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CH_2-Units on (Poly-)ethylene Glycol Radially Dehydrate Cytoplasm of Resting Skinned Skeletal Muscle

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Observing the optical cross-section and electron micrographs of mechanically skinned fibres of frog skeletal muscle, we found that ethylene glycols (EGs) of small (mono-, di-, tri- and tetra-EGs; M_r 62–194) and medium (poly-EGs; M_r 900 and 3350) molecular weights efficiently dehydrate the fibres to shrink them radially without microscopic inhomogeneity. The medium-sized poly-EGs at 30% weight/weight concentration absorbed almost all the evaporable water from the fibre. Passive tension measurement at near slack sarcomere spacing indicated that this dehydration by EGs did not accompany longitudinal fibre shrinkage. Chemically relevant fully hydric alcohols (glycerol, threitol, ribitol and mannitol; M_r 92–182) showed no appreciable dehydrating ability on fibres. An intimate correlation was found between fibre dehydration and CH_2-concentration of the solutions. Viscosity measurements indicated that the hydrodynamic radii of the alcohols were comparable to those of the small EGs. Therefore, hydrodynamic radii are not a primary determinant of the dehydrating ability. Additionally, CH_2-concentration of EGs but not alcohols was found to correlate intimately with the measured viscosity of the bulk solution of EGs. These results suggested that the interaction between water molecules and CH_2-units in crowded cytoplasm of skeletal muscle affects cytoplasm as a whole to realize anisotropic fibre shrinkage.

Key words: cytoplasm, hydration, polyethylene glycol, skeletal muscle, skinned fibre.

Abbreviations: EGs, ethylene glycols; PEG, polyethylene glycol; PVP, polyvinylpyrrolidone; Ms, methanesulfonic group -CH_3SO_3; $C_{w/v}$, weight/volume concentration; [η_c], viscosities at various weight/volume concentration; [η_0], viscosities of plain relaxing solution; [η], intrinsic viscosity; R_h, hydrodynamic radius.

Cytoplasm of biological cells is crowded with various molecules. Biophysics of such crowded milieu is essential for the understanding of life (*1*). A skinned fibre of skeletal muscle, which is a demembranated cell preparation (*2*), is an ideal experimental model of general cytoplasm. The functional state of the fibre can be controlled by incubating solution to one of the resting, contracting and rigor states.

In the fibre, myoproteins are condensed to a level empirically unattainable with the centrifugation of purified myoproteins. In addition, the main component of the fibre, myofibril, consists of regular repeats of longitudinal sarcomere structure and radial hexagonal lattice of myofilaments (*3*). The spacing of both repeats during experiments can be monitored or estimated by optical techniques without fixing the specimen (*3–6*).

We recently evaluated the state of cytoplasmic water of resting skeletal muscle by ^{1}H-NMR (*7*, *8*) and found that cytoplasmic solution consists of several components of different water states. Since efflux of macromolecular solutes from the cell upon skinning was considered to have marked effects on the heterogeneous states of cytoplasmic water (*8*), it is of interest to study the effect of exogenous solutes on skinned fibres.

As for inert macromolecular solutes such as polyvinylpyrrolidone (PVP-K30, M_r 40,000) and Dextran T-500 (M_r 500,000), they are well documented to dehydrate skinned fibres (*9*). The dehydration is generally ascribed to the osmotic effect due to the physical exclusion of the macromolecules from the myofilament lattice. On the other hand, inert small molecules such as ethylene glycol and glycerol have been generally considered to penetrate freely into the cytoplasm of skeletal muscle without osmotically dehydrating the fibres (*10*). One exception is that, in the case of skinned barnacle muscle, Clark *et al*. (*11*) reported that several small neutral organic molecules including glycerol dehydrate the muscle. They hypothesized the dehydration to be a consequence of enhanced association of fixed charges on myofilaments with the counter ions to reduce cytoplasmic osmolyte in the presence of the organic solutes. However, the cause of the enhanced association, and the effects of possible alteration in the hydration of constituent proteins and the small organic molecules have not been considered.

In the present study, we aimed a systematic survey of the effect of ethylene glycol and its polymers (ethylene glycols; EGs), and fully hydric alcohols such as glycerol on the volume of resting skinned fibres. Several workers have studied the effect of EGs on the specific interaction between actin and myosin heads in skinned skeletal muscle at contracting and rigor conditions (*10–13*). However, they paid limited attention to the effect of

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Table 1. **A list of tested small and medium-sized molecules.**

Tested solutes		molecular weight	structural formula	origin
Ethylene glycols (EGs)	Ethylene glycol (EG)	62	$H\text{-}(CH_2CH_2O)_n\text{-}OH$ ($n=1$)	Wako pure chemincal industries, Tokyo, Japan
	Diethylene glycol (di-EG)	106	$H\text{-}(CH_2CH_2O)_n\text{-}OH$ ($n=2$)	Kanto Kagaku, Tokyo
	Triethylene glycol (tri-EG)	150	$H\text{-}(CH_2CH_2O)_n\text{-}OH$ ($n=3$)	Tokyo Kasei Kogyo, Tokyo
	Tetraethylene glycol (tetra-EG)	194	$H\text{-}(CH_2CH_2O)_n\text{-}OH$ ($n=4$)	Sigma-Aldrich, Missouri, USA
	Polyethylene glycol (PEG900)	900	$H\text{-}(CH_2CH_2O)_n\text{-}OH$ ($n=20$)	Sigma-Aldrich
	Polyethylene glycol (PEG3350)	3,350	$H\text{-}(CH_2CH_2O)_n\text{-}OH$ ($n=76$)	Sigma-Aldrich
Fully-hydric alcohols	Glycerol	92	$CH_2OH\text{-}(CHOH)_n\text{-}CH_2OH$ ($n=1$)	Sigma-Aldrich
	DL-threitol	122	$CH_2OH\text{-}(CHOH)_n\text{-}CH_2OH$ ($n=2$)	Sigma-Aldrich
	Ribitol	152	$CH_2OH\text{-}(CHOH)_n\text{-}CH_2OH$ ($n=3$)	Wako pure chemincal industries
	D-(-)-manniol	182	$CH_2OH\text{-}(CHOH)_n\text{-}CH_2OH$ ($n=4$)	Kanto Kagaku
Dextran	Dextran 9k	9,000	$[C_6H_{10}O_5]_n$ ($n=56$)	Kanto Kagaku
	Dextran 488k	488,000	$[C_6H_{10}O_5]_n$ ($n=3012$)	Kanto Kagaku

EGs on whole fibre volume. The present study focuses on resting fibre volume as an ideal representative of the volume of cytoplasm in general. The present correlation analysis of the fibre-dehydrating ability of EGs with their chemical structure, hydrodynamic radii and bulk solution viscosity suggested a unique dehydrating mechanism probably characteristic of general cytoplasm.

MATERIALS AND METHODS

Preparation of Mechanically Skinned Fibres—Skinned fibres (*2*) were mechanically prepared from sartorius muscle of *Rana catesbeiana*. A special care was taken to peel as little myofibrils as possible with the cell membrane to obtain skinned fibres homogeneous along their entire length. Prior to measurements, the fibre was treated with relaxing solution containing 0.5% triton-X100 for 15 min to destroy the internal membrane systems, and sarcomere spacing was adjusted with the help of the laser diffraction technique to 2.4 μm, except in the case of tension measurement where the spacing was adjusted to near slack sarcomere spacing of 2.0–2.1 μm.

Solutions—Relaxing solution was composed of (in mM) KMs 26.1, $Mg(M_S)_2$ 5.7, Na_2ATP 4.4, CP 10, EGTA 10 and PIPIES 20, adjusted to pH 7.0 at 20°C, where Ms stands for methanesulfonic group $-CH_3SO_3$. When one of the test solutes listed in Table 1 was solved in the relaxing solution, pH was re-adjusted to 7.0 with HMs. None of the test solutes required pH readjustment by more than 0.2 units. The test solute concentrations were expressed in weight/weight percentages unless otherwise stated.

Measurement of Cross-Sectional Area—Cross-sectional areas of resting skinned fibres were measured microscopically as described in Takemori (*14*) except that a CCD camera equipped with an image analysing system (HCC-574, Flovel, Tokyo, Japan) was used. The outline of the fibre was traced on a cathode-ray tube display for 4–6 times for each measurement. In most cases, the area was measured at a fixed cross-section of the fibre, although the area did not vary significantly along the fibre (±0.7%). The solution was exchanged by draining the bathing relaxing solution in the experimental trough to replace with the next solution within a second. All the measurements were performed at room temperature of 20°C.

Electron Microscopy—Skinned fibres were tied to insect pins to avoid shorting, and then incubated in the relaxing solution with or without EGs (tri-EG and PEG3350) for more than 10 min at 4°C. To fix the fibres, 2% glutaraldehyde was introduced into the incubating relaxing solution, and the fibres were left incubated for more than 1 h at 4°C. Fixed fibres were washed with 0.1 M phosphate buffer (pH 7.4) and postfixed for 1–2 h with 1% OsO_4 in the phosphate buffer. Dehydration and Epon embedding were performed using the standard procedures. Thin (60 nm) sections, stained with uranyl acetate and lead citrate, were observed under H-7500 electron microscope (operating voltage, 80 kV; Hitachi, Japan).

Passive Tension Measurement—Passive tension of skinned fibres was measured with a force transducer (UL-2, NMB, Tokyo) to which a fibre end was connected through a tungsten wire. A manipulator held the other fibre end to enable adjustment of sarcomere spacing monitored by laser diffraction. Each fibre end was tied with a silk monofilament. During the tension measurement, the fibre was soaked in a trough thermoregulated to 4°C.

Viscosity Measurement and Estimation of Hydrodynamic Radius R_h—Viscosities [η_c] at various weight/volume concentrations ($C_{w/v}$) of the test solutes were measured with Ostwald viscometer at 20°C and are expressed relative to that of plain relaxing solution [η_0]. Here, weight/volume concentration was adopted instead of weight/weight percentages. Einstein viscosity relation (*15*) was then used to estimate the hydrodynamic radius (R_h) from the intrinsic viscosity [η], which is the extrapolated value of $(\eta_c - \eta_0)/(\eta_0 C_{w/v})$ at infinite dilution, with an assumption that the hydrated solute molecule takes a spherical contour of radius R_h.

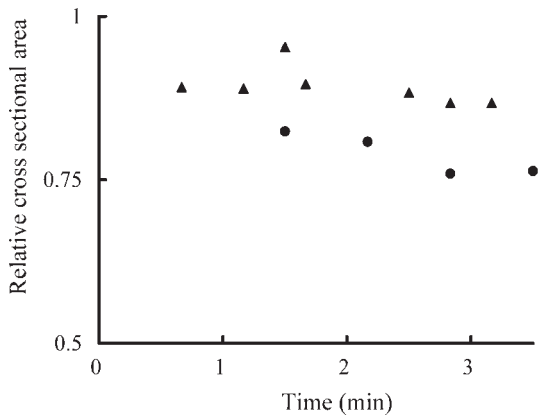

Fig. 1. **Representative time courses of the relative cross-sectional area of mechanically skinned fibres.** Triangles and circles represent the cross-sectional area after immersion in relaxing solution with 20% tri-EG and 5% PEG3350 from plain relaxing solution, respectively.

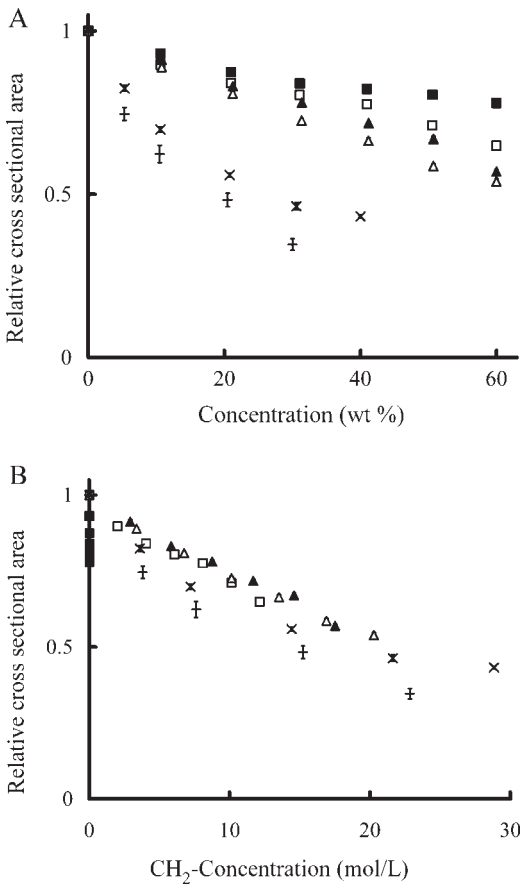

Fig. 2. **Cross-sectional areas of skinned fibres relative to the original value in plain relaxing solution.** Mono-EG, closed squares; di-EG, open squares; tri-EG, closed triangles; tetra-EG, open triangles; PEG900, crosses and PEG3350, pluses. Plots are made against the weight/weight concentration of EGs in panel A, against the molar concentration of CH_2-unit without attached –OH in panel B. Attached bars represent standard errors of the mean (SEM).

RESULTS

EGs Dehydrate Fibres—The observed cross-sectional area of resting skinned fibres decreased with the concentration increase of any of the tested EGs ranging from small EGs (mono-, di-, tri- and tetra-EGs) to medium-sized poly-EGs (PEG900 and 3350). We could not follow the precise time course of the fibre shrinkage because of the refractory turbulence following the solution exchange. However, regardless of the size of EGs, fibre shrinkage seemed to follow roughly exponential time courses of time constants <60 s as representatively shown in Fig. 1. Therefore, 10 min was allowed after any solution exchange to attain steady cross-sectional area. In some cases, area measurement was repeated 20 min after the solution exchange to confirm that the cross-sectional area had reached a plateau within 10 min.

After a series of area measurements at increasing concentration steps of test solute, the cross-sectional area in plain relaxing solution was measured again to examine the reversibility of the shrinkage. The area recovered almost completely to 92–96% of the original. That is, only a small fraction of fibre volume was shrunk irreversibly.

When the measured cross-sectional areas are plotted against the concentration of EGs (Fig. 2A), it is readily seen that EGs of higher molecular weight have higher efficiency for fibre dehydration to shrink the fibres. Note that the medium-sized EGs, PEG900 and PEG3350, shrank the fibres to 43 and 35% of the initial volume at the tested maximal concentration (40 and 30%), respectively. Experiments at higher concentration were difficult because of high viscosity.

Plotting of the same data against the molar concentration of CH_2-unit void of directly attached –OH merges the most of the relationships into a single unified relationship (Fig. 2B). Although some improvement in unifying the relationship is achieved by plotting the data against the molar concentration of CH_2-unit with partial counting of the terminal CH_2-unit to which –OH is attached (not shown), the relationship for PEG3350 is still out of the unified relationship. Each of the CH_2-units on PEG3350 seems to possess almost twice the dehydrating ability of the ordinary CH_2-unit. In any event, the plot (Fig. 2B) strongly indicates that the dehydrating ability of EGs is intimately correlated with the concentration of CH_2-units irrespective of their number on a single molecule in principle.

Uniformity of the Radial Shrinkage—To examine the microscopic uniformity of the fibre shrinkage, we observed electron micrographs of resting fibres in the presence of tri-EG at 30 and 60%. The shrinkage of the myofilament lattice as well as intermyofibrillar space was homogeneous across the cross-section (Fig. 3A–C). In longitudinal sections, thick and thin filaments regularly aligned in parallel with each other without a sign of local coagulation even in the presence of tri-EG (Fig. 3D and E). Despite the effective shrinkage of the lattice, tri-EG showed no significant effects on the length of thick filaments, and the width of M-lines on the micrographs.

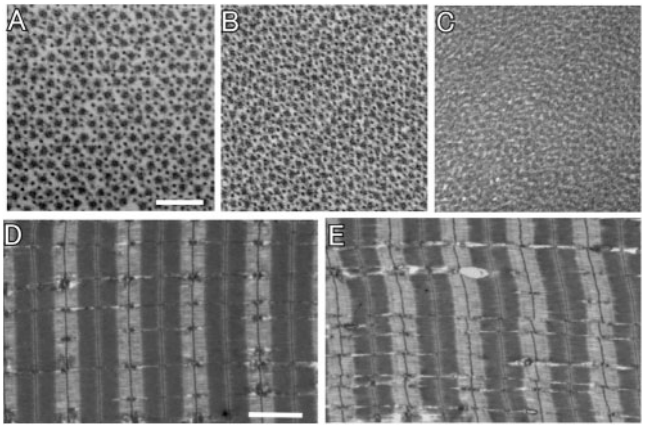

Fig. 3. **Electron micrographs of skinned fibres without EGs (A, D), with 30% tri-EG (B) and with 60% tri-EG (C, E).** The white bar in panel A is a 0.1-μm-scale bar for panels A–C. The bar in panel D is 2-μm-scale bar for panels D, E.

The only deterioration found in the micrographs in the presence of tri-EG is that Z- and M-lines do not run perpendicularly to the myofilaments. This deterioration is likely to be an artifact due to the difficulty in slicing the specimens. The electron micrographs in the presence of 30% PEG3350 were similar to those in the presence of 60% tri-EG (data not shown).

Passive Tension—To examine if EGs shrink skinned fibres not only radially but also longitudinally, we performed passive tension measurement. Sarcomere spacing was set near the slack sarcomere spacing (2.0–2.1 μm) to reduce specific effects of EGs on the elastic components of sarcomere such as connectin/titin (*16*, *17*), and to allow resistance-free sliding of myofilaments. No appreciable change in passive tension was observed, and the fibre did not show any sign of the change in slack sarcomere spacing in the solution of the small EGs up to 50% and of the medium-sized EGs up to 30% (data not shown). Together with the electron micrographs (Fig. 3D and E), the results indicate that dehydrating effect of EGs works anisotropically.

Dehydration Ability of Relevant Molecules—Since mono-EG is one of the fully hydric alcohols, we tested other fully hydric alcohols; glycerol, threitol, ribitol and mannitol (Fig. 4). Surprisingly, they showed no shrinking ability at all, although their molecular weights are comparable to those of small EGs. This clearly indicates that molecular weight is not a primary determinant of the fibre-dehydrating ability of EGs.

For a comparison purpose, we examined the fibre-dehydrating effect of dextran 488k (M_r 488,000) and dextran 9k (M_r 9,000). They were less effective compared with the medium-sized EGs on weight/weight concentration basis. The effect of dextran 488k seemed to reach a maximum at 7.5% concentration with shrinkage to 64% of the original fibre volume, and leveled off with further concentration increase (Fig. 4).

Hydrodynamic Radii (R_h)—Although it is shown earlier that molecular weight is not a primary determinant of the fibre-dehydrating ability, there still remains a possibility that fibre dehydrating small EGs have larger

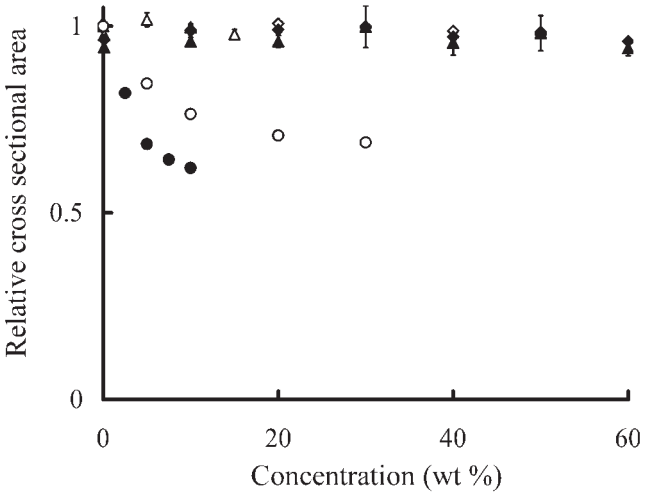

Fig. 4. **Cross-sectional areas of skinned fibres plotted against the concentration of the fully hydric alcohols and dextrans.** Fully hydric alcohols (glycerol, closed diamonds; threitol, open diamonds; ribitol, closed triangles and mannitol, open triangles), dextran 9k (open circles) and dextran 488k (closed circles). Cross-sectional area is expressed relative to the original value in plain relaxing solution. Attached bars represent SEM.

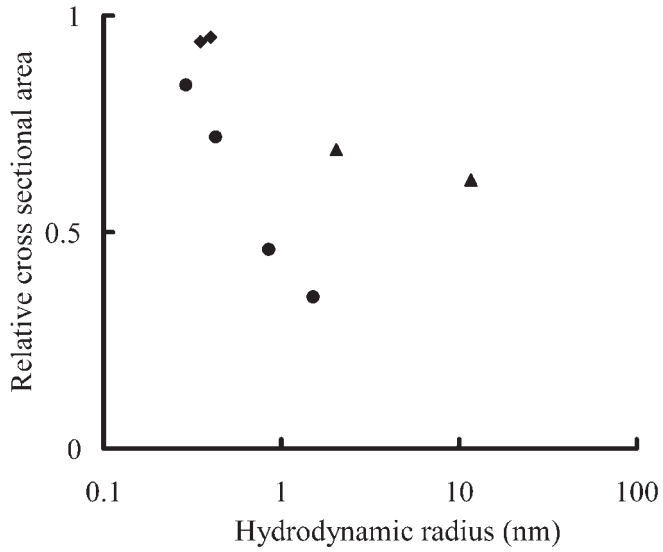

Fig. 5. **Relation between hydrodynamic radius (R_h) of test solutes and fibre cross-sectional area at 30% solute concentration.** The areas are presented relative to the original value in plain relaxing solution. For dextran 488k, the decrease in cross-sectional area with dextran concentration is assumed to be leveled off at 7.5% concentration. Circles represent EGs (left to right; mono-EG, tetra-EG, PEG900 and PEG3350); diamonds, ribitol and mannitol; and triangles, dextran 9k and dextran 488k.

R_h than non-fibre dehydrating fully hydric alcohols. However, estimated R_h from viscosity measurements indicated that the fully hydric alcohols have comparable R_h to those of the small EGs (Fig. 5).

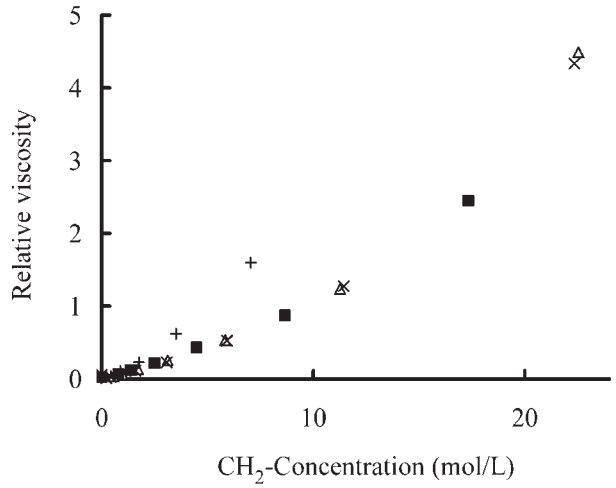

Fig. 6. **Viscosity of the solution of EGs (mono-EG, closed squares; tetra-EG, open triangles; PEG900, crosses and PEG3350, pluses) against the molar concentration of CH_2-unit.**

Viscosity of Bulk Solutions—In search of cue characteristics other than R_h, we plotted relative viscosity of the bulk solutions against the molar concentration of CH_2-units excluding the terminal CH_2-units (Fig. 6). The diverged relationships on the plot against the weight/weight concentration (not shown) merged almost completely into a single relationship. The relationship for PEG3350 is again out of the unified relationship, suggesting that CH_2-units of PEG3350 possess twice the ability to make the solution viscous. Recall that the deviation of the relationship for PEG3350 in Fig. 2B suggested almost twice the fibre-dehydrating ability of CH_2-units on PEG3350. These indicate that both the fibre-dehydrating ability and the viscosity of the bulk solution of EGs commonly involve the same characteristics of CH_2-units.

DISCUSSION

Fibre-Dehydrating Ability of Small and Medium-Sized Molecules—In the present study, we found that EGs of small and medium-sized molecule efficiently shrank the resting mechanically skinned fibres in the radial direction only. This anisotropic effect was almost completely reversible leaving only a small fibre fraction to be shrunk irreversibly. This small fraction would reside in the intermyofibrillar space as reported and discussed in Umazume *et al*. (*5*). Majority of the reversible shrinkage mainly reflects the dehydrating effect of EGs on the myofilament lattice, which almost fills the cytoplasm of frog skeletal muscle.

Several previous workers have studied the effect of EGs on the specifically interacting actin and myosin. Chinn *et al*. (*10*) reported that PEG300 and 4000 at 5% little affected the width of contracting fibres. In our measurements, 5% PEG3350 shrank the relaxing fibre cross-section to 75%, meaning decrease in fibre width by 13%. This is not necessarily a contradiction because myofilament lattice spacing during contraction would be largely affected by the formation of the contractile crossbridges (*6*). Clarke *et al*. (*13*) reported that 50% mono-EG decreased the rigor myofilament lattice by 5%. In our measurement, 50% mono-EG shrank the relaxing fibre cross-section to 80%, meaning 11% decrease in lattice spacing assuming a proportional relationship between the lattice spacing and the fibre width (*5*). This is consistent with the report of Umazume *et al*. (*6*) that rigor cross-bridge formation decreased the radial compliance of the myofilament lattice.

We found no dehydrating ability at all for glycerol up to 60% concentration, although Clark *et al*. (*11*) reported that 0.6 M (6% w/w) glycerol dehydrated chemically skinned fibres of resting barnacle muscle by 20%. The most outstanding difference of their experiment from ours is the sparsely distributed myofilaments in skinned barnacle muscle (*11*). If the dehydration of barnacle muscle is mediated by the association of counter ions with electric charges on the myofilaments as hypothesized by them, frog skeletal muscle would respond differently. This is because ionic strength has an opposite effect on the frog lattice spacing from what is theoretically expected (*5*). In any event, we consider that, as a model of cytoplasm in general, our mechanically skinned frog muscle would be more relevant than the markedly swollen barnacle muscle.

The reversible fibre dehydration exerted by EGs is not due to simple physical exclusion of the molecules from the cytoplasm for the following reasons. First, R_h of the EGs is sufficiently small (Fig. 5; 0.29 nm for mono-EG) compared with the dimension of myofilament lattice [30–40 nm; (*5*, *6*, *18*)]. Secondly, the fully hydric alcohols of comparable R_h showed no shrinking ability (Fig. 4). Thirdly, in ordinary skinned fibre experiments, molecules that are naturally larger than the small EGs (such as ATP; M_r 507) diffuse into the fibre from surrounding solution to sustain contraction. Fourthly, the maximal shrinkage attained by PEG3350 reached 35% of the original volume, which is close to the non-volatile volume fraction of the fibre [25%; (*7*)], while osmotic dehydration attained by macromolecular dextran 488k leveled off at 64% of the original fibre volume (Fig. 4), which is consistent with the previous workers (55–65%; 6, 18).

Solute Depletion Mechanism—Then what is the dehydrating mechanism of the small and medium-sized EGs?

We initially expected that solute depletion mechanism (*19*) would shrink resting fibres. That is, small molecules that are sterically excluded from the gap between nearby macromolecules osmotically dehydrate the gap to mediate aggregation of the macromolecules. In the case of aggregation of red blood cells, Armstrong *et al*. (*20*) found a distinct threshold R_h of 4 nm for the inert aggregation mediators of various kinds. In the case of the present fibre-dehydrating ability, however, we not only failed to find a threshold R_h but also found that molecules as small as mono-EG could efficiently dehydrate the fibre.

From the correlation analysis we further found that each CH_2-unit on EGs is the independent unit of the fibre-dehydrating ability (Fig. 2B). It should be noted here that, so far as present analysis is concerned, the

minimal unit could be an EG-monomer instead of each CH_2-unit. Here, we prefer a CH_2-unit because a similar correlation between fibre-dehydrating ability and CH_2-concentration was found for poly-hydric alcohols such as butanediols and butanetriol (*21*).

The solute depletion mechanism requires the depleting solute to be substantially larger than the solvent water molecules even if water molecules are handled explicitly rather than a continuum (*22*). Since a CH_2-unit or an EG-monomer is as small as a water molecule as our R_h measurements indicated, the simple solute depletion mechanism could not account for the dehydrating ability of EGs. Another or modified mechanism that works with the mixture of similarly sized inert particles is necessary. Independence of the dehydrating effect of each CH_2-unit from intra-molecular linkage indicates that the mechanism does not require freedom for three-dimensional translation of the perturbing particles.

Fibre-Dehydrating Mechanism—To further resolve fibre-dehydrating mechanism of EGs, we consider the following four arguments:

(i) The dehydration is not due to any sub-sarcomeric localized effect of EGs such as aggregation of neighbouring myofilaments and of M and Z-lines because the hexagonal lattice structure of myofilament and intermyofibrillar structure were homogeneously dehydrated in the present electron micrographs (Fig. 3). Our preliminary experiments indicated that the dehydration with EGs accompany decreased longitudinal elastic compliance of the fibre as in the case of evaporative dehydration. Although this decreased compliance may reflect some kinds of sustained interaction between intra-sarcomere components including elastic filaments (connectin/titin), we consider that any interactions characteristic of actin and myosin would not be the primary cause of the dehydration. This is because in the states of rigor and contraction where specific interaction of actin and myosin (including the weak binding) exists, EGs were reported to have smaller effect on fibre width and myofilament lattice (*10*, *13*). In addition, to ascribe the remarkable dehydration induced by PEG900 and 3350 to any characteristic attraction between myosin and actin, either lever arms or S1 heads of myosin molecules should directly cross-link thin filament to the thick filament backbone even if minimal diameters are adopted for them (*23–27*). This seems to be an unlikely situation if concentration-dependent homogeneous dehydrating effect of EGs is taken into account. Moreover, since intermyofibrillar space was dehydrated as well (Fig. 3), the dehydration mechanism should involve general biomolecules.
It should be noted here that the present results do not exclude a possible contribution of some specific interaction (such as weakly binding) between actin and myosin to the fibre dehydration. Rather, the continuously CH_2-concentration-dependent and potent dehydrating effect of EGs strongly suggests that EGs non-specifically affect the whole fibre constituents rendering any assumption of a selective effect of EGs on actin and myosin (*10*, *13*) debatable.

(ii) We successfully account for the lack of dehydrating ability of the fully hydric alcohols larger than glycerol regarding the hydrophobic CH_2-unit void of directly attached hydrophilic –OH as an active dehydrating unit. To account for the dehydrating ability of fully hydric mono-EG, however, exceptional partial evaluation of the terminal CH_2-units is necessary as described in 'Results' section. The synergistic neutralizing effect of –OH groups on the neighbouring CH_2-units may provide a systematic account for the overall dehydrating activity. Note that an explicit synergistic effect of CH_2-unit was found only in PEG3350 (Fig. 2B).

(iii) Although the bulk solution viscosity of EGs intimately correlates with the fibre-dehydrating ability of EGs through CH_2-concentration (Figs. 2B and 6), one cannot directly ascribe the dehydrating ability to the diffusion of EGs, because the correlation was limited for the solutions of EGs, and as viscous solutions of fully hydrated alcohols showed no dehydrating ability (Fig. 4). In addition, viscosity as well as diffusion in cytoplasm might be significantly different from that of the bulk solution, because cytoplasm is so crowded with molecules such as proteins and the penetrated EGs.

(iv) Observing association of paired probe proteins in crowded solution, Kozer *et al*. (*1*) reported that monomers on polymer in solution interact with each other as if they were independent monomers. According to them, this monomer-like interaction emerges only in condensed regime in their experiment because mutual interactions between the probe proteins and the simple solute depletion mechanism predominate at dilute and semi-dilute regimes, respectively. It is reasonable to consider that their monomer-like interaction has a similar background to the present dehydrating activity of independent CH_2-units because cytoplasm of skeletal muscle is already crowded with various molecules.

Tentative Hypothesis—The close correlations of both fibre-dehydrating ability and the bulk-solution viscosity of EGs with CH_2-concentration indicate that the fibre-dehydrating ability involves contribution from solute–solute and solute–solvent interactions at finite EG concentration as essential parts. Therefore the fibre dehydration by CH_2-units would involve substantial force reaching beyond R_h. Recently, evidence for ultra-long-ranged interactions between hydrophobic moieties is accumulating (*28*). We currently hypothesized that the substantial radius of hydrated molecule at finite concentration or in the soup such as cytoplasmic solution can be larger than R_h and can be anisotropic. Correspondingly, our preliminary observation of ^{1}H-NMR signals of water and di-EG indicated that averaged di-EG concentration of cytoplasmic solution is about 10% lower than that of the bathing bulk solution of 40% weight/weight concentration (Takemori, S. and Kimura, M., unpublished data). Since cytoplasmic solution of skeletal muscle as well as general cells is not uniform but can be categorized into several components characterized by spin–spin relaxation

rates of ^{1}H-NMR signals (*7*, *8*, *29*, *30*), EG-concentration would distribute differently among bulk bathing solution and the cytoplasmic solution components. To test our hypothesis, advanced experiments to estimate the EG-concentration and the substantial radius of hydrated EGs in each cytoplasmic solution components are awaited. We infer that in cytoplasm of skeletal muscle, hydration shell around hydrophobic CH_2-unit would be extended with extra hydration to align anisotropically so as to realize anisotropic fibre shrinkage.

CONCLUSION

Each CH_2-unit on EGs acts to dehydrate the fibre independently of its intra-molecular linkage while fully hydric alcohols larger than the mono-EG did not. The mechanism of fibre dehydration by the CH_2-unit requires a novel or modified theory that works with cosolute almost equally sized with water molecules. Differential hydration likely plays a key role. To understand the behaviour of hydrophobic and hydrophilic moieties in cytoplasm in general, the behaviour of dehydrating CH_2-unit and the neutralizing –OH unit in skeletal muscle myoplasm will provide an ideal model.

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