A MELTING POINT FOR THE BIREFRINGENT COMPONENT OF MUSCLE

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ABSTRACT

The A filament of the striated muscle sarcomere is an ordered aggregate of one or a few species of proteins. Ordering of these filaments into a parallel array is the basis of birefringence in the A region, and loss of birefringence is therefore a measure of decreased order. Heating caused a large decrease in the birefringence of glycerinated rabbit psoas muscle fibers over a narrow temperature range ($\sim$ 3°C) and a large decrease in both the birefringence and optical density of the A region of *Drosophila melanogaster* fibrils. These changes were interpreted as a loss of A filament structure and were used to define a transition temperature (T_{tr}) as a measure of the stability of the A region. Since the transition temperature was sensitive to pH, ionic strength, and urea, solvent conditions which often affect protein structure, it is an experimentally useful indicator for factors affecting the structure of the A filament. Fibers from glycerinated frog muscle were less stable over a wide pH range than fibers from glycerinated rabbit muscle, a fact which demonstrates a species difference in structure. Glycerinated rabbit fibrils heated to 70°C shortened to about 40% of their initial length. The extent of shortening was not correlated with the loss of birefringence, and phase-contrast microscopy showed that this shortening occurred in the I region as well as in the A region. This response may be useful for studying the I filament and actin in much the same way that the decrease in birefringence was used for studying the A filament and myosin. The observations presented show that some properties of muscle proteins can be studied essentially *in situ* without the necessity of first dispersing the structure in solutions of high or low ionic strength.

INTRODUCTION

I intend to show in this paper that heat can be used in studying the structure of the A region of striated muscle. This region is characterized by filaments composed largely of the protein myosin which, because of their birefringence, can be optically isolated in a state approaching that found in vivo.

Changes in the state of aggregation and orientation of many proteins can be induced by heat, since most are stabilized by relatively weak noncovalent bonds. These changes may be independent of, correlated with, or in addition to, changes in the conformation of individual molecules or parts of molecules. Chemical modification, solvent properties, and competitive bonding agents, each studied as a function of the rate of

heat denaturation, or less desirably of a denaturation temperature, can modify interactions and give some indication of the types and importance of "bonds" which determine a given macromolecular structure (1).

Birefringence and X-ray diffraction are two properties of the A region of striated muscle which have been used to detect heat-induced changes in aggregate structure. Vles (2) observed a decrease in the birefringence of freshly isolated muscle from the thorax of the beetle, *Dytiscus*, from the abdomen of the crayfish, and from unidentified frog and mussel muscles when these were heated to about 50°C. Shortening of the muscle complicated the interpretation of the birefringence changes. Astbury (3) and also Rudall (4) have similarly noted changes in the wide angle X-ray diffraction pattern of frog striated muscle heated at about 60°C and of actomyosin films when these were heated to above 40°C.

The following work shows that changes detectable with the light microscope accompany the heating of striated muscle and uses these changes to define parameters which affect the stability of the A filaments.

MATERIALS AND METHODS

Flight muscle fibrils were obtained from an Oregon R strain of *Drosophila melanogaster*. Preparations of fibrils were made by teasing pieces of thoracic muscle in a drop of a solution containing 0.1 M KCl, 5 mM $MgCl_2$, 2.5 mM EDTA[1], 5 mM potassium phosphate, and 2.5 mM sodium ATP[2] at a final pH of 7.0. A cover glass with silicone grease[3] on two opposite edges was placed on the drop and pressed to make the fibrils stick to the cover glass. Free fibrils, sarcosomes, and other debris were then washed away before the isolating medium was exchanged for a solution containing 0.1 M KCl and 5 mM potassium phosphate at pH 7.0 and then for the desired experimental medium. Finally, the preparation was sealed with silicone grease. Phase-contrast observations were made with an NA 1.25 phase objective in conjunction with a long working distance condenser. Polarized light observations were made with a prism-equipped polarizing microscope by utilizing an NA 0.65 objective, an NA 0.5 objective used as a condenser, and a mica compensator. I observed preparations of fibrils being heated by using a slide with a transparent resistance film (Arthur H. Thomas, Co., Philadelphia, Pennsylvania).

[1] EDTA, ethylene diamine tetraacetate.

[2] ATP, adenosine triphosphate.

[3] Silicone grease, Dow-Corning High Vacuum, Midland, Michigan.

Rabbit psoas muscle was separated into bundles of fibers which were tied to sticks and placed in ice cold 50% glycerol containing 5 mM potassium phosphate, 20 mM potassium chloride, and 2 mM EDTA at a final pH of 7.0. The fibers were placed in fresh glycerol solution 24 hr later and stored at −10°C for 6 wk to 1 yr. Before use, small fiber bundles (~ 0.2 mm in diameter) were equilibrated in 0.1 M KCl containing 5 mM potassium phosphate at pH 7.0, for at least 1 hr. Single fiber lengths about 5 mm long were teased from these bundles and mounted without flattening between a slide and cover glass. The preparation was then sealed with a silicone grease.

Glycerinated frog muscle was prepared from the lower hind leg of a frog (*Rana pipiens*). Skin and the gastrocnemius muscle were removed, after which the leg was tied to a straight stick. The glycerination procedure was the same one used for rabbit psoas muscle, except that the frog muscles were not separated into smaller bundles and were left attached to their insertions. Preparations of frog fibers were usually made from the peripheral part of the soleus muscle in the same manner as rabbit psoas fiber preparations.

Glycerinated frog and rabbit muscle fibers were observed with a polarizing microscope at a magnification of 200, a particular region of the fiber where the birefringence was uniform was noted, and the retardation and fiber width at this point were measured. After these initial measurements, the slide was heated for 2 min, and then the retardation and width were measured again.

Slides were heated by immersion in a beaker of water which was slowly stirred. The temperature decrease of the bath over a 2 min period at 50°C was about half a degree, and the nominal temperature was considered to be the initial temperature. Experiments with heating times longer than 2 min were performed in a bath regulated to ±0.1°C.

I measured birefringence with a quartz wedge using a 10 × NA 0.25 objective as a condenser in conjunction with a 20 × NA 0.5 objective and a 10 × filar micrometer. A water-cooled GE AH-6 mercury arc used with a Kodak 77 A filter served as the source.

Reagent grade inorganic chemicals and distilled water were used throughout. Mersalyl[4] was obtained from Winthrop Laboratories, New York, New York, ATP from Pabst Research Laboratories, Milwaukee, Wisconsin, *N*-acetyl tryptophan and *N*-acetyl histidine from California Corporation for Biochemical Research, Los Angeles, California; sodium deoxycholate from Difco Laboratories, Inc. Detroit, Michigan, and sodium dodecylsulfate was a commercial grade preparation from E. H. Sargent & Co., Chicago, Illinois. The pH of all solutions was determined at room temperature and this value was used as the

[4] Mersalyl (Salyrgan), salicyl-(γ-hydroxymercuri-β-methoxypropyl)-amide-*O*-acetic acid.

nominal pH at other temperatures. The effect of temperature on the phosphate buffer generally used was not studied, but the work of Bates and Acree (5) indicates that the change in pH between 24° and 60°C will be small at pH 7.

RESULTS

Drosophila

Changes Observed by Light Microscopy

PHASE-CONTRAST MICROSCOPY: The phase-contrast appearance of a freshly isolated *Drosophila* fibril is shown in the first photograph of Fig. 1. H and I zones are clear, with the I zone being the wider of the two. A Z line may be visible but is rarely obvious, while each half A band has a suggestion of three or four faint striae which may be optical artifacts.

When a *Drosophila* fibril was heated, a characteristic sequence of changes was visible by phase-contrast microscopy. Fig. 1 shows the pattern of changes as the fibril was heated to progressively higher temperatures in 0.1 M KCl at pH 7. After an initial decrease in diameter, the first sign of change was in the H zone which became wider. As this zone continued to widen, an M line became visible and gradually denser, giving the appearance of a Cm band. The denser regions remaining at the edges of the A region in adjacent sarcomeres gave the appearance of doublets centered on the Z line. With further heating, these doublets often merged into a single band. A similar pattern of changes was seen in 0.1 M KCl at pH 6 and at pH 4 and in 0.1 M KCl containing 2.5 mM ATP + 5 mM $MgCl_2$ + 2.5 mM EDTA at pH 7.0. When fibrils in 0.1 M KCl at pH 8.5 were heated, the changes which occurred were similar but not so clear and may have been complicated by extraction. The use of letters to refer to the striae of heated muscle is a convenience in describing the relative position and density of a region and does not imply that the striae of heated muscle are necessarily structurally comparable to the striae of unheated muscle.

There was little decrease in sarcomere length (less than 2% in Fig. 1) when fibrils were heated in 0.1 M KCl at pH 7 to temperatures at which there were marked cytological changes, but shortening to about 90% of the initial length was often seen when fibrils were heated in 0.1 M KCl at pH 8.5.

POLARIZED LIGHT MICROSCOPY: The image of relaxed *Drosophila* fibrils in polarized light is complex, as shown in Fig. 2. The structural significance of this image is uncertain and fibrils which have been dehydrated in acetone and immersed in nitrobenzene may provide a more direct image of the intrinsic structure of the myofibril. Acetone caused a decrease in fibril diameter of roughly 60% to a diameter of about 1 μ, and the nitrobenzene gives an approximate refractive index match.[5] Such fibrils do not show hyperstriae in the A region, a triplet Z structure, or a birefringent M line. Instead, the A region is more evenly birefringent, although a slightly less birefringent central region can often be detected.

In order to compare the phase-contrast and the polarized light appearances of fibrils after heating, preparations of isolated *Drosophila* fibrils in 0.1 M KCl were heated to a given temperature for 2 min. They were then observed by phase-contrast microscopy to determine the range of sarcomere patterns characteristic of the preparation. The same slides were then dehydrated in acetone, immersed in nitrobenzene, and examined with polarized light. Variability among fibrils within a preparation occurred and appeared to be a property of the fibrils rather than of large regions within the preparations.

A comparison of polarized light and phase-contrast images for heated fibrils is shown in Fig. 3. The first change in pattern seen by phase contrast was widening of the H zone at about 48°C (0.1 M KCl, pH 7, 2 min heating). This zone was not obviously present in polarized light at 48°C, and a large, easily noticed decrease in birefringence was not seen until about 52°C. At this temperature the center of the sarcomere and the ends of the A region appeared more "stable" than the intervening regions. With further heating, the birefringence decreased everywhere, but the same pattern was retained.

In Vivo Comparison

The relation of the structure of isolated fibrils to fibril structure within the animal was studied by heating intact flies with a 2 min immersion. No cytological signs of strong shortening in terms of Cz and Cm bands were seen in fibrils from

[5] The refractive index of nitrobenzene is intermediate to the refractive indices of acetone-dehydrated *Drosophila* muscle fibrils. When one observes a fibril with green polarized light at 25°C by phase-contrast microscopy, it is possible to see images which are matched, overmatched, or undermatched, depending on the plane of polarization.

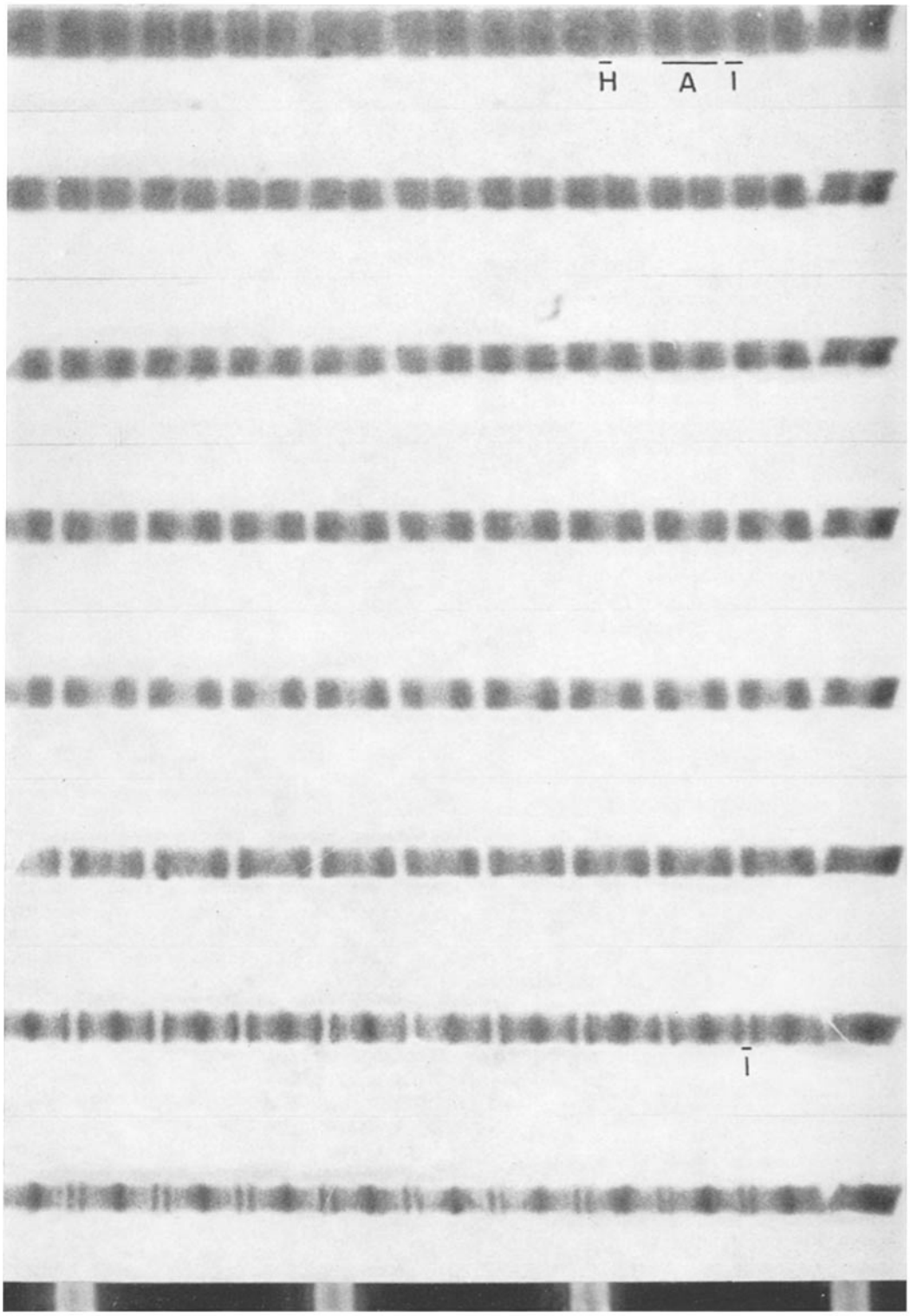

FIGURE 1 Changes seen when a fibril from *Drosophila* indirect flight muscle was slowly heated from 24°C to about 70°C in 0.1 M KCl at pH 7. The first photograph in the series was taken at room temperature. Phase-contrast. Scale divisions are 10 μ. × 2600.

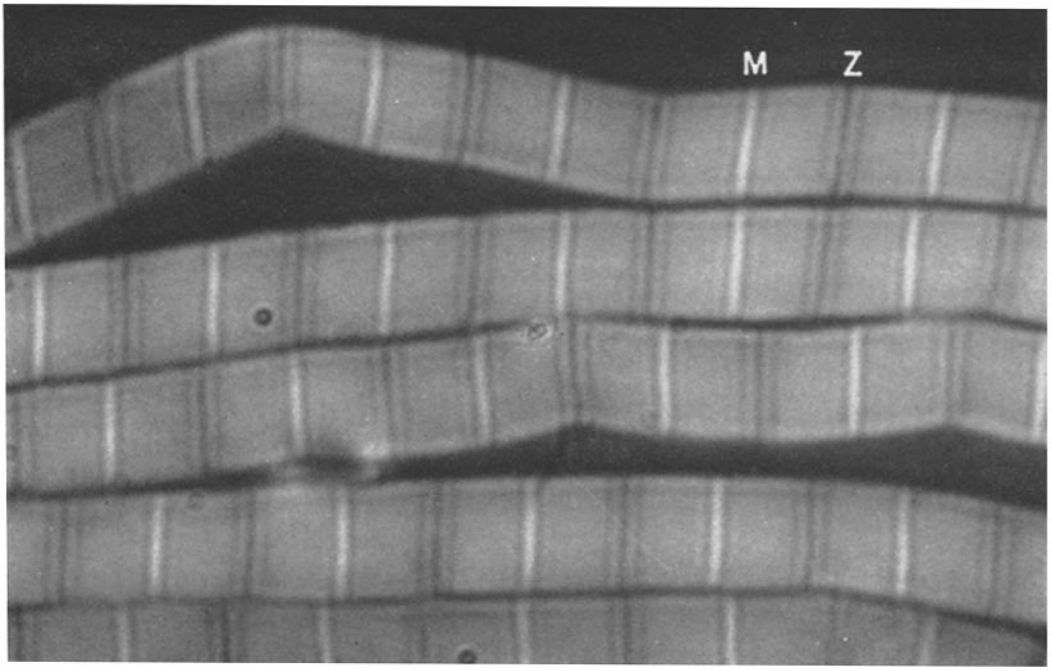

FIGURE 2 Teased fibrils from *Drosophila* indirect flight muscle in a solution containing 0.1 M KCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 2.5 mM ATP, and 5 mM potassium phosphate at pH 7. The photograph was taken in polarized light using a rectified NA 1.25 objective loaned by Dr. S. Inoué. The brightest line is at the level of the M line. Polarized light. × 6000.

heated animals. The sarcomere pattern was comparable to the pattern of fibrils which had been heated after isolation, except for unusual striation patterns due to stretching. A salt solution which would permit a precise comparison of fibrils could not be defined. Isolated fibrils heated in a solution containing 0.1 M KCl, 5 mM phosphate, 5 mM $MgCl_2$, 2.5 mM EDTA, and 2.5 mM ATP at a final pH of 7.0 showed a large decrease in birefringence at about 52°C, while whole flies showed a large decrease in birefringence on heating to about 54°C. The rough agreement shows that the structure of isolated fibrils does not differ radically from that of fibrils in vivo. Isolated fibrils heated in the solution described above had about the same transition temperature as fibrils heated without the 2.5 mM ATP.

CONTRACTILITY

Fibrils isolated from *Drosophila* indirect flight muscle have two demonstrable responses, one of shortening and one of lengthening, which are presumed to reflect contraction and relaxation, respectively (6, 7). By varying the suspending media while watching a fibril in the microscope, it is possible to induce and detect these responses reliably. Using this method, both responses were lacking in fibrils from flies which had been heated to 44°C for 2 min, while some fibrils from flies heated to 42°C for 2 min were seen to respond.

SOLVENT EFFECTS

The effects of several solvent conditions on the stability of the A region were examined to determine whether or not these might be useful in defining the structure of the A filament.

The data presented are based on determining a "transition temperature" (T_{tr}) which was defined as that temperature of heating for 2 min which gave some characteristic cytological change. A well defined increase in H zone width was the usual endpoint. There was variability in this response which limited the reproducibility to about ±1.5°C.

pH: Between pH 6 and pH 8 there was a change of 8°C in the transition temperature, and fibrils had a higher transition temperature at pH 6 than at pH 8. A transition temperature was not determined below pH 4 and above pH 10, since the A region was unstable at room temperature (23°C). These data are included later in Fig. 9. Fibril diameter decreased below pH 7, presumably as the isoelectric point was approached, and increased at alkaline pH's. Diam-

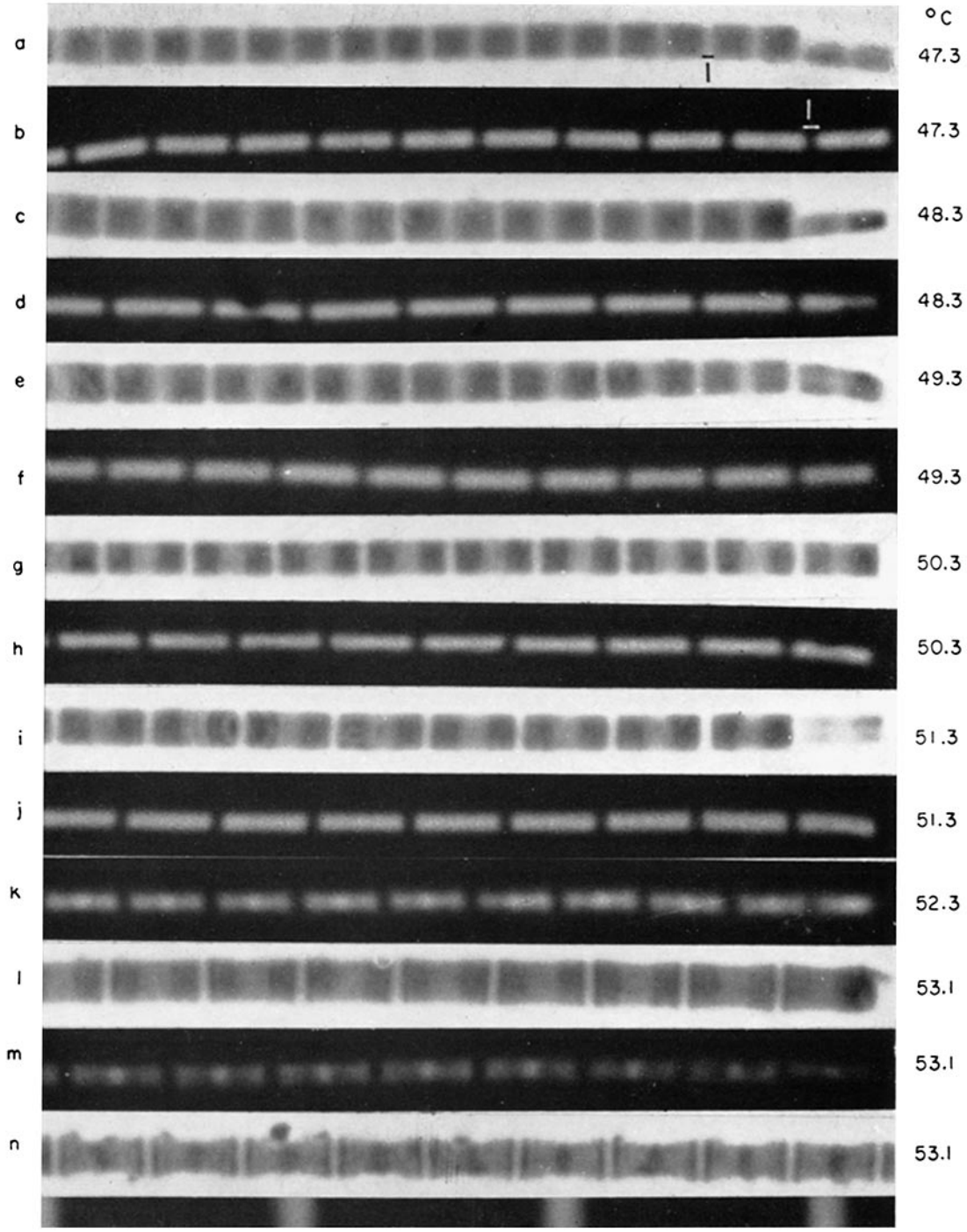

FIGURE 3 *Drosophila* fibrils in 0.1 M KCl at pH 7 were heated in an unregulated water bath for 2 min at the initial temperatures shown in the figure. The phase-contrast photographs (*a*, *c*, *e*, *g*, *i*, *l*, *n*) are of fibrils immersed in 0.1 M KCl, while the polarized light photographs (*b*, *d*, *f*, *h*, *j*, *k*, *m*) are of fibrils in the same preparations after being dehydrated in acetone and immersed in nitrobenzene. Scale divisions are 10 μ. × 2700.

eter changes between pH 5 and pH 9 appeared reversible despite some extraction at pH 9.

CATIONS: Sodium, potassium, and lithium chlorides at pH 7 reduced the transition temperature in a similar way at concentrations of 0.2 and 0.4 molar. Calcium and, less markedly, magnesium chlorides at equivalent ionic strengths decreased the thermal stability even more.

ANIONS: Potassium iodide, sodium thiocyanate, and potassium phosphotungstate were more effective than potassium chloride in reducing the thermal stability of the A region (Fig. 4).

Several organic anions which act as denaturing agents were studied. The A region of *Drosophila* fibrils was extracted at room temperature by low concentrations of sodium deoxycholate (10 mM) and of sodium dodecylsulfate (0.1 mM) in 0.1 M KCl at pH 7. Sodium salicylate, potassium *N*-acetyltryptophan, and potassium benzoate, all at pH 7, were also more effective than potassium chloride in weakening the structure of the A region (Fig. 5).

Potassium *N*-acetylhistidine and carnosine did not appear to differ from an equivalent concentration of potassium chloride, and 3 mM colchicine in the presence of 0.1 M potassium chloride also had no specific effect.

GLYCEROL: The presence of glycerol in 0.1 M KCl at pH 7 caused an increase in the thermal stability of the A region. Concentrations tested were 2 and 5 M, and these caused an increase in transition temperature of 3° and 8°C, respectively.

UREA: The A region was extracted at room temperature in a 2 M solution of urea containing 0.05 M KCl at pH 7.

In summary, fibrils teased from the indirect flight muscle of *Drosophila* show well defined structural changes when heated to temperatures of about 50°C. These changes are visible by phase-contrast microscopy and by polarized light microscopy. The temperature at which cytologically visible changes in structure occur is sensitive to solvent factors affecting protein structure and suggests that the structure of the A region of *Drosophila* flight muscle can be partially analyzed by this method.

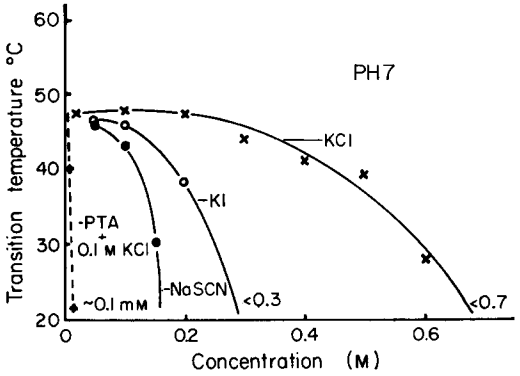

FIGURE 4 Transition temperatures of *Drosophila* fibrils in solutions containing inorganic anions. All solutions contained 5 mM potassium phosphate, in addition to the other ions. PTA is potassium phosphotungstate at pH 7. The concentration of salt giving a T_{tr} of 22°C was less than 0.3 M for KI and less than 0.7 M for KCl since the A region was extracted at 22°C by 0.3 M KI and by 0.7 M KCl.

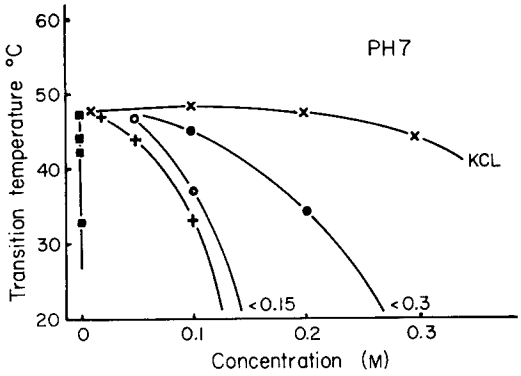

FIGURE 5 Transition temperatures of *Drosophila* fibrils in solutions containing a variety of organic anions. All solutions contained 5 mM potassium phosphate. —○— potassium benzoate; —●— potassium *N*-acetyltryptophan, + potassium salicylate; —■— sodium deoxycholate + 0.1 M KCl. The A region was extracted at room temperature by 0.3 M potassium benzoate and by 0.15 M potassium *N*-acetyltryptophan so that the concentrations corresponding to a T_{tr} of 22°C will be less than these values.

Vertebrate Muscle

The following section deals primarily with the response of glycerinated rabbit psoas muscle to heat. The advantages of using glycerinated rabbit muscle are the ease of teasing long fibers of fairly uniform size, birefringence, and sarcomere length, the ease of exchanging solutions without affecting the contractile state, and the information available as to the properties and amino acid composition of the myofibrillar proteins (8). Many contractile properties of intact muscle

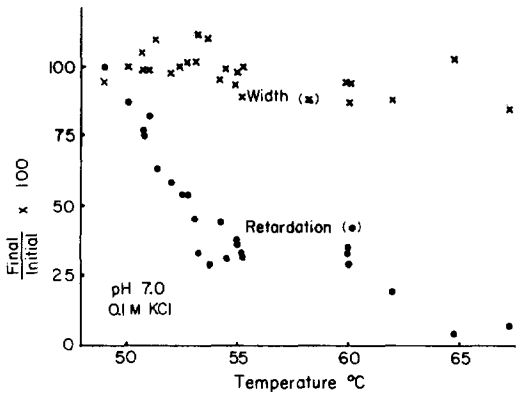

FIGURE 6 The effect on fiber width and retardation of heating glycerinated rabbit psoas fibers for 2 min at pH 7.

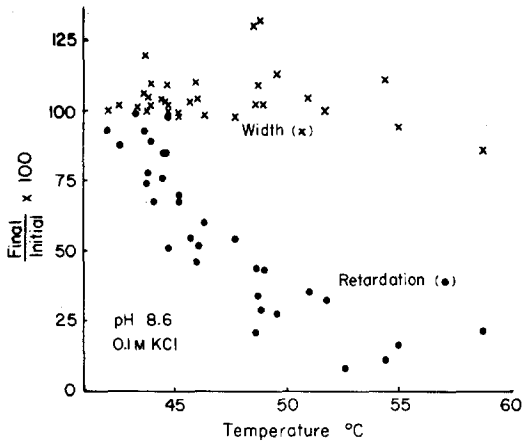

FIGURE 7 The effect on fiber width and retardation of heating glycerinated rabbit psoas fibers for 2 min at pH 8.6.

have been demonstrated with glycerinated rabbit fibers (9) including the ability to "relax" (10), indicating that glycerination has little effect on their potential ability to function. Probably the best indication that the structure of the A filament is not changed on glycerination is the similarity in salt extractability of presumably identical proteins from fresh and from glycerinated muscle (11).

EFFECT OF HEAT ON BIREFRINGENCE

Heating glycerinated rabbit fibers caused a large decrease in birefringence. From phase-contrast observations on fibrils the loss of birefringence was not associated with a marked change in the density of the A region. Observations with the polarizing microscope showed that the decrease in birefringence was not due to disorientation of fibrils, or to a large increase in fiber diameter or length. Unpublished electron microscope observations show that heating in the range where birefringence disappears causes the compact structure of the A filament to break down into a fibrous mass without easily detectable filamentous organization. Loss of birefringence and of electron microscopically detectable A filament structure has been previously shown to occur when myosin is specifically extracted from rabbit muscle (12). For these reasons the loss of birefringence on heating muscle is considered to reflect change in the aggregate structure of myosin.

Birefringence changes resulting from heating are presented as the per cent decrease in the initial maximum retardation. This was possible because changes in fiber width were relatively small, with the exception of values determined near pH 5 and pH 6.

Figs. 6 and 7 show the relation between the change in retardation with temperature of heating at pH 7 and at pH 8.6.

In these experiments, a transition temperature was defined as that temperature of heating which gave a 20% decrease in the initial birefringence (retardation). The effect of prolonged heating on this transition temperature was studied in 0.1 M KCl at pH 7. With a heating time of 22 hr, the transition temperature was 5°C lower than that determined for a 2 min heating time. The actual difference was probably less, since there was a shift in pH toward more alkaline values which would decrease the apparent transition temperature. A precise determination of the effect of prolonged heating requires the use of other methods, such as electron microscopy, to detect changes in filament structure directly and of larger solvent volumes to keep the pH constant.

In the work to be described, the time of heating used was always 2 min. A fiber preparation was heated only once, even though there was evidence that heating several degrees below the transition temperature for short periods had little effect on the transition temperature. All the observations were made on glycerinated fibers from a single bundle of fibers from one rabbit. This bundle had been stretched to give a sarcomere length of about 3 μ. Psoas fibers from two other rabbits were studied much less extensively and gave no indi-

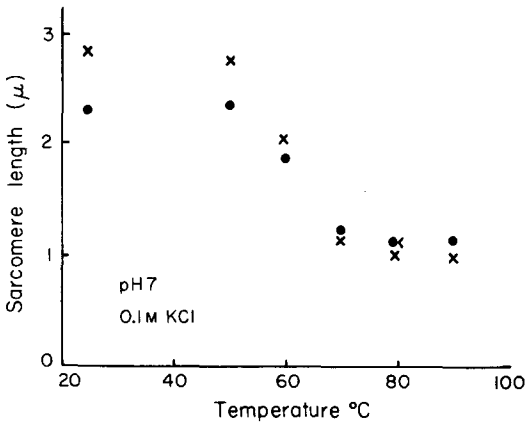

FIGURE 8 The effect on sarcomere length of heating glycerinated rabbit psoas fibrils for 2 min. Shortening below 1 μ probably occurred but was not detected because of the difficulty in counting short sarcomeres. Fibrils with an initial average sarcomere length of about 3 μ are shown by the Xs, and those with an initial length of about 2.3 μ are shown by (—●—).

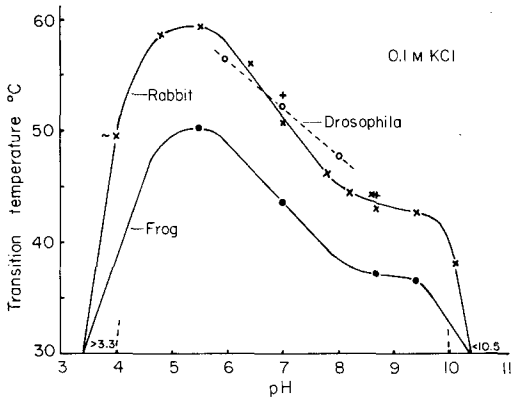

FIGURE 9 The transition temperatures of glycerinated rabbit psoas fibers, glycerinated frog leg fibers, and freshly teased *Drosophila* fibrils at different pHs are shown. The nominal pH was determined at room temperature. All points were determined in 0.1 M KCl + 5 mM phosphate, except the two (+) points which were of rabbit psoas fibers in 0.1 M KCl + 10 mM Tris.

cation that the responses which I shall describe are unique.

REVERSIBILITY: Fibers heated in 0.1 M KCl at pH 7 to temperatures causing a decrease in birefringence gave no indication of recovery of birefringence when kept in the same solution at room temperature for periods up to 24 hr.

LENGTH CHANGES

Heating glycerinated rabbit psoas fibers at temperatures *above 55°C* has been shown by Varga (13) to cause shortening. This observation was verified as shown in Fig. 8 but could not be demonstrated in all cases when observing fiber segments or fibrils which were attached to the cover slip; presumably the force developed or the fibril strength was too weak to overcome this slight resistance, at least for long sarcomeres. Phase-contrast microscopy showed that this shortening involved both the I and the A regions. Similar cytological changes have been noted in muscle soaked in 0.1% benzoquinone followed by treatment with 2 M KI at 80°C (14). Shortening appears to reflect changes in the I filament but is not easy to work with experimentally. As mentioned, the fibrils do not shorten consistently, the transition range is not sharp, and time of heating is an important parameter. Since extensive shortening occurred only at temperatures above those at which the birefringence was lost, it does not appreciably affect the determination of the transition temperature.

CONTRACTILITY

Glycerinated fibers shorten in the presence of magnesium ions and ATP. Fibers heated for 2 min in 0.1 M KCl showed a gradual decrease in the amount of ATP-induced shortening until at about 52°C the fibers did not noticeably shorten.

SOLVENT EFFECTS

pH: The effect of pH on the transition temperature of glycerinated rabbit psoas and frog leg muscle is shown in Fig. 9. Measurements were made in 0.1 M KCl containing 5 mM phosphate and the nominal pH was measured at room temperature. The curves of T_{tr} vs. pH for frog and rabbit muscles are of the same general shape showing a maximum stability at about pH 5, a decrease in thermal stability between pH 6 and pH 8, and instability at room temperature below pH 4 and above pH 10. Frog muscle was less stable than rabbit muscle over the entire pH range. These data show that one or more ionizable groups with a pK of 7 has an effect on the stability of the A region. The imidazole nitrogen of histidine is a likely possibility but other molecules with a pK in this range, which includes free and esterified phosphates, cannot be eliminated. Ionizable groups other than those titrating

near pH 7 are obviously important in determining the structure of the A region as might be expected from the high proportion of charged and of polar amino acids in myosin (8). A low concentration of phosphate was used for buffering but did not appreciably affect the curve since 0.01 M Tris gave comparable values of T_{tr}.

Szent-Györgyi and Borbiro (15) observed that the breakdown of light meromyosin in urea had a marked pH dependency similar to that expected from the titration of the imidazole nitrogen of histidine. This observation may be related to the pH dependency described above.

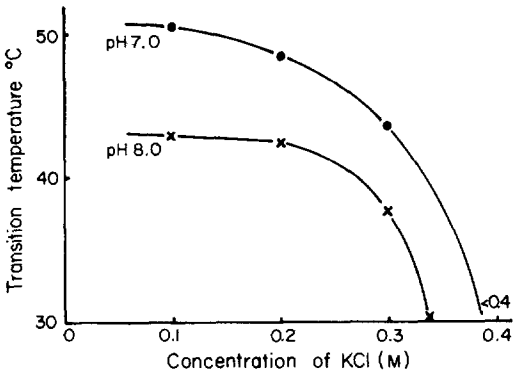

FIGURE 10 The effect of potassium chloride concentration on the transition temperature of glycerinated rabbit psoas muscle.

Measurements made at pHs 4.8, 5.5, and 6.4 were corrected for a decrease in fiber diameter by assuming that the number of parallel birefringent rods per unit of fiber cross-section varies inversely at the square of the radius, and that the thickness of the fiber varies as the radius. This correction ignores changes in form birefringence. At pH 5.5, where the diameter decrease was a maximum (28%), the effect of the correction was to reduce the observed transition temperature by about 2°C.

POTASSIUM CHLORIDE: The effect of potassium chloride at pH 7 and at pH 8.7 is shown in Fig. 10. Myosin isolated from rabbit skeletal muscle is soluble in 0.3 M KCl but is normally extracted at a higher ionic strength (16). The decreased stability of the A region in 0.3 M KCl seems, at best, to agree roughly with the known solubility characteristics of myosin after isolation.

UREA: Urea in 0.1 M KCl at pH 7 and pH 8.8 clearly had an effect on A filament structure at concentrations of 1 and 2 M (Fig. 11). Small changes in the optical rotation of myosin dissolved in 0.5 M KCl have been observed with 2 and with 4 M urea (17, 18), but extreme changes in optical rotation and separation of subunits are not seen except at higher concentrations.

SODIUM DODECYLSULFATE: Sodium dodecylsulfate (0.5 mM in 0.1 M KCl at pH 7) caused a 7°C decrease in the transition temperature of rabbit psoas muscle. This was in contrast to its effect on *Drosophila* fibrils where a concentration of 0.1 mM sodium dodecylsulfate slowly extracted the A region at room temperature.

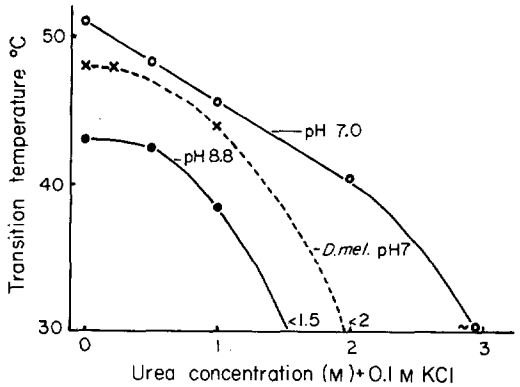

FIGURE 11 The effect of urea in the presence of 0.1 M KCl + 5 mM phosphate at pH 7 on the transition temperature of glycerinated rabbit psoas muscle and on freshly teased *Drosophila* fibrils. That urea concentration giving a T_{tr} of 30°C was less than 1.5 M (< 1.5) for rabbit fibers at pH 8.8 and less than 2.0 M (< 2.0) for *Drosophila* fibrils at pH 7.

GLYCEROL, ETHYLENE GLYCOL, AND METHANOL: The birefringence of glycerinated rabbit fibers in 0.1 M KCl containing glycerol, ethylene glycol, or methanol was less sensitive to heat than in 0.1 M KCl alone (Fig. 12). Several other proteins (19) and protein structures (20, 21) also show increased stability in glycols.

Ethylene glycol has been shown to change the optical rotation of myosin in solution but not that of light meromyosin (22). At high concentrations of ethylene glycol (10 and 15 M in Fig. 12) there was no suggestion of a decrease in the stability of the A region in glycerinated fibers. There were changes in the dimensions of these heated fibers as to both length and width, making the values of the transition temperature in glycerol and ethylene glycol less precise than at lower temperatures. However, fibers heated to 70°C in high concentrations of glycerol or ethylene glycol

and then resuspended in 0.1 M KCl at pH 7 and reheated (Fig. 12) gave an apparently normal response at 52°C. A 50% v/v glycerol solution has a glycerol molarity of 6.9.

MERSALYL: This reagent is considered to bind sulfhydryl groups specifically at pH 7 (23). Exposure for 1 hr to 1 mM mersalyl in 0.1 M KCl at pH 7 lowered the transition temperature of both frog and rabbit muscles by about 10°C when tested at pH 7 in 0.1 M KCl after excess mersalyl had been washed out. It is interesting that mersalyl has an effect on the stability of rabbit muscle

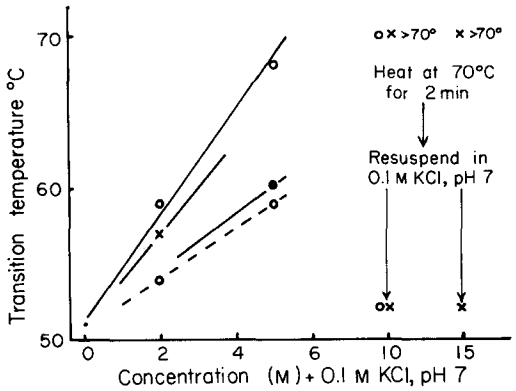

FIGURE 12 Effects of glycerol (—○—), ethylene glycol (—×—), and methanol (—●—), each in the presence of 0.1 M KCl + 5 mM phosphate at pH 7, on the transition temperature of glycerinated rabbit psoas fibers. The effect of glycerol + 0.1 M KCl on the transition temperature of freshly teased *Drosophila* fibrils is also shown (- - -○- - -). Fibers in 10 M and 15 M glycerol or ethylene glycol did not give any indication of loss of birefringence when heated to 70°C for 2 min. As shown on the right side of the figure, fibers which were heated to 70°C under these conditions when resuspended in 0.1 M KCl had a transition temperature near 52°C. On this basis the transition temperature of fibers in 10 M and 15 M ethylene glycol or glycerol is greater than 70°C.

since light meromyosin fraction I, a fragment of myosin with a molecular weight of 120,000, may be a major part of the A filament structure (16) and yet does not contain any of the sulfhydryl groups found in myosin (24).

The data presented have some relation to the noncovalent forces which structure the components of the A filament or the A filament itself, but the low specificity in terms of bonds affected, the gross similarity in the kinds of bonding at all levels of structure, and the lack of a good reference model do not permit an explicit interpretation. An indication of the kinds of bonds within proteins which may be affected by the experimental conditions used here and their relation to protein structure can be found in the work of a number of authors (1, 25, 26, 27).

Rupture of the weak intermolecular bonds which stabilize protein aggregates may precede changes in secondary and tertiary structure and lead to a nematic state. It is, however, unlikely that this will be generally true, under a variety of solvent conditions, for a large complex protein aggregate such as the A filament. Following an initial loss of A filament aggregate structure, or of some part of it, one can picture possibilities for new conformations and for aggregation in new ways. In muscle, one or many of these changes is reflected in the decrease in birefringence.

The protein remaining in the A region after heating may show how closely extensive changes in protein conformation are related to the loss of aggregate structure as reflected by decreased birefringence. Changes in solubility have been used as an indication of such changes although more precise methods for their detection do exist.

Glycerinated rabbit fibrils which had been heated in 0.1 M KCl at pH 7 were treated for several minutes with 0.5 M potassium iodide at pH 7. The effect on the A region was observed by phase-contrast microscopy. In unheated muscle, 0.5 M KI extracts the A region and much of the I filament material (12), while in muscle heated to 55°C for 2 min little or none of the A material appeared to be extracted by 0.5 M KI. The first signs of decreased solubility were seen in fibrils heated to 48°C in which a small amount of material in the center of the A region was not extracted (these fibrils had good H zones). The correlation between heat-induced birefringence changes and decreased solubility demonstrates that temperatures which reduce the birefringence have effects on protein structure in addition to disaggregation. This was also shown by the *gradual* decrease in strength of ATP-induced shortening seen when fibrils were heated at temperatures below those where the birefringence decreased.

Attempts to analyze muscle function in terms of the dimensional changes that occur on heating have been made by several investigators, notably Florey, Mandelkern, and Hoeve (28). The effects

described would appear to involve gross changes in A and I filament structure.

Species differences in the thermal stability of intact frog and rabbit muscle as well as in the heat denaturation of actomyosin from frog and rabbit muscle have been cited by Mirsky (29). Rabbit muscle and rabbit actomyosin are stable at temperatures about 5°C above those which affect frog muscle and frog actomyosin. Similarly, this report has shown that the transition temperature for the birefringence of rabbit muscle was about 7°C above that of frog muscle.

An optimistic view of the kind of thermal study considered in this paper suggests that it is a means of examining single, small structures without the necessity for dissociation or the formation of a uniform suspension containing large numbers of the structure considered, is applicable at both the light and electron microscope levels, and contains elements of chemical specificity. Such study does not appear to be especially applicable to providing a quantitative description of a structure, but does appear to be useful in distinguishing between structures, in giving some chemical indication of how the structures differ, and possibly in dissecting compound structure. I might emphasize that there are few ways of studying structure at the electron microscope level, and it is at this level that one anticipates finding biologically significant macromolecular aggregates. In particular, thermally induced changes may be useful in discriminating between classes or regions of structure which are chemically and/or physically heterogeneous but which display a similar appearance, such as may exist in the arrays of membranous, granular, and filamentous elements found within cells.

I should like to express my appreciation to the Department of Biological Structure of the University of Washington and particularly to Dr. John Luft for the opportunities and facilities provided. This work was supported by United States Public Health Service Grant No. NB 00401 administered by Dr. John Luft.

Received for publication 17 November 1965.

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