Spherocytic Hemolytic Disease During Magnesium Deprivation in the Rat

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Young male rats (115 g) were maintained on diets containing 4–8 mg of magnesium per 100 g of diet for 12 wk. By 3 wk the characteristic features of magnesium deprivation developed, including decreased plasma and tissue magnesium concentration, growth retardation, ruffled fur, patchy dermatitis, irritability, hyperemia of acral parts, onychymegaly, and in the most severely restricted, premature death. By 7 wk of deprivation, evidence of a hemolytic state existed and thereafter reticulocytosis, spherocytosis, shortened $^{51}$Cr red cell survival, erythroid hyperplasia of the bone marrow, and mild anemia were present. Erythrocytes during magnesium deficiency were characterized by decreased intracellular magnesium, glucose utilization, lactate production, ATP, and 2,3-DPG concentration. A progressive decrease in red cell deformability as measured by cell elastometry occurred. The reduction in lactate production and in ATP concentration due to magnesium deficiency may be causal in the development of rigid spherocytes with shortened survival in vivo. In addition, the shape and deformability alteration of the red cell may be due to defective membrane construction in a magnesium-deficient environment.

Considerable experimental and clinical data describing the effects of magnesium lack have been gathered. Magnesium is important as a cofactor for ATP generation during glycolysis, and for ATP utilization in the red cell. Moreover, magnesium is an integral constituent of the red cell membrane. However, the possible effects of magnesium deficiency on the erythron have not been well documented. Anemia during magnesium deprivation has been noted in some but not in other studies. When anemia has been observed, studies have not been performed to exp-.
amine the role of increased red cell destruction or decreased production in its
genesis. Moreover, studies of the membranes, enzyme content, glycolytic
metabolism or hemoglobin of red cells from chronically magnesium-deficient,
anemic animals have been lacking.

The following studies were conducted to examine the alterations in the red
cell which occur with sustained magnesium deprivation.

**MATERIALS AND METHODS**

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.),
weighing 100 to 130 g, were divided into four groups and placed on one of four exper-
imental diets. The basal diet for each group was a “magnesium-free” diet to which was
added zero, 2, 4, or 65 mg of magnesium per 100 g of diet and then prepared as color-
coded pellets (General Biochemical Company, Chagrin Falls, Ohio). Since the “magnesium-
free” diet provided by General Biochemical had a magnesium content of 4 mg/100 g when
assayed in our laboratory, magnesium content of the final diets was 4, 6, 8, and 69
mg/100 g. Therefore, the groups are referred to throughout this paper as Diet-4, Diet-6,
Diet-8, and Diet-69.

The animals, kept in individual stainless steel cages, were allowed unrestricted quantities
of diet and distilled deionized water. Animals were weighed and their clinical findings
recorded at various intervals over a 12-wk period.

Blood samples were obtained from the tail at 3, 7, and 12 wk and collected in tubes
containing sodium heparin shown to be free of magnesium. Plasma and red cell
magnesium, hematocrit, reticulocyte counts, blood smears, and red cell deformability were
studied sequentially at 3, 7, and 12 wk in animals from each dietary group. Some animals
were sacrificed by exanguination from aorta for studies involving larger volumes of blood
(i.e., red cell sodium, potassium, and glycolytic rate). In some cases, the six determinations
noted above were also made.

Packed red cell volume was measured in duplicate in a microhematocrit centrifuge.
Reticulocyte counts were made from smears prepared from blood exposed to new methylene
blue. Peripheral blood smears were prepared with Wright’s stain. In addition, blood was
diluted in 0.15 M NaCl-0.010 M Tris Cl buffer, pH 7.4, containing 1% albumin and
erthrocytes examined by phase contrast microscopy. Erythrocyte counts were determined
with a Model B Coulter particle counter using a 70-μ orifice. Lower thresholds were
determined by plotting volume distribution. Mean cell volume was determined by the use
of a standard formula based on red cell count and packed cell volume.

Plasma creatinine and urea nitrogen were determined using Technicon autoanalyzer
methodology N-38A. Plasma inorganic phosphorus was measured by modification of the
phosphomolybdate acid method of Fiske and SubbaRow.12 Plasma and red cells washed
thrice in 0.17 M NaCl-Tris Cl were extracted with 10% trichloracetic acid and magnesium
concentration determined using a Perkin-Elmer Model 303 atomic absorption spectrophotom-
eter with a stoichiometric air-acetylene flame. Red cell sodium and potassium was
measured on blood washed thrice with 0.10 M MgCl2-.0010 M Tris Cl, pH 7.4, using
flame photometry with lithium chloride as an internal standard.

Red cells from blood collected in sodium heparin were sedimented by centrifugation
at 1200 g. The buffy coat was removed and the red cells were suspended to a hematocrit
of approximately 25% in autologous plasma. White cell counts of the final suspensions were
less than 1000 per cu mm. The blood was adjusted to a final glucose concentration of
approximately 180 mg/100 ml, incubated at 37°C in a Dubnoff shaker water bath in air.
After 30 min (zero time) and after 3 hr, 0.9-ml samples were removed and added to
1.6 ml of ice cold 6% perchloric acid and centrifuged at 2000 g. The perchloric acid
extracts were then neutralized with 0.2 ml ice cold 5M potassium carbonate, centrifuged
at 2000 g and stored frozen at −20°C until assayed.13 Blood glucose was assayed by the
glucose oxidase method,14 red cell lactate by the lactic dehydrogenase method,15 red cell ATP
by the “backwards” glyceraldehyde phosphate dehydrogenase method,16 and red cell 2,3-
DPG by Schröter and Heyden’s modification17 of the method of Krimsky.18
Red cell deformability was measured with a cell elastimeter with a micropipette orifice of measured internal diameter. The elastimeter was modified by LaCelle\textsuperscript{19} from the apparatus described by Mitchison and Swann\textsuperscript{20}. Micropipettes with an internal diameter of 2.4 to 2.6 µ were used. The negative pressure in mm H\textsubscript{2}O required to draw an entire red cell into the micropipette was recorded. On each sample studied, 50–100 cells were tested. The examiner was unaware whether the sample was from control or from magnesium-deficient rats.

Red cell survival was measured on three animals who had been on an 8 mg/100 g and three animals who had been on a 69 mg/100 g magnesium diet. One ml of blood was obtained from the inferior vena cava at laparotomy performed under a stringent aseptic technique using light ether anesthesia. The blood was anticoagulated with modified Squibb A-C-D solution and incubated with 10 µCi Na\textsubscript{51}CrO\textsubscript{4} at 37°C for 20 min and ascorbic acid added. The abdominal incision was closed after the blood was reinjected through the inferior vena cava and hemostasis was assured. Blood samples were obtained at 24 hr and every 1 to 4 days thereafter until T ½ was reached. Plasma radioactivity was negligible at 24 hr. A 100-µl blood sample was obtained at each bleeding for determination of radioactivity and hematocrit. Hemostasis after sampling was meticulously maintained. Corrections were made for blood removed during periodic sampling, which resulted in an increase in T ½ of 3–10%. All six animals were alive, active, and feeding normally with healed laparotomy incisions well after the end of the survival studies.

**RESULTS**

**Clinical Observations:** The magnesium-deprived rats exhibited clinical signs of deficiency, including irritability and hyperexcitability, hyperemia of the ears and feet, edema, ruffled fur, patchy dermatitis, and retarded growth. In addition, it was noted that the toenails of the rats on a magnesium-deficient diet grew longer than those of control animals. The effects of magnesium deficiency were more severe and tended to occur earlier in the more severely magnesium-restricted rats, as indicated by the rate of increase of body weight (Fig. 1). Ten rats in each dietary group who had only the standard hematologic studies at 3, 7, and 12 wk were used to calculate death rate. Premature death oc-

![Fig. 1.—Mean body weights. The fraction of rats surviving at 6 and 12 wk are shown in parentheses.](image-url)
SPHEROCYTIC HEMOLYTIC DISEASE

Fig. 2.—Mean plasma and red cell magnesium concentration.

curred in animals who received less than 8 mg of magnesium per 100 g of diet (Fig. 1). The death rate of magnesium-deficient rats may have been increased by handling and periodic phlebotomy and does not represent the death rate of rats left undisturbed on such diets.

Plasma and Red Cell Magnesium: A reduction in plasma and red cell magnesium occurred with age in both control and magnesium-deprived animals (Fig. 2). The most severely magnesium-restricted rats developed reduced blood magnesium concentrations most rapidly; however, by 12 wk of study all three magnesium-restricted groups had similar concentrations of red cell or plasma magnesium. This may be explained in part by the selective loss

Fig. 3.—Distribution of packed cell volume, percent reticulocytes, and percent spherocytes.
by death of the most severely affected rats who would be expected to have the most deviant chemical values. At 12 wk of study the plasma magnesium in magnesium-deficient animals was about 50% and the red cell magnesium 65% of control animals.

**Plasma Inorganic Phosphorus, Urea Nitrogen, and Creatinine:** Mean (± SE) plasma inorganic phosphorus concentration was mildly decreased in the magnesium-deficient (5.40 ± 0.16) as compared to control (6.10 ± 0.30) rats. Plasma urea nitrogen (19.9 ± 0.77 mg/100 ml) and creatinine (0.58 ± 0.053 gm/100 ml) concentrations were normal in a sample of magnesium-deficient animals studied.

**Hematocrit, Reticulocytes, Spherocytes:** The magnesium-deprived animals had a significant decrease in mean hematocrit as compared to controls (Fig. 3). Of the magnesium-deficient animals, 44% had a packed cell volume less than the lowest value for control rats. The most severely magnesium-restricted rats were often frankly anemic. In addition, a highly significant increase in the number of reticulocytes occurred. Magnesium-deprived rats had a mean reticulocyte count of 5.3% as compared with 1.2% in control animals (Fig. 3). Spherocytosis was also frequent in magnesium-deficient animals. As shown in Fig. 4. red cells from control rats had central depressions, although the cells frequently appeared stomatocytic, with slit-like central depressions or bowl-

**Fig. 4.—** Phase contrast photomicrographs of red cells from a Diet-69 rat (A, × 640; C, × 400) and Diet-6 rat (B, × 640; D, × 400). Note the frequency of small, smooth, refractile cells without central concavity (microspherocytes) in the blood of the Diet-6 rat (B and D). These photographs were made on rats chosen randomly from control and magnesium-deficient groups and from microscopic fields selected randomly for photographing.
like shapes, which were evident while the red cells were tumbling. Magnesium-deficient animals exhibited frequent smooth microspherocytes. Such red cells were smaller in diameter, were highly refractile, and lacked a central concavity. The magnesium-deficient rats had 17.4% of red cells as microspherocytes by phase contrast microscopy whereas 1.9% of red cells of Diet-69 animals were microspherocytes (Fig. 3). The mean and SE value of red cell volume was 51.6 ± 1.8 cu µ in six randomly selected rats from Diet-69 group as compared with 58.5 ± 2.8 cu µ in six randomly selected rats from Diet-8 group.

*Cellular Deformability:* By 3 wk on the experimental diets, magnesium-deficient rats had a slightly increased proportion of spherocytic cells and a slight increase in the proportion of cells with increased resistance to deformation. Over the next 9 wk, a progressive increase in the proportion of spherocytes and in cells resistant to entry into the micropipette orifice occurred (Fig. 5). Spherocytosis and marked resistance to deformation were highly correlated. Of further interest was a shift of the entire population of red cells,

![Graph](image-url)

*Fig. 5.—Distribution of negative pressure required to aspirate entire cell into micropipette of 2.4–2.6 µ diameter at 3, 7, and 12 wk of diet. The mode present at 20 mm of H₂O pressure is an artifact due to the inclusion of all cells with deforming pressures equal to or greater than 20 mm H₂O in that group.*
regardless of apparent shape, to a higher negative pressure by 12 wk. This increase indicated that cells that appeared morphologically normal (non-spherocytic) also had an increase in resistance to deformation.

Na\textsuperscript{51}CrO\textsubscript{4}-RBC Survival Studies: Na\textsuperscript{51}CrO\textsubscript{4}-RBC survival curves were determined on three control and three magnesium-deficient (Diet-8) rats that had not been bled previously. The T \% of Na\textsuperscript{51}CrO\textsubscript{4}-labeled red cells was 14.9, 16.0, and 17.3 days in the three control animals and 8.4, 9.5, and 14.3 days in the three magnesium-deficient rats.

Red Cell Glycolysis, ATP, and 2,3-DPG: Red cell glucose utilization was mildly decreased in magnesium-deficient animals. By 7 wk, 13 of 16 magnesium-deficient rats tested had glucose utilization rates less than the lowest control values (Fig. 6A). Red cell lactate production was also reduced in magnesium-deficient rats (Fig. 6B). Lactate production appeared to be more severely affected in the most magnesium-deprived animals. In some of the Diet-4 group, lactate production was reduced to 50% of control animals.

Red cell ATP concentrations were decreased significantly in the animals on magnesium-deficient diets (Fig. 6C). The rats fed a normal diet had a mean ATP concentration of 0.95 mmol/liter RBC compared to mean concentrations of 0.69, 0.58, 0.33 mmol/liter RBC in rats on Diet-8, Diet-6, and Diet-4, respectively.

Red cell 2,3-DPG was decreased in magnesium-deficient rats (Fig. 6D). This was most notable in the rats on more stringent magnesium-deficient diets. Mean 2,3-DPG concentrations were 9.10 mmol/liter RBC in rats in the Diet-69 group as compared with 7.23, 6.54, and 5.41 mmol/liter RBC in animals on Diet-8, Diet-6, and Diet-4 respectively.

Lactate:glucose ratios were greater than two in control animals (2.47 ± 0.054), indicating an additional source other than glucose for lactate pro-

Fig. 6.—Red cell (A) glucose utilization, (B) lactate production, (C) ATP, and (D) 2,3-DPG. The results in the control group (Diet-69) did not differ at 7 and 12 wk of study. The results presented for magnesium-deficient rats were measured at 7 and 12 wk of study.
duction in the rat red cell. Magnesium deficient rats had a reduction in lactate: glucose ratios by 7 wk of study. Diet-4 rats had the lowest ratios (1.72 ± 0.093), whereas Diet-6 (2.18 ± 0.068) and Diet-8 (2.16 ± 0.153) rats had intermediate ratios.

**DISCUSSION**

The clinical syndrome of magnesium deprivation observed in the rat in our studies was very similar to the classical description of Kruse and co-workers. Kruse and colleagues and later investigators interested in magnesium deprivation have not directed their attention to the possible effects of magnesium deficiency on red cell turnover. Decreased blood hemoglobin concentration has been observed in magnesium deficient guinea pigs and, more recently, pregnant rats. Kruse and co-workers noted a decreased packed red cell volume in magnesium-deficient dogs, although the values reported were very erratic from day to day. The failure to observe anemia in one study in rats can be explained by the short period of observation. The increase in reticulocytes noted was discounted by the investigator as experimental variation. The pathogenesis of the mild anemia that developed in guinea pigs and rats was not ascertained. Sylim-Rapoport and co-workers have reported reduced red cell glucose utilization in the rat and reduced red cell ATP concentration in the absence of decreased glucose utilization in the dog during magnesium deprivation. However, further characterization has not been made of the anemia that develops during magnesium deficiency.

Magnesium is an important cofactor in the enzymic utilization of glucose by the Meyerhof-Embden and pentose phosphate pathways in the red cell. Since these sequences of enzymatic reactions are responsible for the eventual generation of ATP in the red cell, a deficit in ATP generation as a result of magnesium deficiency is predictable. The lactate:glucose ratio of 2.5 in normal rats indicates that, unlike the human red cell, 20% of lactate production is in excess of glucose utilization and must be derived from another substrate. In magnesium-deprived rats, the reduced rate of lactate production may involve reduced metabolism of this alternate pathway as well as anerobic glycolysis. The decrease in red cell 2,3-DPG during magnesium deficiency may also be explicable by the decrease in overall glycolysis as well as by an alteration in the reaction rates of the two enzymes composing the Rapoport-Luebering pathway. The decrease in organic phosphate, especially 2,3-DPG would increase hemoglobin-oxygen affinity and, therefore, could contribute to tissue hypoxia. In addition to the deleterious effects of the reduction in 2,3-DPG on tissue oxygenation, stimulation of erythroid marrow would be heightened for a given red cell mass.

Studies have indicated that ATP, the major energy source in mature erythrocytes, is responsible for maintenance of cell shape, deformability, and thereby for cell survival in the microcirculation. The precise role of ATP in maintenance of membrane integrity has not been defined. Presumably the stability of lipoprotein and glycoprotein components of the membrane is dependent on the transduction of energy from ATP by as yet ill-defined enzymic
systems. In the magnesium-deficient animal, a curtailment of lactate production and ATP generation may result in a critical shortage of high-energy phosphates for the maintenance of cell shape, leading to a more spherical cell, which is more rigid and subject to premature destruction. Also, it is possible that the membrane of red cells in chronically magnesium-deficient rats may be intrinsically abnormal. This could result from a deficiency of intramembranous magnesium, which may play an important role as a membrane stabilizer, or from a defect in membrane synthesis in earlier stages of red cell development (erythroblast) because of the possible effect of magnesium deficiency on steps in protein synthesis. The progressive increase in resistance to deformation of the entire red cell population may reflect such an alteration. The decrease in deformability of the discoidal red cells in magnesium-deficient rats may relate also to the increased prevalence of reticulocytes and macrocytes, which are known to be slightly less deformable than the average red cell. Moreover, recent evidence has linked ATP depletion to calcium accumulation and enhanced red cell rigidity. Since calcium extrusion in the red cell is an active process dependent on ATP, the magnesium-deficient red cell may not maintain normal calcium efflux because of ATP depletion or altered membrane characteristics. The site or sites of destruction of the magnesium-deficient spherocyte and the possible amelioration of the shortened red cell survival by splenectomy have not yet been studied.

The temporal appearance of the effect of magnesium depletion on the red cell is governed in part by the efflux rate of magnesium from the circulating cell. The fall in red cell magnesium seen in the magnesium-deficient rats was too rapid to be due to replacement by a new population of low-magnesium red cells, unless more accelerated hemolysis and increased red cell turnover was present very early in the experimental period. Contrary to studies in man, plasma to red cell exchange of a substantial magnitude has been observed in the rat in influx studies with the radioactive metal. Although efflux studies with radioactive magnesium have not been reported, the parallel fall in plasma and red cell magnesium suggests the dependence of the latter on the former in the rat. The lag in development of maximum spherocytosis until weeks after the presence of maximum red cell magnesium reduction in the rat may indicate that part of the effect of magnesium deficiency on the shape change may be the result of defective membrane synthesis in the erythroblast in a magnesium-deficient environment.

Although we have demonstrated several aberrations in metabolism in the magnesium-deficient red cell of the rat, it is difficult on the basis of our data to locate the precise site or sites of derangement in red cell lactate generation and ATP synthesis. This would require study of glycolytic intermediates in the red cell of the magnesium-deficient rat. Although one could predict an effect on enzymatic steps, such as pyruvate kinase activity, known from in vitro studies to be dependent on magnesium, other effects may be anticipated in the face of decreases in ATP and 2,3-DPG concentration. Moreover, only circumstantial evidence allows us to infer that the changes in cell shape and deformability are a direct result of ATP depletion or of defective membrane synthesis. Further study of glycolytic intermediates, energy metabolism, and
the properties of membranes from magnesium-deficient red cells should provide more precise answers to these several questions.

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