Calcium permeability and ATPase activities of red blood cells of magnesium deficient rats

Edmond Rock, Elyett Gueux, Christine Cubizolles, and Yves Rayssiguier

Institut National de la Recherche Agronomique, Laboratoire des Maladies Métaboliques, 63122, Saint Genès Champanelle, France

Decreased intracellular magnesium and potassium and increased sodium levels were shown in the erythrocytes of weanling rats fed a low magnesium diet for 8 days. Magnesium-dependent ATPase activity was significantly increased in membranes obtained from Mg-deficient cells and the (Ca^{2+})- and ($Na^+ + K^+$)-stimulated activities were similar to control values. Calcium flux measurements clearly show that passive permeability was significantly increased in red blood cells from Mg-deficient rats. Moreover, initial rates of calcium influx and to a lesser degree calcium efflux were increased in ghosts prepared from Mg-deficient erythrocytes. In agreement with our previous findings on the physico-chemical parameters of these membranes, the present results strongly suggest that permeability properties rather than the pump activities are primarily affected by dietary magnesium deficiency. (J. Nutr. Biochem. 5:351–355, 1994.)

Keywords: magnesium deficiency; rats; erythrocytes; membrane; (Ca^{2+}-Mg^{2+})-ATPase; (Na^+-K^+)-ATPase; calcium flux

Introduction

Magnesium is the second most abundant metal ion in cells, and its role in cell functions and integrity has been recently reviewed.[1] Magnesium deficiency, which occurs in humans,[2] has been extensively studied in laboratory animals. The secondary effects of magnesium deficiency on cell constituents are well recognized. These include loss of potassium and accumulation of sodium and calcium.[3–5] The observation that in the Mg-deficient state the cellular content of potassium is abnormally low while sodium and calcium accumulate intracellularly is consistent with functional impairment of the ATP-dependent cell membrane pumps when Mg is in short supply.[6] However, direct evidence of such impairment in red blood cells is lacking.[7] On the other hand, recent studies indicate that Mg affects the physico-chemical properties of membranes.[8–12] These data support the hypothesis that damage to membrane lipid bilayer could be the primary lesion underlying the cellular disturbances that occur in Mg-deficiency. Fluorescence polarization was used to compare the fluidity of RBC membranes from Mg-deficient and control rats. Erythrocyte membranes of magnesium-deficient rats were more fluid than those of control rats.[9] Although magnesium can directly influence membrane fluidity, the changes during its deficiency in vivo are mainly mediated via disturbances in lipid metabolism.[11,13] Thus, the possibility exists that modification in cell membrane fluidity may lead to increased cell membrane permeability[14] to passive ion fluxes and cause the cellular potassium depletion and intracellular sodium and calcium accumulation resulting from Mg-deficiency.

Methods and materials

Animals and diets

The institution's guide for the care and use of laboratory animals was used. Weanling male Wistar rats weighing 65 g were randomly divided into control and Mg-deficient groups. The deficient group was fed 30 mg/kg Mg-containing diet, whereas the control group was pair-fed the normal Mg (960 mg/kg) diet. Otherwise, the synthetic diets contained (in g/kg) 200 casein, 3 DL-methionine, 705 sucrose, 50 corn oil, 35 mineral mixture, and 10 vitamin mixture, as previously described.[9] Distilled water was provided ad libitum during the 8-day experimental period.

Preparation of erythrocytes and ghosts

Blood was withdrawn under pentobarbital anesthesia and the red blood cells were collected and washed by centrifugation at 1300*g* for 15 min. Plasma membranes were prepared by the method of

Address reprint requests to Dr. E. Rock at the Laboratories des Maladies Métaboliques, I.N.R.A., 63122, Saint Genès Champanelle, France.
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Hanahan and Ekholm[15] using Tris HCl buffer (172 mM), pH 7.6. Washed erythrocytes were lysed with hypotonic Tris HCl buffer (11 mM) pH 7.6, and the membranes sedimented by centrifugation at 25,000*g* for 15 min at 4° C. The ghosts were washed with hypotonic buffer before resuspending in the latter buffer at about 3 mg/mL of protein. Protein concentration was determined by the Lowry method.[16] For the measurements of calcium fluxes, the membranes were washed and resuspended in isotonic buffer containing 50 mM Hepes, 150 mM KCl, pH 7.4.

Metal determinations

Metals were determined with a Perkin-Elmer series 400 atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, CT USA) as previously described.[8] Magnesium was measured by atomic absorption analysis in lanthanum chloride solution containing 1.0 g La/L; potassium and sodium by flame emission in cesium chloride solution containing 1.0 g Cs/L. Plasma was analyzed directly after dilution with the appropriate solution and erythrocytes after hemolysis in desionised water.

Ca-ATPase activity

Ca-ATPase activity was determined at 37°C using 0.5 mL of an assay medium containing 150 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 2 IU lactate dehydrogenase, 2 IU pyruvate kinase, and 50 mM Hepes adjusted to pH 7.4. The reaction was started by addition of 50 μg proteins and the absorbance changes were recorded at 340 nm. EGTA (2 mM) was added in order to determine the basal activity. Calcium concentrations were varied (0 to 5 μM) by additions of different volumes of 10 mM $CaCl_2$. The basal level of ATPase activity was subtracted from the ATPase activity in the presence of calcium to obtain the calcium-dependent ATPase activity. The activity was expressed as μmoles Pi/h/mg protein.

(Na-K)-ATPase activity

(Na-K)-ATPase activity was determined by a coupled optical test at 340 nm at 37°C as described in[17] with slight modifications. The assay medium contained 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 2 IU lactate dehydrogenase, 2 IU pyruvate kinase, 3 mM $MgCl_2$, 2 mM ATP, 1 mM EDTA, 100 mM NaCl, 10 mM KCl, and 50 mM Tris-HCl pH 7.2. The reaction was started by addition of 20 μg/ml protein. (Na-K)-dependent ATPase activity was calculated by subtracting the total activity with the basal one determined in the absence and in the presence of 1 mM ouabain, respectively. The activity is expressed in μmoles Pi/h/mg protein.

Ouabain binding

100 μg of proteins were incubated 20 minutes at 37° C in a mixture containing 50 mM Tris HCl, 1 mM EDTA, 3 mM $MgCl_2$, 100 mM NaCl, and 10^{-5} M [^{3}H]ouabain (31 Ci/mmol) in the presence or in the absence of 3 mM ATP. The proteins were then filtered through 0.65 μm Millipore DAWP filter and washed three times with 5 mL of cold buffer. Radioactivity was determined in the filters by liquid scintillation counting. Results were expressed in pmoles/mg protein. Specific binding was calculated by subtracting the amounts of dpm counted in the presence of ATP with those determined in its absence.[18]

Measurements of ^{45}Ca-influx in the erythrocytes

Washed erythrocytes were adjusted to a hematocrit of about 25% in 50 mM hepes, 145 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM Na_2HPO4, 5 mM glucose pH 7.4. ^{45}Ca (2 μCi/μmol) was added to a concentration of 1.2 mM at 37° C. At specified times, aliquots of 0.5 mL of cells were removed, washed, and precipitated with 0.5 mL of 10% trichloroacetic acid. After centrifugation, radioactivity was measured in the supernatant. The pellet was solubilized in 0.1 N NaOH and the proteins were determined using the Lowry method.[16]

Measurements of ^{45}Ca-influx and efflux from erythrocyte membranes

Calcium influx was determined with 50 μg membranes incubated at room temperature with buffer containing 5 mM $^{45}CaCl_2$. At different times, an aliquot was filtered through 0.65 μm Millipore DAWP filter and washed three times with 5 mL of cold buffer containing 5 mM unlabeled $CaCl_2$ to replace labeled calcium ions bound to the external side of membranes. The radioactivity contained in the filter was determined by liquid scintillation counting.

For the efflux measurements, washed membranes were first incubated overnight at 4° C in buffer containing 5 mM $^{45}CaCl_2$. About 50 μg proteins were filtered through 0.65 μm Millipore DAWP filter. The filter was rinsed under vacuum with 800 μL of Hepes buffer containing 5 mM of unlabeled $CaCl_2$. From the amount of radioactivity retained by the filter, we determined the initial content of calcium inside the ghosts. Calcium release was initiated by incubation of washed membranes with 800 μL of calcium-free Hepes-buffer. After different incubation times, the buffer was removed by vacuum. The filter was rinsed with 5 mL of Ca-free buffer and the amount of radioactivity was determined by liquid scintillation counting.

Statistical analysis

Results are expressed as mean ± SEM. Comparison of mean values was tested by Student's *t* test for statistical significance. The values were considered significantly different at $P < 0.05$.

Results

At the end of the experimental period, the mean final body weight of Mg-deficient rats was significantly lower than the control ones (83 ± 2 versus 90 ± 3 g for six animals in each group; $P < 0.01$). Plasma magnesium level dropped from 0.82 ± 0.02 in control rats to 0.12 ± 0.01 mM in Mg-deficient rats. The effect of magnesium deficiency on the intracellular ion contents of red blood cells is shown in *Table 1*. Magnesium and potassium levels were significantly decreased, whereas the sodium content was increased in the cells obtained from Mg-deficient rats.

The membranes of red blood cells from control and Mg-deficient rats were purified and used to study the ATPase activities associated with either the (Na^+-K^+) or (Ca^{2+}) pumps. As shown in *Table 2*, ($Na^+ + K^+$)-ATPase activity from Mg-deficient membranes did not differ significantly

Table 1 Intracellular ion contents of red blood cells

	Magnesium	Sodium	Potassium
Control	3.75 ± 0.02	3.28 ± 0.05	121.2 ± 3.0
Mg-deficient	2.72 ± 0.16	3.77 ± 0.08	112.6 ± 2.1
P	<0.01	<0.01	<0.01

Values shown are mean ± S.E. for six control and six Mg-deficient rats. The data are expressed in mmoles/L. *P* values were calculated with reference to control.

Table 2 ATPase activities of red blood cell membranes of control and Mg-deficient rats

		Total activity	Basal activity	Ion(s)-stimulated activity
(Na/K)-ATPase	Control	2.41 ± 0.12	1.03 ± 0.06	1.37 ± 0.09
	Mg-deficient	2.73 ± 0.13	1.23 ± 0.06*	1.50 ± 0.09
(Ca)-ATPase	Control	1.85 ± 0.05	1.06 ± 0.03	0.78 ± 0.06
	Mg-deficient	2.04 ± 0.09	1.34 ± 0.05**	0.70 ± 0.08

The activities were expressed in μmoles Pi/h/mg of protein.
Total activity was determined in the presence of (100 mM NaCl + 10 mM KCl) or 5 μM free calcium.
Basal activities were determined in the presence of 1 mM ouabain and 2 mM EGTA for (Na/K)- and (Ca)-ATPase activities respectively.
Ions-stimulated activity represents the difference between total and basal activities.
Values are mean ± SE for six control and six Mg-deficient rats. The asterisks indicate significant differences from controls.
*$P < 0.05$.
**$P < 0.01$.

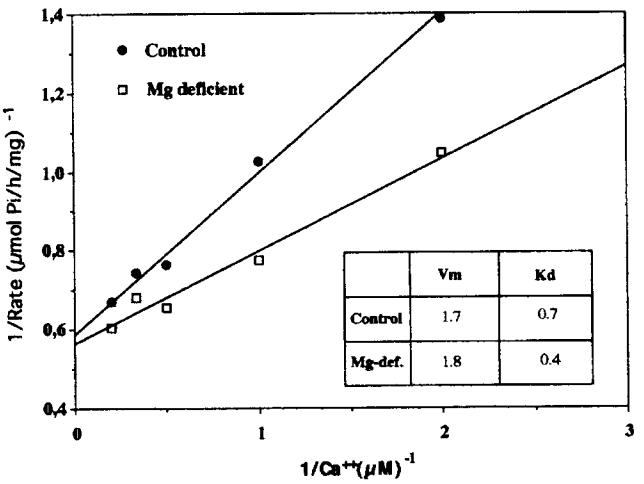

Figure 1 Double reciprocal plot of calcium ATPase activity versus calcium concentration. Membranes were prepared from control (●) and Mg-deficient (□) red blood cells. Each point represents the mean values of three different preparations. From the linear curve fitting were determined the maximum velocity and the affinity constant (Inset). V_m and Kd were expressed in μmoles Pi/h/mg protein and in μM, respectively.

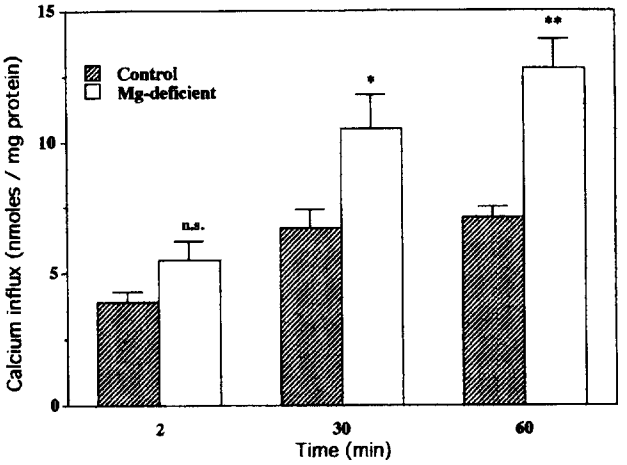

Figure 2 Passive calcium influx in intact red blood cells obtained from control (▨) and Mg-deficient (□) rats. Values are given as nmoles of calcium accumulated per mg protein. Mean ± SE for six different preparations. The asterisks indicate significant differences from controls: *$P < 0.05$; **$P < 0.01$.

from controls either in total or in $(Na^+ + K^+)$-dependent activity. Basal or (Mg^{2+})-ATPase activity, measured in the presence of specific inhibitor (1 mM ouabain) is significantly higher in Mg-deficient membranes as compared with control ones. The concentration of $(Na^+ + K^+)$-ATPase molecules was investigated through their capacity to bind ouabain specifically. No differences were observed in [^{3}H] ouabain binding (0.22 ± 0.03 versus 0.22 ± 0.02 pmol/mg protein for six control and six Mg-deficient membranes, respectively). The calcium-stimulated ATPase activity was assayed in the presence of 5 μM free calcium. No significant differences were observed between control and Mg-deficient membranes. The basal activity, measured in the presence of 2 mM EGTA, is again significantly higher in Mg-deficient membranes as compared with controls *(Table 2)*.

We further investigated the kinetic properties of (Ca^{2+})-ATPase activity of red blood cell membranes from control and Mg-deficient rats. *Figure 1* presents data as Lineweaver-Burk plots of activity versus calcium concentration. In control membranes, the calculated values of the maximal velocity (V_{max}) and the apparent affinity, K_m, is about 1.7 μmoles Pi per hour per mg protein and 0.7 μM, respectively *(Figure 1)*. The membranes from Mg-deficient cells showed similar maximal rate (1.8 μmoles Pi per hour per mg protein), but the affinity of the pump to calcium decreased to about 0.4 μM (Figure 1).

Membrane permeability was assessed by measuring [^{45}Ca] fluxes, either through intact cells or the isolated membranes. Incubation of red blood cells with 1 mM calcium in the outside medium resulted in an accumulation of calcium inside the cells. The initial amount of calcium accumulated after less than 2 minutes incubation is not significantly different between Mg-deficient and control cells. The amount of calcium accumulated by Mg-deficient cells became significantly higher when compared with control cells after 30 and 60 minutes incubation *(Figure 2)*. Using the values obtained after 30 minutes incubation, an apparent rate of 93 and 166 pmol/min/mg protein was obtained for control and Mg-

deficient membranes, respectively. *Figure 3* depicts passive calcium influx through isolated membranes from control and Mg-deficient cells. The initial rate is threefold higher in Mg-deficient membranes as compared with controls. The amount of calcium accumulated after 2 minutes incubation is about two fold higher in membranes prepared from Mg-deficient versus control cells. However, the total calcium accumulated after 1 hour incubation was not different between control and Mg-deficient membranes (13.0 ± 0.36 versus 13.0 ± 0.78 nmole/mg protein).

The passive calcium efflux was determined after overnight incubation of membranes with 5 mM $^{45}CaCl_2$ and dilution in the same incubation medium without calcium. The initial content of intravesicular calcium was about 15 nmoles per mg protein for Mg-deficient and control membranes. Data were therefore expressed as percentage of calcium released within the times of analysis. The results from *Figure 4* show that within 1 minute 25% and 15% of passively accumulated calcium was released by Mg-deficient and control membranes, respectively. This higher initial rate of calcium efflux from Mg-deficient membranes did not affect the total amount of calcium released after a 10-minute reaction time, which represented about 50% of calcium accumulated for both types of membranes.

Discussion

It is known that long-term magnesium deficiency affects the levels of intracellular Na, K, and Ca ions[3-5] in cells of most of the tissues analyzed. Modifications of Na and K levels may appear early, as observed in our model of short-term acute Mg-deficiency. The threefold higher rate of accumulation of labeled calcium by freshly isolated cells from Mg-deficient rats strongly suggests that increased calcium permeability may cause the increased intracellular calcium level usually observed in Mg-deficient tissues. In long-term deficiency in rats and hamsters, it was shown that imbalanced levels of sodium and potassium resulted from alterations of

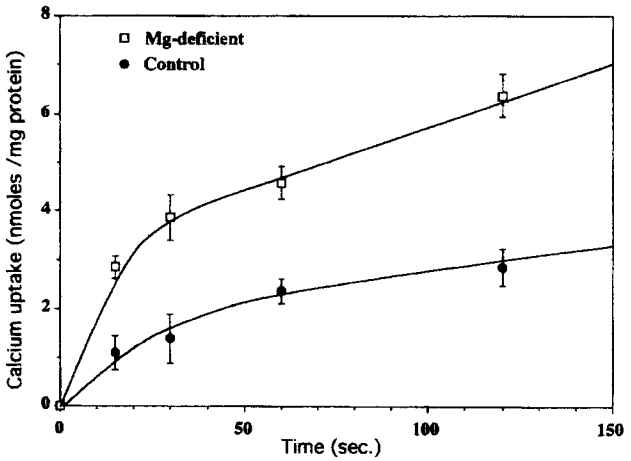

Figure 3 Passive calcium influx in red blood cell membranes obtained from control (●) and Mg-deficient (□) rats. Values are given as nmoles of calcium accumulated per mg protein. Mean ± SE for four different preparations.

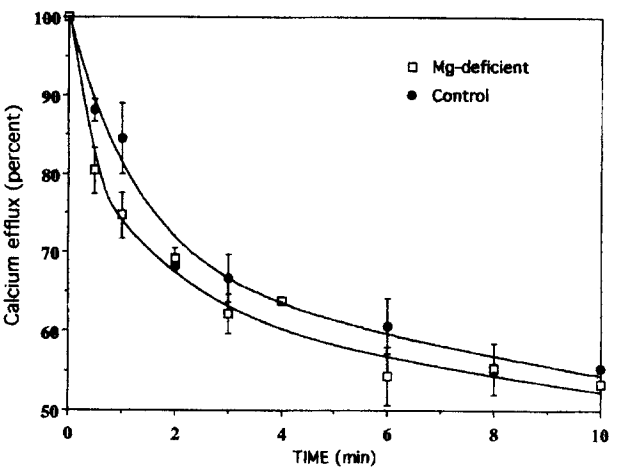

Figure 4 Calcium efflux from preloaded red blood cell membranes prepared from control (●) and Mg-deficient (□) rats. Mean ± SE from four different preparations.

sodium-potassium pump activity.[19,20] Indeed, (Na/K)-ATPase in microsomal fractions of rat hearts has been shown to be linearly dependent on the amount of magnesium in the diet,[19] and digoxin treatment, an inhibitor of (Na,K)-ATPase, induced a dose-dependent increase in myocardial lesion.[20] Inhibition of (Na/K)-ATPase activity has been attributed to the decrease in intracellular magnesium pool that is necessary to activate the enzyme.[6] However, we have demonstrated that in short-term deficiency enzymatic activity was essentially normal despite increased sodium content and decreased potassium levels in Mg-deficient cells. Moreover, erythrocytic depletion of intracellular magnesium led to activation of (Mg)-dependent ATPase activity, such as is observed in the presence of ouabain or EGTA. Therefore, in our model dietary Mg deficiency seems to affect membrane permeability rather than pump activities. Recent studies on skeletal muscle (using Mg^{2+}-sensitive K^+ channels) support this hypothesis, because potassium depletion was induced by increased K^+ efflux, rather than by inhibition of (Na^+-K^+) pump mediated K^+-influx.[5]

On the other hand, increased intracellular calcium may be explained by the significantly high influx rate observed in the entire cell as well as in the isolated membranes of Mg-deficient erythrocytes. The greater rates of calcium influx and efflux from Mg-deficient membranes show that the overall membrane permeability was affected in these membranes. This greater permeability could be a direct consequence of the damage to the lipid bilayer previously shown in membranes from Mg-deficient red blood cells.[9] The decreased magnesium levels and the altered cholesterol to phospholipid ratio mediate the increased fluidity of Mg-deficient membranes. The substantial increase in the influx:-efflux rates suggests that the inward calcium flux is increased in Mg-deficient cells. Involvement of calcium channels in calcium-overloading of Mg-deficient tissues has been proposed to explain the protection afforded by calcium channel blockers against the myocardial necrosis[20] and mortality[21] of Mg-deficient animals. Thus, accumulation of calcium by Mg-deficient cells or membranes does not result from

impairment of the calcium efflux system, as Ca-ATPase activity was similar in Mg-deficient and control cells and membranes. Moreover, Mg-deficient membranes had the highest apparent enzymatic affinity for calcium.

The present results, demonstrating an enhanced permeability of Mg-deficient membranes without apparent perturbations in pumps-associated ATPase activities, are in agreement with perturbations of physical and chemical properties of membrane lipid components induced by magnesium deficiency.[9–12]

References

1 Romani, A. and Scarpa, A. (1992). Regulation of cell magnesium. *Arch. Biochem. Biophys.* **298,** 1–12

2 Durlach, J. (1988). *Magnesium in clinical practice,* (J. Durlach, ed.), John Libbey, London, UK

3 Whang, R. and Welt, L.G. (1963). Observations in experimental magnesium depletion. *J. Clin. Invest.* **42,** 305–313

4 Ghazi, A.G. and Heaton, F.W. (1975). Changes in cellular composition during magnesium deficiency. *Biochem. J.* **152,** 609–615

5 Dørup, I. and Clausen, T. (1993). Correlation between magnesium and potassium contents in muscle: role of Na^+-K^+ pump. *Am. J. Physiol.* **264,** C457–C463

6 Heaton, F.W. (1990). Role of magnesium in enzyme systems. In *Metal ions in biological systems,* (H. Sigel and A. Sigel, eds.), p 119–133, Marcel Dekker, Inc, New York, Basel

7 Günther, T., Vormann, J., Averdunk, R., and Ising, H. (1982). Irreversible alteration of Na^+ and K^+ transport in erythrocytes from magnesium-deficient rats. *Magnesium Bull.* **1,** 37–41

8 Heaton, F.W., Tongyai, S., Motta, C., Rayssiguier, Y., and Gueux, E. (1987). Changes in erythrocyte membrane during magnesium deficiency. *Nutr. Res.* **7,** 655–663

9 Tongyai, S., Rayssiguier, Y., Motta, C., Gueux, E., Maurois, P., and Heaton, F.W. (1989). Mechanism of increased erythrocyte membrane fluidity during magnesium deficiency in weanling rats. *Am. J. Physiol.* **257,** C270–C276

10 Freedman, A.M., Mak, I.T., Stafford, R.E., Dickens, B.F., Cassidy, M.M., Muesing, R.A., and Weglicki, W.B. (1992). Erythrocytes from magnesium-deficient hamsters display an enhanced susceptibility to oxidative stress. *Am. J. Physiol.* **262,** C1371–C1375

11 Kummerow, F.A. (1992). Hypothesis: possible role of magnesium and calcium in the development of structure and function of the plasma membrane in mammalian cells and in human diseases. *J. Am. Coll. Nutr.* **11,** 410–425

12 Rayssiguier, Y., Gueux, E., and Motta, C. (1991). Magnesium deficiency: effect on fluidity and function of plasma and subcellular membranes. In *Magnesium: A Relevant Ion,* (B. Lasserre and J. Durlach, eds), p 311–319, John Libbey, London, UK

13 Rayssiguier, Y. (1990). Magnesium and lipid metabolism. In *Metal ions in biological* systems, (H. Sigel and A. Sigel, eds.), p 341–358, Marcel Dekker, Inc. New York, NY USA

14 Shinitzky, M. (1984). Membrane fluidity and cellular functions. In *Physiology of membrane fluidity,* (M. Shinitzky, ed.), p 1–52, CRC Press, Boca Raton. FL USA

15 Hanahan, D.J. and Ekholm, J.E. (1974). The preparation of red cell ghosts. In *Methods in Enzymology,* (S. Fleisher and L. Packer, eds), p 168–172. Academic Press, New York, NY USA

16 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275

17 Thomas, C.E. and Reed, D.J. (1990). Radical-induced inactivation of kidney Na+,K+-ATPase: sensitivity to membrane lipid peroxidation and the protective effect of vitamin E. *Arch. Biochem. Biophys.* **281,** 96–105

18 Narahara, H.T., Vogrin, V.G., Green, J.D., Kent, R.A., and Gould, M.K. (1979). *Biochim. Biophys. Acta* **552,** 247–261

19 Fischer, P.W.F. and Giroux, A. (1987). Effects of dietary magnesium on sodium-potassium pump action in the heart of rats. *J. Nutr.* **117,** 2091–2095

20 Ahmad, A. and Bloom, S. (1989). Sodium pump and calcium channel modulation of Mg-deficiency cardiomyopathy. *Am. J. Cardio. Pathol.* **2,** 277–283

21 Sakaguchi, H., Sahaguchi, R., Ishiguro, S., and Nishio, A. (1992). Beneficial effects of a calcium antagonist, nifedipine, on death and cardiovascular calcinosis induced by dietary magnesium deficiency in adult mice. *Magnesium Res.* **5,** 121–125