X-RAY DIFFRACTION OBSERVATIONS OF CHEMICALLY SKINNED FROG SKELETAL MUSCLE PROCESSED BY AN IMPROVED METHOD

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ABSTRACT Whole frog sartorius muscles can be chemically skinned in ~2 h by relaxing solutions containing 0.5% Triton X-100. The intensity and order of the X-ray diffraction pattern from living muscle is largely retained after such skinning, indicating good retention of native structure in fibrils and filaments. Best X-ray results were obtained using a solution with (mM): 75 K acetate; 5 Mg acetate; 5 ATP; 5 EGTA; 15 K phosphate, 2% PVP, pH 7.0.

Equatorial X-ray patterns showed that myofibrils swell after detergent skinning, as also observed after mechanical skinning. This swelling could be reversed by adding high molecular weight colloids (PVP or dextran) to the extracting solution. By finding the colloid osmotic pressure needed to restore the in vivo interfilament spacing (3% PVP, 4×10^4 mol wt) the swelling pressure was estimated as 35 Torr in a standard KCl-based relaxing solution. The swelling pressure and the extent of swelling were less when acetate replaced chloride as the major anion.

Detergent-skinned muscle lost the constant-volume relation between sarcomere length and lattice spacing seen in intact muscle.

Changes in A band spacing were paralleled by changes in I band-Z line spacing at a constant sarcomere length.

After detergent skinning, $I_{1,0}$ rose while $I_{1,1}$ fell, a change in the relaxing direction. Since raising the calcium ion concentration from pCa 9 to pCa 6.7 was without effect on equatorial or axial X-ray patterns, we concluded that these intensity changes were not due to calcium-dependent cross-bridge movement but rather to disordering of thin filaments in the A band.

INTRODUCTION

Skinned muscle fibers are those where the sarcolemma has been removed or disrupted, exposing the contractile apparatus to experimental solutions. Such muscle models have been very useful tools for study of the contractile mechanism. Mechanical skinning by dissection of the sarcolemma from single fibers (Natori, 1954) is not as widely used as are chemical skinning methods, such as glycerination (Szent-Gyorgyi, 1951) or glycerol-treatment followed by nonionic detergent (Julian, 1971). Chemical skinning is especially suited for preparing specimens for X-ray diffraction study because whole muscles or muscle bundles can be used; their greater scattering mass enables study of myofilament structure and behavior impracticable with single fibers. We undertook the work reported here because others have shown that rabbit psoas muscle prepared by glycerination (Rome, 1972) or by glycerol-detergent procedures (Lymn and Huxley, 1973; Lymn, 1975*a*) recovers resting native structure only poorly when the glycerol rigor medium is replaced by aqueous relaxing solution. Even though this preparation is mechanically relaxed and no trace of rigor remains in the X-ray patterns,

the 430 Å myosin layer line series is very weak. Since these layer lines indicate ordering of the cross-bridge arrangement in resting muscle (Huxley and Brown, 1967; Haselgrove, 1975), we judged that too much disorder was present in glycerinated psoas to encourage study of myosin filament structure or cross-bridge behavior. For this reason, we tried to develop a better way to chemically skin vertebrate muscle.

We have found that whole frog sartorius muscles are rapidly and irreversibly skinned by soaking them in a relaxing solution containing the nonionic detergent Triton X-100. This preparation, using a muscle which gave poor results with conventional glycerination (Reedy, unpublished observations), shows unprecedented retention of the crystalline order of live muscle. This development opens new possibilities for studies coordinating mechanical, biochemical and structural behavior in a freely accessible, highly-ordered muscle preparation.

MATERIALS AND METHODS

Specimen Preparation

Sartorius muscles (~1-mm thick) were dissected from *Rana pipiens*. After control X-ray patterns were taken in Ringer's, muscles were tied to plastic frames with threads around the pelvic bone and tibial tendon. They were then soaked 15 min in 25 ml of ice-cold relaxing solution in a 35-ml vial. Then, membrane destruction was begun by adding Triton X-100, usually to 0.5%, and the muscles continuously agitated on a rotator at 5 rpm at 4°C until time for the X-ray exposure. Stirring was found necessary for retention of relaxed structure; inversion during rotation of the partly filled vial produced good stirring. 12–18 h overnight extraction was usual, but the range from 0–60 h was studied. At 60 h a strong relaxed pattern could still be obtained.

The rate at which a solution penetrated the muscle was determined from the time course of lattice spacing changes in sequential equatorial photographs. Exposure durations from 5–30 min were used depending on the speed of the process under study. Solutions were changed either by switching to a new perfusion reservior or by addition of Triton to the circulating solution (See Fig. 4, for example). Up to eight exposures were recorded serially at 1-cm intervals on the same sheet of film; film could be shifted manually in <15 s. Using *in situ* perfusion, time sequence studies of lattice swelling showed demembranation to be complete in ~2 h. Such *in situ* extraction was used in some cases (Fig. 1 *a* and *b*) to insure that the same portion of the specimen was diffracted before and after skinning.

Plain (no detergent) relaxing or rigor solution, buffered near pCa 9 with EGTA (free magnesium ion was varied between 0 and 1 mM), did not skin frog skeletal muscle, although such solutions have been reported to skin mammalian fibers after prolonged immersion (Wood et al., 1975; Eastwood et al., 1979). The relaxed X-ray pattern survived many days in a KAc/PO_4/EGTA "rigor" saline (a solution which quickly produces rigor in skinned muscles by washing out ATP) or in KAc relaxing solution, and the lattice swelling characteristic of skinned muscle did not occur until detergent was added. Moreover, X-ray patterns did not show any important differences between resting states in Ringer's and in these high-potassium (depolarizing) media (compare Fig. 2 *a* and *b*). Resting structure is expected from the mechanical behavior of frog twitch fibers, which contract for a few seconds and then relax and become inexcitable when depolarized by high potassium (Hodgkin and Horowicz, 1960).

X-ray Exposures

Live or extracted muscles were mounted in a closed acrylic chamber (3-ml capacity) which was perfused continuously at 15 ml/min with solution. Recirculating flow from a 50-ml reservoir in ice was maintained with a Pall syringe pump (Pall Corp. Glen Cove, N. Y.). Peltier cooling held the solution bathing the muscle at 12–15°C in most experiments. The effect of temperature was not systematically studied. A pair of windows in the flow cell, sealed with 25-μm thick Mylar, provided passage for X-rays

or for a laser beam used in sarcomere length determination. To minimize X-ray absorption by excess solution, the gap between windows and muscle was minimized. This diffracting region of the specimen was shown by dye studies to be the least accessible to perfusate. Experience indicated that relaxation in these relatively thick specimens required good superfusion with relaxing solution. On occasion, signs of contracture or rigor appeared in the window region; data from these experiments were discarded.

An Elliott GX 6 rotating Cu anode tube (Diano Corp., Woburn, Mass.) loaded at 5–6 kW/mm^2 was the X-ray source. Most X-ray patterns were made using a bent quartz crystal monochromator camera giving a line focus, and using a 27-cm specimen-to-film distance (see Fig. 2). On this camera a strong axial pattern could be recorded in ~2 h, an equatorial pattern in ~0.5 h. Occasionally, higher resolution photographs (see Fig. 1) requiring ~24 h were made with a point-focus 47-cm mirror-monochromator camera as described by Huxley and Brown (1967). Spacings were measured with a Scherr-Tumico ×10 profile projector (Scherr-Tumico, Inc., St. James, Minn.) and routinely calibrated by reference to the 143.4 Å meridional spacing of relaxed muscle (Haselgrove, 1975). Direct calibration of the cameras agreed to within 0.4%. A band lattice spacings (d_m) are given as the center-to center spacing of the thick filaments calculated from the 1,0 spacing ($d_m = d_{10}/\sin 60°$). When necessary, intensities were measured with a Joyce-Loebl microdensitometer (Model IIIC) (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England).

Sarcomere Length

Sarcomere length (SL) was measured by diffraction of a 2-mW HeNe laser beam, on a vertical bench, calibrated with a 10-μm microscope stage micrometer. In most experiments muscles were first mounted in the X-ray cell and the SL then adjusted to 2.05–2.25 μm. In one series of experiments where lattice spacing dependence on SL was studied, a somewhat different method was adopted. Muscles were stretched on plastic frames to various SLs, extracted, and X-rayed in a special flow cell which accomodated the frame. Afterwards, specimens were fixed with glutaraldehyde in relaxing solution; SL was determined as the average of 60–80 fibers individually sampled from the region that had been X-rayed. This length differed only slightly from that estimated by laser diffraction of the live muscle.

Solutions

Ringer's solution for live muscles contained (mM); 115 NaCl; 2.5 KCl; 1.8 $CaCl_2$; 3 sodium phosphate buffer pH 7.0. The compositions of some relaxing solutions are listed in Table I. ATP (grade II), EGTA, 10,000-or 40,000 -mol wt polyvinylpyrollidone (pharmaceutical grade, abbreviated PVP-10 or PVP-40), and 40,000 mol wt dextran (dextran-40) were from Sigma Chemical Co., St. Louis, Mo. MOPS buffer (morpholinopropane sulfonic acid) was from Calbiochem (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.). Triton X-100 ("reagent grade," Research Products International, Elk Grove Village, Ill.) was routinely used as supplied. Batch treatment of 25% Triton X-100 stock solution with ion-exchange resin (Rexyn 201, Fisher Scientific Co., Pittsburgh, Pa.) which reduced low but detectable aldehyde and peroxide contaminants did not demonstrably improve X-ray patterns.

Detergent Selection

In preliminary studies, a broad sampling of various commercial nonionic detergent families (Triton, Brij, Lubrol, Tween, Plurafac, Emulphogene, etc.) was assayed microscopically and by centrifugation for the speed and completeness with which they hemolyzed human red blood cells (RBC), solubilized RBC ghosts, and removed visible membranous components from homogenates of insect, frog, and rat muscle. Detergents were tested at 1% in standard salt (solution 1, less ATP). By all measures, Triton X-100 was among the best, which also included Triton N-101, Triton CF-21, Plurafac RA-30, and Emulphogene BC610. Lubrol WX (Julian, 1971) and Brij 58 (Orentlicher et al., 1974) were not as generally effective. Tween 80 (Abbott and Chaplain, 1966) was relatively inactive in several tests. Because of its easy availability, lack of binding to cytoplasmic proteins (see review by Helenius and Simons, 1975), and effectiveness, Triton X-100 was adopted as the standard detergent for these extraction studies.

RESULTS

Axial Patterns

Our principal finding is illustrated in Fig. 1. It shows that chemical skinning by extraction in Triton relaxing solution preserves, with only a small loss of intensity, native resting structure of the thick and thin filaments. Patterns like these were routinely obtained and are clearly a great improvement over the best results obtained with glycerinated rabbit psoas (Rome, 1972; Lymn and Huxley, 1973; Lymn, 1975*a*). The muscles were definitely skinned, because after exposure to detergent, the relaxed pattern depends on exogenous MgATP; when the extraction was carried out in the absence of added MgATP, a rigor pattern was then obtained.

In relaxing solution, no change was seen in the spacing of the meridional spot at 143.4 Å. Although the total intensities of the before-and-after patterns appear quite similar, the skinned patterns are not as sharp. This is especially apparent as broadening on the equator and higher row lines. Meridional reflections are not much broadened by this effect, but the 4th, 5th, and 6th orders of 430 Å are weaker than in living patterns. The principal actin reflection at 59 Å is weakened as well. Swelling of the myofilament lattice after skinning (see Table I) has caused the myosin layer lines to be sampled closer to the meridian.

About 30 different skinning and relaxing solutions, and over 400 X-ray photographs were examined in the search for an optimal procedure. Solution effects on structure were evaluated by visually comparing the axial X-ray diffraction patterns from skinned specimens with those recorded from the same muscle or its contralateral mate before detergent treatment. The

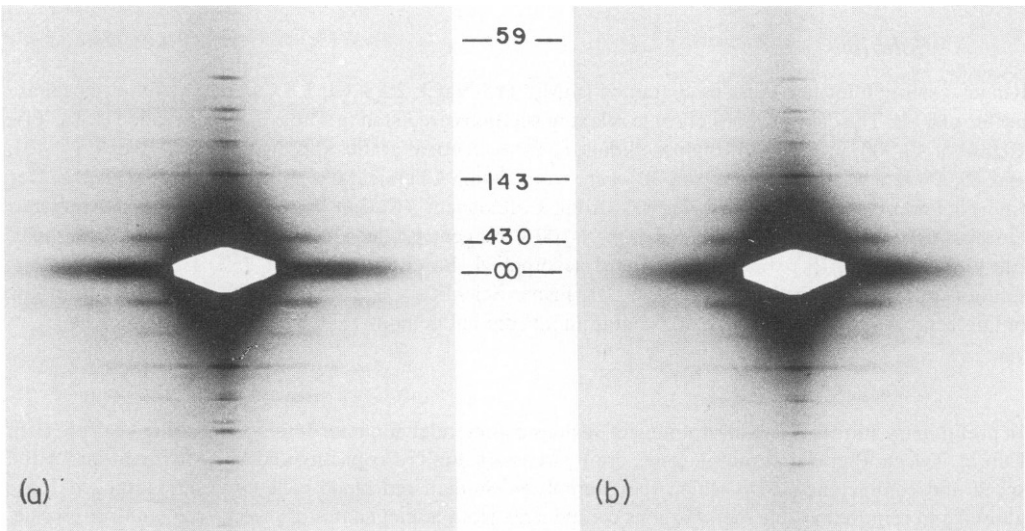

FIGURE 1 Effect of skinning on axial X-ray diagrams of a frog sartorius muscle shown live in Ringer's (*a*), and skinned (*b*) by 0.5% Triton X-100 in relaxing solution 5. Exposure time for both pictures was ~21 h. A first order compensation for the intensity loss expected when myofibrils swell on skinning was made by allowing a 0.25-mm solution layer between the live muscle and the Mylar window. Sarcomere length was 2.1 μm; temperature was 8–10°C. These prints of X-ray patterns were made without masking (Lymn, 1975*a*), burning or dodging.

strong diagram routinely obtainable from resting live frog sartorius gives an easily documented standard to aim for (Fig. 1 *a*).

Experience indicated that acetate solutions (see Table I) gave higher yields of strong, clear patterns like that pictured in Fig. 1 *b*) than did chloride-based media. For the same reason, an ionic strength of 0.154 or less rather than 0.190 (solution 1) was preferred as was the use of phosphate rather than MOPS as the pH buffer. By exchanging acetate for chloride, the preferred relaxing solution (solution 5) routinely gave good-to-excellent X-ray results, whereas solution 1, 2, or 3 often gave only poor-to-fair results. Solution 5, especially when supplemented with 2% PVP-40 or 4% dextran-40 gave occasional patterns almost indistinguishable from living muscle, whereas this was never the case with chloride-based media.

Equatorial Patterns

In contrast with axial patterns where skinned muscles appear essentially the same as live muscles, equatorial diagrams brought out distinguishing features of the skinned preparation. (Equatorial diagrams indicate side-spacing and regularity in the myofilament lattice, and give some information about the radial position of myosin cross-bridges.) Four significant effects were seen in equatorial patterns of skinned muscle. (*a*) When living muscles were skinned by detergent the myofilament lattice swelled markedly. (*b*) Myofibrillar swelling could be controlled by the colloid osmotic pressure of water-soluble polymers added to the relaxing media. (*c*) The higher equatorial orders, indexing on 1,0 and 1,1 were weakened or lost. (*d*) The intensity ratio $I_{1,1}/I_{1,0}$ fell (i.e., changed in the relaxing direction).

All the relaxing solutions tested after Triton skinning (see Table I and Fig. 2 *c*) produced swollen interfilament spacings compared to living muscle in Ringer's. The swelling of detergent-skinned whole frog sartorius duplicates the behavior of mechanically skinned single fibers from frog (Matsubara and Elliott, 1972) and crayfish muscle (April et al., 1971, 1972). And just as the myofilament lattice of mechanically skinned fibers no longer maintains constant volume during length changes (Matsubara and Elliott, 1972; April and Wong, 1976), so have we found that detergent skinned whole muscles also lose this property of live

TABLE I
COMPOSITION OF SOME RELAXING SOLUTIONS AND THE THICK FILAMENT SPACINGS (d_m) THEY INDUCE IN TRITON-SKINNED FROG SARTORIUS MUSCLE

Solution	KCl	KAc	Phosphate buffer	MOPS buffer	d_m	Ionic strength
1	100	—	20	—	488 ± 2 (4)	0.190
2	75	—	15	—	482 ± 0 (2)	0.154
3	100	—	—	20	478 ± 5 (7)	0.138
4	—	100	20	—	448 ± 3 (6)	0.190
5	—	75	15	—	462 ± 4 (7)	0.154
6	—	100	—	20	454 ± 2 (2)	0.138
7	3	70	—	20	453 ± 5 (9)	0.118
Ringer's			(see Methods)		409 ± 10 (28)	0.130

Concentrations are millimolar. In addition to the ingredients listed, all solutions contained 5 mM each of: K_2EGTA, $MgCl_2$ or $Mg(acetate)_2$, and $Na_2K_{1.4}ATP$. pH was set to 7.0 at 23°C. X-ray patterns were obtained at 12–15°C. Sarcomere length was 2.05–2.25 μm.
d_m (in Å) is shown ± 1 SD, followed, in parentheses, by the number of muscles tested.

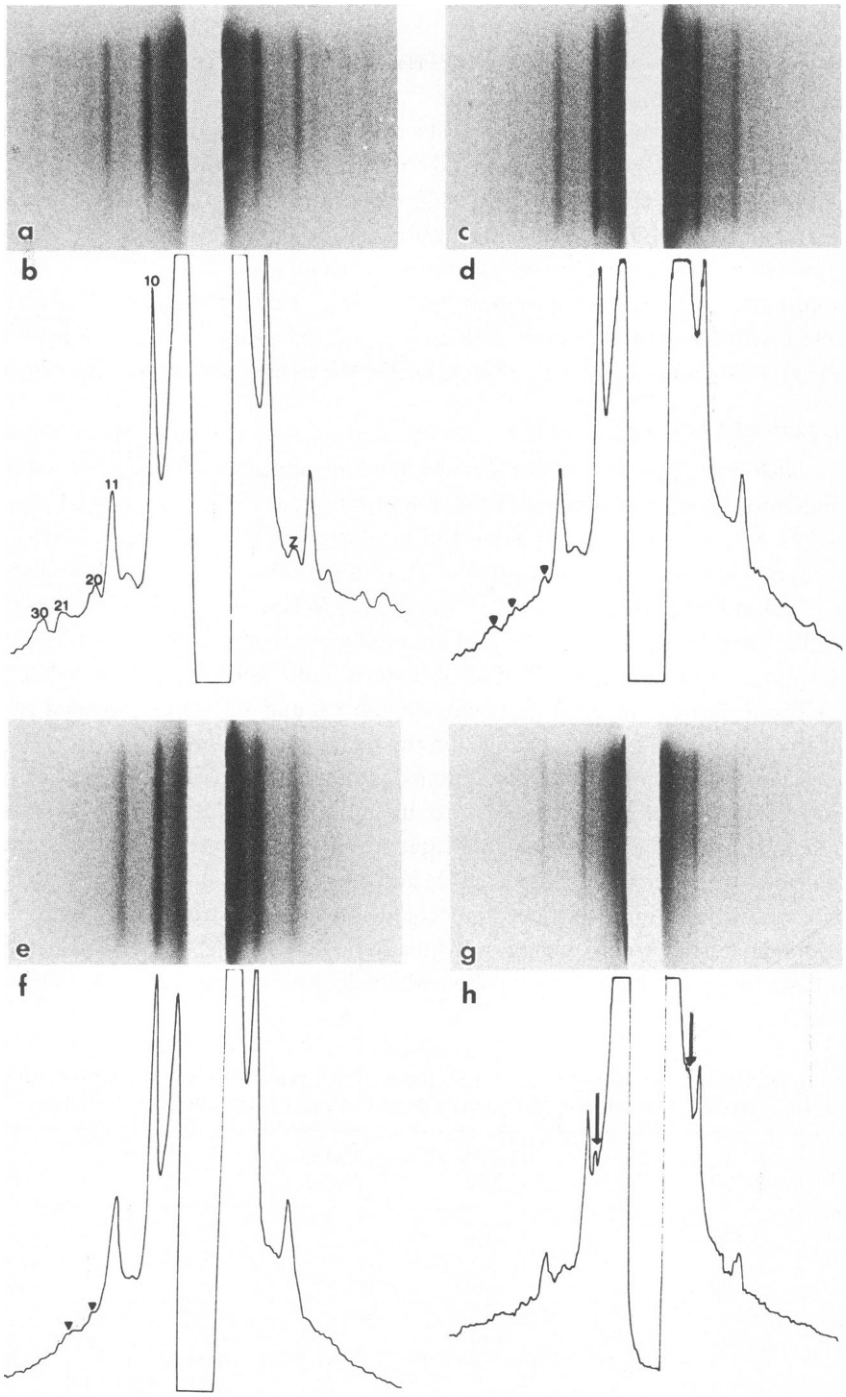

FIGURE 2 Equatorial photographs and densitometric scans of muscles to show skinning-swelling and colloid-shrinking of filament lattice. Temperature, 12–15°C. Exposures, 0.5 h. Sarcomere length, 2.1 μm.(*a* and *b*) Live, in Ringer's d_m = 410 Å. Note strength of higher orders indexing on an hexagonal lattice and presence of nonindexible reflection (*Z*). (*c* and *d*) Live, depolarized in relaxing solution 4, d_m = 419 Å. (*e* and *f*) Triton-extracted in relaxing solution 4, d_m = 443 Å. Note that $I_{1,0}/I_{1,1}$ is decreased compared to the live values and the higher orders are much weaker. (See arrowheads). (*g* and *h*) Triton-extracted in solution 1, with 4% PVP included. Most of the myofibrils have shrunken to d_m 398 Å after 6.5 h exposure to added colloid, but a small fraction, d_m = 483 Å, (see arrows) presumably at the center of the muscle were only later to experience shrinking action of colloid diffusing in from the bath (e.g., Fig. 4). The final shrunken spacing was stable but the lattice could be reswollen by removing PVP from the bath.

muscle. As shown in Fig. 3, detergent-skinned muscles react to length changes in a fashion markedly different from the constant-volume behavior of intact muscle (Elliott et al., 1963). Interfilament spacing (d_m) was only slightly reduced by stretching up to SL 2.6–2.8; beyond this point d_m fell sharply with further extension. Over the SL range studied, d_m fell 107 Å in contrast with 61 Å calculated for the constant volume case.

The largest effect of ionic variations was that chloride-based solutions caused substantially more lattice swelling than those in which acetate was the principal anion (d_m averaged 483 ± 5.0 Å in chloride and 454 ± 6 Å in acetate). It will be shown below that chloride relaxing solutions caused more swelling because they induce a greater apparent swelling pressure in the lattice. Within each of these two groups, no consistent effects on lattice spacing of ionic strength, buffer anion, or [free Mg^{2+}] were seen. The anion-specific effect is presently without explanation. The difference may arise from effects on water structure as hypothesized by von Hippel and Schleich (1969) to account for the orderly specific ion effects on the structure and function of numerous proteins, including myosin.

Effects of Colloids

Many equatorial diffraction patterns were obtained with PVP or dextran added to the skinning solution to examine their effect on filament spacing in the myofibrils. This followed up on the finding by light microscopy that mechanically skinned fibers became shrunken by colloid-containing relaxing solution (Maughan and Godt, 1974, 1978; Godt and Maughan,

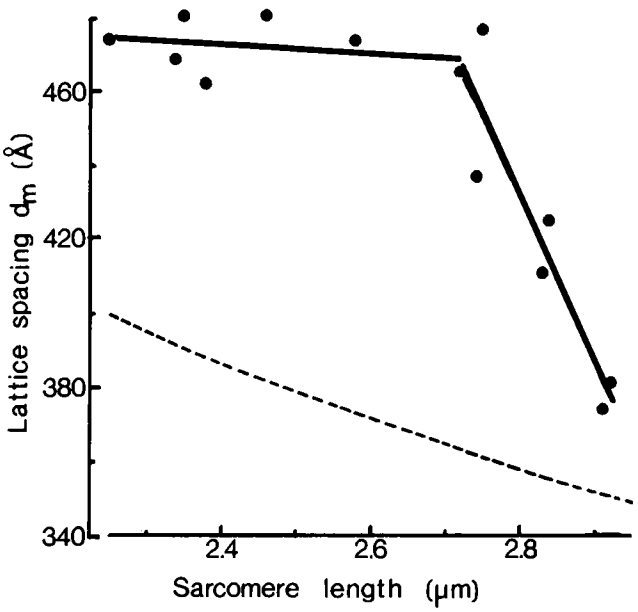

FIGURE 3 A band lattice spacing dependence on SL in relaxed, skinned frog sartorius (●) is far from the constant volume behavior reported by others in living frog sartorius. Each data point is from a different skinned sartorius measured in relaxing solution 1. For illustrative convenience, two least-square lines have been fitted to the data. No physical meaning is assigned to this representation. The constant volume case (dashed line) is calculated from $d_m^2 \cdot SL$ = constant, taking the constant as 3.6×10^9 Å^3 from our measurements in Ringer's (Table I). SL was measured from individual fibers as described in the text.

1977). We wanted to verify their assumption that this shrinkage affected the filament lattice. We also hoped that reversal of myofibrillar swelling in skinned muscles might repair or prevent the equatorial intensity changes mentioned above. Both polymers were found to cause concentration-dependent lattice shrinkage but neither restored the in vivo equatorial pattern entirely, although some improvement was noted when an isovolumic concentration (see below) of colloid was included in the relaxing solution.

Squeezing of myofibrils by these polymers clearly has a colloid osmotic basis. That is, it shows a marked dependence on particle size, rather than on concentration or chemical structure; PVP-10 caused less than half the shrinkage at four times the molar concentration than did PVP-40. The most direct interpretation of this observation is that the higher molecular weight polymer is more effectively excluded from the lattice and therefore exerts a greater osmotic pressure. A subsequent report will detail the response to colloid by the lattice. For the present, we wish only to report our values for the apparent swelling pressures of the relaxed filament lattice, estimated indirectly by osmotic stress experiments (Godt and Maughan, 1977; Maughan and Godt, 1974, 1978; April et al., 1977). We found that the swelling pressure in the skinned muscle lattice was greater in chloride. That is, the lattice is not only more swollen in chloride than acetate, it also takes more PVP or dextran to shrink the lattice back to its in vivo spacing. It took 3% (wt/vol) PVP-40 in chloride (solution 1) but only 2% in acetate (solution 5). Using dextran the concentrations needed were twofold higher, 6% and 4% respectively. These weight-percentages can be converted to osmotic pressure by using Vink's (1971) membrane osmometer measurements. The corresponding pressures are 35 and 23 Torr with PVP and 87 and 46 Torr for dextran. The higher estimates from the dextran experiments may indicate that the dextran used, although the same weight-average molecular weight as the PVP, contains fewer long-chain molecules and therefore is less effectively excluded from the lattice. Godt and Maughan (1977) observed similar differences between PVP and dextran.

The range of estimates, 23–87 Torr, is much lower than the 195 Torr estimated by Godt and Maughan (1977). We have consulted with them about this difference. They agree that

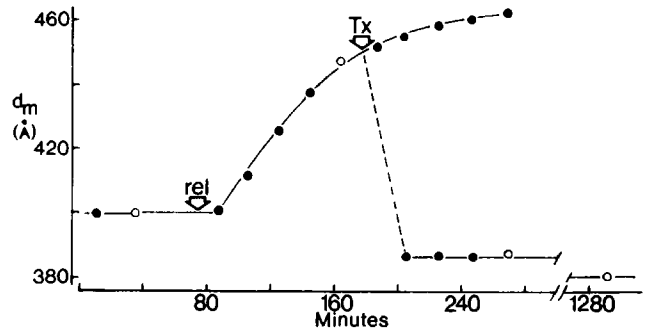

FIGURE 4 Lattice spacing changes induced by KCl and Triton in the presence of 6% PVP. For the first two exposures the muscle was in Ringer's (SL = 2.35 μm, 15°C). At arrow (*rel*) the circulation of relaxing solution 1 containing 6% PVP-40 but without Triton was begun. Sequential 20 min exposures were obtained thereafter. Data are plotted at the midpoint of their time interval. At *Tx*, 0.5% Triton was added (from a 25% stock). Addition of Triton triggered, after a short delay, appearance and growth of a second 1,0 reflection from a shrunken lattice. Open circles correspond to patterns reproduced in Fig. 4.

their use of silicone oil to establish the "original" cross sectional area caused their overestimate of how much PVP it takes to return skinned fibers to their original size. They have since observed, as have we, that silicone oil (even when "water-saturated") gradually dehydrates skinned fibers leading to a substantial underestimate of diameter. Paraffin oil is much better in this respect since isolated fibers do not shrink much even when left several hours in it.

Although three laboratories (Godt and Maughan, 1977; Maughan and Godt, 1974, 1978; April et al., 1977; this paper) have shown shrinking of relaxed skinned fibers by colloids, Millman and co-workers (Millman and Racey, 1977; Millman and Wakabayashi, 1979) could not demonstrate lattice shrinking with relaxed muscle after their skinning procedure but only with muscle in iodoacetate rigor shown to possess damaged membranes. Resolution of this disparity may depend on criteria used to indicate "skinning." Millman (personal communication) has used the swelling which occurs on exposure to their EGTA-containing

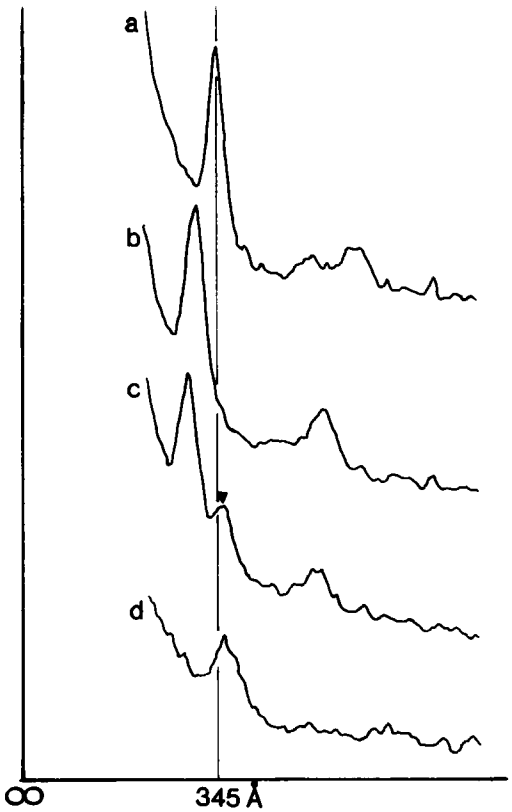

FIGURE 5 Tracings of selected equatorial patterns from time-course experiment of Fig. 3. (*a*) Ringer's; the vertical line indicates 345 Å position for the 1,0 reflection to help comparison with subsequent traces. (*b*) Tracing made 92 min after exposing the specimen to KCl-based relaxing solution. (*c*) Tracing made 91 min after Triton was added to perfusate. Arrow marks the 1,0 spacing at 335 Å seen in addition to that at 400 Å. (*d*) Tracing made 1,114 min after detergent addition; the swollen spacing has been replaced entirely by the reflection centered at 329 Å. The spacing remained unchanged when measured a further 22 h later (not shown).

relaxing solution as his criterion for skinning. It is possible that such relaxed muscles were not skinned but only swollen by the large "K·Cl product" (Boyle and Conway, 1941) of their standard relaxing solution (very similar to our solution 1). If the muscle were not skinned, the colloid, being impermeant, would be unable to act directly upon the myofibrils. The experiment depicted in Figs. 4 and 5 validated this hypothesis. Soon after replacing the Ringer's with a KCl-based relaxing solution the filament lattice began swelling despite the presence of 6% PVP-40, which was unable to counter the large Donnan-osmotic pressure of the KCl borne across the intact plasma membranes of the muscle cells. Only after the membranes were dissolved by adding 0.5% Triton to the perfusate, could the colloid exert direct pressure on the swollen myofibrils. After a short delay a new 1,0 peak at 335 Å appeared which gradually grew larger at the expense of the reflection at ~400 Å (Fig. 5 *c*) as PVP diffused in from the bath. At long times (Fig. 5 *d*), all fibrils had shrunken to the spacing first shown by those fibrils first affected at the surface of the muscle.

It is interesting and puzzling that changes in A band spacing were also paralleled by changes in the I band. The reflection designated "Z" in Fig. 2 *b* seems to arise from the ordering of the thin filaments into a square array as they enter the Z line (Elliott et al., 1967) and this gives a measure of I band spacing. Measurements have been made of the ratio d_Z/d_{10} over the range for d_{10} between 338 and 416 Å at a constant sarcomere length of 2.1 μm. Lattice spacing changes were produced in either live or skinned muscle by changes in the tonicity of the physiological saline, the ionic composition of the skinning solution, or by the addition of colloids. The ratio was found to have a constant value of 0.72 ± .02 (N = 10). This value agrees closely with 0.71 ± 0.02 reported by Yu et al. (1977) in live frog muscle when spacing changes were produced by stretching. Our result shows that A band and Z line spacings change in a coordinated way, independent of sarcomere length changes or the presence of a volume-limiting plasmalemma. The reasons for this are not obvious; on the contrary, differences in protein composition and structure between A, I, and Z bands might lead one to expect different responses to a change of anion or to osmotic squeezing by a given colloid solution.

Lattice Disorder

Equatorial patterns of skinned muscle whether in colloid or in simple relaxing salines invariably showed two distinctive changes in equatorial intensities. First, higher orders of the 1,0 and 1,1 reflections were weakened (Compare Fig. 2 *a* and *c*) indicating increased lattice disorder. Second, a change occurred (opposite in direction to that seen in contraction or rigor) in the intensities of the 1,0 and 1,1 reflection: I_{10} went up and I_{11} went down. At first this change was interpreted in terms of cross-bridge movement. We hypothesized that the unphysiologically low calcium level (~pCa 9) in the relaxing solutions had caused detachment of the fraction of the cross-bridges which are attached in both live resting muscle (Hill, 1968) and at pCa 7, but not pCa9, in skinned muscle (Moss et al., 1976). This hypothesis was tested by exposing skinned frog muscles to pCa 6.7 (made by adding 1.67 mmol/liter $CaCl_2$ to solution 5). Single fiber transducer experiments showed pCa 6.7 to be below the contraction threshold. Lowering pCa to 6.7 brought about no change whatever in either the axial or meridional intensities either in ordinary skinning solutions or with colloid added to restore normal filament spacing. We think therefore that the equatorial intensity changes are not due

to cross-bridge movement but rather to lattice irregularity. The equations for the intensity of the 1,0 and 1,1 reflections (Elliott et al., 1963) indicate that if the thin filaments are displaced from their locus upon the 1,1 planes, then reciprocal equatorial intensity changes similar to those observed with skinned frog muscle will follow. (J. Haselgrove's consultation on this matter is gratefully acknowledged.) The magnitude of the intensity change was substantial; in the technically best five before-and-after experiments the intensity ratio ($I_{1,1}/I_{1,0}$) of skinned muscles dropped, on average, to 55% (± 17% SD) of that measured before skinning . The initial intensity ratio found in Ringer's was 0.43 (± 0.08 SD), very close to that found by others (Haselgrove and Huxley, 1973; Lymn, 1975*b*; Haselgrove et al., 1976; Podolsky et al., 1976).

DISCUSSION

General Conclusions

We draw several conclusions from our results with detergent skinned frog skeletal muscle. A simple, defined medium can replace the complex sarcoplasm with only small effects on native structure. Therefore the fine structure of frog myofibrils and myofilaments does not depend on unknown cytoplasmic factors, nor are these proteinaceous structures significantly perturbed by potent membrane solubilizers such as Triton X-100. Further, since good if not excellent resting structure appeared on occasion in numerous relaxing solutions including all those in Table I, it is likely that mechanically skinned fibers in commonly used relaxing solutions also possess reasonably native myofilament structure.

It is not known whether any functional loss accompanies weakness or absence of the resting layer line pattern from relaxed muscles. It is clear that mechanically competent fibers, able to relax and contract in appropriate solutions, can be prepared by procedures which leave scarce or no myosin layer line intensity detectable from relaxed bundles or whole muscles. To take an example which helped motivate our study, when detergent-relax treatment was preceded by brief glycerination, as used to prepare mechanically acceptable fibers by Julian (1971), our X-ray patterns from whole frog sartorius in relaxing solution did not show myosin layer lines. But such layer lines remain a requisite for certain structural studies. The reversible loss of myosin layer line intensity during contraction (Huxley and Brown, 1967) indicates a change in the cross-bridge lattice that can only be investigated in systems which give clear layer lines from the start.

Using frog muscle which readily gives excellent living low-angle X-ray patterns has allowed us to document by an objective physical method the value of the detergent-relaxing solution approach to demembranation. We recognize of course that nonionic detergents have received extensive use in muscle studies (e.g., Julian, 1971; Solaro et al., 1971; Orentlicher et al., 1974; Taylor, 1976; Small, 1977, Gordon, 1978) as well as for preparing "cytoskeletal" models of nonmuscle cells (e.g., Gibbons and Gibbons, 1972; Small and Celis, 1978). What is distinctive in our successful procedure with frog muscle, is that skinning is carried out under relaxing conditions, avoiding both rigor induction and exposure to glycerol. Preliminary trials of the method with rabbit psoas have been as disappointing as the results with glycerinated psoas.

Functional tests showed that detergent treatment does not harm the contractile or regulatory properties of the chemically skinned myofibril. Single-fiber transducer studies

showed that a 2-h incubation in Triton-relaxing solution did not affect the maximal calcium-activated isometric tension compared to mechanically skinned fibers. Perhaps because the sarcoplasmic reticulum was dissolved, Triton left the fibers more sensitive to calcium, shifting the pCa-tension relationship to higher pCa by 0.5 pCa units.

Well-regulated myofibrils, capable of 100-fold ATPase activation by Ca^{2+}, have been prepared from frog muscles subjected to 30 min Triton extraction (Magid and Mumma, 1979); similar calcium sensitivity survives overnight extraction in Triton as well.

Lattice Disorder

The lattice disorder which we have observed in equatorial X-ray patterns of detergent skinned muscle fibers as a fall in $I_{1,1}/I_{1,0}$ and weakness of higher orders has also been seen by others in electron micrographs of mechanically (April et al., 1971, 1972) and chemically (Huxley, 1968; Eastwood et al., 1979) skinned muscle. Efforts are continuing in our laboratory to find a remedy for this unwelcome consequence of muscle skinning.

Since our results indicate that changes in the intensity of equatorial reflections can arise from lattice disorder, apart from any cross-bridge movements, the possible role of such disturbances in producing the equatorial changes studied in contracting muscle (e.g., Haselgrove and Huxley, 1973; Podolsky et al., 1976) must be considered in interpreting such data. Direct inference of cross-bridge behavior from intensity changes in equatorial diffraction patterns can be valid only if no changes in lattice regularity have occurred.

Lattice Swelling and Shrinking

When muscle cells are skinned the filament lattice abruptly swells and the constant volume behavior of intact fibers is lost. We feel that the most satisfactory explanation for these effects is that fibrils in vivo are constrained to a constant volume by the osmotic barrier established by a functional sarcolemma (Matsubara and Elliott, 1972). We interpret the shrinking action of colloids in the isolated lattice as an osmotic effect, colloids replacing the osmotic pressure normally provided by the effective impermeability of NaCl in the extracellular fluid. This nonequilibrium status of the myofilament lattice (April et al., 1972; April, 1975*a* and *b*), reflected as a 35-Torr swelling pressure, would be substantial driving force for cellular edema. Marked cell swelling has been noted after cell membrane injury (Robinson, 1975) and metabolic fatigue (Gonzales-Serratos et al., 1978).

Concluding Remarks

In conclusion, our results demonstrate that a simple, defined medium can sustain the structure of living myofibrils at rest. Experiments made possible by our skinned, relaxed whole muscle preparation should help in investigations of muscle questions. At present, we are using this material to study the effects on relaxed structure of: exposure to glycerol mixtures, fixatives, colloids, rigor solutions, ATP analogues, and long-term storage.

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REFERENCES

ABBOTT, R. H., and R. A. CHAPLAIN. 1966. Preparation and properties of the contractile element of insect fibrillar muscle. *J. Cell Sci.* **1**:311–330.

APRIL, E. W. 1975 *a*. Liquid-crystalline characteristics of the thick filament lattice of striated muscle. *Nature (Lond.)* **257**:139–141.

APRIL, E. W. 1975*b*. The myofilament lattice: studies on isolated fibers. IV. lattice equilibria in striated muscle. *J. Mechanochem. Cell Motil.* **3**:111–121.

APRIL, E. W., M. FARRELL, and J. SCHREDER. 1977. Osmotically-induced changes in the filament lattice of skinned striated muscle fibers. *Biophys. J.* **17**:174 *a*. (Abstr.).

APRIL, E. W., and D. Wong. 1976. Non-isovolumic behavior of the unit cell of skinned striated muscle fibers. *J. Mol. Biol.* **101**:107–114.

APRIL, E. W., P. W. BRANDT, and G. F. ELLIOTT. 1971. The myofilament lattice: studies on isolated fibers. I. The constancy of the unit-cell volume with variation in sarcomere length in a lattice in which the thin-to-thick myofilament ratio is 6:1. *J. Cell Biol.* **51**:72–82.

APRIL, E. W., P. W. BRANDT, and G. G. ELLIOTT. 1972. The myofilament lattice: studies on isolated fibers. II. The effects of osmotic strength, ionic concentration, and pH upon the unit-cell volume. *J. Cell Biol.* **53**:53–65.

BOYLE, P. J., and E. J. CONWAY. 1941 Potassium accumulation in muscle and associated changes. *J. Physiol.* **100**:1–63.

EASTWOOD, A. B., D. S. WOOD, K. L. BOCK, and M. M. SORENSON. 1979. Chemically skinned mammalian skeletal muscle. I. The structure of skinned rabbit psoas. *Tissue Cell.* **11**:553–566.

ELLIOTT, G. F., J. LOWY, and C. R. WORTHINGTON. 1963. An X-ray and light-diffraction study of the filament lattice of striated muscle in the living state and in rigor. *J. Mol. Biol.* **6**:295–305.

ELLIOTT, G. F., J. LOWY, and B. M. MILLMAN. 1966. Low-angle X-ray diffraction studies of living striated muscle during contraction. *J. Mol. Biol.* **25**:31–45.

GIBBONS, B. H., and I. R. GIBBONS. 1972. Flagellar movement and adenosine triphosphatase activity in sea urchin sperm extracted with Triton X-100. *J. Cell Biol.* **54**:75–97.

GODT, R. E., and D. W. MAUGHAN. 1977. Swelling of skinned muscle fibers of the frog. *Biophys. J.* **19**:103–116.

GORDON, A. R. 1978. Contraction of detergent-treated smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.* **75**(7)3527–3530.

HASELGROVE, J. C., and H. E. HUXLEY. 1973. X-ray evidence for radial cross-bridge movement and for the sliding filament model in actively contracting skeletal muscle. *J. Mol. Biol.* **77**:549–568.

HASELGROVE, J. C. 1975. X-ray evidence for conformational changes in the myosin filaments of vertebrate striated muscle. *J. Mol. Biol.* **92**:113–143.

HASELGROVE, J. C., M. STEWART, and H. E. HUXLEY. 1976. Cross-bridge movement during muscle contraction. *Nature (Lond.)*. **261**:606–608.

HELENIUS, A., and K. SIMONS. 1975. Solubilization of membranes by detergents. *Biochim. Biophys. Acta.* **415**:29–79.

HILL, D. K. 1968. Tension due to interaction between the sliding filaments in resting striated muscle. The effect of stimulation. *J. Physiol.* **199**:637–684.

HODGKIN, A. L., and P. HOROWICZ. 1960. Potassium contractures in single muscle fibres. *J. Physiol.* **153**:386–403.

HUXLEY, H. E. 1968. Structural difference between resting and rigor muscle: Evidence from intensity changes in the low-angle equatorial X-ray diagram. *J. Mol. Biol.* **37**:507–520.

HUXLEY, H. E., and W. BROWN. 1967. The low-angle X-ray diagram of vertebrate striated muscle and its behavior during contraction and rigor. *J. Mol. Biol.* **30**:383–434.

JULIAN, F. J. 1971. The effect of calcium on the force-velocity relation of briefly glycerinated frog muscle fibres. *J. Physiol.* **218**:117–145.

LYMN, R. W. 1975*a*. Low-angle X-ray diagrams from skeletal muscle: the effect of AMP-PNP, a non-hydrolyzed analogue of ATP. *J. Mol. Biol.* **99**:567–582.

LYMN, R. W. 1975*b*. Equatorial X-ray reflections and cross arm movement in skeletal muscle. *Nature (Lond.)*. **258**:770–772.

LYMN, R. W., and H. E. HUXLEY. 1973. X-ray diagrams from skeletal muscle in the presence of ATP analogs. *Cold Spring Harbor Symp. Quant. Biol.* **37**:449–453.

MAGID, A., and M. MUMMA. 1979. Properties of frog myofibrils prepared by a novel method. *Biophys. J.* **25**:18 *a*. (Abstr.).

MATSUBARA, I., and G. F. ELLIOTT. 1972. X-ray diffraction studies on skinned single fibres of frog skeletal muscle. *J. Mol. Biol.* **72**:657–669.

MAUGHAN, D. W., and R. E. GODT. 1974. Role of Donnan-osmotic forces in skinned muscle fibers. *Fed. Proc.* **33**:401. (Abstr.).

MAUGHAN, D. W., and R. E. GODT. 1978. Interfilament pressure opposing radial compression of relaxed skinned skeletal muscle cells. *Biophys. J.* **21**:62 *a*. (Abstr.).

MILLMAN, B. M., and T. J. RACEY. 1977. Osmotic shrinkage of the filament lattice in frog semitendinosus muscle. *Biophys. J.* **17**:175 *a*. (Abstr.).

MILLMAN, B. M., and K. WAKABAYASHI. 1979. Shrinking of the muscle filament lattice in polymeric solutions. *Biophys. J.* **25**:111 *a*. (Abstr.).

MOSS, R. L., M. R. SOLLINS, and F. J. JULIAN. 1976. Calcium activation produces a characteristic response to stretch in both skeletal and cardiac muscle. *Nature (Lond.).* **260**:619–621.

NATORI, R. 1954. The property and contraction process of isolated myofibrils. *Jikei Med. J.* **1**:119–126.

ORENTLICHER, M., J. P. REUBEN, H. GRUNDFEST, and P. W. BRANDT. 1974. Calcium binding and tension development in detergent-treated muscle fibers. *J. Gen. Physiol.* **63**:168–186.

PODOLSKY, R. J., R. ST. ONGE, L. YU, and R. W. LYMN. 1976. X-ray diffraction of actively shortening muscle. *Proc. Natl. Acad. Sci. U.S.A.* **73**:813–817.

ROBINSON, J. R. 1975. Colloid osmotic pressure as a cause of pathological swelling of cells. *In* Pathobiology of Cell Membranes Vol. 1. B. F. TRUMP and A. V. ARSTILLA, editors Academic Press, Inc., New York.

ROME, E. 1972. Relaxation of glycerinated muscle: low-angle X-ray diffraction studies. *J. Mol. Biol.* **65**:331–345.

SMALL, J. V. 1977. Studies on isolated smooth muscle cells: the contractile apparatus. *J. Cell Sci.* **24**:327–349.

SMALL, J. V., and J. E. CELIS. 1978. Filament arrangements in negatively stained cultered cells: the organization of actin. *Cytobiologie.* **16**:308–325.

SOLARO, R. J., D. C. PANG, and F. N. BRIGGS. 1971. The purification of cardiac myofibrils with Triton X-100. *Biochim. Biophys. Acta.* **245**:259–262.

SZENT-GYORGYI, A. 1951. Chemistry of Muscular Contraction. Academic Press, Inc. New York.

TAYLOR, D. L. 1976. Quantitative studies on the polarization optical properties of striated muscle. I. Birefringence changes of rabbit psoas muscle in the transition from rigor to relaxed state. *J. Cell Biol.* **68**:497–511.

VINK, H. 1971. Precision measurements of osmotic pressure in concentrated polymer solutions. *Eur. Polymer J.* **7**:1411–1419.

VON HIPPEL, P. H., and T. SCHLEICH. 1969. Ion effects on the solution structure of biological macromolecules. *Acc. Chem. Res.* **2**:257–265.

WOOD, D. S., J. ZOLLMAN, J. P. REUBEN, and P. W. BRANDT. 1974. Human skeletal muscle: properties of the "chemically skinned" fiber. *Science (Wash. D. C.).* **187**:1075–1076.

WRAY, J., P. VIBERT, and C. COHEN. 1978. Actin filaments in muscle: pattern of myosin and tropomyosin/troponin attachments. *J. Mol. Biol.* **124**:501–521.

YU, L. C., R. W. LYMN, and R. J. PODOLSKY. 1977. Characterization of a non-indexible equatorial X-ray reflection from frog sartorius muscle. *J. Mol. Biol.* **115**:455–464.