Magnesium in Single Skeletal Muscle Cells of *Balanus*

ERNEST PAGE, BERT A. MOBLEY, MARGARET JOHNSON, and JUDY E. UPSHAW

From the Departments of Medicine and Physiology, The Pritzker School of Medicine, The University of Chicago, Chicago, Illinois 60637

ABSTRACT Single skeletal muscle cells of *Balanus* contain 48 ± 1 mmoles magnesium/kg dry weight. Although ^{28}Mg can be shown either to enter the cells or to be bound to the cell surface within less than 10 min, only 2.1 ± 0.3 % of cellular or cell surface Mg exchanges with this isotope even after several hours. Glycerinated cells washed out in Tris buffer at low ionic strength retain ~70 % of the Mg present in intact cells. About 85 % of this Mg is removed by extraction with KCl or NaCl at concentrations of K and Na which prevail in intact cells, as well as by pyrophosphate, Tris-ATP, or reduction of the ionized Mg concentration to 1 μM. Lowering the ionized Mg concentration to 0.1 μM does not further reduce the Mg content of glycerinated cells. The pH dependence of KCl-inextractable Mg suggests that more than one class of binding sites is involved. A significant fraction of the KCl-inextractable Mg bound to glycerinated cells fails to exchange with ^{28}Mg even after long equilibration. It is suggested that this fraction may be actin-bound Mg incorporated into the thin filaments during the polymerization of actin.

Magnesium ion plays an important role in the myofibrillar processes of contraction and relaxation (Weber and Winicur, 1961; Weber and Herz, 1963; Weber, Herz, and Reiss, 1969; Dancker, 1970; Hotta and Bowen, 1970). Nevertheless, remarkably little is known about the state of Mg in striated muscle cells and about the behavior of this ion under various conditions. Early studies by Bozler (1955) and Hasselbach (1957) indicated that a fraction of cellular Mg is bound to the myofibrils. Measurements of the exchange of Mg with ^{28}Mg in frog skeletal muscles (Gilbert, 1960) showed that the sarcolemma is permeable to Mg, but that as much as 75% of the Mg in these muscles fails to exchange with ^{28}Mg even after a very long equilibration.

The giant skeletal muscle cells of *Balanus*, introduced by Hoyle and Smyth (1963), have recently been used for extensive studies of ion transport and electrophysiological phenomena (Hagiwara and Naka, 1964; Hagiwara and Takahashi, 1967; Hagiwara, Takahashi, and Junge, 1968; Brinley, 1968;

188

McLaughlin and Hinke, 1966, 1968). These cells are up to 0.2 cm in diameter and several centimeters long. They are composed of a sarcolemma enclosing a mass of striated myofibrils, with relatively insignificant amounts of mitochondria, sarcoplasmic reticulum, and nuclei (McNeill and Hoyle, 1967; Selverston, 1967). Because of its large size, this preparation presents an opportunity to study the properties of Mg in single striated muscle cells. In the experiments described here we have taken advantage of this opportunity to measure the content, exchange, and binding of Mg in intact and glycerinated cells.

Portions of this work have been previously reported (Page, Mobley, and Lewis, 1970).

METHODS

Barnacles of the genus *Balanus* obtained from Puget Sound (C. Vandersluys, Friday Harbor, Wash.) were shipped by air to Chicago and maintained in an aquarium (Aquarium Systems, Inc., Wickliffe, Ohio) containing seawater at 12°C. Multiple single muscle cells were isolated from the depressor scutorum rostralis at their tendinous insertion; their other (muscular) insertion into the shell was left intact until the end of the experiment. Single muscle cells thus dissected were the starting material for two types of experiments: (a) incubation of intact cells with radioactive tracers in solutions of different chemical composition, followed by chemical and radioactive analyses; and (b) glycerination of the muscle, followed by equilibration with radioactive or nonradioactive solutions of variable chemical composition.

Experiments with Intact Cells

Single cells of a cell bundle were freed from their connective tissue attachments to one another by blunt dissection leaving intact their muscular insertion into the shell. This procedure yielded a muscle bundle containing many cells freely floating at their tendinous ends. Each cell could be excised by holding its free tendinous end with a forceps and cutting its muscular insertion near the shell with a pair of fine scissors. The muscle bundle was incubated at 22–24°C in a small glass vessel containing a solution of the desired composition for as long as required by the experimental design. The vessel was stirred with a magnetic stirrer. At the designated time, cells were removed, drained against the side of the vessel without blotting, and weighed immediately on a Cahn recording microbalance. Alternate muscles were (a) extracted without drying for the chemical determination of Mg, and, when applicable, for measurement of radioactivity; or (b) dried in a vacuum oven at 90°C, reweighed, and, in selected instances, ashed for the subsequent determination of their K and Na contents. Correction for extracellular volume and for surface contamination with the incubating solution was made by including inulin-^{14}C or sucrose-^{14}C in the bathing medium and comparing the ^{14}C content of the muscle extract with that of a unit volume of bathing solution.

The intracellular content of Mg was measured in cells incubated in a control (artificial) seawater solution at pH 7.2, in otherwise identical solutions at pH 4 and

pH 10, in Ca- and Mg-free artificial seawater containing EDTA, and in artificial seawater whose Mg concentration had been increased by raising the external Mg concentration from 12 to 24 mM. The exchange of cellular Mg for ^{28}Mg was measured as a ^{28}Mg influx by incubating the muscle bundle in the control seawater labeled with ^{28}Mg and sucrose-^{14}C and assaying the radioactivity of cells removed sequentially at short intervals over a period of 2–3 hr.

Experiments with Glycerinated Cells

For experiments with glycerinated cells, the individual cells of the depressor scutorum were dissected free in the control seawater solution and left attached to the shell as described above. The bundle was then placed in a special Lucite glycerinating chamber which was initially filled with seawater. In the chamber the shell was immobilized by clamping it with a sliding Lucite clamp; each cell was then stretched to approximately *in situ* length by tying a silk thread to its tendon, threading the tie through a small hole in a plate perforated with many small holes, and attaching the thread to an anchor in the compartment of the chamber on the other side of the perforated plate. After the muscles were thus fixed in place, the seawater was poured out of the chamber and replaced by glycerinating solution at 3–4°C. The chamber was rinsed with several volumes of glycerinating solution and the cells were left in this solution for 24 hr at 3–4°C. The chamber was then transferred to a freezer at −14°C for from 2 wk to as long as 6 wk. For studies of Mg content and exchange, glycerinated cells were cut free from the shell and treated as described below. Glycerinated cells prepared in this way contracted without exception on exposure to MgATP in the presence of traces of Ca ion as low as 10^{-9} M (i.e., in the presence of 4 mM EGTA).

All subsequent experiments on the glycerinated cells were done in a cold room at 3–4°C. After removal from the glycerinating solution cells were first washed free of glycerol for 15 min in a large volume of a solution which will be referred to as the primary rinse. They were then equilibrated for a period of from 16 to 36 hr in a solution which will be referred to as the secondary rinse.

The primary rinse had the same ionic composition as that of the equilibrating solution. After equilibration with a solution of a given ionic composition, the Mg and other ions not firmly bound to the glycerinated cell were removed by a secondary rinse for 1 hr in a solution containing no Mg, and usually no K or Na, the solution being buffered with Tris or TES at the same pH as that used for equilibration. (In selected experiments, the Mg-free secondary rinse contained 150 mM KCl.) This experimental plan, which resembles that of Bozler (1955), was adopted to measure bound Mg without contamination from Mg in the aqueous solution filling the interstices between binding sites, or Mg in the solution adhering to the surface of the tissue. Preliminary experiments, in which the secondary rinse (with two or more changes of solution) was carried out for 30 and 60 min, respectively, showed that the washout of unbound Mg was complete within 30 min.

Equilibration was done in polypropylene test tubes set circumferentially in a rack around a magnetic stirrer (Tri-R Instruments, Inc. [Rockville Center, N.Y.], Stirrer NS-7 and Table MT-72). This arrangement permitted the simultaneous stirring of

eight test tubes each containing two cells in 10 ml of solution. The cells were suspended in the middle of the solution by a silk tie around their tendinous end and protected from contact with the magnetic spinbar in the bottom of the tube by taping the thread around their tendinous end to the outside of the tube. At the end of the secondary rinse the glycerinated cells were drained quickly against the glass side of the vessel, then weighed and analyzed as described for intact cells. In these experiments the volume of the incubation vessel was effectively infinite relative to that of the glycerinated cells.

Solutions

The composition of the artificial seawater for experiments with intact cells was (in mmoles/liter): NaCl 450, KCl 8.0, $CaCl_2 \cdot 2H_2O$ 20, $MgCl_2 \cdot 6H_2O$ 12, Tris maleate 2, N-acetylglycine 2. The Mg and Ca concentrations are those recommended by Hoyle and Smyth (1963) and used by Hagiwara and Naka (1964); these Mg and Ca concentrations are, respectively, about one-quarter times and twice those of normal seawater. The pH was brought to 7.2 by the addition of NaOH, the final Na concentration being 454 mM. In selected experiments the seawater was modified in one of several ways including (a) isosmolal substitution of NaCl for all of the Mg in the solution, (b) raising the Mg concentration to 24 mM by isosmolal substitution of $MgCl_2$ for NaCl, (c) omitting both Mg and Ca and adding 2 mM sodium ethylenediamine-tetraacetate (EDTA), again at constant total osmolality and pH, (d) lowering the pH to 4.0 by the addition of HCl, and (e) raising the pH to 10.0 by the addition of NaOH.

The glycerinating solution was prepared by adding 0.242 g of Tris (hydroxymethyl) aminomethane (Trizmabase, Sigma) and an appropriate volume of 1 N HCl to 500 ml of glycerol and by making up to a volume of 1000 ml with distilled water. The pH of the glycerinating solution and of all the solutions used for experiments on glycerinated muscles was adjusted to 7.2 at the experimental temperature of 3–4°C. Because the alkali metal cations have important effects on the Mg content of the glycerinated cells, the use of KOH or of NaOH in the adjustment of pH was avoided; upward and downward adjustments were instead made by using organic buffers without significantly altering the ionic strength. In later experiments, the glycerinating solution was buffered with TES buffer (N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid) (Good et al. [1966]). For experiments in which the pH was systematically varied from 3.5 to 8.5, a series of buffers were prepared by adding the required amount of solid Tris base to a 5 mM solution of glycylglycine. A buffer containing 150 mM KCl at pH 10.1 was prepared using histidine adjusted with KOH. All solutions were made with distilled water which was passed through a deionizer and then redistilled with an all-glass still.

Analyses

After weighing, cells were either extracted without drying, or dried, reweighed, and transferred to 10 ml covered quartz crucibles (Thermal American Fused Quartz Co., Montville, N. J.) for ashing at 550°C in a muffle furnace. Cells were extracted for 48

hr on a shaker in polypropylene test tubes with caps. The extractant was 1.0 ml of the same solution that served as a blank and diluent in the spectrophotometric determination of Mg and Ca. (This solution, which is a modification of that previously employed by Krames and Page (1968), is made as follows: 11.73 g La_2O_3 are dissolved in 50 ml of concentrated HCl; to this are added sequentially 58.4 g NaCl, 500 ml distilled H_2O, and 50 g trichloroacetic acid, and sufficient distilled water to make 1 liter. The concentrated stock solution thus obtained is diluted 1:10 with distilled water.) In experiments with ^{28}Mg or ^{42}K, a polypropylene test tube containing one cell immersed in 1 ml of extractant solution was counted in the well of a gamma scintillation counter and its radioactivity compared with that of 1 ml of the bathing solution. After extraction aliquots of the extractant were taken for measurement of total Mg and ^{14}C. Mg was determined on a Jarrell-Ash atomic absorption spectrophotometer (Jarrell-Ash Co., Waltham, Mass.).

Previous experience has shown that if fresh muscles are dried and ashed or extracted, recovery of Mg is incomplete because of the formation of effectively insoluble phosphate compounds. In the present work it was observed that, unlike fresh muscle, glycerinated muscle can be dried and ashed with maximal recovery, presumably because the glycerination has extracted the potentially insoluble organic and inorganic phosphates. The techniques for compensating interferences with the Mg determination have been published by Krames and Page (1968). In all analyses the experimental sample presented to the atomic absorption spectrophotometer was immediately preceded and followed by one of a large selection of standards. The standards between which the experimental sample was bracketed gave a signal within 5 % of that of the sample. Between each sample and standard, the flame was rinsed with distilled water to preserve as nearly constant a flame temperature as possible. In this way, the stability of the aspiration and flame could be checked for each sample. Analyses were performed with an air–acetylene flame using standards between 0.25 ppm and 1.0 ppm of Mg, a range in which the instrument was very sensitive to Mg. The ratio (mean ± SD) of replicate paired determinations was 0.995 ± 0.005 at a concentration of 1 ppm and 1.00 ± 0.01 at 0.25 ppm. The corresponding figure for Ca (to which the instrument is less sensitive) was 1.00 ± 0.02 at a concentration of 1 ppm. When intact cells were analyzed in this way, their Mg content per unit dry weight was observed to be the same for cells varying in dry weight over a fivefold range. Protein was measured by the method of Lowry et al. (1951). Techniques for measurement of ^{14}C, K, and Na have been previously described (Page, 1962; Page and Page, 1968).

Reagents

The sources of reagents were as follows: Tris, TES, HEPES, N-acetyl-glycine, Tris maleate, Tris ATP, and sodium ATP from Sigma Chemical Company (St. Louis, Mo.), inulin-^{14}C–carboxylic acid and sucrose-^{14}C from New England Nuclear Corp. (Boston, Mass.), ^{28}Mg from Brookhaven National Laboratory, and ^{42}K from the Isoserve Division of Cambridge Nuclear Corporation (Cambridge, Mass.). The decay scheme of ^{28}Mg has recently been revised by Alburger and Harris (1969).

RESULTS

Experiments with Intact Cells

CELLULAR Mg CONTENT

Fig. 1 is a plot of the cellular Mg contents of four muscles against the duration of incubation in the control seawater solution at 22–24°C. The figure shows that the Mg content of cells from a given barnacle remains constant for several hours. Gilbert (1960) has suggested that Mg is actively transported out of muscle cells; if so, it is conceivable that the effectiveness of the pump might diminish during prolonged incubation, with a resultant cellular accumulation of Mg. No evidence for such an accumulation was found.
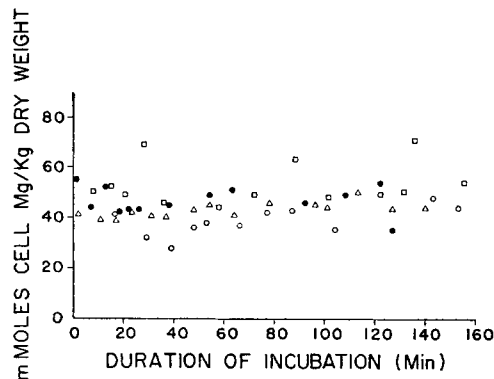

FIGURE 1. Cellular magnesium content of cells from four barnacles as a function of the duration of incubation at 22–24°C, $[Mg]_o = 12$ mM.

Fig. 1 shows significant differences in the Mg contents of cells from the same barnacle, and somewhat greater differences in the Mg contents of cells from different barnacles. The Mg contents of 77 cells from 8 barnacles have been averaged for all times to give a mean Mg content of 48 ± 1 mmoles/kg dry weight (Table I).

Since Gilbert (1961) reported that the Mg content of frog skeletal muscle decreased below and increased above physiological pH, we have examined the cellular Mg contents at pH 4.0 and 10.0; at both pH values cellular contents did not change with time of incubation; their mean values (Table I) fall within the distribution of the controls. Table I also shows that incubation in isosmolal solutions modified by omitting Mg or by raising the Mg concentration to twice that of the control seawater produced no significant change in the average Mg contents, nor did the Mg contents vary with time under these conditions (five control cells incubated in normal seawater ($[Mg]_o = 12$ mM) and taken from the same muscle bundle as the cells incubated in 24 mM Mg had a cellular Mg content of 42 ± 3 mmoles/kg dry weight; the apparently

TABLE I

Mg CONTENT OF CELLS UNDER VARIOUS CONDITIONS*

Experimental conditions	Cellular Mg content	No. of barnacles	No. of cells
	mmoles cell Mg/kg dry weight		
Controls (seawater with 12 mM Mg)	48 ± 1	8	77
Mg-free isosmolal seawater	45 ± 2	1	12
24 mM Mg isosmolal seawater	41 ± 4	1	10
pH 4.0	44 ± 2	1	10
pH 10.0	48 ± 2	1	9
Mg-free, Ca-free, EDTA			
All cells	57 ± 4	2	18
Spontaneous contracture	57 ± 7	2	8
Not contracted	59 ± 5	2	10

* Except for experiments in Mg-free solutions, the cellular Mg content has been obtained by subtracting the Mg content of the inulin space from the total Mg content of the fiber.

low value for the 24 mM Mg solutions is therefore not significant.) Table I also presents the mean cellular contents for cells incubated in isosmolal Ca-free, Mg-free seawater containing 2 mM EDTA. Nearly half the cells contracted spontaneously under these conditions. There was no decrease in the cellular content of Mg and no difference in the Mg contents of contracted and uncontracted cells from the same muscle bundle. (In fact, the Mg content per unit dry weight of this group of muscles was, for undetermined reasons, higher than in the controls.) At the same time, there were significant changes in the total contents of Na and K regardless of the contractile state, with the Na content increasing from control values of 259 ± 15 (n = 10) to 503 ± 34 (n = 12), and the K content falling from 514 ± 10 to 444 ± 28.

EXCHANGE OF CELLULAR OR CELL SURFACE Mg WITH ^{28}Mg

Fig. 2 presents a representative experiment showing the uptake of ^{28}Mg by cells from one barnacle fiber bundle immersed in ^{28}Mg-labeled control seawater. The figure shows that the equilibration of both the total and the cellular Mg attains a steady value by the time the first measurement can be made, and certainly within less than 20 min; thereafter it does not usually increase significantly. In such experiments with 29 cells from 4 barnacles we have found that an average of only 0.021 ± 0.003 of cellular or cell surface Mg exchanges with ^{28}Mg even after long incubations. If this Mg fraction is in fact intracellular, it is possible to calculate that the intracellular concentration of exchangeable Mg in barnacle muscle would not exceed 309 μM/kg cell water. However, an alternative interpretation is that all or part of the isotopic Mg does not enter the cells but is instead bound to the cell surface.

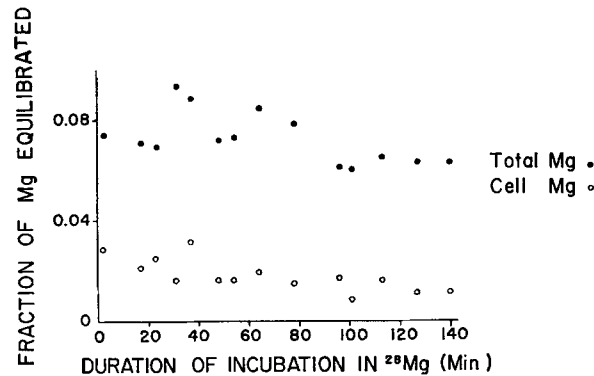Although the fraction of cellular or cell surface Mg exchanging is very close

FIGURE 2. Fraction of total Mg (upper curve) and cellular or cell surface Mg (lower curve) equilibrated with ^{28}Mg in the incubation medium as a function of time in ^{28}Mg-labeled seawater, $[Mg]_o = 12$ mM.

to zero, there is no doubt that a small but significant amount of Mg associated with the cell does exchange. The evidence for this was that in the four experiments (each of which involved analysis of multiple cells) the difference between the total content of ^{28}Mg and the ^{28}Mg content of the sucrose space was without exception a positive quantity; that is, there was invariably a small, but significant amount of ^{28}Mg either in the cells or bound to the cell surface. The relatively small amount of radioactivity thus taken up by the cells precluded a more definitive kinetic study of its transport or binding.

Experiments with Glycerinated Cells

EFFECTS OF GLYCERINATION ON CELL COMPOSITION AND PERMEABILITY

Table II summarizes some of the effects of glycerination on barnacle muscle cells. The data in the table allow one to refer the Mg content of glycerinated cells to that of intact cells. Such a calculation must take into account that the units of reference commonly used for expressing the ion content of muscle cells (the dry weight or the protein content) are unstable under the conditions used by us; that is, that the dry weight and protein content of intact cells are not identical to the corresponding quantities after glycerination, since glycerination extracts protein and other components of the dry weight.

Table II also shows that glycerination destroys the selective permeability of the plasma membrane and of other membrane-limited systems. The evidence for the loss of selective permeability includes the increase in the sucrose space, the loss of K associated with the intact cell, and the fact that cells will contract on exposure to ATP under appropriate conditions. The conclusion that cellular membrane systems have lost their effectiveness as osmotic barriers is further supported by the finding that neither the sucrose space nor the total

TABLE II

COMPARISON OF GENERAL FEATURES OF
INTACT AND GLYCERINATED CELLS*

	Intact cells (22–24°C)	Glycerinated cells (4°C)
Weight total water/wet weight	0.796 ± 0.003 (48, 5)	0.919 ± 0.003 (43, 5)
Weight water in sucrose space/total water	0.172 ± 0.009 (48, 5)	0.93 ± 0.02 (10, 1)‡
Weight protein/dry weight	0.46 ± 0.02 (12, 2)	0.65 ± 0.03 (10, 2)
Mmoles K/kg dry weight	514 ± 10 (10, 1)	Trace (5, 1)
Dry weight after glycerination		0.642 ± 0.005 (20, 1)
Dry weight before glycerination		

* First figure in parentheses gives total cells measured; the second figure gives number of barnacles. The data on glycerinated cells were obtained after washing out the glycerol in Tris buffer at 4°C.

‡ Sucrose spaces were measured after 72 hr of equilibration.

water content of glycerinated cells changed when the external osmolality was raised by the addition of 100 mM $MgCl_2$.

CONTENT AND EXCHANGE OF BOUND Mg AT PHYSIOLOGICAL pH

As an initial approach to the study of Mg in glycerinated single muscle cells we utilized a technique similar to that applied by Bozler (1955) to whole frog skeletal muscles. In this approach glycerination, primary rinse, equilibration, and secondary rinse are all, unless otherwise indicated, done in Mg-free solution. (The term Mg-free solution as used here means a solution to which no Mg has been added; contamination with traces of Mg in other reagents was doubtless present. Measurement by atomic absorption spectrophotometry suggested that the contamination was usually of the order of 10^{-6} M and did not exceed 10^{-5} M. Mg^{++} was controlled in selected experiments by the use of a Mg-EDTA buffer.) Bozler's technique has the advantage that it introduces no exogenous Mg into the tissue. Its disadvantage is that some of the Mg normally bound by cellular structures may be lost by exposure of the cell to Mg-free solutions.

Table III gives the Mg contents of glycerinated cells equilibrated under various conditions. The table also shows the fraction of Mg which has exchanged with ^{28}Mg after 24 hr at 3–4°C. As in the glycerinated frog muscle described by Bozler, equilibration with buffered or unbuffered distilled water to which no inorganic cations have been added leaves a relatively large amount of Mg bound to the glycerinated cells. It can be calculated from the data of Tables II and III that the 52 mM Mg/kg dry weight remaining in glycerinated cells equilibrated with Tris buffer (Table III) represents ~70% of the Mg content of the intact (unglycerinated) cell. From 83–85% of the Mg in the glycerinated cell can be extracted by exposure to solutions contain-

TABLE III

CONTENT AND EXCHANGE OF ENDOGENOUS Mg
IN GLYCERINATED CELLS (4°C, pH 7.2)

Equilibrating solution		Duration of equili-bration	Mg concentration	Mg content	Fraction of Mg exchanged with ^{28}Mg in 24 hr
		hr	*mM*	*mmoles/kg dry weight*	
Unbuffered distilled water	(5)	1	0*	45 ± 6	—
Tris buffer	(4)	18	0*	52 ± 9	—
150 mM KCl	(4)	18	0*	5.5 ± 0.2	—
20 mM NaCl	(4)	18	0*	8.9 ± 0.4	—
Tris buffer + ^{28}Mg	(10)	24	0.09–0.12	41 ± 3	0.9 ± 0.1
150 mM KCl + ^{28}Mg	(8)	24	0.13–0.14	8.3 ± 0.4	0.411 ± 0.003
1 mM Na$_4$P$_2$O$_7$	(6)	24	0*	6.7 ± 0.7	—
5 mM Tris ATP	(4)	24	0*	5.9 ± 0.8	—

Figures in parentheses give number of cells.
* No Mg added.

ing K and Na at the approximate concentrations in which these two alkali metal cations occur in the intact cell: 150 mM KCl and 20 mM NaCl, respectively; other concentrations were not examined. The residual Mg represents 10–12% of the Mg content of the intact cell. Exploratory experiments in which the muscles were extracted with KCl for 6, 18, and 24 hr showed that the amount of residual Mg was the same at each of the three equilibration times. A similar amount of Mg is extracted from glycerinated cells by lowering the ionized Mg concentration ($[Mg^{++}]$) to 1 μM (Fig. 3), by Na pyrophosphate, or by Tris ATP (Table III).

It was of interest to determine whether the extraction of bound Mg by a KCl solution of approximately physiological K concentration resulted in significant binding of K to the glycerinated cell. In order to examine this question, the experiment was repeated in the presence of 150 mM KCl labeled with ^{42}K. In nine such cells from two barnacles the mean content of ^{42}K-labeled K was 100 ± 3 mmoles/kg dry weight. Although this result is not statistically different from the number of equivalents of Mg extracted, the scatter of the data precludes a decision as to whether the stoichiometry of the cation exchange is exact.

A stoichiometry in which the equivalents of K bound exactly match the equivalents of Mg extracted is not, in any case to be expected, because 150 mM KCl also extracts bound Ca: The Ca content of glycerinated cells washed out in Tris buffer was 24 ± 3 mmoles/kg dry weight (*n* = 5) before extraction with KCl, and 4 ± 1 mmoles/kg dry weight (*n* = 5) after extraction with KCl. The amount of Ca bound to glycerinated cells both before and after extraction with KCl can be reduced by incorporating 5 mM MgCl$_2$ in
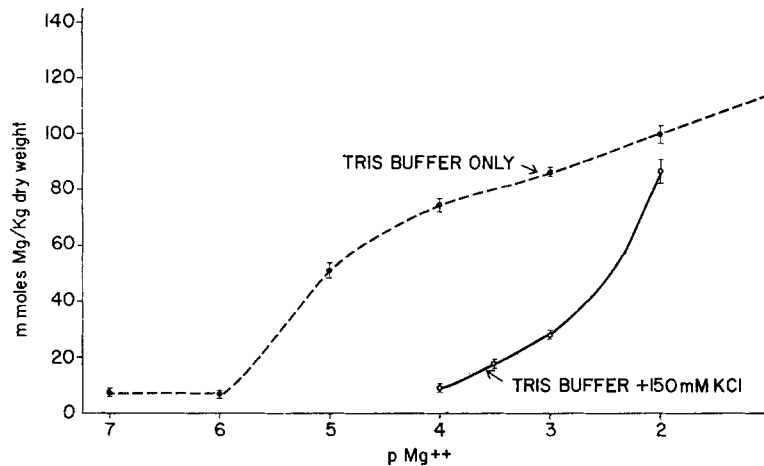
FIGURE 3.   Mg content of glycerinated cells as a function of the Mg concentration of the incubation medium (3–4°C, pH 7.2). Upper curve, Mg content at low ionic strength in inorganic cation-free buffer. Lower curve, corresponding values in 150 mM KCl. Incubation time 24 hr. Values for pMg 6–7 were obtained in the presence of a magnesium-EDTA buffer. The number of experiments in the absence of KCl at pMg 1, 2, 3, 4, 5, 6, and 7 were, respectively, 10, 10, 12, 6, 6, 8, and 6; in the presence of KCl there were six experiments at each point.

the glycerinating solution. Under these conditions the Ca content (millimoles per kilogram dry weight) after washing the cells out in inorganic cation-free Tris buffer was 7.5 ± 0.5 (n = 6); the corresponding figure after extraction with 150 mM KCl was 2.57 ± 0.03 (n = 6).

Exploratory experiments in which the muscles were equilibrated with [28]Mg for 6 and 24 hr, respectively, showed that the fraction of bound Mg exchanged with [28]Mg did not increase between 6 and 24 hr. Table III shows that, to within the precision of the measurement, the Mg bound to the glycerinated cells washed out in low ionic strength Tris buffer (pH 7.2) exchanged with [28]Mg. After removal of KCl-extractable Mg by exposure to 150 mM KCl about 60% of the residual Mg was inexchangeable in 24 hr. While the absolute Mg content is subject to considerable variation between cells, the existence of a substantial fraction of inexchangeable Mg in KCl-extracted cells appears firmly established.

The results shown in Table III indicate that the Mg content of glycerinated cells consists of at least two fractions: A small, tightly bound fraction which is only partially exchangeable at an external Mg concentration of 130 μM, that is, at a Mg concentration of the same order of magnitude as that of the exchangeable Mg in intact cells; and a much larger fraction, characterized by its extractability by KCl, NaCl, pyrophosphate, and Tris-ATP, as well as by the fact that it is exchangeable with [28]Mg.

DEPENDENCE OF Mg CONTENT ON Mg CONCENTRATION

Fig. 3 is a plot of the Mg content against the external Mg concentration. The values for Mg concentrations of 0.1–10 μM (pMg 7.0–5.0) refer to the ionized Mg concentration calculated for mixtures of $MgCl_2$ with EDTA, the total concentration of Mg being 1 mM. In the absence of KCl the Mg bound to the glycerinated cells approaches a value of about 80 mmoles/kg dry weight at the exchangeable Mg concentration (309 μM) which prevails in intact cells. At Mg concentrations higher than this value the Mg content increases in an approximately linear way. Below an external Mg concentration of 309 μM more than 90% of the Mg bound at 309 μM is progressively lost, until between pMg 6 and 7 the Mg content becomes independent of pMg.

In the presence of 150 mM KCl the Mg-binding curve is shifted to the right: Under these conditions the Mg content at an external Mg concentration of 100 μM is as low as that found in 1 μM Mg solution in the absence of KCl.

pH DEPENDENCE OF Mg CONTENT

Fig. 4 is a plot of the Mg content of glycerinated cells equilibrated 24 hr in solutions whose pH was varied from 3.5 to 10.1. The experiments were done both in the presence and absence of 150 mM KCl; no exogenous Mg was added to the equilibrating solution. Under these conditions the Mg content in the absence of KCl at a physiological pH was somewhat lower than usual, perhaps because a glycylglycine-Tris buffer was substituted for the usual
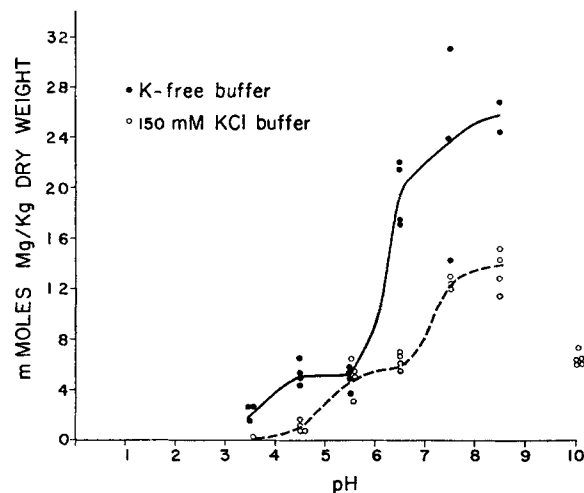

FIGURE 4. pH dependence of Mg content in glycerinated cells(3–4°C). Upper curve, Mg content at low ionic strength in inorganic cation-free buffer. Lower curve, corresponding values in 150 mM KCl. Incubation time 24 hr.

Tris or TES buffers. Nevertheless, the pH dependence of the Mg content is apparent. At pH 5.5 the Mg contents in the presence and absence of KCl are approximately equal; at all other values of the pH the Mg content is strikingly less in the presence of KCl. In the absence of KCl the curve has plateaus from pH 4.5 to 5.5 and from pH 7.5 to 8.5. In the presence of 150 mM KCl there is again a plateau between pH 7.5 and 8.5, but the plateau which occurs from pH 4.5 to 5.5 in the absence of KCl is shifted by 1 pH unit to pH 5.5–6.5. As the result of this shift the half-maximal value for the binding sites which saturate at pH 7.5–8.5 lies at about pH 7. At pH 10.1 the Mg content in the presence of 150 mM KCl has decreased to the same magnitude as at pH 6.5.

DISCUSSION

Residual Mg Present after Extraction with KCl

Although about 70% of the Mg present in intact cells remains bound to glycerinated cells after rinsing the cells in distilled water or low ionic strength buffer, most of this Mg is lost on exposure to KCl or NaCl at K or Na concentrations comparable to those which prevail in intact cells. Similar amounts of Mg are removed by anions which bind Mg, for example, by pyrophosphate or ATP. The nature of the cellular structures to which the KCl-extractable Mg is bound in the glycerinated cell remains undetermined.

It seems probable that the residual Mg present after extraction with KCl is bound to myofibrillar protein. Fig. 3 shows that the Mg so bound is not further reduced when the Mg^{++} concentration is lowered from 10^{-6} to 10^{-7} M. The pH dependence (Fig. 4) suggests that at least two classes of binding sites may be involved; and the measurements of [28]Mg exchange indicate with good statistical reliability that a significant fraction of KCl-inextractable Mg fails to exchange with [28]Mg. Weber, Herz, and Reiss (1969) have proposed that myofibrillar actin may contain Mg which is bound during the polymerization of actin. In this connection, the present finding that a fraction of Mg bound to KCl-extracted glycerinated cells fails to exchange with [28]Mg may be compared with the observation by Weber et al. (1969) that a part of myofibrillar Mg does not exchange with Ca. This comparison suggests that the [28]Mg-inexchangeable fraction of Mg which remains in glycerinated cells after extraction with 150 mM KCl may be bound to actin.

REFERENCES

ALBURGER, D. E., and W. R. HARRIS. 1969. Decay scheme of Mg[28]. *Phys. Rev.* 185:1495.
BOZLER, E. 1955. Binding of calcium and magnesium by the contractile elements. *J. Gen. Physiol.* 38:735.

Brinley, F. J. 1968. Sodium and potassium fluxes in isolated barnacle muscle fibers. *J. Gen. Physiol.* **51**:445.

Dancker, P. 1970. The binding of calcium and magnesium to actomyosin and its modification by natural tropomyosin. *Arch. gesamte Physiol. Menschen Tiere (Pfluegers).* **315**:198.

Gilbert, D. L. 1960. Magnesium equilibrium in muscle. *J. Gen. Physiol.* **43**:1103.

Gilbert, D. L. 1961. Effect of pH on muscle calcium and magnesium. *Proc. Soc. Exp. Biol. Med.* **106**:550.

Good, N. E., D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Singh. 1966. Hydrogen ion buffers for biological research. *Biochemistry.* **5**:467.

Hagiwara, S., and K. Naka. 1964. The initiation of spike potential in barnacle muscle fibers under low internal Ca^{++}. *J. Gen. Physiol.* **48**:141.

Hagiwara, S., and K. Takahashi. 1967. Surface density of calcium ions and calcium spikes in the barnacle muscle fiber membrane. *J. Gen. Physiol.* **50**:583.

Hagiwara, S., K. Takahashi, and D. Junge. 1968. Excitation-contraction coupling in a barnacle muscle fiber as examined with voltage clamp technique. *J. Gen. Physiol.* **51**:157.

Hasselbach, W. 1957. Die Bindung von Adenosindiphosphat, von anorganischem Phosphat und von Erdalkalien an die Strukturproteine des Muskels. *Biochim. Biophys. Acta.* **25**:562.

Hotta, K., and W. J. Bowen. 1970. Contraction and ATPase activity of glycerinated muscle fibers and myofibrillar fragments. *Amer. J. Physiol.* **218**:332.

Hoyle, G., and T. Smyth. 1963. Neuromuscular physiology of giant muscle fibers of a barnacle, *Balanus nubilus* Darwin. *Comp. Biochem. Physiol.* **10**:291.

Krames, B., and E. Page. 1968. Effects of electron microscopic fixatives on cell membrane of the perfused rat heart. *Biochim. Biophys. Acta.* **150**:24,

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

McLaughlin, S. G. A., and J. A. M. Hinke. 1966. Sodium and water binding in single striated muscle fibers of the giant barnacle. *Can. J. Physiol. Pharmacol.* **44**:857.

McLaughlin, S. G. A., and J. A. M. Hinke. 1968. Optical density changes of single muscle fibers in sodium-free solutions. *Can. J. Physiol. Pharmacol.* **46**:247.

McNeill, P. A., and G. Hoyle. 1967. Evidence for superthin filaments. *Amer. Zool.* **7**:483.

Page, E. 1962. Cat heart *in vitro.* III. The extracellular space. *J. Gen. Physiol.* **46**:201.

Page, E., B. A. Mobley, and M. Lewis. 1970. Mg content and exchange in intact and glycerinated barnacle skeletal muscle. Abstracts of the Biophysical Society 14th Annual Meeting. Baltimore, Maryland. 224a.

Page, E., and E. G. Page. 1968. Distribution of ions and water between tissue compartments in the perfused left ventricle of the rat heart. *Cir. Res.* **22**:435.

Selverston, A. 1967. Structure and function of the transverse tubular system in crustacean muscle fibers. *Amer. Zool.* **7**:515.

Weber, A., and R. Herz. 1963. The binding of calcium to actomyosin systems in relation to their biological activity. *J. Biol. Chem.* **238**:599.

Weber, A., R. Herz, and I. Reiss. 1969. The role of magnesium in the relaxation of myofibrils. *Biochemistry.* **8**:2266.

Weber, A., and S. Winicur. 1961. The role of calcium in the superprecipitation of actomyosin. *J. Biol. Chem.* **236**:3198.