$^{-2}$)

k : rate constant for transport (in s$^{-1}$)

K$_{\text{1/2}}$ : free ion concentration at which half maximal activity occurs (in mmol.l$^{-1}$)

n : number of observations

P : probability

Q$_{a}(t)$ : quantity (of magnesium) in compartment a at time t (in mol, mmol, μmol or nmol)
\( q_a(t) \) : quantity of tracer in compartment a at time t (in Bq)

\( R_a(t) \) : counting rate at time t (in counts.s\(^{-1}\), counts.s\(^{-1}.g\(^{-1}\) or counts.s\(^{-1}.l\(^{-1}\))

\( r \) : correlation coefficient

\( S_a(t) \) : specific activity of compartment a at time t (in Bq.mol\(^{-1}\))

\( t \) : time (in s, min, h or day)

\( t_e \) : time at the end of the experiment (in s, min, h or day)

\( t_{1/2} \) : half-life of radioisotope (in s, min, h or year)

\( t_0 \) : time at the begin of the experiment (in s, min or h)

\( V_a(t) \) : volume at time t (in l)

\( V_{\text{max}} \) : maximum rate of entry (in nmol.min\(^{-1}.mg\(^{-1}\))

\( v_a \) : sample volume out of compartment a (in l)

\( W_a \) : wet weight (in kg or g)

\( dW_a \) : dry weight (in kg or g)

\( \epsilon \) : counting efficiency (in counts.s\(^{-1}.Bq\(^{-1}\))

\( \psi \) : electrical potential difference (in mV)
2) COMPARTMENT RELATED SUBSCRIPTS

b : bone compartment
c : scales compartment
f : fish compartment or food compartment
h : hard tissue
i : interstitial fluid compartment
p : plasma compartment
s : soft tissue compartment
w : water compartment
DISCUSSION RELATED PUBLICATIONS AND CONTRIBUTIONS TO INTERNATIONAL MEETINGS

1) PUBLICATIONS


Kolar, Z.I., J.A. van der Velden, G. Flik & J.J.M. de Goeij (accepted for publication) $^{27}\mathrm{Mg}^{2+}$ and $^{28}\mathrm{Mg}^{2+}$: Preparation and use as radiotracers in magnesium
physiology oriented studies of tilapia" in: Magnesium, a relevant ion? Book of the Congress - 3rd European Congress on Magnesium.

2) CONTRIBUTIONS TO INTERNATIONAL SCIENTIFIC MEETINGS


DANKWOORD

Tijdens het onderzoek en de voorbereiding van dit proefschrift hebben velen mij geholpen. Allen die een bijdrage hebben geleverd aan het tot stand komen van dit proefschrift wil ik hierbij hartelijk bedanken. Zonder deze hulp was het tot stand komen van dit proefschrift niet mogelijk geweest.


Tenslotte wil ik mijn ouders bedanken, die mij de mogelijkheid hebben geboden om te studeren en natuurlijk Astrid voor haar altijd aanwezige belangstelling en bemoediging tijdens mijn promotieonderzoek.
CURRICULUM VITAE

Joan van der Velden werd geboren op 14 november 1958 te Geldrop. Na het behalen van zijn 2e graads lesbevoegdheid Biologie aan het Mollerinstituut (Nieuwe Leraren Opleiding) te Tilburg begon hij in 1983 met de studie Biologie aan de Katholieke Universiteit Nijmegen. In oktober 1987 legde hij het doctoraal examen ("oude stijl") af met als hoofdvakken Vergelijkende Dierfysiologie (Prof. Dr. S.E. Wendelaar Bonga) en Aquatische Oecologie (Prof. Dr. C. den Hartog) en als bijvak Chemische Cytologie (Prof. Dr. G.W.F.H. Borst Pauwels). Vanaf november 1987 was hij werkzaam als assistent in opleiding bij de afdeling Radiochemie van het Interfactultair Reactor Instituut, Technische Universiteit Delft. Het in dit proefschrift beschreven onderzoek is verricht op de afdeling Radiochemie van de Technische Universiteit Delft, op de afdeling Experimentele Dierkunde van de Katholieke Universiteit Nijmegen, op de afdeling Radiochemie van het Nationaal Instituut voor Kernfysica en Hoge-Energiefysica te Amsterdam, en op de vakgroep Experimentele Dierkunde van de Universiteit van Amsterdam.