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STUDIES ON WATER IN BARNACLE MUSCLE FIBRES II. ROLE OF IONS AND ORGANIC SOLUTES IN SWELLING OF CHEMICALLY-SKINNED FIBRES

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SUMMARY

Single muscle fibres from the giant barnacle, *Balanus nubilis*, were chemically skinned (2% Tween 20), then equilibrated for 40 h in salt solutions ranging in ionic strength from 0·025 to 0·6 M at pH 7·0. The water content of the fibres and the net charge on the myofilaments increased with increasing salt concentration. Cation accumulation in the fibres was about equal to anion exclusion at all salt concentrations. When an organic solute (trimethylamine oxide, glycine, alanine, serine, proline, or glycerol) in the concentration range from 0·1 to 0·6 M was added to the salt solution, cation accumulation increased and water content decreased. Myofilament architecture was disrupted when the fibres were equilibrated in high salt (> 0·4 M) solutions and preserved when 0·5 M-triethylamine oxide was also added. The results are consistent with the view that organic solutes enhance the association between the fixed charge sites and their counterions. This hypothesis is examined quantitatively using the Oosawa relationship between the volume and the counterion association for cylindrical polyelectrolytes. The results illustrate that organic solutes can influence fibre volume in a way other than through osmo-regulation.

INTRODUCTION

The volume of an intact animal cell is determined by its water content, which in turn depends upon the osmotic value of colloids and small solutes within the cell. In vertebrates, ions comprise about two-thirds of the intracellular osmotic solutes (Conway, 1957), and hence have been the primary focus of studies on cell volume regulation (MacKnight & Leaf, 1977). Reversible inhibition of the ion transport function of the plasma membrane leads to swelling, which has been explained as an osmotic effect owing to an excess of Na influx over K efflux (Rixon & Stevenson, 1956; Kleinzeller & Knotkova, 1964). Swelling also occurs when membrane-damaged muscle fibres are placed in dilute salt solutions (April, Brandt & Elliott, 1972; Godt & Maughan, 1977), the volume change being related to changes in the spacing of the myofilament lattice (Rome, 1968; April *et al.* 1972). Such swelling has been interpreted in terms of a new Donnan-osmotic equilibrium between the charged myofilaments and the freely diffusible ions (Godt & Maughan, 1977).

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Besides electrolytes, however, the cytoplasm of animal cells contains numerous small organic solutes which are osmotically active. In the frog or rat muscle fibre, these solutes, mainly phosphagen, dipeptides and amino acids, constitute one-third of the osmotic activity (Conway, 1957). Among marine invertebrates, where the total osmotic pressure is three times that of most vertebrates, the intracellular electrolyte concentrations are only slightly higher than those of vertebrates, the remaining osmotic difference (600–700 m-osmol) being made up by amino acids and quarternary amines (Schoffeniels, 1976; Clark & Hinke, 1980). Significantly, when euryhaline invertebrates are subjected to hypo-osmotic stress, cell volume regulation is accomplished mainly by lowering the content of these organic solutes rather than that of the electrolytes in the cytoplasm (Shaw, 1958; Clark, 1968; Freel, Medler & Clark, 1973; Schoffeniels, 1976). Thus, organic solutes are a second cell volume regulating mechanism, in addition to the ion transport system.

A specific advantage of nitrogenous organic solutes over most salts at high osmotic activities is their lack of a deleterious effect on soluble enzymes (Clark & Zounes, 1977). Here we turn to their effects on cell volume regulation. The water content and solute distribution have been determined in membrane-damaged (chemically skinned) single fibres of the giant barnacle (*Balanus nubilis*) equilibrated with solutions of varying ionic strength and organic solute concentration. In addition to testing organic solutes characteristic of marine invertebrates, we also examined the effects of two other solutes accumulated by organisms that experience water stress: glycerol, found in frost-resistant insects (Asahina, 1966) and in marine and halophilic green algae (Borowitzka & Brown, 1974); and urea, found in coelacanths (Lutz & Robertson, 1971), in chimaeroids (Robertson, 1976), in elasmobranchs (Robertson, 1975) and in terrestrial anurans (McClanahan, 1972). In agreement with the findings of others, we observe that the equilibrium swelling is enhanced by increasing salt concentrations. Such swelling, however, is suppressed by increasing concentrations of all of the organic solutes tested except urea. This new observation raises the possibility that the cytoplasmic organic solutes have an important role not only as osmotic effectors, but also in stabilizing the myofilament lattice spacing, and even in maintaining the integrity of the myofilaments themselves.

METHODS

Preparation of fibres

Fresh barnacles (*Balanus nubilis*) were maintained and dissected as described in the preceding paper (Clark & Hinke, 1980). Bundles of scutal depressor muscle fibres were placed for 30 min in each of two modified barnacle Ringer solutions at 2–4 °C. The first solution was typical barnacle Ringer (Hoyle & Smyth, 1963; Gayton & Hinke, 1968), but with the Ca^{2+} omitted; the second solution was similar but with 2 mM-EGTA (ethylene bis (oxyethylene nitriol) tetra-acetic acid) added. This procedure lowered the free Ca^{2+} sufficiently to prevent fibre contractions during subsequent treatments. Single fibres were then dissected free from one another, cleaned of adhering connective tissue and fat, cut free from the tendon and baseplate, and placed for a further hour in Ca^{2+}-free Ringer containing 5 mM-EGTA. Each fibre was re-

moved from the solution, gently blotted and quickly weighed to establish the fibre's 'initial wet weight'. The 'initial dry weight' was calculated from the water content of companion fibres. The fibres were then shaken for 40 h at 2 °C in an experimental solution which was changed three times, each wash being at least 50 × the volume of the fibres.

Each fibre was then removed, gently blotted on a series of filter papers until no further water stain appeared, then weighed to establish the 'final wet weight'.

When the ionic content was to be determined, the fibre was dried to constant weight (at 70 °C for 48 h), extracted with organic solvents to remove fat (Clark & Hinke, 1980), redried and reweighed. This 'final dry weight' differed from the first dry weight by about 3%. Each fibre was then digested for 1 week in 4 ml of 0·75 N-HNO_3, after which the digest was diluted to known volume. This procedure permitted chloride and cation analyses to be made on the same fibre. No increase in cation recovery was obtained following digestion of fibres in more concentrated acid.

No final dry weight was determined on fibres destined for organic solute determination. The final water content of these fibres was taken as equal to that obtained on the companion fibres used for the determination of ionic content. In general, 20 fibres were equilibrated with each solution, of which half were used for water and ion determinations and half for organic solute determinations.

Experimental solutions

The solutions were prepared from analytical grade salts, grade A organic solutes and glass-distilled water. All experimental solutions contained 2% Tween 20 (a nonionic detergent) to disrupt membranes, 5 mM-EGTA and 25 mM-Tris-propionate. In most experiments, the Tris buffer maintained the pH between 6·9 and 7·1 throughout the experiment, but in some experiments (see Results) wider deviations of pH occurred.

That Tween 20 is capable of disrupting both the plasma and internal membranes and of establishing an equilibrium state between the fibre and the bath was determined by the following observations: any change occurring in fibre volume and appearance were discernible within a few hours after transfer to experimental solutions and no further changes occurred after 24 h (the 40 h equilibration period being chosen for convenience); no intact membranes were observed in electron micrographs, regardless of the state of the myofilaments (see below); the membrane potential of treated fibres decreased from −70 to −20 mV in about 30 min; and intrafibre Na, K, Cl and amino acid concentrations approached the bath concentrations in about 10 h.

The basic experimental salt solution (1x) contained 150 mM-KCl, 50 mM-NaCl and 10 mM-$MgCl_2$. These concentrations were halved, doubled or tripled to produce 0·5x, 2x and 3x salt solutions. The selected cation ratios conform approximately to those found in the intact fibre (Hinke, Caillé & Gayton, 1973). In one experiment, propionate was substituted for various amounts of chloride to check if fibre water content might be dependent on the choice of anion.

Organic solutes – trimethylamine oxide (TMAO), glycine, alanine, serine, proline, glycerol, and urea – were added to either buffered detergent or the above salt solutions, at concentrations ranging from 0·1 to 0·6 M.

The osmolarity of each experimental solution was determined using a Fiske osmometer (Clark & Hinke, 1980).

Chemical analysis

All analyses were performed both on fibres and on subsamples of the experimental solutions at the end of the equilibration period, thus permitting determination of the equilibrium distribution ratio of each solute between fibre and bath.

Cation concentrations were determined by flame spectrophotometry, using a Varian Techtron (AA5) unit. Sodium and magnesium were measured by absorption spectrometry and potassium by emission spectrometry. To compensate for interferences, the standards contained concentrations of acid and of other ions similar to those expected in the samples, and experimental values were interpolated from the slope between the nearest bracketing standards.

Chloride was determined using a Buchler-Cotlove amperometric titrator. Except for fibres equilibrated in solutions nominally devoid of ions, the extracts contained suffiicient amounts of chloride such that the largest error encountered was $\pm 5\%$.

The concentration of TMAO in fibres equilibrated with this organic solute was estimated by the method described in the previous paper (Clark & Hinke, 1980). This method is restricted to samples which contain more than 10 μmoles of TMAO, a condition which was met only when the fibre was relatively large (50 mg wet weight) and the bath concentration of TMAO was relatively high ($> 0{\cdot}25$ M). The partition of TMAO between fibre and bath at lower TMAO concentrations was assumed to have the same ratio ($\sim 1{\cdot}05$) as was observed at higher concentrations. Amino acids were estimated colorimetrically with ninhydrin using the methods of Clark (1972) following extraction of the fibres in cold 80% ethanol (Clark & Hinke, 1980).

The concentration of propionate, glycerol or urea in fibres equilibrated in the presence of one of these solutes was determined by the isotope method. The ^{14}C-labelled solute (New England Nuclear Co.) was added to the last change of the experimental solution, which meant that the fibres were exposed to the isotope for at least 12 h. Following wet and dry weight determinations, the fibres were hydrated and digested in Soluene 350 (Packard) for 24 h. Isotope activity was determined using a Nuclear Chicago (Searle) Mark II liquid scintillation system; counts were corrected for background and for quenching by recounting after the addition of an external standard. By assuming equality in the specific activities between the fibre and the bath, the solute concentration in the fibre was calculated from the known solute concentration in the bath.

Data treatment

To help in exploring the relationship among water, solutes and non-soluble solids within the fibre, all results have been expressed in terms of the non-soluble dry weight of the fibre. This necessitated correcting the measured dry weight of a fibre for its content of salts and other soluble molecules, the amount of which varied significantly from one treatment to another. Because salt concentrations were measured in individual fibres, the dry weight of a fibre could be precisely corrected for salt contribution. The correction for measured organic solutes was made using the average concentration obtained on a set of simultaneously treated companion fibres. The correction for other organic compounds (EGTA and buffer) was made by assuming that they partition equally between bath and fibre. Finally, in experiments wher

amino acids were not measured independently, correction was made for approximately 5 g residual amino acids per kg fibre water, an amount found in preliminary studies to remain in the fibre even after prolonged exposure to an amino acid-free medium.

The excess accumulation of mobile ions in a fibre was calculated in a similar way to that of Hinke, Caillé & Gayton (1973). For the ith ion, the excess ion accumulation in the fibre, ν_i in m-mol/kg nonsolute dry weight, can be defined as

$$\nu_i = [(C_i)_f - (C_i)_b] \cdot \left[\frac{\text{kg fibre water}}{\text{kg non-soluble dry weight}}\right], \quad (1)$$

where $(C_i)_f$ and $(C_i)_b$ are the m-mol concentrations of the ith ion in the fibre and the bath, respectively. The total excess of mobile ions in a fibre becomes $\Sigma\nu_i$, where i equals Na, K, Mg, Cl and propionate.

The magnitude of the fixed charge, $z_r\nu_r$ associated with the nonsolute dry weight can be obtained from the electroneutrality statement, $z_r\nu_r + \Sigma z_i\nu_i = 0$, where z_i is the valency of the ith ion. Thus, the net fixed charge (in m-equiv/kg) on the non-soluble solid in the fibre becomes

$$z_r\nu_r = -(\nu_{Na} + \nu_K + 2\nu_{Mg} + \nu_{Cl}). \quad (2)$$

Electron microscopy

The following procedure, adapted from Brigden, Spira & Hinke (1971), yielded adequate and reproducible fixation of *Balanus* muscle. Fibres were stretched to resting length on pieces of thin cardboard and were cut longitudinally (except fresh fibres) to facilitate penetration of fixative. They were immersed first in a Ca^+-free barnacle Ringer solution containing 3% glutaraldehyde for 30 min, then in a similar Ringer solution containing 6% glutaraldehyde for a further 5 h. Following several rinses in 0·1 M-cacodylate buffer (pH 7·0), the fibres were immersed overnight in the same buffer solution, then post-fixed for 1 h in 1% osmium tetroxide in 0·1 M-cacodylate buffer. After further rinsings, the fibres were dehydrated in alcohol before small pieces were cut for embedding in Epon. All fixation and rinsing procedures were carried out at 0·4 °C. Thin sections were cut on a Porter-Bloom ultramicrotome and examined, with or without uranyl acetate staining, in a Philips 300 EM electron microscope.

RESULTS

From the naturally occurring solutes that participate in intracellular osmoregulation, TMAO was selected as the model organic solute to study because it carries no charge, yet is highly polar (dipole moment ~5). Thus TMAO should not directly affect the fixed charge in the fibre nor the resulting Donnan distribution of ions.

Results with TMAO

(*a*) *Effect of ionic strength on fibre water content.* The upper curve in Fig. 1 shows the effect of ionic strength on the water content of membrane-damaged fibres relative to their content of non-soluble solids, mainly myofilament proteins. Water content in-

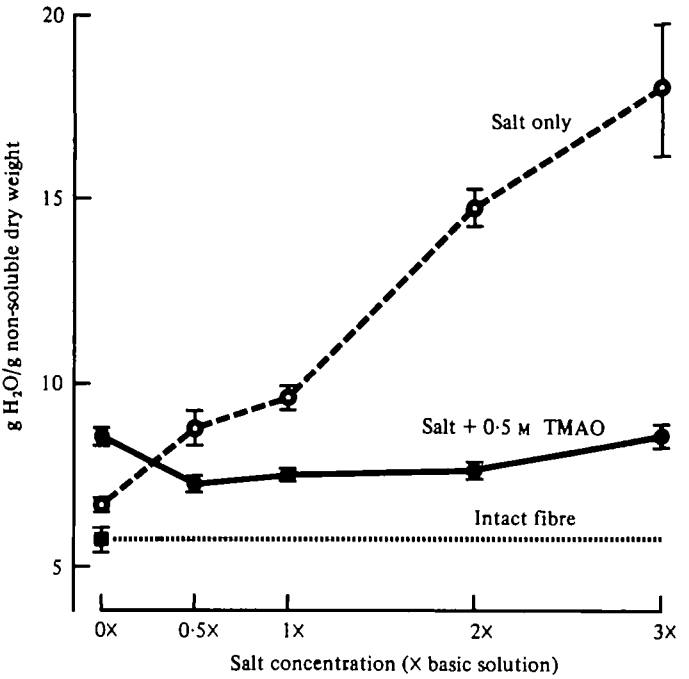

Fig. 1. The water content of membrane-damaged fibres relative to non-soluble dry weight after 40 h equilibration in different salt concentrations: in the absence of TMAO (upper, dashed curve), and in the pressure of 0·5 M-TMAO (lower, solid curve). For comparison, the dotted line, labelled 'intact' indicates the water content of fresh fibres (untreated with Tween 20) in normal barnacle Ringer. Vertical bars = ±1 S.E.M. (n = 8 to 10 fibres for each point on the graph).

creased almost linearly with ionic strength, resulting in an enormous swelling of fibres in 3x salt solution; such fibres appeared swollen and opaque. Even at very low ionic strengths, membrane-damaged fibres were significantly more hydrated than intact fibres in barnacle Ringer, indicated by the horizontal line in Fig. 1. Inexplicably, the amount of swelling observed in skinned fibres varied considerably from one barnacle to another, even in the absence of added solutes (other than buffer). Thus it was not possible to make absolute comparisons of fibre water content from one experiment to the next, although relative effects were consistent.

Analyses of the treated fibres indicated that only at high ionic strength (3x) was there a measurable loss of non-soluble dry weight, probably because of partial solubilization (salting-in) of the contractile proteins. At lower ionic strengths, the non-soluble dry weight of salt-treated fibres was similar to the non-soluble dry weight of freshly dissected fibres. The relatively high Mg^{2+} concentration (30 mM) in the 3x solution may explain why more salting-in of the contractile proteins did not occur at this high ionic strength (~0·6 M) (Sarkar, 1950).

In the presence of 0·5 M-TMAO, there was no swelling of fibres as salt concentration was increased (solid curve, Fig. 1). These fibres were translucent and indistinguishable from intact fibres. This TMAO concentration is similar to the total concentration of nitrogenous organic solutes found within cells of marine invertebrates.

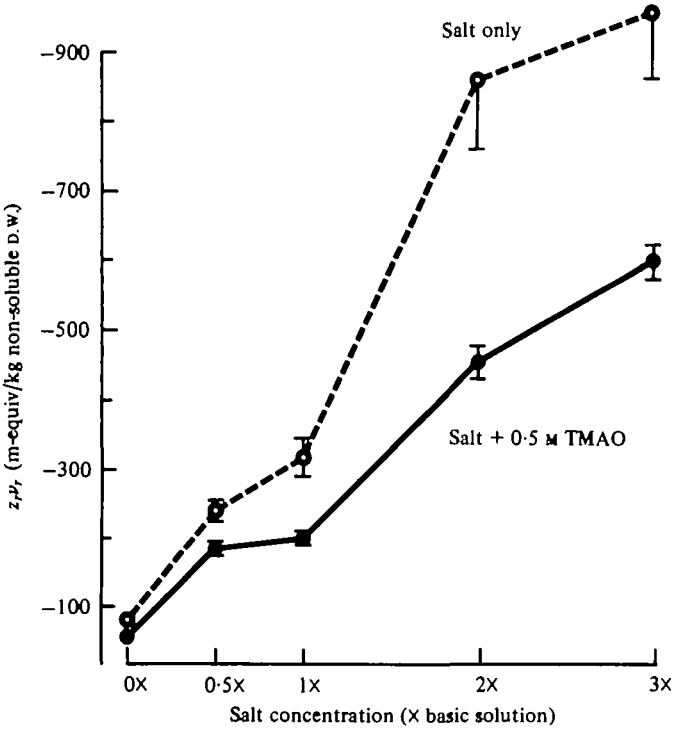

Fig. 2. Effect of salt concentration on the myofilament charge as defined by equation 2 in text. Data from same fibres as for Figs. 1 and 3. Vertical bars = ±1 S.E.M. (n = 8 to 10 fibres).

(*b*) *Effect of ionic strength on net fixed charge.* The charge on the myofilament proteins, $z_r\nu_r$, increased with increasing salt concentration, approximately tripling with a three-fold increase in ionic strength (upper curve, Fig. 2). This is much greater than the effects of ionic strength observed by Mihalyi (1950) on the titration curves of crystalline myosin; we have no explanation for this difference except that other proteins are also present in our muscle fibres. For the experiments shown in Fig. 2 (solid curve), the net charge on the proteins was somewhat lower at all salt concentrations when 0·5 M-TMAO was added. This apparent decrease in $z_r\nu_r$ may be partly because the final pH of the TMAO-containing solutions (after 40 h equilibration) was consistently about 0·2 pH units lower than that of the corresponding salt solutions, all of which were near pH 7·0. In another experiment (Fig. 4*B*, upper curve), where the pH of the equilibrating solution ranged from 6·97 in the absence of TMAO to 7·06 in the presence of 0·6 M-TMAO, the increasing TMAO concentration appeared to have a negligible effect on $z_r\nu_r$.

(*c*) *Effect of ionic strength on ion distribution.* Fig. 3 shows the total excess ions, $\Sigma\nu_i$ in fibres as a function of ionic strength. The lower (dashed) curve illustrates that there was very little alteration in the balance between cation accumulation and anion exclusion with increasing salt concentration, despite the large increase in net protein charge shown in Fig. 2. There was only a modest excess of ions in the fibres, amounting to around 100 m-mol/k non-solute dry weight. In contrast, the upper (solid) curve

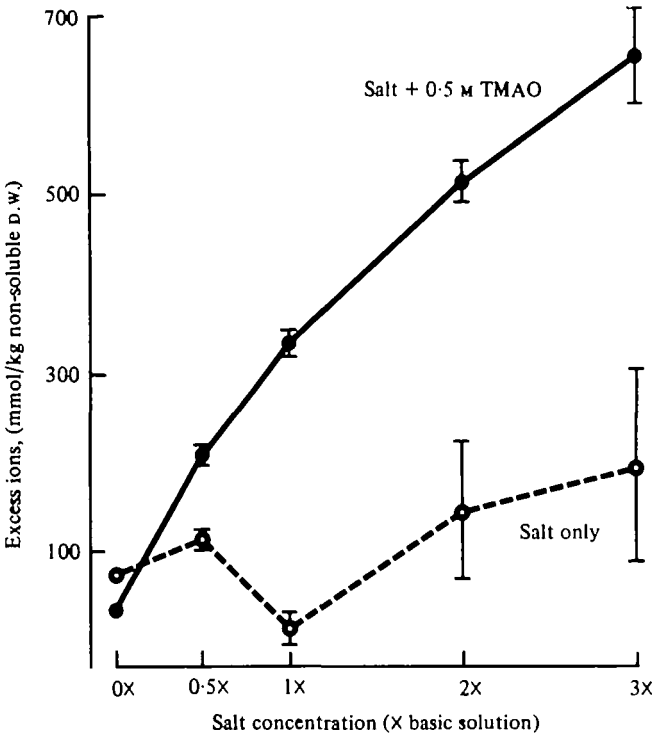

Fig. 3. Effect of salt concentration on the excess ion ($\Sigma\nu_i$) accumulation in the fibre, where ν_i for each ion is obtained from equation (1) in text. Data from same fibres as for Figs. 1 and 2. Vertical bars = ± 1 S.E.M. (n = 8 to 10 fibres).

shows that TMAO significantly altered the ion distribution between fibres and bath. The increase in $\Sigma\nu_i$ with ionic strength is owing in part to an increase in cation accumulation and in part to a decrease in anion exclusion.

(*d*) *Effects of increasing TMAO at constant ionic strength.* To examine the effectiveness of TMAO in reversing the influence of inorganic ions on fibre swelling, fibres were equilibrated at physiological salt concentrations (1x), in the presence of 0·1 to 0·6 M-TMAO (Fig. 4, Table 1). Fig. 4*A* clearly shows a reduction in fibre swelling with increasing concentrations of TMAO, the water content of the membrane-damaged fibres was similar to the water content of intact fibres (see Fig. 1). Fig. 4*B* (lower curve) shows that there was an increase in the number of excess ions ($\Sigma\nu_i$) in the fibre as the TMAO concentration increased, even though the net charge on the proteins (Fig. 4*B*, upper curve) changed comparatively little.

The changes in cation accumulation and anion exclusion with increasing TMAO in the fibre are shown in Table I. When TMAO was absent (1x salt only), $\Sigma\nu_i$ was close to zero indicating that cation accumulation ($\nu_{Na}+\nu_K+\nu_{Mg}$) approximately equalled anion exclusion ($-\nu_{Cl}$). As the concentration of TMAO was increased in the salt solution, however, the cation accumulation increased (especially ν_{Na} and ν_K) while $-\nu_{Cl}$ decreased.

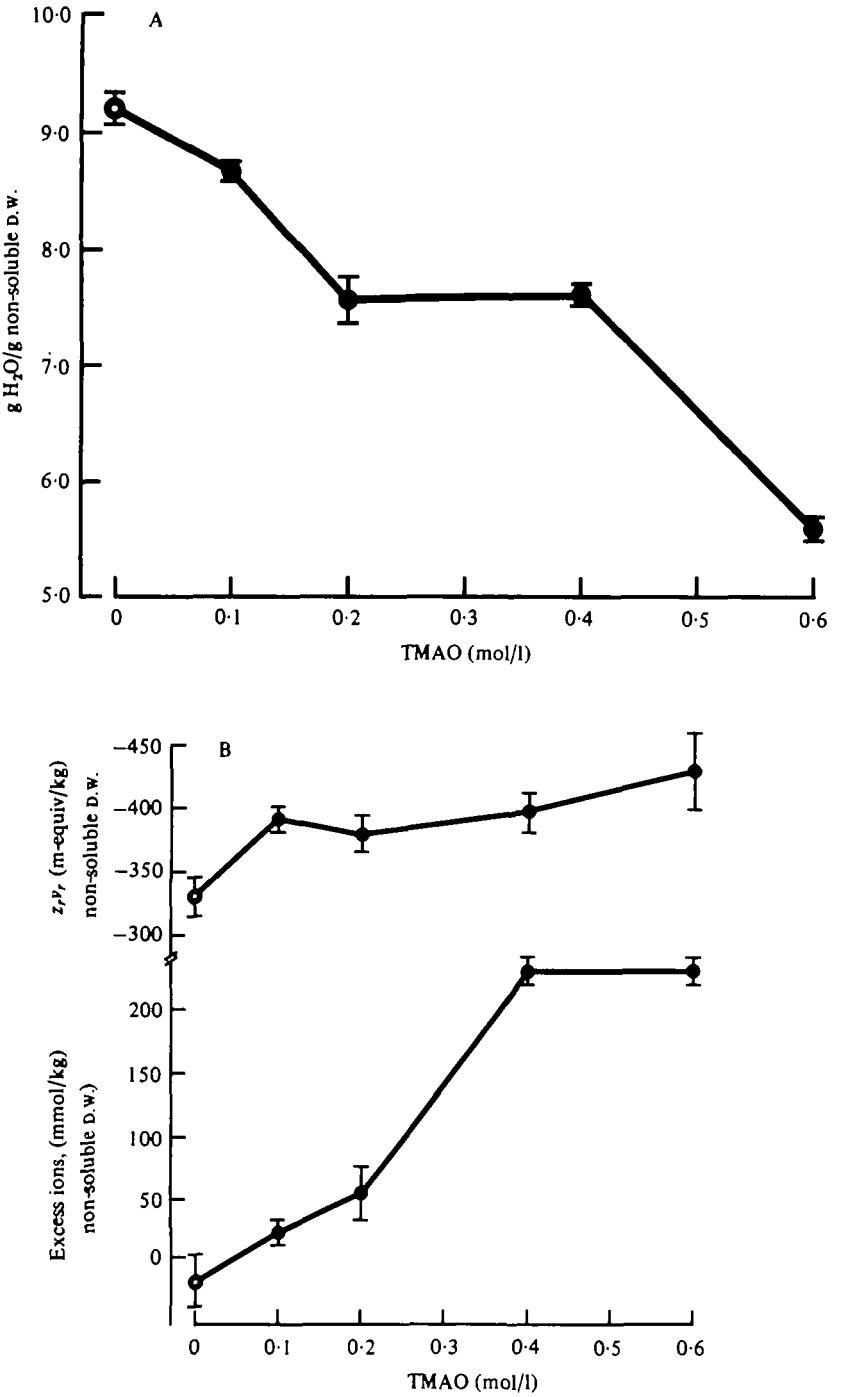

Fig. 4. A. Effect of increasing TMAO (in 1x salt solution) on the water content of membrane-damaged fibres. B. Effect of increasing TMAO on the excess ion content (lower curve) and on the myofilament charge (upper curve) of membrane-damaged fibres. Vertical bars = ±1 S.E.M. (n = 8 fibres for each point).

Table 1. *Effect of increasing TMAO concentration on fibre ion content and fixed charge at constant (1x) ionic strength*

(Mean ± S.E.; n = 8.)

Equilibration solution*	Total ions in fibre: Measured Σ (ions) (mM/l)	Ratio fibre/bath	$z_r\nu_r$ (m-equiv/kg)†	Excess individual ions (mmol/kg)†: ν_{Na}	ν_K	ν_{Mg}	ν_{Cl}	$\Sigma\nu_i$
1x salt	435·4	1·00	−333	14·9	69·1	50·2	−148·7	−15
	±2·5		±16	±3·2	±7·6	±0·6	±18·6	±23
1x salt + 0·1 M-TMAO	439·6	1·01	−392	24·2	106·5	51·2	−158·9	23
	±1·3		±11	±2·3	±7·6	±1·4	±9·2	±11
1x salt + 0·2 M-TMAO	443·6	1·02	−378	20·0	122·2	53·1	−142·3	53
	±2·7		±17	±5·6	±3·7	±0·7	±16·2	±20
1x salt + 0·4 M-TMAO	466·4	1·07	−394	52·1	173·7	55·7	−56·7	225
	±1·5		±16	±3·7	±4·1	±0·6	±13·0	±12
1x salt + 0·6 M-TMAO	477·3	1·09	−425	42·5	192·3	59·9	−70·4	224
	±2·3		±30	±5·7	±11·0	±2·5	±12·0	±10

* Final pH of all solutions = 7·0 ± 0·1.
† kg = kilogram of nonsoluble dry wt in fibre.

The nearly constant net charge ($z_r\nu_r$) of about 400 m-equiv/kg non-soluble solids in the presence of TMAO at pH 7 (Table 1) equals 40 equivalents of negative charge for every 10^5 g of contractile protein. In comparison, Lewis & Saroff (1957) calculated a net charge of -50 equiv/10^5 g of purified myosin A and Collins & Edwards (1971) calculated a capacity of -33 equiv/10^5 g of protein in glycerinated frog muscle.

(*e*) *Effects of propionate substitution for chloride.* In another experiment, propionate was substituted for varying amounts of chloride in the equilibrating solution to see if this altered the swelling effects of the salt solutions and the reversal of such effects by TMAO. As shown in the upper half of Table 2, such substitution reduced fibre swelling to some extent (12·9 to 9·7 g H_2O/g non-soluble dry weight), and increased $\Sigma\nu_i$ (88 to 138 mmol/kg). In these respects, propionate substitution appears to mimic the effects of TMAO.

Propionate substitution complemented the effects of TMAO in reducing the swelling of the fibre (9·1 to 7·3 g H_2O/g non-soluble dry weight), but antagonized the marked increase in $\Sigma\nu_i$ (367 to 230 mmol/kg) brought about when TMAO was added to chloride-containing salt solutions (Table 2, lower half). Note that in both the absence and the presence of TMAO, propionate reduced $z_r\nu_r$ about equally (-475 to -345 m-equiv/kg), suggesting that this organic anion may be suppressing proton dissociation from some fixed charge sites.

(*f*) *Electronmicroscopy of membrane-damaged fibres.* Fig. 5 provides typical examples of cross-sections of membrane-damaged fibres equilibrated for 40 h in salt solutions with and without the addition of 0·5 M-TMAO. The left hand series of sections show

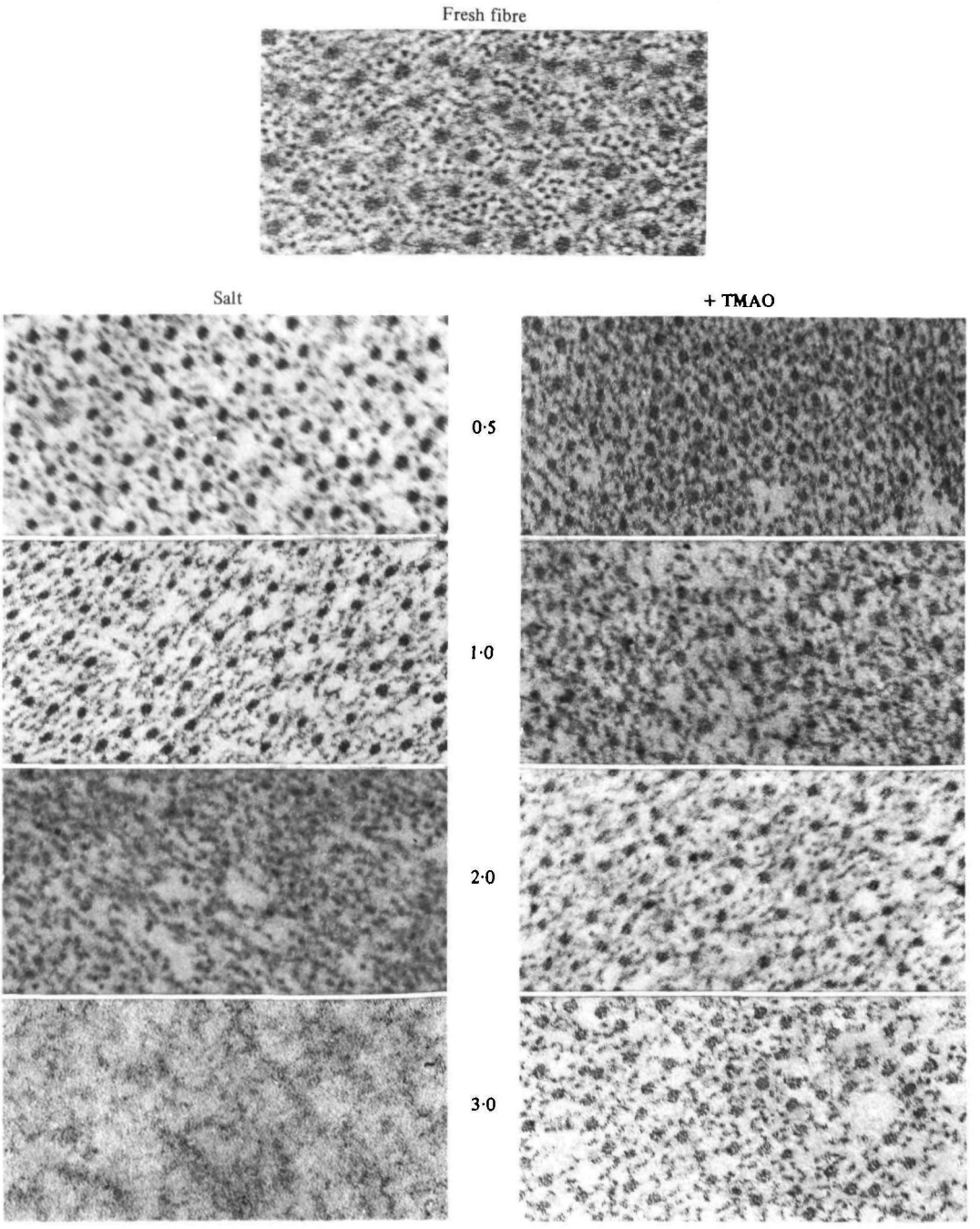

Fig. 5. Typical electronmicrographs of cross sections of myofibrils, in the region of thick-thin filament overlap. Top, intact fibre. Remainder are fibres equilibrated for 40 h in experimental solutions of the relative ionic strengths shown. Salt-only solutions for the four left sections; salt + 0·5 M-TMAO solutions for the four right sections. (108,000 × magnification).

Table 2. *Effect of replacing Cl with propionate on fibre water, ion content and fixed charge at constant (1x) ionic strength*

(Mean ± S.E.; n = 10.)

Equilibration solution*	Fibre hydration g H_2O / g nonsolute dry wt	Total ions in fibre: Measured Σ (ions) (mM/l)	Total ions in fibre: Ratio fibre/bath	$z_r\nu_r$ (m-equiv/kg)	Excess individual ions (mmol/kg): ν_{Na}	ν_K	ν_{Mg}	ν_{Cl}	ν_{Pr}	$\Sigma\nu_i$
Salt only										
12 mM-propionate	12·9 ± 0·4	450·7 ± 2·2	1·02	−475 ± 29	49·7 ± 5·4	163·1 ± 11·0	45·9 ± 2·0	−145·5 ± 21·4	−24·9 ± 0·9	88 ± 25
62 mM-propionate	11·9 ± 0·4	447·5 ± 1·0	1·02	−518 ± 14	55·8 ± 3·2	178·6 ± 5·5	49·0 ± 1·0	−95·9 ± 11·0	−89·9 ± 2·9	98 ± 12
182 mM-propionate	9·7 ± 0·2	452·7 ± 2·9	1·03	−340 ± 13	43·7 ± 2·3	148·3 ± 6·2	31·3 ± 0·9	22·4 ± 10·0	−107·7 ± 2·4	138 ± 14
Salt + 0·5 M-TMAO										
12 mM-propionate	9·1 ± 0·2	452·7 ± 2·9	1·09	−476 ± 10	86·7 ± 5·0	245·0 ± 6·7	59·6 ± 0·8	−19·0 ± 6·7	−5·7 ± 0·1	367 ± 9
62 mM-propionate	8·9 ± 0·1	471·3 ± 1·5	1·07	−407 ± 18	75·2 ± 2·1	180·0 ± 10·9	51·3 ± 1·0	−38·2 ± 8·3	−11·5 ± 0·1	257 ± 10
182 mM-propionate	7·3 ± 0·1	469·8 ± 3·4	1·07	−345 ± 8	64·5 ± 2·0	169·5 ± 3·6	35·8 ± 0·5	19·6 ± 6·1	−59·1 ± 0·5	230 ± 8

* Final pH of all solutions = 7·0 ± 0·1.

that without TMAO there was a loss of integrity of the myofilament architecture, that began in 2x strength salt solution (compare with section of intact fibre at top) and was virtually complete at 3x; distinct thick and thin filaments are no longer visible. This is consistent with the well-known observation that contractile proteins are soluble at high (0·6 M) salt concentrations. Dry weight analyses, however, indicate that the losses of myofilament proteins were relatively small even at 3x ionic strength, probably owing to the maintenance of a constant ratio of Mg to monovalent cations in all bathing solutions, which prevented complete solubilization (Sarkar, 1950). The right hand series of sections illustrates that TMAO in the salt solutions helps to preserve myofilament architecture, even up to 3x ionic strength. Not too much importance is given to the relative diameters of the thick filaments since Hoyle, McNeill & Selverston (1973) reported variations in the diameter of the thick filaments in different parts of the sarcomere in fresh barnacle fibres.

Results with other organic solutes

(*a*) *Amino acids.* Four neutral amino acids, glycine, alanine, serine, and proline, that are normally found at relatively high concentrations inside cells of marine invertebrates were studied for their effects on fibre swelling net fixed charge and ion distribution in membrane-damaged fibres. Fresh barnacle muscle contains about 280 mM-glycine and about 50 mM-proline (Clark & Hinke, 1980). The equilibrating solutions in this experiment contained salt at 1x ionic strength and an amino acid in the range of 0·5 to 0·6 M. In contrast to TMAO which tends to concentrate to 1·05x in the fibre, the amino acids are excluded; fibre content ranged from 0·88x for glycine to 0·92x for proline.

As Fig. 6 shows, all four amino acids behave similarly to TMAO in reducing the degree of hydration in the fibre, and in increasing the total ion accumulation ($\Sigma\nu_i$) without altering the net fixed charge ($z_r\nu_r$). The results indicate that glycine is the least effective and proline is the most effective of the four in reducing the swelling effect of the salts.

(*b*) *Glycerol and urea.* The effects of 0·2 to 0·6 M-glycerol or urea upon fibre swelling were studied in the 1x salt solution. Like TMAO (Fig. 4), glycerol reduced the water content of membrane-damaged fibres (Fig. 7*A*) and increased total excess ion accumulation without greatly affecting the protein net charge (Fig. 7*B*). Like amino acids, glycerol was somewhat excluded from the fibres (0·86x). The effect of urea was different in that it did not reduce the swelling of the fibres but it did increase the total excess ion accumulation. Urea tended to distribute equally between the fibres and the bath (0·98x).

DISCUSSION

Our results may be summarized as follows. When membrane-damaged barnacle muscle fibres are exposed to increasing salt concentrations they swell and myofilament architecture is disrupted. The swelling is accompanied by an increase in net fixed negative charge ($z_r\nu_r$), but there is little change in ion distribution between the fibres and bath. If TMAO, or one of several amino acids, or glycerol (but not urea) is also present in the bathing medium, swelling and disruption of myofilament architecture

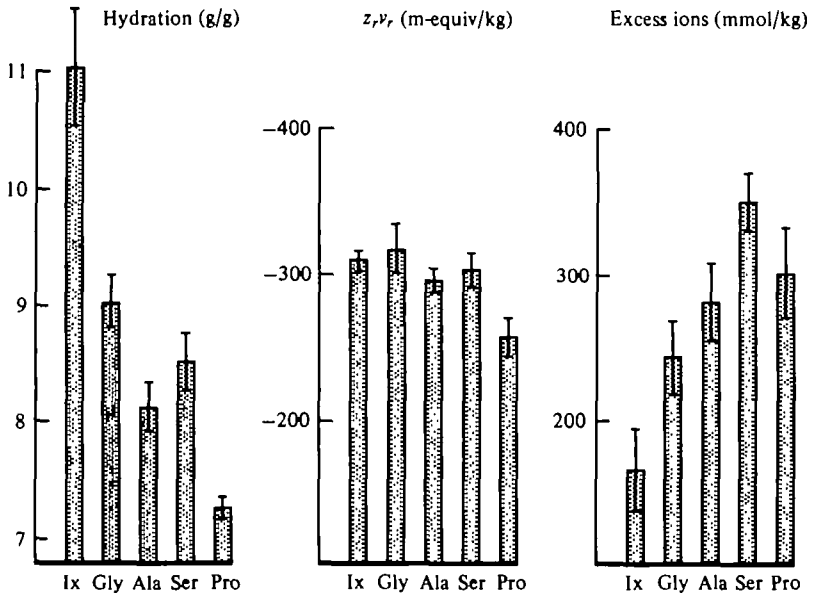

Fig. 6. The water content (hydration), the net filament charge ($z_r\nu_r$) and the excess ion ($\Sigma\nu_i$) content of membrane-damaged fibres equilibrated for 40 h in experimental solutions: Ix equals basic salt solution only; Gly equals Ix salt + 0·5 M-glycine; Ala equals Ix salt + 0·5 M-alanine; Ser equals Ix salt + 0·5 M-serine; Pro equals Ix salt + 0·5 M-proline. Vertical bars = ± 1 S.E.M. (n = 10 fibres).

are suppressed, even though $z_r\nu_r$ remains unchanged. The presence of these solutes also results in a rise in the relative amounts of both cations and anions in the fibre with increasing ionic strength.

Our interpretation of these results is considered in four steps: (1) the effect of myofilament charge on fibre water content; (2) the effect of ionic strength on myosin charge; (3) the effect of organic solutes on counterion association; and (4) the role of organic solutes in volume regulation of living cells.

Effect of myofilament charge on fibre water content

As noted in the Introduction, X-ray diffraction studies have shown that volume changes in both intact and membrane-damaged muscle fibres are reflected in changes in the lattice spacing of the contractile filaments (Rome, 1968; April, Brandt & Elliott, 1972; Matsubara & Elliott, 1972). We, too, interpret the volume changes reported here in terms of the water content of the myofilament lattice.

The equilibrium volume of such a lattice of charged filaments may be calculated either on the basis of a Donnan equilibrium (Collins & Edwards, 1971) or as a liquid crystal whose dimensions are a balance between van der Waals' attractive forces and electrostatic repulsive forces (Bernal & Fankuchen, 1941; Elliott, 1968). Both models predict volume changes with variations in pH and ionic strength of the intervening medium. In simplest form, Donnan equilibrium principles provide the following relationship among the net fixed charge in a fibre ($z_r\nu_r$), its water content (g H_2O/g

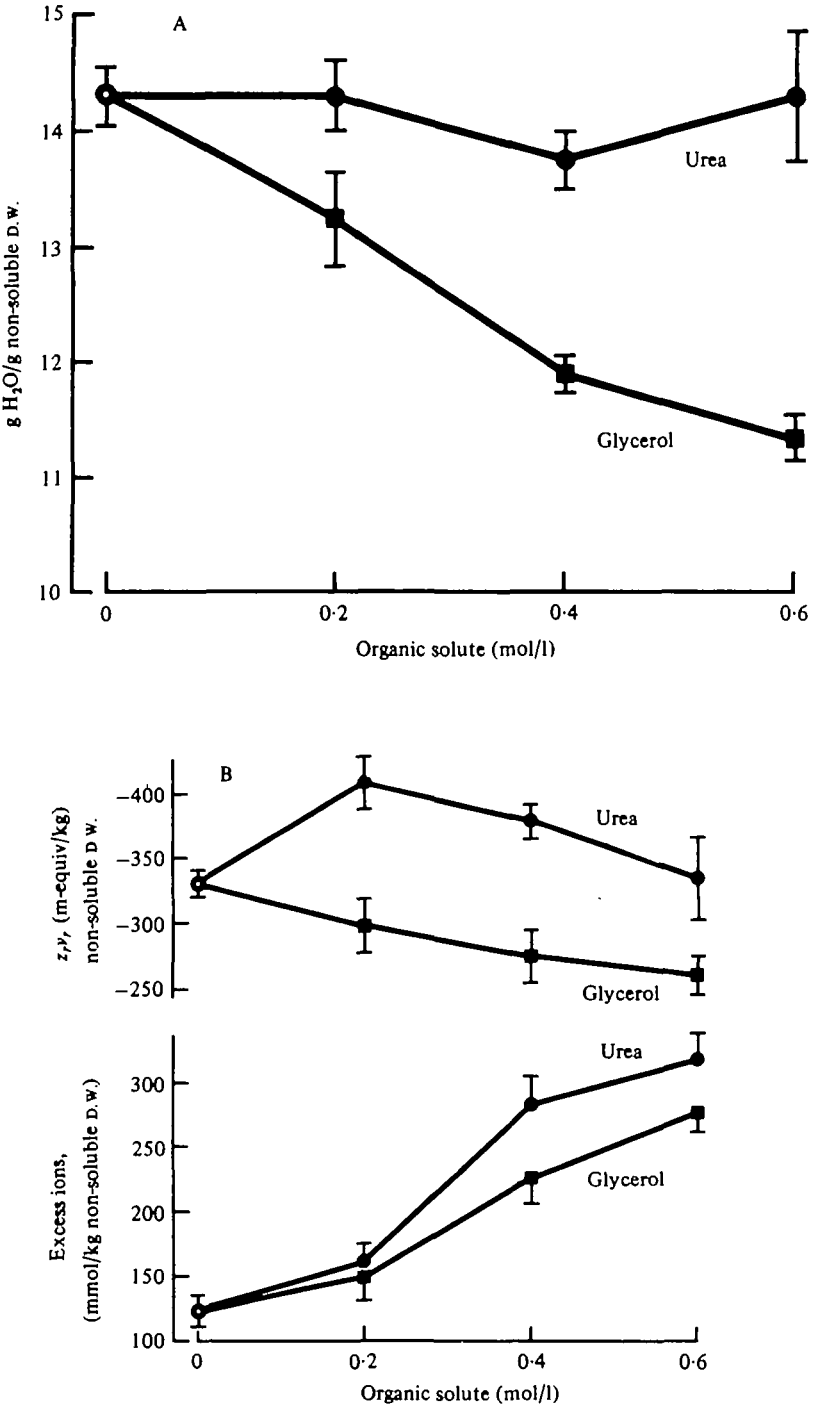

Fig. 7. Effect of glycerol and urea on the water content (Fig. 7A), the net filament charge (Fig. 7B, upper curves) and the excess ion content (Fig. 7B, lower curves) of membrane-damaged fibres (40 h equilibration). Each experimental solution contained the basic (1x) salt solution. Vertical bars = ± 1 S.E.M. (n = 10 fibres for each point).

dry wt.), the salt concentration of the bath (C_0), and the Donnan potential (E_D) between the lattice and the surrounding medium (Collins & Edwards, 1971):

$$z_r\nu_r = \left[\exp\frac{E_D F}{RT} - \exp\frac{-E_D F}{RT}\right] \cdot \left[\frac{\text{g } H_2O}{\text{g dry wt}} \cdot C_0\right]. \quad (3)$$

For constant values of $z_r\nu_r$ and E_D, equation (3) predicts an inverse relation between fibre volume and external salt concentration. (This equation assumes a constant ratio of ion activity coefficients between the fibre and the bath at all ionic strengths.)

Changes in pH have been found by others to bring about the expected changes in volume of membraneless fibres, presumably owing to titration of fixed charge, but increase in ionic strength causes swelling rather than shrinking (Rome, 1968; April, Brandt & Elliott, 1972). With increasing salt concentration, membrane-damaged barnacle muscle also swells, in a very striking fashion (upper curve, Fig. 1). In terms of equation (3), this swelling can be explained by either a decrease in E_D with ionic strength, or by an increase in $z_r\nu_r$.

Although these two effects are not mutually exclusive, we suggest that much of the swelling with increasing ionic strength in barnacle muscle is due to increasing net negative charge, as shown in Fig. 2. A similar increase in fixed charge with increasing ionic strength has been observed recently by Caillé (1979) in barnacle fibres constrained to constant volume. In 3x basic salt solution ($\sim$0·6 M), $z_r\nu_r$ reaches a value of -940 mequiv/kg non-soluble dry weight, or -94 equiv/per 10^5 g of myosin, well within the total number of free carboxyl groups ($\sim 165/10^5$) in myosin (Mihalyi, 1950). With such large changes in net fixed charge capacity, equation (3) predicts swelling rather than shrinking of the myofilament lattice with increasing ionic strength. In fact, if the data from Fig. 1 and Fig. 2 for salt-only fibres are combined and inserted into equation (3), it is possible to predict a decrease in E_D (from -2 to -1 mV) as the salt concentration, C_0, is raised from 1x to 3x basic salt solution. Any deviation from equation (3) obtained by simultaneous measurements of E_D would indicate the magnitude of other factors, such as counterion binding ($z_r\nu_r$ too high), non-solvent water (g H_2O/g dry wt. too high), or the effects of salt on reducing the van der Waals' attractive forces, as suggested by April, Brandt and Elliott (1972).

The effect of ionic strength on myosin charge

Although in barnacle muscle only about 36 % of myofilament protein is myosin (see below) this is the most studied of the contractile proteins and forms the basis of this discussion. We assume actin and other myofilament proteins behave somewhat similarly.

Most of the models developed to account for the lattice-spacing of cylindrical polyions such as the thick filament lattice of muscle assume negligible changes in the dissociation of protons with ionic strength (see, for example, Brenner & McQuarrie, 1973). This assumption at first appears to be confirmed by the negligible effect of ionic strength on the myosin dissociation curves described by Mihalyi (1950). On the other hand, Sarkar (1950) observed shifts in the isoelectric point of myosin of more than three pH units between KCl solutions of 0·025 M ($\sim$ 0x) and 0·6 M ($\sim$ 3x). These he explained as due to binding of K ions at low ionic strength and of Cl ions at

high ionic strength. The change in $z_r\nu_r$ with ionic strength, seen in our Fig. 2, however, is not compatible with this explanation and we conclude that the assumed convergence at pH 5·76 of isoionic points (in the sense of 50% of titratable protons) indicated by Mihalyi cannot be correct.

An increase in net charge with increasing ionic strength is in accordance with the well known solubility of myosin that occurs at high salt concentrations (Noda & Ebashi, 1960; Huxley, 1963; Josephs & Harrington, 1966). In our electronmicrographs, we observed an increasing disruption of thick filaments as salt concentration was raised from ~0·4 M (2x) to ~0·6 M (3x) (Fig. 5, left). The failure of the myofilaments to undergo complete solubilization at these high ionic strengths we attribute to the maintenance of a constant proportion of Mg to other cations in the bath. As shown by Sarkar (1950), at concentrations of 10 to 30 mM, Mg greatly broadens the pH range within which myosin exhibits reduced solubility.

Thus, the result of increasing ionic strength on myofilament charge is exhibited in two steps; first swelling of the lattice at physiological ionic strengths, followed by disruption of the myofilaments at excessive ionic strengths.

The effect of organic solutes on counterion association

The clear-cut effect of small organic solutes such as TMAO, amino acids, glycerol, and propionate in reversing the effects of ionic strength on fibre swelling (Figs. 1, 4*A*, 6, 7*A*; Table 2) and on the disruption of myofilament architecture (Fig. 5, right) is a new observation not predicted by previous investigators. The effects of TMAO (Fig. 4*A*) and of glycerol (Fig. 7*A*) on water content are approximately linear with the concentration of these solutes in the bath. Furthermore, the concentration of TMAO (0·5 to 0·6 M) required to suppress swelling at all ionic strengths is comparable with the sum of the nitrogenous solutes in the intact fibre (Clark & Hinke, 1980).

TMAO, amino acids, and glycerol do not appear to be acting by suppressing salt induced proton dissociation from myofilament proteins, since the net negative charge, $z_r\nu_r$, is similar in their presence or absence (Figs. 4*B*, 6, 7*B*). On the other hand, all of these solutes appear to facilitate univalent cation accumulation within the fibre and reduce anion exclusion (Figs. 3, 4*B*, 6, 7*B* and especially Table 1). (Note that propionate appears to act in a different way (Table 2); except for favouring Cl accumulation, it has little effect on ion distribution, apparently exerting its main effect on fibre hydration through reduction of $z_r\nu_r$).

In searching for the mechanism by which organic solutes affect myofilament hydration, we note that the solutes employed have no net charge (TMAO, glycerol, neutral amino acids) or are of the wrong charge (propionate) to screen directly the negative fixed charge. Although TMAO is slightly concentrated (1·05x) in the fibre, amino acids (0·88x to 0·92x) and glycerol (0·86x) are somewhat excluded. Thus, these solutes do not appear to be acting by binding directly to the myofilaments.

According to Donnan theory, in the absence of ion binding, the distribution ratio of cationic species between fibre and bath should be the reciprocal of that for the anionic species. Using the data from which Figs. 4*A*, *B* and Table 1 were generated, we can compare the ratios found in fibres in 1x salt solutions containing no TMAO with those found in a solution containing 0·6 M-TMAO, as shown in Table 3. (We hav

Table 3. *Ratios of the distribution of univalent cations and anions between fibre and bath in* 1*x salt solutions without TMAO and with* 0·6 *M TMAO added*

	$\frac{g\ H_2O}{g\ nonsolute\ dry\ wt}$	$\frac{[Na+K]_{fibre}}{[Na+K]_{bath}}$	$\frac{[Cl]_{bath}}{[Cl]_{fibre}}$
no TMAO	9·21	1·05	1·07
0·6 M-TMAO	5·58	1·23	1·06

ignored the Mg ion distribution since this is so little affected by TMAO, presumably because much of the Mg is bound; also, we group Na and K ions together for convenience, having found no consistent selectivity for one ion over the other in these fibres.) As already noted (see Fig. 3), when only salts are present, the cations and anions contribute about equally to the distribution of mobile charges between bath and fibre, as to be expected if none of the ions are bound. When TMAO is added, the distribution ratio of Cl ions remains unchanged; thus the apparent decrease in Cl ion exclusion ($+\nu_{Cl}$) shown in Table 1 may simply be a result of decreasing fibre water content. On the other hand, the univalent cation ratio is much higher than predicted from the Cl distribution, leading to an excess of + 170 m-equiv/kg myofilament dry weight. This leads us to propose that organic solutes enhance the association of univalent cations with the fixed charge sites which they screen.

In order to estimate the fraction of the myofilament fixed charge that would need to be affected by the presence of organic solutes to achieve the observed changes in hydration, we have employed the relationship provided by Oosawa (1971) between the degree of dissociation (β) of mobile counterions and the apparent volume fraction (ϕ) of cylindrical charged macromolecules in an aqueous system:

$$ln\left(\frac{1-\beta}{\beta}\right) = ln\left(\frac{\phi}{1-\phi}\right) + \beta Q.\ ln\left(\frac{1}{\phi}\right). \tag{4}$$

The Q term in this equation is defined as:

$$Q = \frac{{e_0}^2}{\epsilon_0 kTd}, \tag{5}$$

where d is the average distance between charge sites on the macromolecule, e_0 is the electronic charge, ϵ_0 is the dielectric constant of the solvent, k is the Boltzman constant and T is absolute temperature. Clearly, Q determines the intensity of counterion binding and is directly related to the total number (n) of fixed charges or inversely related to d ($d = l/n$ where l is length of macromolecule).

An average value for d in our muscle system can be estimated from the $z_r\nu_r$ values (e.g. Fig. 4*B*) combined with knowledge of the dimensions of the thick and thin filaments, and their ratio in the myofibril. In a morphological study of barnacle muscle, Hoyle *et al.* (1973), measured the average dimensions of thick filaments to be 5·2 μm long and 140 Å diameter, and of thin filaments to be 7·0 μm long and 80 Å diameter. They also estimated the thin/thick filament ratio to be about 4·5 which suggests a weight ratio of 64/36% for the thin and thick filaments in the barnacle fibre. If one accepts from Fig. 4*B* that in 1x basic salt solution the total net charge on

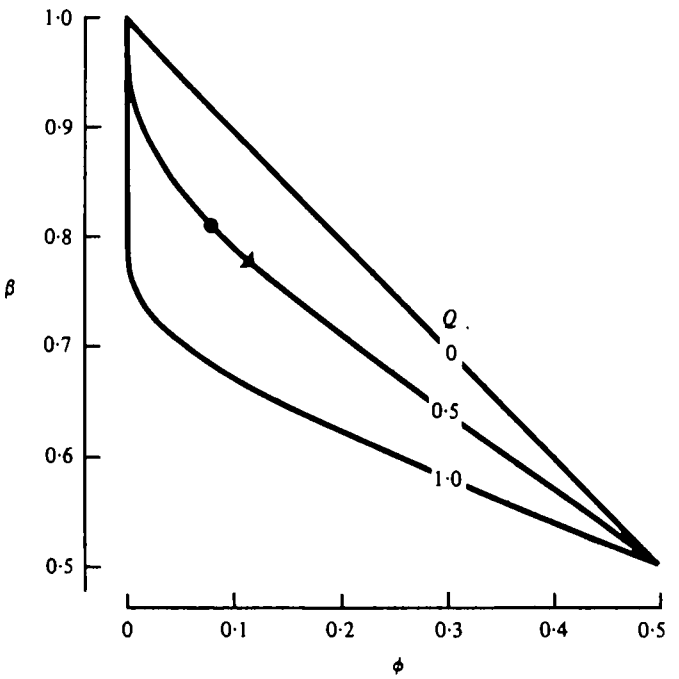

Fig. 8. The degree of dissociation (β) of the charged groups as a function of the apparent volume (ϕ) of cylindrical macromolecules in aqueous solution, obtained from equation 4 using three values for Q (equation 5). The point corresponds to the water content of a fibre in 1x salt +0·1 M-TMAO (Fig. 5A) and the arrowhead corresponds to that of a fibre in 1x salt+0·6 M-TMAO (Fig. 5A).

the filaments is -400 m-equiv/kg protein and assigns half of this charge to thick filaments and half to thin filaments, then d becomes 8·8 Å on the thick filaments and 15·6 Å on the thin filaments. Using the thin/thick ratio of 4·5, we calculate an average Q value of about 0·5. The value of ϕ for a given fibre treatment was obtained from the water content per g non-soluble dry weight, assigning a density of 1·0 to the water and of 1·3 to the myofilament protein (Hinke, 1970).

Fig. 8 illustrates the relationship between counterion dissociation (β) and the apparent volume fraction of the macromolecule (ϕ) at three Q values. On the Q = 0·5 curve, the point coincides with the water content of a fibre in 1x salt solution + 0·1 M-TMAO and the arrowhead coincides with the water content of a fibre in 1x salt solution + 0·6 M-TMAO (Fig. 4*A*). The corresponding decrease in β is from 0·81 to 0·77. This exercise indicates that when Q = 0·5 then only a 4·7% increase in counterion association is required to induce a 33% decrease in the fibre water content. Ignoring the effects of changes in the dielectric constant of the medium upon the addition of various solutes is unlikely to have a significant effect on the predicted water content since, as shown in Fig. 8, even large errors in Q do not greatly affect the fractional change in β over the range of water content observed in the fibres.

The mechanism by which organic solutes may effect the postulated changes in counterion binding is not clear. We note that most models for the lattice volume

cylindrical charged macromolecules assume constant properties for the intervening solution, ignoring any special hydration requirements of the macromolecular surface (Oosawa, 1971; Brenner & McQuarrie, 1973). On the other hand, it has repeatedly been shown that between 20 and 30% of myoplasmic water in intact barnacle muscle behaves as 'nonsolvent' water (Hinke, 1970; Caillé & Hinke, 1974; Pezolet, Pigeon-Gosselin, Savoie & Caillé, 1978), and it seems probable that much of this water is located at the surface of the myofilament proteins. Possible changes in the properties of this water, induced by selected organic solutes, could affect the degree of counterion association, and hence the distribution of mobile ions between fibre and bath.

It is tempting to propose that the actions of these solutes are somehow due to their polar nature. Yet the results with urea (Fig. 7*A*, *B*) show that not all polar solutes are equivalent; urea causes increased ion acumulation in muscle fibres but does not reduce swelling. Likewise, compared to amino acids and TMAO, which are nonharmful or even activating for enzymes (Clark & Zounes, 1977; Yancey & Somero, 1979), urea is deleterious, but its effects can be countered by other nitrogenous solutes, especially methylamines (Yancey & Somero, 1979). In those vertebrates that utilize urea as an osmotic effector, there is always a significant intracellular concentration (> 100 mM) of TMAO and betaine (Lutz & Robertson, 1971; Robertson, 1975, 1976). At this stage, we are left with the empirical observation that certain solutes in nature (amino acids, betaines, taurine, glycerol) and in the test tube (ammonium sulphate, sucrose) are biologically preferred to most inorganic ions, to urea, and perhaps to other solutes as well, without being able to explain the basis for their different effects.

Role of organic solutes in volume regulation of living cells

These experiments suggest that TMAO, certain amino acids, and glycerol may regulate the volume of an intact cell in two independent and opposite ways. Accumulation of these solutes in cells under conditions of water loss (e.g. high external osmotic pressure or desiccation through evaporation or freezing) assists in osmotic volume regulation, as discussed in the Introduction; shrinkage owing to water loss is prevented. Similarly, swelling of fibres when external osmotic pressures are lowered is compensated by loss of these organic solutes from the cell. As a simple osmotic effector, urea is also utilized by a number of vertebrate groups.

It is usually observed, however, that muscle cell volume regulation in euryhaline organisms is imperfect (see, for example, Freel, 1978). This may be due in part to the sharp fall in the ratio of organic solutes/ions within these fibres during osmotic dilution, resulting in an increased counterion dissociation and hence a greater myofilament lattice spacing. Likewise, the swelling regularly observed on damage to the Na-pump or to the sarcolemma of a fibre bathed in physiological salt solution may be due not only to the osmotic entry of water following the inward diffusion of previously excluded Na ions (April, 1975; Godt & Maughan, 1977; MacKnight & Leaf, 1977); it may also be due to the loss of organic solutes or to a failure to retain them through a Na-coupled transport system (Schultz & Curran, 1970). This would result in an increased proportion of inorganic ions to (1) organic acids which, like propionate, may help suppress $x_r v_r$; and (2) to nitrogenous solutes which, except for urea, may suppress swelling through counterion binding.

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