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THE CONCENTRATION OF IONIZED MAGNESIUM IN BARNACLE MUSCLE FIBRES

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SUMMARY

1. The total Mg in isolated fibres of *Balanus aquila* was 10·5 m-mole/kg wet wt.

2. The intracellular free Mg was measured by a null point method using Eriochrome Blue as an indicator of free Mg, and internal dialysis with solutions of varying ionized Mg concentrations. The results indicated a free Mg of 6 mM or 4·2 m-mole/kg wet wt. in the intracellular water immediately surrounding the dialysis capillary.

3. The ATP concentration was estimated to be 4·9 m-mole/kg wet wt.

4. A tentative partitioning of Mg among various intracellular constituents based on present data combined with published work by others is (m-mole/kg wet wt): free, 4·2; MgATP, 4·2; myofibrillar bound, 1; residual (presumably bound to arginine phosphate and phosphate) *ca.* 1.

INTRODUCTION

The measurement of internal ionized Mg concentration is important for several reasons. The cytoplasmic level of ionized Mg in effect regulates the activity of enzyme systems requiring magnesium. The active form of high energy nucleotides of biological interest, i.e. ATP, is actually the Mg complex rather than the free form. Since the concentration of the Mg complex depends upon the free Mg, measurement of the latter parameter gives information about the effective concentration of high energy compounds in the cytoplasm. Finally a knowledge of internal ionized Mg permits calculation of the electrochemical gradient, a quantity useful in considering the energy requirement for active Mg transport.

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A variety of techniques has been used to estimate ionized Mg. One commonly used method measures the equilibrium concentrations of reactants and products of enzymatic reactions whose apparent equilibrium constants are known to depend upon Mg ions. Referring the experimental constant to a standard curve in which apparent equilibrium constants have been determined *in vitro* at known ionized Mg concentrations permits an indirect estimate of free Mg *in situ*. Nanninga (1961) calculated the free Mg in frog muscle by considering the binding constants of all compounds, e.g. nucleotides, proteins, anions, etc., known to complex with Mg. Free Mg was estimated in barnacle muscle by Ashley & Ellory (1972) using a replacement microinjection technique to determine the concentration of diffusible Mg in the sarcoplasm. By subtraction of the calculated concentration of Mg complexed to intracellular constituents from the diffusible Mg, it was possible to estimate the free Mg. A fourth approach has been to estimate ionized Mg by analysing the effect of Mg quenching on the light emitted by the Ca-aequorin reaction (C. C. Ashley, unpublished, quoted by Ashley & Ellory, 1972).

Recently a null point method of measuring free Mg in squid axons has been described using the relatively selective Mg binding dye, Eriochrome B (Brinley & Scarpa, 1975), which does not depend upon either measurement of a Mg complex or a dissociation constant. The measurements reported here for barnacle muscle fibres were obtained using a modification of this technique to increase its sensitivity.

METHODS

Apparatus

The technique used for measuring ionized Mg ions in a barnacle muscle fibre is similar to that previously used for the determination of Mg^{2+} in squid giant axons (Brinley & Scarpa, 1975). It consists of the microinjection of a metallochromic indicator sensitive to changes in ionized Mg^{2+} into the cytosol of the fibre and in the subsequent detection of changes in the absorbance under various conditions through sensitive dual wave-length microspectrophotometry.

The apparatus used in these experiments was a time-sharing multichannel spectrophotometer designed and built at the Johnson Foundation, University of Pennsylvania. The optics are illustrated schematically (not to scale) in Fig. 1 and consist of (*a*) a light source, provided by a stabilized 48 W tungsten-iodine lamp (6 A), (*b*) a 8·7 cm diameter disk containing four 2·2 cm interference filters (40–60% T, 1–2 nm halfband width, from Omega Optical Co., Brattleboro, Vt.) driven at variable speeds up to a few kHz by compressed air directed on the vanes. The wheel is mounted on a shaft which is machined at one end to form four apertures for a GaAsP light emitting diode contained inside. Two stationary axial rings, each housing two phototransistors are mounted over the enclosed shaft which are illuminated during the rotation of the shaft by the four apertures, to produce pulses every 90° rotation of the disk, (*c*) a collimating lens which focuses the light of the

lamp after the passage through each filter on a bundle of minute optical fibres which diverge on a horizontal row at the level of a Plexiglass bridge mounted across the barnacle fibre, (*d*) a photomultiplier tube (E.N.I. 9425 B), and (*e*) electronic circuitry to generate a gate for each filter synchronized to the rotation of the disk shaft position as described above. Other features of the circuit include a four-channel amplifier, four gain equalization gates, four detector gates and a dark current clamp gate.

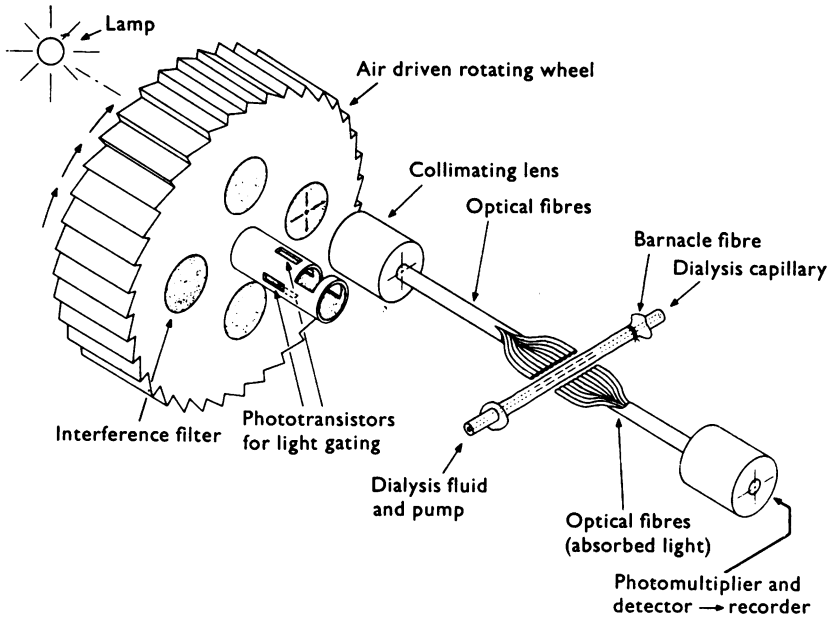

Fig. 1. Schematic diagram of the optical system used for Mg^{2+} measurements. Chamber for holding barnacle fibre not shown. Details discussed in text.

In the initial experiments in the present series, the barnacle muscle fibre, cannulated at either end, was suspended between the light pipe assembly which consisted of a linear array of fifteen 1 mm plastic monofilament optical guides (Dolan-Jenner, Melrose, Mass.) as previously described. The distance between the entrance and exit light pipes was approximately 10 mm. Positioning of the muscle fibre in the beam for maximum absorption proved to be critical, and in addition a good deal of light was lost by scattering from the surface of the fibre.

In a second series of experiments, the plastic monofilament optical fibres were incorporated into lucite blocks which fitted in grooves in the side of the dialysis chamber. After the barnacle muscle fibre had been positioned in the slot, the entrance and exit light pipes could be independently slid into close approximation to the surface of the muscle fibre. With this arrangement, the separation of the entrance and exit light pipes was never more than 1·5–2 mm. In addition, the diameter of the exit light pipe was increased to 3 mm, to collect more scattered light. These improvements in technique resulted in almost an order of magnitude improvement in signal to noise ratio in the second series of experiments.

Three wave-lengths were monitored simultaneously, the isosbestic intensity of the free and bound form at 566 nm, and the pair 580–550 nm. Changes in absolute absorbance at 566 nm were a useful measure of change in total dye concentration and over-all absorption of the fibre (position, light scattering). The pair 580–550 nm was chosen because the differential absorbance at this pair measures Mg^{2+} and is insensitive to Ca (Scarpa, 1974).

Principle of the method

The null point method of determining free Mg, depends upon being able to detect a change in the differential spectrum of Eriochrome B, located in the cytoplasm of the cell, when the concentration of cytoplasmic Mg is perturbed by dialysing the interior of the fibre with solutions mimicking the ionic composition of sarcoplasm but containing varying concentrations of free Mg. If the free Mg concentration in the dialysis solution is the same as that in the cytoplasm, dialysis does not change the internal concentration of Mg and therefore produces no change in the differential spectrum. However if there is a mismatch in the free Mg concentrations in dialysate and cytoplasm, then this fact is reflected in a change in differential absorbance.

Although this method worked satisfactorily for squid axons (Brinley & Scarpa 1975), when similar measurements were attempted in barnacle muscle fibres, the technique lacked sensitivity. The lack of sensitivity probably resulted because of the larger size of the barnacle fibre compared to squid axon. Since the initial absorbance change, which is used to indicate the null point, depends upon the fractional change in Mg–EB produced by dialysis, locating this null point is relatively less sensitive in large fibres.

Additional sensitivity was obtained in the following manner. An isotonic mixture of EGTA (250 mM) + KTES (80 mM) designated A3D, was used as dialysis solution (see Table 1) with varying amounts of magnesium (0–80 mM). Fortunately the *in situ* effective dissociation constant for Mg-EGTA is high enough (*ca.* 30 mM) so that Mg can be effectively buffered in the mM range with reasonable concentrations of total EGTA and total Mg.

Although no specific comparisons of the permeability of the porous capillary to Mg and EGTA were done, previous studies have indicated that the permeability of Ca and EDTA were about equal (Brinley & Mullins, 1974).

When such a mixture is dialysed through the porous capillary and allowed to diffuse into the sarcoplasm, the fractional volume of sarcoplasm subjected to buffer stabilization of ionized Mg is greater than if the dialysate contained an unbuffered Mg solution. Therefore if the Mg concentration of the magnesium EGTA dialysate does not match the ionized Mg in the sarcoplasm exactly, a greater fraction of dye will show a change in differential absorbance, during the initial minutes of dialysis. Thus the use of Mg-EGTA in the dialysate increases the amount of absorbance change produced by a given mismatch between ionized Mg in the dialysate and in the sarcoplasm.

A series of four *in vitro* dialysis done to test the method is shown in Fig. 2. A solution of CTT (composition given in Table 1) containing Eriochrome Blue and 4 mM-Mg (used as artificial sarcoplasm) was placed inside a glass capillary 1 mm inside diameter. At the time indicated by the vertical arrow labelled D at the left of the Figure, dialysis was begun with A3D containing the amounts of total Mg indicated on the right hand part of the Figure. When the dialysis mixture contained 47 mM total Mg (calculated ionized Mg $\sim$ 6 mM) there was little change in differential absorbance signal over a 15 min period. However when the dialysate contained more or less than this amount, there was an obviously detectable change in absorbance which could be observed within 1–2 min after dialysis was begun.

These preliminary experiments indicated that the technique was feasible, and formed the basis of calibration experiments which permitted us to convert total Mg concentration in the dialysate into ionized Mg concentration in the sarcoplasm.

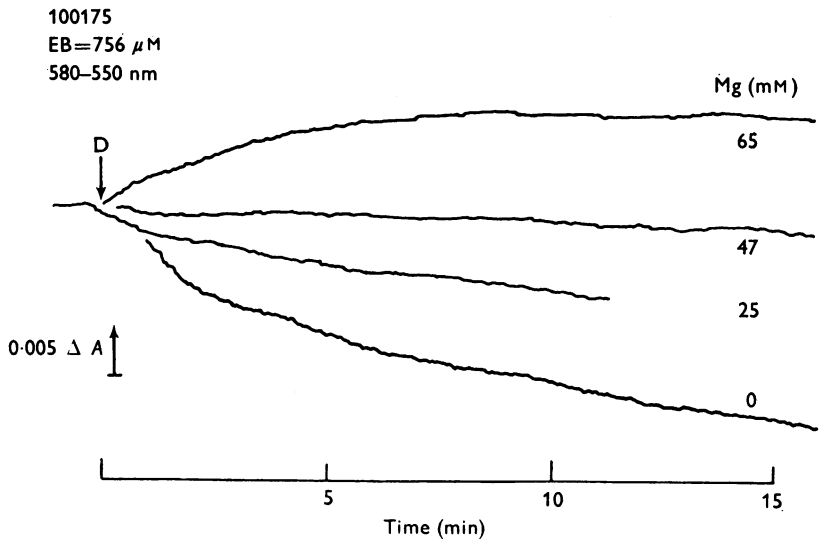

Fig. 2. *In vitro* test of the method used in these experiments. A 1 mm inside diameter glass capillary was positioned in the apparatus as for the barnacle experiments. The capillary was flushed with a solution of CTT containing 4 mM free Mg. Vertical arrow labelled D indicates onset of dialysis with the EGTA test mixture containing indicated amounts of *total* Mg. Mismatch between free Mg in glass capillary and in dialysate is indicated by direction and magnitude of the initial absorbance change after the onset of dialysis. Figure is composite of four separate runs with fresh solution placed in glass capillary before each run. Starting times have been superimposed from separate records. Direction of arrow on absorbance scale indicates direction of increasing Mg in external solution in glass capillary.

TABLE 1. Solutions (mM)

	K	Na	Mg	Ca	Cl	Isethionate	D-Aspartate	TES	Glycine	EGTA	pH
Barnacle saline	10	452	32	10	541	0	0	5	0	0·1	7·5
Ca-free saline	10	460	32	0	534	0	0	5	0	0·1	7·5
Mg-free saline	10	447	0	42	541	0	0	5	0	0·1	7·5
CTT (dialysate)	267	30	0	0	0	204	86	8	204	0·2	7·3
CBT (dialysate)	267	30	0	0	0	290	0	8	204	0·2	7·3
A3D (dialysate)	0	0	0	0	0	0	0	80	0	250	7·3

Dye washout

Since the dialysis capillary is slightly permeable to Eriochrome B, continuous dialysis could be expected to wash some dye out of the capillary and might confuse the interpretation of the records. The result of an experiment to study this effect is shown in Fig. 3.

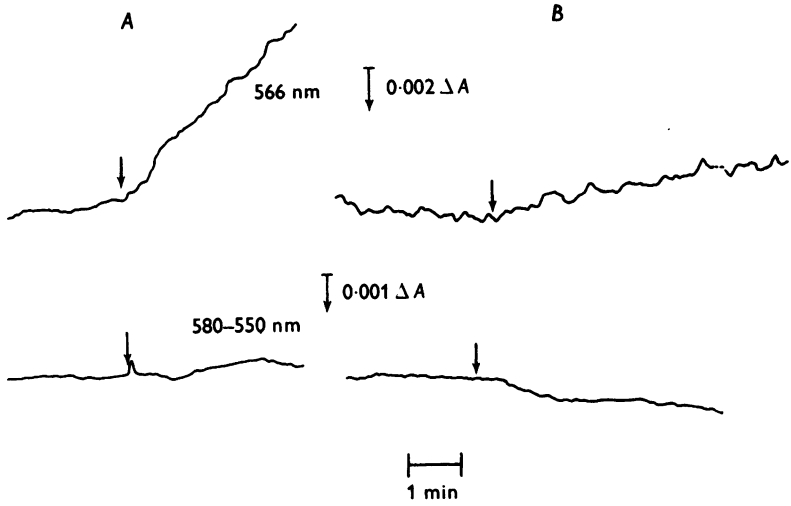

Fig. 3. Absorbance change produced by dye washout. Dialysis capillary in glass cannula 1·1 mm inside diameter. External solution: CTT + 5 mM-Mg + 500 μM-EB. Vertical arrow indicates onset of dialysis. Arrow on absorbance scale indicates absorbance increase (566 nm) or decrease (580–550 nm). The composition of CTT is given in Table 1. *A*, capillary dialysed with solution of CTT (5 mM-Mg) without Eriochrome B. Differential signal (580–550 nm) is unchanged indicating no change of ionized magnesium in the surrounding fluid in the glass cannula. The isosbestic point (566 nm) deflects in direction indicating loss of dye from the optical path. *B*, similar procedure to part *A* except the dialysis solution was identical to the fluid in the glass cannula. Differential spectrum (580–550 nm) shows essentially no change. Isosbestic intensity (566) also shows little change with continued dialysis indicating that dye is being lost from the optical path much less rapidly.

In this experiment the dialysis capillary was positioned inside a glass cannula 1·1 mm in diameter containing CTT + 5 mM-Mg + 500 μM Eriochrome B. The dialysing solution was also CTT + Mg with or without Eriochrome B. Fig. 3*A* shows that dialysis with CTT plus Mg produced a prompt deflexion in the isosbestic intensity, 566 nm, in the direction of loss of dye. In the experiment of Fig. 3*B*, the dialysing solution was replaced by the same solution as in the cannula. As the Figure shows, the change in absorbance at 566 nm was reduced by a factor of 10. The rate of dye washout seen in Fig. 3*A* which was comparable to that observed *in situ* was equivalent to a dye loss of about 0·5%/min.

The differential traces shown at the bottom of Fig. 3 were much less affected by dye washout than the absolute absorbance measured at 566 nm.

pH mismatch

Inasmuch as the dissociation constant for Mg Eriochrome B, as well as the integral spectrum, is rather pH sensitive, it was important to match the pH of the dialysis solution with that of the calibrating solutions and the sarcoplasm to avoid artifacts due to pH differences.

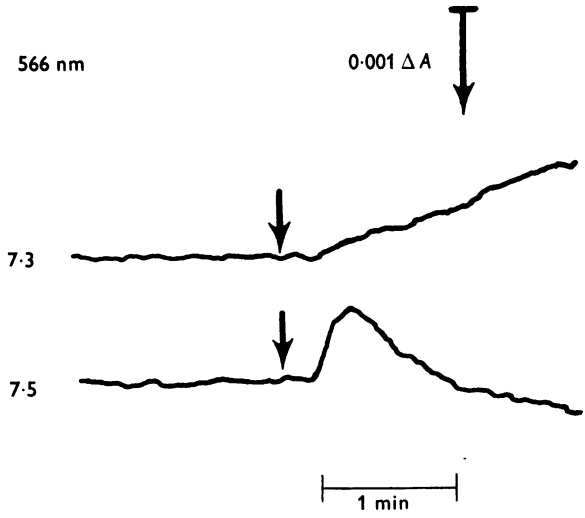

Fig. 4. Effect of pH of internal solution on isosbestic absorbance (566 nm). Dialysis capillary in glass cannula. Internal and external solutions contained CTT plus 5 mM-Mg. External solution was initially at pH 7·3 in both runs. Dialysis with internal medium of pH 7·3 produced base-line shift similar in slope and direction to that seen with dialysis of the barnacle muscle *in vivo*. Dialysis with internal solution of pH 7·5 produced entirely different artifact. Vertical arrows indicate onset of dialysis. Arrow on absorbance calibration indicates direction of absorbance increase.

The pH of the internal dialysate was adjusted to pH 7·3 on the basis of recent measurements of the pH of barnacle sarcoplasm (Boron & Roos, 1976). Although our measurements were not designed to measure the internal pH, the sensitivity of the Eriochrome B spectrum to pH results in a significant 'pH artifact' at 566 nm if the pH of internal and external solutions differ. This artifact is illustrated in Fig. 4. In this calibration experiment, the pH of the calibrating solution (CTT + 5 mM-Mg) was kept at pH 7·3 whereas the pH of the dialysate (CTT + 5 mM-Mg) was set at either pH 7·3 or 7·5.

When the pH of the two solutions matched as indicated in the upper part of Fig. 4, the 566 nm absorbance showed a typical dye washout change directly the dialysis was begun. However when the pH of the external solution was 0·2 pH unit more alkaline, a qualitatively different artifact was observed.

Since the artifacts observed *in situ* dialysis were invariably of the configuration

illustrated in the upper part of Fig. 4, i.e. pH 7·3 rather than pH 7·5, we infer that the internal pH of our barnacle fibres was close to that value reported by Boron & Roos and the pH of all internal solutions was adjusted to pH 7·3.

Experimental material and procedures for dialysis

Large specimens of *Balanus aquila* were obtained from Pacific Biomarine Company, Venice, California. Animals were stored in refrigerated recirculated artificial sea water (Instant Ocean, Aquarium Associates).

Muscle fibres were isolated either from the *depressor scutum lateralis* or *rostralis*. Where possible, the rostralis muscles were used because these fibres were much more nearly circular in cross-section. After a fibre had been dissected free from the bundle, it was lifted up into air and cut at the end near the shell attachment. The barnacle was then transferred rapidly to another dish containing a small amount of saline ringed in Vaseline. The barnacle muscle was placed in the saline drop with its ends extending beyond the Vaseline. The tendon end was then cut, and both cut ends were allowed to dry for several minutes. The fibre was then transferred to the dialysis chamber and allowed to lie on a square of smooth filter paper soaked with barnacle saline. After the barnacle fibre had been cannulated at either end, the chamber was flooded with saline, the fibre drawn taut and positioned in the centre of the chamber.

The fibre was then microinjected either twice, or more usually three times with a solution of Eriochrome B (10–20 mM) dissolved in 10 mM-K-TES, buffered to pH 7·3. The length of fibre injected was 20 mm, which corresponded to the length of the light pipe assembly. Final concentration of Eriochrome B in the sarcoplasm, assuming diffusional equilibration and no binding, was 400–700 μM. During this period of time the fibre was kept immersed in Ca-free barnacle saline, because previous reports (Russell & Blaustein, 1974; Zacharovà & Zachar, 1967) indicated that the fibres are less prone to contract spontaneously in Ca-free saline.

After the microinjection had been completed, a plastic porous capillary was passed through the centre of the fibre using techniques previously described. The length of the porous region was about 20 mm. The capillary was positioned so that the porous region extended for at least 2 mm beyond the injected region on either end to ensure that during subsequent dialysis the injected region experienced only radial concentration gradients. The end of the porous region was positioned some 2–4 mm from the cut and tied end of the fibres. This was done to minimize longitudinal diffusion of magnesium from the damaged ends of the fibre into the central dye containing region where the ionized Mg was to be measured.

After positioning the fibre, the plastic tubing leading to the porous capillary was filled with the selected dialysis solution. Great care was taken in filling the tubing to make sure that dialysate was not inadvertently pushed through the porous region. After the capillary had been filled, a base line was obtained for several minutes and then the dialysis was started. About 15–20% of the experiments had to be rejected at this point because pressure artifacts at the beginning of dialysis made the record at 580–550 nm impossible to interpret. The standard rate for dialysis was 1·2 μl./min. In a few experiments the dialysis pump was turned off, and the solution in the dialysis capillary allowed to equilibrate for approximately 30–45 min with the sarcoplasm. The dialysis capillary was then flushed and refilled with A3D containing a different concentration of total Mg and the dialysis repeated. Because of the possibility that the free Mg in the sarcoplasm could be significantly perturbed by the presence of Mg-EGTA introduced during the first dialysis, little importance was attached to a second or subsequent dialyses, but the procedure was useful to demonstrate that the method could discriminate between dialysis solutions

containing relatively small differences in the amount of total Mg added. All experiments were performed at 25° C.

Solutions and reagents

The composition of external and internal solutions is shown in Table 1. Eriochrome B was purchased from Sigma Chemical Company and twice recrystallized before use. The differential spectrum of the dye has been published (Scarpa, 1974).

Determination of total cellular Mg

Freshly isolated barnacle fibres were washed for varying periods in Mg-free barnacle saline and then briefly blotted on filter paper. A 20–50 mg section was then cut from the centre region of the fibre, weighed and transferred to a 15 ml. tissue grinder. The sample was then homogenized by grinding in 2 ml. 1 N-HCl, or in a few cases by wet ashing in 2 N-HNO_3. After homogenization the sample was diluted to 10 ml. and analysed with a Perkin–Elmer model 305B atomic absorption spectrophotometer.

Several samples were made up in 20 mM $LaCl_3$ solution and the absorption compared with La containing standards. The results of these analysis were not significantly different from those without added $LaCl_3$ indicating no phosphate interference, in agreement with Slavin (1968).

ATP determinations

Isolated single barnacle muscle fibres were ground with sand in a small test tube containing 0·5 ml. 0·01 N-HCl in an icebath. After complete dispersion of the muscle, the solution was diluted to 10 ml. with distilled water. A 10 μl. aliquot of this volume was analysed by the luciferin luciferase firefly method using apparatus previously described (Mullins & Brinley, 1967). The method was standardized by the use of comparable volumes of freshly made Na-ATP solution.

RESULTS

Cellular Mg content of barnacle muscle fibres

The results of twenty-one analyses on three barnacles are shown in Fig. 5. The cellular content is plotted versus soak time in Mg-free barnacle saline. The three fibres which were dipped in the Mg-free solution for 5 sec or less, and plotted on the zero of the abscissa, gave obviously higher values than those washed for longer periods. The difference is presumably due to Mg contained in the clefts which was washed out with longer soaks. In agreement with Page, Mobley, Johnson & Upshaw (1971) and Ashley & Ellory (1972) there was no significant loss of Mg except for that contained in the clefts during the period in Mg-free solution. The average for 18 fibres rinsed 2 min or longer was 10·5 m-mole/kg wet wt.

ATP analysis

Fig. 6 shows the results of analysis of nine freshly isolated fibres. The ATP content averaged 4·9 m-mole/kg sarcoplasm in fresh muscle fibres. Fig. 6 also shows the time course of loss of ATP as a function of time spent in barnacle saline containing 2 mM cyanide + 1 mM iodoacetic acid. The

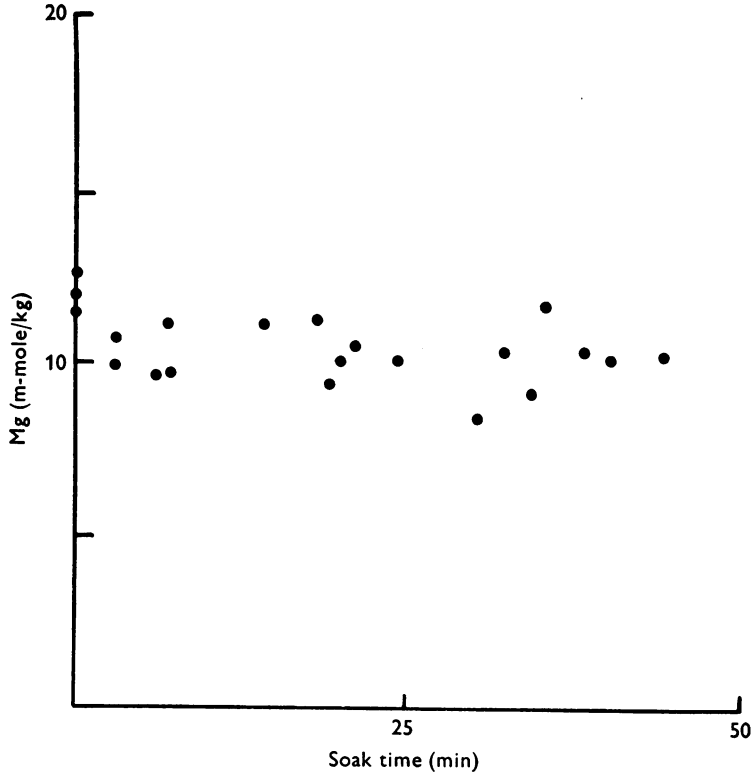

Fig. 5. Cellular magnesium content of isolated barnacle muscle fibres as a function of soak time in Mg-free barnacle saline.

Figure shows that the ATP content is maintained for about 2 hr under these conditions. At the end of 3·5 hr there is a clearly significant reduction of ATP content in approximately half the fibres analysed. By the end of 6·5 hr in cyanide + iodoacetic acid, the ATP content had been reduced to less than 1 μmole/kg. The symbol X represents the mean of seven fibres. Presumably the ATP is maintained for the first 2–3 hr of incubation by the phospho-arginine transphosphorylase reaction which would phosphorylate ADP at the expense of phosphoarginine. The ATP content is maintained for a much longer period than in squid axons, where Caldwell

(1960) noted a clear decrease in ATP concentration after 1 hr in cyanide. The longer period of maintenance of ATP content undoubtedly reflects the higher concentration of phosphoarginine in muscle compared to nerve. Lundsgaard (1931) reports a value of about 29 m-mole/kg tissue for muscle fibres isolated from *Maia*.

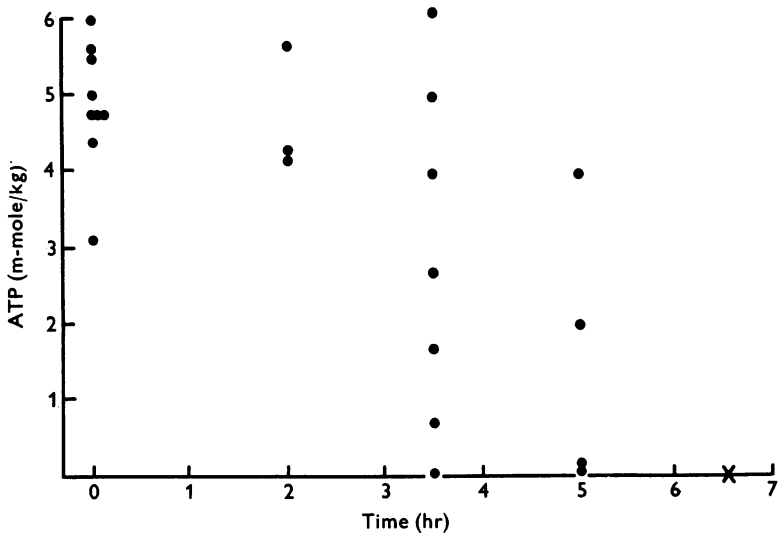

Fig. 6. ATP content (mmole/kg) sarcoplasm) in isolated barnacle muscle fibres immersed for varying periods in barnacle saline containing 2 mM cyanide plus 1 mM iodoacetic acid. × on the abscissa at 6·5 h represents the mean concentration in seven muscle fibres, which was less than 1 μM. The mean content of ATP in fresh fibres was 4·9 m-mole/kg sarcoplasm. Temperature 25° C.

Measurement of absorbance change in barnacle fibres during dialysis

A. *Initial series, bridge mounting of light pipes*

The results of a representative experiment are shown in Fig. 7. The vertical arrow indicates the beginning of dialysis. The top trace shows the result of dialysing with the standard dialysis solution (A3D) plus 47 mM added Mg. Dialysis with this amount of added Mg produced virtually no change in the differential absorbance. This result could indicate that the match of ionized Mg in sarcoplasm and dialysate was very close. On the other hand it could reflect lack of sensitivity or a porous region impermeable to Mg. That these latter two explanations for the result illustrated in the upper trace are incorrect is shown by the bottom two traces. The centre trace shows the result of a second dialysis on the same fibre. In this trial the fibre was dialysed with the standard dialysate without any added

Mg. Following a pressure artifact which caused a shift in the base line, the differential absorbance change occurs in a direction indicating loss of Mg from the dialysed region. A third dialysis was performed on this fibre with standard dialysate plus 80 mM-Mg. In this case the direction of differential absorbance change was opposite to that illustrated in the central trace, as would be expected if the free Mg in the dialysed region were increasing.

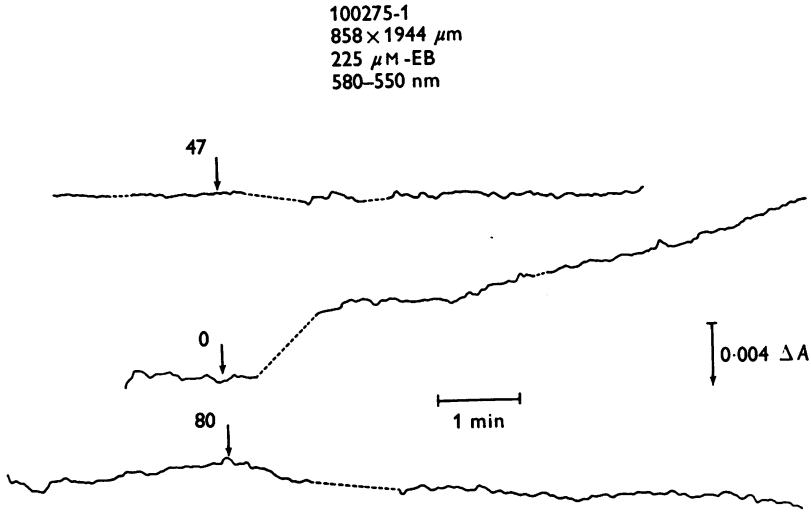

Fig. 7. Absorbance change produced by dialysing a barnacle muscle fibre containing Eriochrome B with a solution of A3D containing various amounts of added Mg. Vertical arrows indicate onset of dialysis; the numbers indicate concentration of total Mg in the dialysate. Arrows on absorbance scale indicates direction of increased ionized Mg in sarcoplasm. Interrupted lines indicate section of trace deleted because of light or movement artifacts.

Inasmuch as the second and third dialysis occurred after the sarcoplasm had been exposed to a significant amount of Mg plus EGTA, these trials provided little quantitative information about the initial Mg concentration. However such trials were useful and occasionally done to show that failure to observe an initial deflexion was not due to lack of sensitivity, or porosity of the dialysis capillary.

In order to determine the sensitivity of this null point method of determining ionized Mg, other experiments were performed in which the initial Mg concentration in the dialysate was deliberately selected to be other than that expected to give a minimal or zero deflexion. When the initial dialysis indicated a mismatch between the ionized Mg in the sarcoplasm and that in the dialysate, some information about the extent of deviation

between the two ionized Mg concentrations could be obtained by measuring the initial slope of the deflexion, since this should be approximately proportional to the difference in the two ionized Mg concentrations. The proportionality is not exact because the null concentration will also be affected by washout of Eriochrome B from the sarcoplasm, as well as changes in the dissociation constant for Mg-EGTA as the dialysate is diluted by diffusion through the sarcoplasm. These complications are considered in more detail in the section on calibration of the null point.

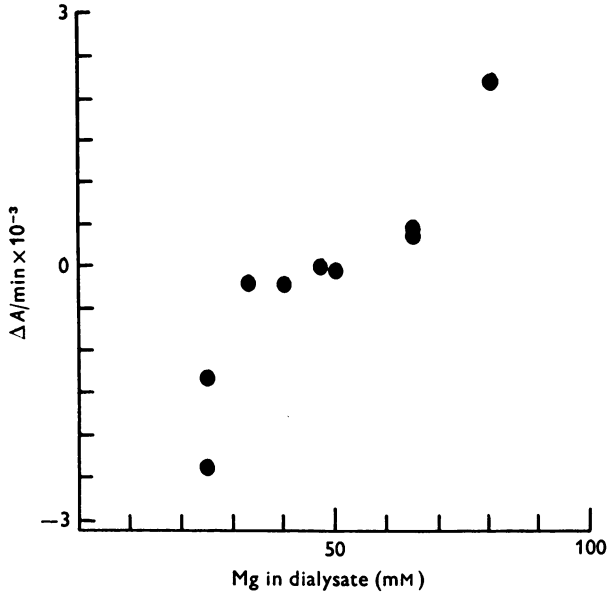

Fig. 8. Collected data from nine fibres showing the magnitude and direction of the initial deflexion (normalized for fibre diameter) plotted against concentration of total Mg (bound and not bound) in the dialysate. Only data from the first dialysis were used.

Fig. 8 illustrates the data obtained from nine muscle fibres in which the initial slope of the absorbance change was measured during the first 2 min of dialysis in an experiment. These initial slopes have been normalized for fibres of different diameter by assuming that the concentration of the dye was that calculated for uniform distribution throughout the sarcoplasm. The data from the initial series indicate that a reasonable null point could be obtained when the dialysate contained between 40 and 50 mM of added Mg.

B. *Second series, sliding block light pipes*

In order to define the null point more exactly, the experiments were repeated using light pipes imbedded in Lucite blocks which could be moved into close approximation to the surface of the muscle fibres (see Methods). This modification increased sensitivity approximately an order of magnitude.

The results of six dialyses are shown in Fig. 9. The Figure shows that the null point method of determining ionized Mg easily discriminates between a 10–15 % change in total Mg in the range of 40–50 mM added Mg in the internal dialysate. Collected results of fourteen experiments are shown in Fig. 10. In four experiments, two dialyses were run on the same fibre and the results obtained with the two dialyses are connected by continuous lines. By interpolation the null point occurs at approximately 47 mM added Mg, i.e. the free Mg in the sarcoplasm of the muscle fibre is the same as that occurring in the dialysate, A3D, containing 47 mM total Mg.

Calibration of the null point

Once it was determined that a null point was obtained when the dialysate contained about 47 mM of added Mg, it was necessary to measure the ionized Mg in dialysate containing 47 mM. This was done by *in vitro* calibrations as follows. A porous plastic capillary was inserted inside a glass tube with an inside diameter of 1 mm. The glass capillary was then flushed with a solution of artificial sarcoplasm (solution CTT or CBT, Table 1) containing Eriochrome B dye in concentrations calculated to be close to that usually present in the sarcoplasm and containing various amounts of total Mg.

In order to avoid any possible complication of variation in the absorbance at 580–550 nm by Ca, all of these artificial sarcoplasm solutions contained approximately 200 μM EGTA to chelate Ca. This concentration had negligible influence on ionized Mg concentration.

After the glass cannula had been thoroughly flushed with either solution CTT or CBT containing dye and magnesium, several minutes of base line were obtained. Dialysis was then begun with the standard dialysate A3D plus 40, 45 or 50 mM added Mg and the direction and magnitude of initial deflexion noted.

Collected results of the calibrations are shown in Fig. 11. In this Figure the rate of absorbance change (units of ΔA/min) is plotted against Mg concentration in the calibrating solution (either CBT or CTT) for various concentrations of total Mg in the A3D. Calibrations obtained with CBT as the external solution are considered more representative of that obtained in sarcoplasm. Solution CTT was used to measure the effect of

aspartate on the apparent binding constant for Mg-EGTA. The null point (i.e. zero rate of absorbance change) indicates a concentration of Mg in the calibrating solution (CBT or CTT) which matches the ionized Mg in the A3D.

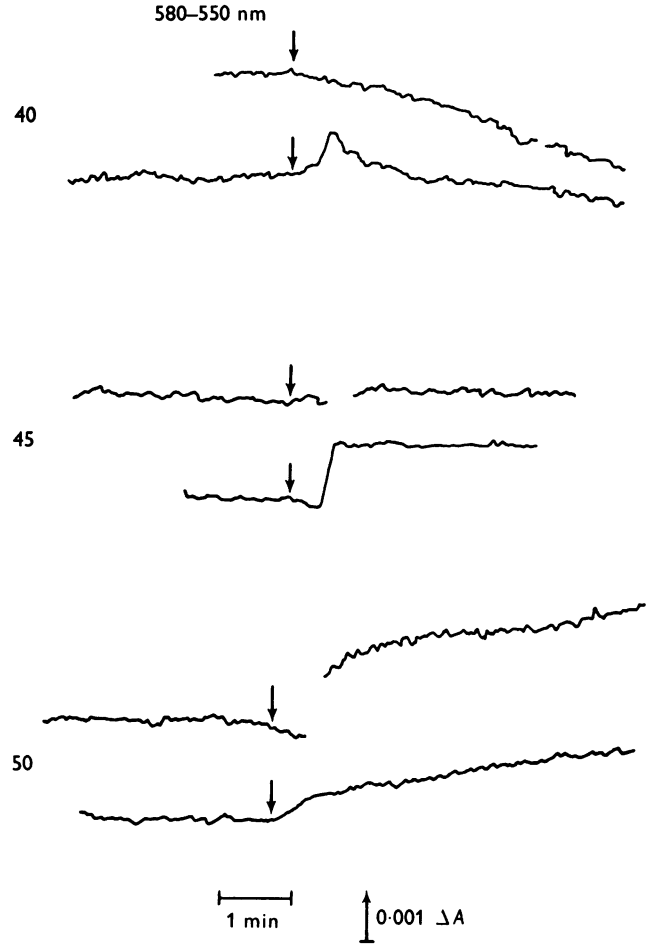

Fig. 9. Composite Figure showing the effect of six separate dialyses in barnacle muscle fibres with varying concentrations of total Mg (bound and unbound) in A3D. Vertical arrows indicate onset of dialysis. Concentration of total Mg (mM) shown at the left of each trace. Direction of arrow on absorbance scale indicates increasing magnesium in sarcoplasm. Dialysis with 45 mM of total Mg produces essentially no deflexion of the 580–566 nm trace indicating a close match between ionized Mg in the dialysate and in the sarcoplasm. Dialysis with either 40 or 50 mM total Mg in the dialysate results in deflexions in the direction indicating either loss or increase of Mg respectively in the sarcoplasm.

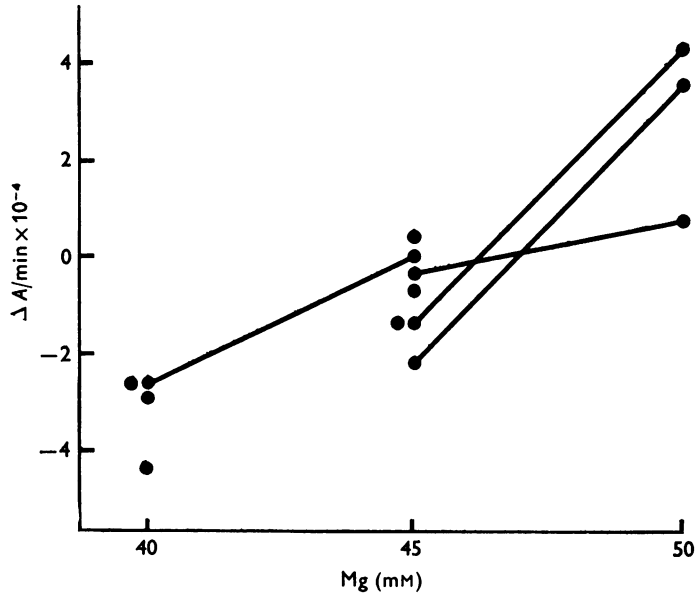

Fig. 10. Collected results of fourteen dialyses on ten fibres, in which the total Mg in the A3 dialysate was varied between 40 and 50 mM. In four experiments two dialyses were done on the same fibre; these are represented by the connected filled circles. Ordinate represents initial slope of the 580–566 nm trace obtained 1–2 min after onset of dialysis.

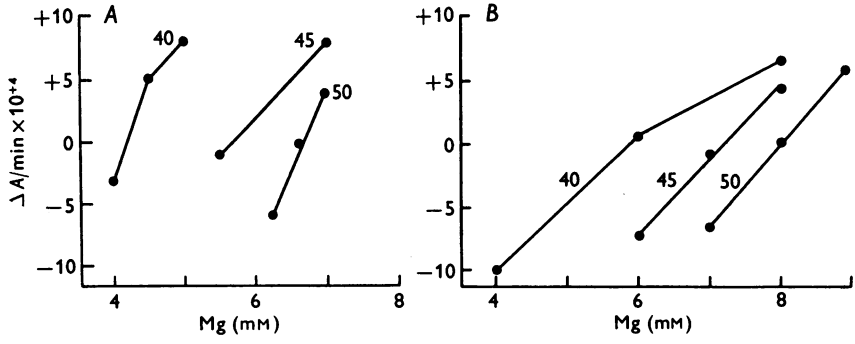

Fig. 11. Results of calibration experiment relating total Mg (bound and unbound) in A3D to Mg in artificial sarcoplasm. *A*, external solution CBT; *B*, external solution CTT. Ordinate is the initial slope of the 580–550 nm trace obtained 1–2 min after onset of dialysis.

The null concentration of ionized Mg corresponding to each of the three levels of total Mg in A3D have been replotted in Fig. 12.

Using the mull point for the *in situ* dialyses obtained from Fig. 10 (47 mM total Mg in the A3D) and the data from Fig. 12 showing null point Mg concentrations as a function of added Mg, allows one to conclude that the free Mg in barnacle muscle fibres is about 6 mM in the intracellular water

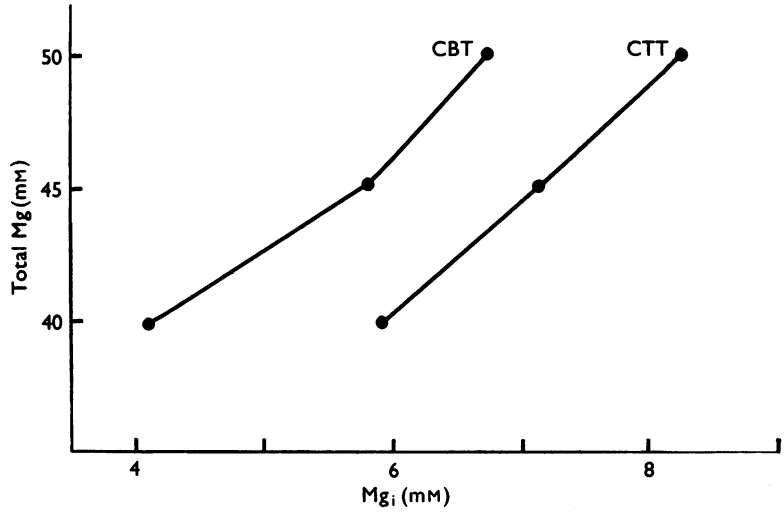

Fig. 12. Plot of null concentration of Mg in external solution (abscissa) *vs.* total Mg (bound and unbound) in A3D (ordinate).

immediately surrounding the dialysis capillary. No measurements of absorbance change were made exactly at the null point (47 mM), but the standard deviation of the distribution of slopes at 45 mM added Mg was $0{\cdot}9 \times 10^{-4}$ ΔA/min (Fig. 10) which corresponds (Fig. 11) to a variation of about 0·2 mM-Mg.

It is important to realize, that the null point, either *in vitro* or *in vivo* is influenced not only by the relative ionized Mg concentrations in the dialysate and the immediately surrounding medium, but also by the rate at which dye is washed out of the sarcoplasm, by changes in dissociation constant of Mg-EGTA as it is diluted by diffusion away from the porous capillary into the surrounding fluid, etc. In using the calibration curves in Fig. 12 to convert the null point measurements into ionized Mg concentration in the space immediately outside the surface of the dialysis capillary, we have explicitly assumed that these influences occurred to the same extent in the sarcoplasm and in the solutions used for calibration.

Effect of external Mg solutions on the internal ionized magnesium concentration

Several fibres were exposed to external solutions containing varying Mg concentrations for periods up to 30 min. Long term drift in the spectrometer made it difficult to quantitate the measurements but if there was any change in free Mg it was less than 5 %.

DISCUSSION

Since the dissociation constants for Mg-EGTA and Mg-EB are strong functions of pH, a difference in pH between the calibrating solutions and sarcoplasm would introduce a significant error into the determination of free Mg in the sarcoplasm. We adjusted the pH of all dialysis solutions to 7·3 on the basis of data by Boron & Roos (1976). Although we made no specific measurement of pH, reasons have been given in the Methods section for believing that no serious mismatch of sarcoplasmic and dialysate pH occurred in these experiments.

The determination of free Mg by the null point method does not require an independent measurement of the Mg-EGTA dissociation constant. However such a constant can be calculated from the data in Fig. 12, since the calibration experiments give a value for free Mg in the dialysate with known total EGTA and Mg, and provide a useful check on the internal consistency of the experiments. The effective dissociation constants calculated for the A3D containing 40, 45 and 50 mM-Mg were 25, 30, and 31 mM respectively. These values agree well with a value (33 mM) calculated from the published dissociation constants for Mg-EGTA assuming that the effect of ionic strength on magnesium EGTA is the same as for Ca-EGTA (DiPolo *et al.* 1976) and provides some evidence that dialysis *per se* and dye washout do not introduce a serious artifact into the measurement.

The data in Fig. 12 also permit a calculation of the apparent binding constant for Mg aspartate, assuming that the increase in free Mg corresponding to a null point for a given total Mg seen in solution CTT compared to CBT reflected magnesium binding by aspartate. The results indicated an apparent binding dissociation constant for Mg aspartate of 200–400 mM in solution CTT. Comparable measurements of the dissociation constant for high ionic strength do not appear to have been made; however this value is relatively close to the value of 500 mM determined for calcium aspartate under comparable conditions (T. Tiffert and F. J. Brinley Jr., unpublished).

It should be noted that binding of dye to sarcoplasmic elements will not affect the measurement of ionized Mg by the null point. Although the

sensitivity of the method (i.e. the ability to detect small changes in slope occasioned by a mismatch in free Mg between dialysate and sarcoplasm) does depend upon the amount of dye in sarcoplasm, the actual end point does not.

One important limitation of the method is that it detects only the ionized magnesium in the immediate vicinity of the dialysis capillary, and does not provide information about the existence or distribution of free Mg in compartments located away from the core of the fibre. However barnacle muscle fibres usually contain deep invaginations running several hundred microns into the interior of the fibre. Since these invaginations occur randomly, one might expect that the dialysis capillary would in some cases have been quite close to sarcolemma, even though it was located nearly axially. Since no large variations in the null point were noted in these experiments, we infer that there is probably not a large radial variation in the free Mg concentration.

The total intracellular Mg in these fibres was 10·5 m-mole/kg wet wt. which agrees closely with the values of 9·6 m-mole/kg wet wt. (Page *et al.* 1971) and 11·6–12·1 m-mole/kg wet wt. (Ashley & Ellory, 1972), obtained on single muscle fibres of *Balanus nubilus*. From data of Shaw (1955), working with muscle fibres of the crab *Carcinus maenas*, it is possible to calculate a value of 9·4 mmole/kg wet wt. (using 73·5 % water content and assuming 3 m-mole/kg wet wt. of extracellular Mg, as reported by Ashley & Ellory (1972) for barnacle fibres).

The latter authors also reported the diffusible Mg (free and bound), as measured by internal sampling of sarcoplasmic fluid, to be 11·5 m-mole/kg wet wt. thus accounting for virtually all of the intracellular Mg. Due to the inherent uncertainty in this method, the result is not inconsistent with the conclusion of Page *et al.* (1971), that about 10 % of the cell Mg in their preparation, i.e. 1 m-mole/kg sarcoplasm, was not extractable by glycerine treatment and was probably bound to the myofibrils. The present direct measurement of free Mg^{2+} is 6 mM, which is somewhat higher than the upper limit of 5 mM estimated by Ashley & Ellory (1972) on the basis of unpublished measurements of Mg interference with calcium induced aequorin luminescence (see also Caldwell, 1969). Since about 20 % of the wet weight in barnacle fibres is occupied by solid (Page *et al.* 1971) and probably about 10 % of the water is either compartmentalized or structurally bound and hence unavailable to Mg, the amount of ionized Mg expressed on a wet weight basis is about 4·2 m-mole/kg.

It is possible to estimate the amount of Mg bound to ATP by using the dissociation constant for ATP at high ionic strength (about 0·7 mM, DeWeer, unpublished) together with the data on ATP content, 4·9 m-mole/kg wet wt. (Ashley & Ellory, 1972 estimated 3·1 m-mole/kg). The

result, 4·2 m-mole/kg, indicates that about 90 % of the ATP is present at MgATP.

The three Mg fractions considered above (m-mole/kg): free Mg (4·2) MgATP (4·2) and myofibrillar bound Mg (*ca.* 1) together account for 9·4 m-mole Mg/kg wet wt.

The remaining Mg (*ca.* 1 m-mole/kg wet wt.) is probably bound to other chemical species such as phosphoarginine and phosphate. Significant binding to amino acids seems unlikely on the basis of the amino acid composition of a single sample of barnacle muscle as determined on an amino acid analyser by Professor Poljak. The results were (m-mole/kg wet wt.): glycine 190, proline 96, arginine 61 (including phosphoarginine), serine 46, alanine 29, valine 12. The effective magnesium binding constants of these amino acids have not been measured under conditions comparable to those of the present experiments, but must surely be very much less than that of aspartate which was estimated to be *ca.* 300 mM in dialysate CTT.

The above partitioning of magnesium cannot apply to a different species of barnacle, *Megabalanus psittacus*, where the total Mg is only 3·2 m-mole/kg wet weight (Keynes, Rojas, Taylor & Vergara, 1973).

The wide variation in free and total Mg in various tissues precludes simple comparisons. The fraction of total Mg in barnacle muscle that is free, about 50 %, is higher than most other tissues although it is the same as in another marine preparation, the squid giant axon (Baker & Crawford, 1972; Brinley & Scarpa, 1975). The percent of free Mg in frog skeletal muscle is about 30 % (Nanninga, 1961) and 30 % in rat brain, liver or kidney cortex (Veloso, Guynn, Oskarsson & Veech, 1973). The free Mg concentration is also somewhat higher in barnacle than for vertebrate tissues, e.g. rodent brain 0·6–1·5 mM (Bachelard & Goldfarb, 1969; Veloso *et al.* 1973), rat muscle 2·8 mM (Günther, 1967), frog muscle 3·4 mM (Nanninga, 1961). The estimate of free Mg in frog muscle, however, may be low, since the calculations from binding constants yielded a free Ca of 0·91 mM. Since the free Ca in muscle is now known to be in the micromolar range presumably a recalculation should yield a lower free Ca by about 0·9 mM and an increased free Mg by a similar amount. The higher concentration of free Mg in squid axon and barnacle muscle fibres may be a consequence of the higher extracellular Mg concentration in the saline solutions used to bathe these fibres compared to vertebrate preparations.

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