ships among all of the agents which effect significant changes in mitochondrial ion transport.

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Chemistry, Microscopy, and Performance of Methylated Glycerol-Treated Muscle Fibers*

William J. Bowen and Virginia L. S. Field

ABSTRACT: This investigation was made to learn more of the procedures, reactions, and products involved in the methylation of glycerol-treated rabbit psoas muscle fibers. It is known from earlier research that dimethyl sulfate methylated glycerol-treated muscle fibers lose their adenosine triphosphatase (ATPase) activity but retain two-thirds of their ability to shorten when ATP is applied. Shortening is also brought about by adenosine diphosphate (ADP), adenosine monophosphate (AMP), and sodium pyrophosphate (PP) which makes it appear that this shortening is due to the binding of the anions of these phosphate compounds to cationic sites of the methylated protein. Anionic sites of the protein are covered by methyl esterification and the fibers become predominantly cationic. Thus this type of shortening is anion induced rather than ATP induced. This appears not to be a general protein phenomenon because methylated collagen fibers did not shorten. Phase-contrast microscopy revealed that the structure of the fibers is reversibly distorted by methylation and subsequent hydration. When ATP or PP are applied to them, the fibers recover from the distortion and show clear I and A bands as well as H zones. Methylation with ^{14}C-labeled dimethyl sulfate revealed that carboxyl groups were implicated in only about two-thirds of the sites methylated. Attempts to identify the other one-third were made by means of chromatography and autoradiography and the results indicate that histidine and lysine residues also are sites of methylation.

It has been reported that methylated glycerol–water extracted fibers of rabbit psoas muscle retain ATP[1]-induced contractile ability and lose ATPase activity (Bowen and Martin, 1964). In those experiments fibers were methylated by exposure to dimethyl sulfate in aqueous buffered solution. Contractility was induced by ATP, ADP, AMP, and PP, all of which possess a large anion. Small anions also caused shorten-

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[1] Abbreviations used: DMS, dimethyl sulfate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; PP, sodium pyrophosphate; PMB, *p*-mercuribenzoate.

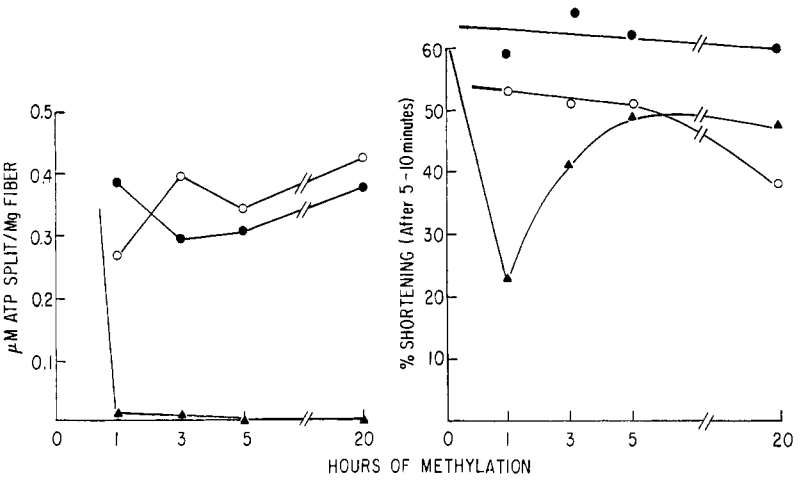

FIGURE 1: The effects of methylation by dimethyl sulfate on the ATPase activity (left) and ATP-induced shortening (right) of glycerinated muscle fibers. Plots show the activities after varying times of methylation in buffered dimethyl sulfate solution compared to control exposure in water only and to the buffer solution (0.028 M). pH in methylating solution decreased to about 2.0. ● = water control. ○ = buffer control. ▲ = methylated.

ing of methylated muscle fibers but to a lesser extent than that caused by large anion (20 *vs.* 50% or more), and the anionic concentration had to be higher than that of the large anions. Since these results provided an instance of ATP-induced contraction of a biological muscle model not accompanied by hydrolysis of ATP the phenomenon has been investigated further.

In the earlier research on this problem, it was noted that glycerinated psoas fiber bundles became hydrated after 16 hr of reaction with dimethyl sulfate (Bowen and Martin, 1964). When they were transferred to water, this hydration increased to the extent that the fibers became nearly invisible due to loss of relative refractive index. When the suspending medium was changed from water to a solution containing large anions (*e.g.*, ATP or PP), the fibers shrank back to pretreatment diameter, regained their relative index of refraction, and underwent shortening of 40–50%. This shortening is the criterion of effect in many of the following experiments.

Methylation was performed by several variations of a basic method which were designed to study aspects of the reaction with the protein. Dimethyl sulfate under the conditions of the reaction provided methyl alcohol and sulfate and its solution contained buffer. The separate effects of these were considered. The sites on the protein molecule which were being modified by the procedures were identified by chromatography and autoradiography as much as possible.

Professor H. E. Huxley (personal communication) raised the question of whether methylation altered the striated structure of the fibers. In other words, are the fibers now behaving like synthetic polyelectrolytes which shorten by changes of ionic conditions, especially those of acids and alkalies (Davies, 1965). These variables have been investigated by examination of the methylated fibers by phase-contrast microscopy.

The results indicate that during treatment with DMS shortening ability is gradually reduced to a low level during the first hour and then recovers with extended treatment (up to 20 hr). The results definitely show that the striated structure is not lost by methylation and they suggest that the reaction with DMS is primarily an esterification of the free carboxyl groups and secondarily a methylation of nitrogens in lysine and histidine. Shortening of methylated fibers appears to be anion induced.

Methods

Glycerol–water-extracted fibers were prepared by the traditional procedure of Szent-Györgyi (1951). Several weeks or months later the glycerine was washed out and the fibers were methylated by the method of Saroff *et al.* (1953) by immersing them in cold, 0.127 M dimethyl sulfate and sodium acetate buffer varying from 0.028 to 0.143 M and from pH 4.2 to 5.0. The DMS solution was prepared by dissolving 1 ml of pure DMS in 60 ml of cold water with thorough shaking and then mixing 50 ml of this solution with 20 ml of 0.1 M sodium acetate. The fibers were routinely left to methylate 16–20 hr but because of the extreme hydration after transfer to water, they were frequently left in the methylating solution for a few days, then transferred to water (several changes) to wash before being used experimentally.

Isotonic contractions of fibers were produced by addition of solutions of ATP or PP, with or without both $MgCl_2$ and Tris-maleate buffer, and were measured on microscope slides placed on a millimeter scale. The pH was 6.6–7.0. ATPase activities were determined in small beakers using two pieces of fiber bundles 60 mm long and approximately 250 μ in diameter and 2.0 ml of a solution of 5 mM ATP, 5 mM

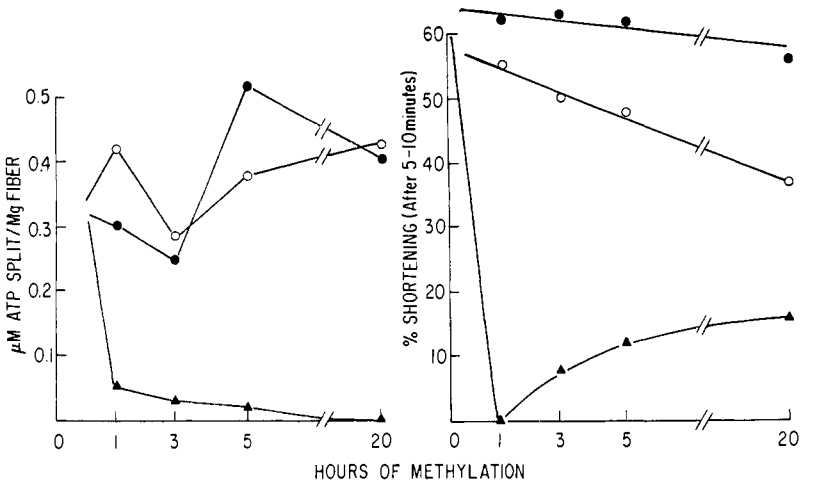

FIGURE 2: Same as Figure 1 except that the final concentration of buffer in the methylating solution and the control was 0.143 M. pH in the methylating solution decreased to about 3.5. See Figure 1 for key.

$MgCl_2$, and 0.02 M Tris-maleate buffer. The ATPase reactivities were performed on a shaker oscillating 80–90 times/min. The amount of ATP split was estimated after 10 min of reaction by measuring the P_i present in the solutions by the Fiske–SubbaRow method. The protein in the reaction mixtures was measured by weighing the washed and dried muscle fiber bundles on a Rodder quartz fiber torsion balance. Fiber weights per reaction mixture ranged from 0.2 to 0.8 mg.

The sites of methylation were investigated by adding 40 μl of ^{14}C-labeled DMS with 3.24×10^7 cpm to the methylating solution made as described above. Fibers were treated overnight, transferred through four washes of water, and oven-dried, and the radiation was estimated with a thin-window counter. After determining that the fibers had acquired radioactivity, some were hydrolyzed overnight with 6 N HCl at 105°. The hydrolysate was flash evaporated, dried, and redissolved repeatedly until a neutral solution was obtained. Then the dry residue was dissolved in 10 ml of water, and a 1-ml portion was dried on a planchet for estimation of the radiation resulting from methylation.

Blocking the functional groups of histidine and tyrosine in actomyosin was done by treating an estimated 10 mg (dry weight) of fibers with diazosulfanilic acid, prepared according to Sarkar *et al.* (1964) in 20 ml of 0.01 M Tris buffer (pH 7.5) for 3 hr at 25°. The reaction was stopped by removing the fibers from the reaction mixture and washing them with several changes of cold water over a 4-hr period. Essentially the same procedure was employed to block the sulfhydryl groups, except 0.5 mg of PMB/10 mg of fibers was substituted for Pauly's reagent.

Two-dimensional paper chromatography using *t*-butyl alcohol–formate–water (70:15:15) as solvent for one direction and *t*-amyl alcohol–lutidine–water (178:178:110) for the other direction (Irreverre and Martin, 1954; Piez *et al.*, 1956) was used to separate the amino acids of the hydrolysates. Spots were located by 0.2% ninhydrin in acetone.

Results

Chemistry of Methylation with Dimethyl Sulfate. During methylation by this means, sulfate ions and methyl alcohol were produced. This ion production was accompanied by a lowering of the pH because 0.028 M sodium acetate was not strong enough to buffer the acidity produced. When the pH of the 0.1 M stock of buffer was 5.0 and the final concentration was 0.028 M, the pH of the methylating solution fell to 3.0–3.5 in 5 hr and to 1.8–2.0 in 20 hr. The effects of this pH change on ATPase activity and ATP-induced shortening as criteria of methylation were investigated. Such investigation was undertaken by varying the concentration of buffer in the methylation from 0 to 0.143 M.

Figures 1 and 2 show that methylation with 0.028 and 0.143 M concentrations of sodium acetate buffer had a similar effect on ATPase activity and isotonic contractions. Methylation for 1 hr produced 86–97% inhibition of the initial ATPase. Further methylation resulted in 100% inhibition. Comparable times of treatment in water and in buffer solutions had no adverse effect on ATPase activities of the fibers. The variations of the plots of ATPase activity are probably due to irregularities of the enzyme activity of fibers rather than to treatment.

Fibers methylated in the presence of the two concentrations of buffer suffered loss of ability to undergo ATP-induced shortening during the first hour of methylation, but the extent of the loss differed considerably with the low and high concentrations. In marked contrast to the effects on ATPase activity, longer exposure to the methylating solution caused reversal of the loss of ATP-induced shortening. This

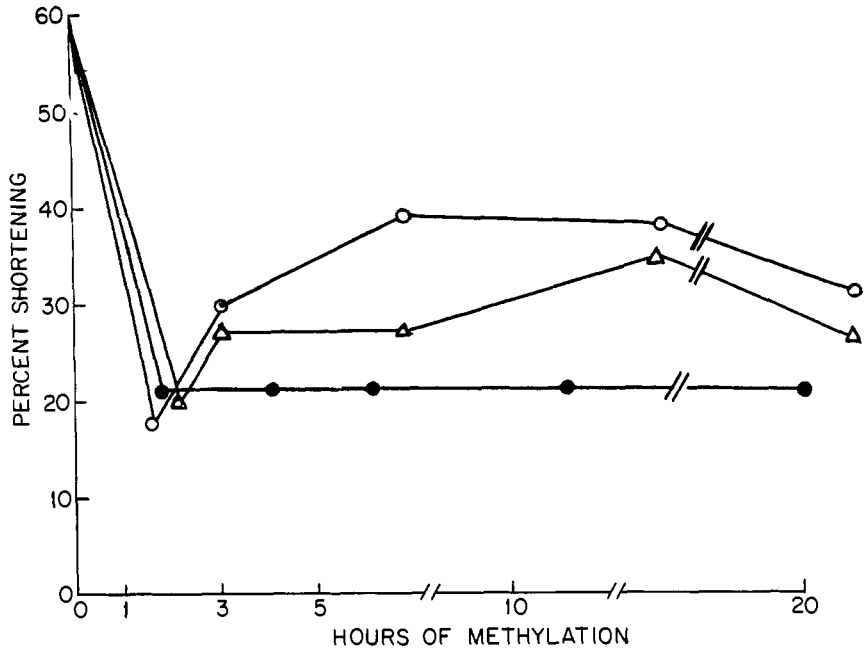

FIGURE 3: Anion (ATP) induced shortening of glycerinated muscle fiber after methylation by dimethyl sulfate with and without sodium acetate as buffer and with and without NaCl as a source of ions. ● = DMS only. ○ = DMS and 0.028 M sodium acetate. Δ = DMS and 0.03 M NaCl.

occurred while the ATPase activity was inconsequential and approaching zero. After 1 hr of methylation in strongly buffered DMS solution, ATP-induced shortening of the fibers was reduced from 60 to 0% loss of length incurred by normal glycerinated fibers; *i.e.*, shortening did not occur. In weakly buffered DMS solution, shortening was reduced from 60–70 to 20–25% loss of the original length (Figures 1 and 2). After 20 hr of methylation, the fibers in both the strongly and the weakly buffered DMS solutions recovered ATP-induced contractile ability so that they shortened 15 and 50%, respectively, of the original length. The ATPase activity of both kinds of fibers was 0 at 20 hr of methylation. Similar amounts of shortening were obtained by using 0.01 M PP instead of ATP solution. Methylation of the fibers was entirely responsible for their shortening in PP solution because this did not occur in normal glycerinated fibers (Bowen and Martin, 1964).

These results show that treatment of glycerinated muscle fibers with DMS in sodium acetate buffer solution which is not strong enough to maintain a pH value above 2.0 causes a reversible loss of ATP-induced isotonic shortening and an irreversible loss of ATPase activity. It appears probable that the fibers bear a preponderance of positive charges because this shortening is induced by multivalent large anions which include, besides ATP and PP, ADP and AMP (Bowen and Martin, 1964). It therefore seems appropriate to term the phenomenon "anion-induced" shortening.

The Role of Ions. The question whether acetate and sulfate ions and extreme acidity play a role in methylation of the fibers was investigated by exposing fiber bundles to unbuffered Na_2SO_4, H_2SO_4, and HCl, with and without $MgCl_2$, and to 0.127 DMS without buffer.

Unbuffered DMS reduced shortening rapidly to about one-third of that induced by ATP in untreated fibers (Figure 3) but the extent of recovery varied from a negative value (*i.e.*, no shortening) to the amount which occurred when the DMS was buffered. Most frequently, the shortening was not affected by time of exposure to DMS as is shown in Figure 3. Closely correlated with the extent of anion-induced shortening was the microscopic appearance of the fibers. Those which did not shorten were devoid of striations and apparently all contractile protein. Only sarcolemma and nuclei were visible. Fiber bundles which would shorten as shown in Figure 3 had striations remaining in some fibers while other fibers were structureless.[2] No explanation of this variation is known, but it appears that sarcolemma is not the contractile protein of methylated fibers.

These results raise the question whether sodium acetate is instrumental in the recovery of anion-induced shortening after methylation by being a buffer or a source of ions. To answer this question, NaCl was substituted for sodium acetate with no buffer present. The results (Figure 3) indicate that fibers in DMS solution with NaCl underwent the initial decrease and subsequent

[2] The striations which remained had the appearance of those of Figure 5C.

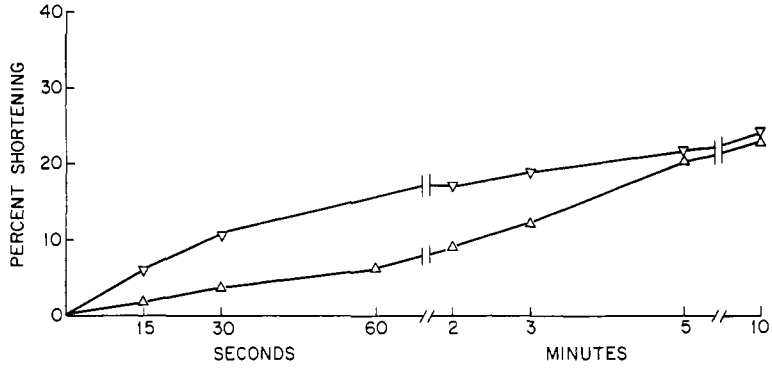

FIGURE 4: Two experiments to illustrate the effect on the rate of shortening of glycerinated muscle produced by 0.02 M sulfate ion from H_2SO_4 instead of from dimethyl sulfate. ▽ = June 17. Δ = July 13.

increase in shortening ability as they did when sodium acetate was added, but the increase was somewhat slower in occurring. This difference in rate occurred in several experiments done at widely separated times.

These results, taken together with the photographic evidence presented below, indicate that methylation of the contractile proteins causes them to become water soluble. When salt is present solubilizing is limited to hydration.

The experiments described above account for several factors of DMS treatment of muscle fibers but not for the effects of SO_4^{2-} and $[H^+]$. Sulfation of the muscle proteins at pH values around 2.0 seemed unlikely; however, to ascertain any possible effect, fibers were exposed to 0.02–0.03 M H_2SO_4 which is pH 1.8–2.0. The fibers soon became translucent like those in buffered DMS solutions, but when they were transferred to water, there was no hydration like that which occurred when fibers treated with DMS were put into water. Microscopic examination showed that they retained their normal striated appearance. When ATP or PP was applied, they shortened 10–20% of their original length (Figure 4).

Treatment with 0.01 N HCl (pH 2.5) produced fibers after 30 min which shortened a maximum of 11% with 2 mM ATP. Since they did not shorten when PP was applied, the small amount of shortening produced by ATP was probably a residual of ATP-induced shortening of glycerol-treated fibers.

Fibers treated with unbuffered DMS, H_2SO_4, and HCl as described above lost their ATPase activity. Since this activity was lost by the treatment with HCl, it is highly likely that the loss of ATPase activity following methylation by DMS is due to the high $[H^+]$. This conclusion is supported by the fact that treatment with 0.02 M Na_2SO_4 at pH 6.5 yielded fibers capable of high rate of ATP-induced shortening (50% in 1 min), the same as occurred with fibers treated with water only. Also, the splitting of ATP was not reduced or increased by Na_2SO_4.

From these results, it can be stated that treatment of glycerinated muscle fibers with buffered DMS alters the fibers so that the anions of ATP or PP cause shortening and that this shortening is associated with the methylation rather than with high acidity. The sulfate ions accompanying the DMS may have a synergistic effect.

Photomicroscopic Studies. Fiber bundles were examined by phase contrast microscopy to ascertain the structure of the methylated and hydrated fibers before and after the application of ATP and PP. Fibers, methylated with DMS in the usual manner, were washed and mounted in water or solutions of ATP and PP on microscope slides under no. 1 cover glasses.

Figures 5A,B are of control fibers (treated in water for the duration of the methylation process) before and after application of ATP. The Z line is not visible after ATP-induced shortening. The lengths[3] of the sarcomeres were decreased (Table I). Of this shortening, less than 10% occurred in the A bands, while more than 90% occurred in the I bands (see Huxley and Hanson, 1957).

Figure 5C shows the microscopic appearance of a methylated and greatly hydrated fiber. The I bands are considerably longer than those of normal fibers (Table I). The A bands appear to be compressed into irregular short lengths.

Figures 5D–F are of fibers which were methylated and then immersed in 10 mM ATP, 4 mM ATP, and 10 mM PP solution, respectively. Figure 5D shows the extensive shortening of the sarcomeres produced by 10 mM ATP only. The total sarcomeric lengths were reduced by the ATP from about 3.0 to about 1.25 μ. The A bands as well as the I bands shortened, but the A bands shortened less than the I bands. When 4 mM ATP with 4 mM $MgCl_2$ and 0.1 M KCl was applied the reduced sarcomeric length was 1.9 μ and most, if not all, of the shortening occurred in the I bands (Figure 5E and Table I). Pyrophosphate (10 mM) resulted in shortening of the sarcomere to 1.6 μ and in more shortening in the I band than in the A band

[3] "Length" is really "height" of a cylindrical section of the muscle fiber. It is used rather than "width" because the latter really is the diameter of the cylinder.

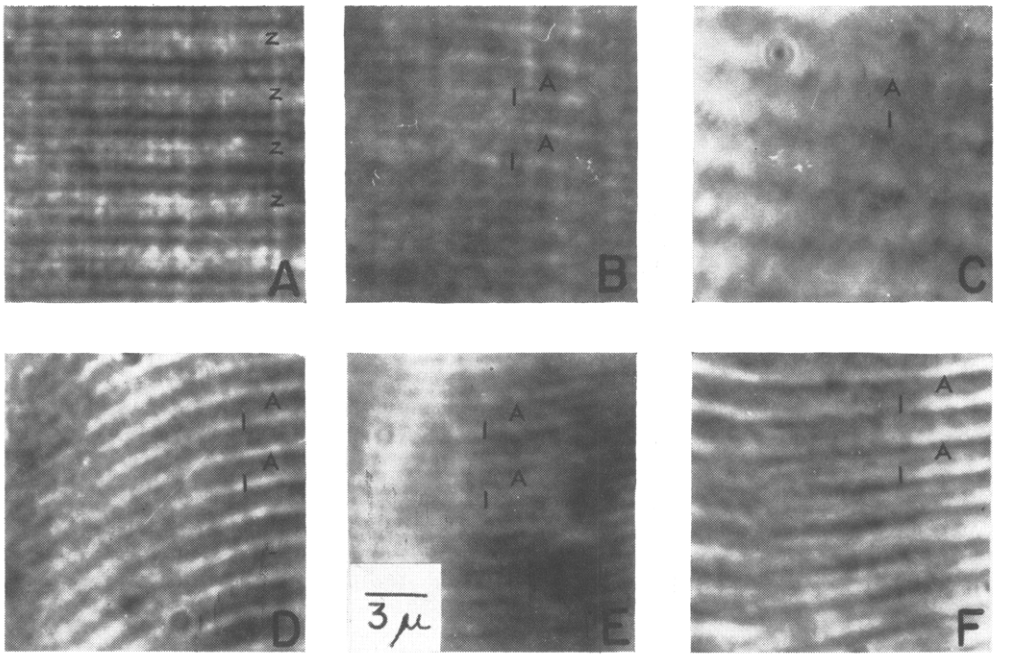

FIGURE 5: Effect of methylation by dimethyl sulfate on the striated structure of glycerol-treated muscle fibers before and after ATP- and pyrophosphate-induced shortening of the fibers. (A) Nonmethylated fiber mounted in 0.044 M KCl; Z lines indicated; (B) nonmethylated fiber shortened by 4 mM ATP; (C) fiber methylated in buffered 0.127 M dimethyl sulfate mounted in water; (D) methylated fiber shortened with 10 mM ATP; (E) methylated fiber shortened with 4 mM ATP, 4 mM $MgCl_2$, and 0.1 M KCl; and (F) methylated fiber shortened with 10 mM sodium pyrophosphate. A and I bands indicated in B–F.

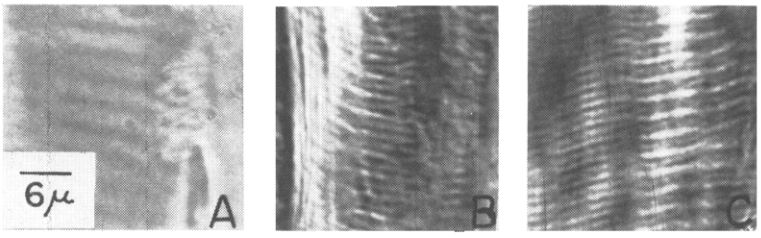

FIGURE 6: Effect of methylation by acidified methanol on the structure of glycerinated muscle before and after application of ATP and sodium pyrophosphate. (A) Fiber methylated and mounted in water; (B) same after application of 2 mM ATP with 2 mM $MgCl_2$ and 0.04 M Tris (pH 6.6); and (C) after application of 10 mM sodium pyrophosphate.

TABLE I: Lengths of A and I Bands and H Zones of Glycerol-Extracted Muscle Fibers of Figure 5 before and after ATP- or PP-Induced Shortening with and without Methylation by Dimethyl Sulfate.

Condn and Treatment of the Glycerinated Fibers	Length (μ)			Length of Sarcomere (μ)
	A	I	H	
Not methylated, before ATP, and mounted in 0.044 M KCl	1.35	1.55	0.6	2.9
Not methylated, after 4 mM ATP, and in 0.044 M KCl	1.25	0.55	0.45	1.8
After treatment with DMS and in water	0.9	2.1	—	3.0
DMS treated and in 10 mM ATP	0.65 to 0.9	0.35 to 0.7	—	1.25
DMS treated, 4 mM ATP and $MgCl_2$ and 0.1 M KCl	1.20	0.55	0.3	1.75
DMS treated, 10 mM NaPP	1.0	0.6	—	1.6

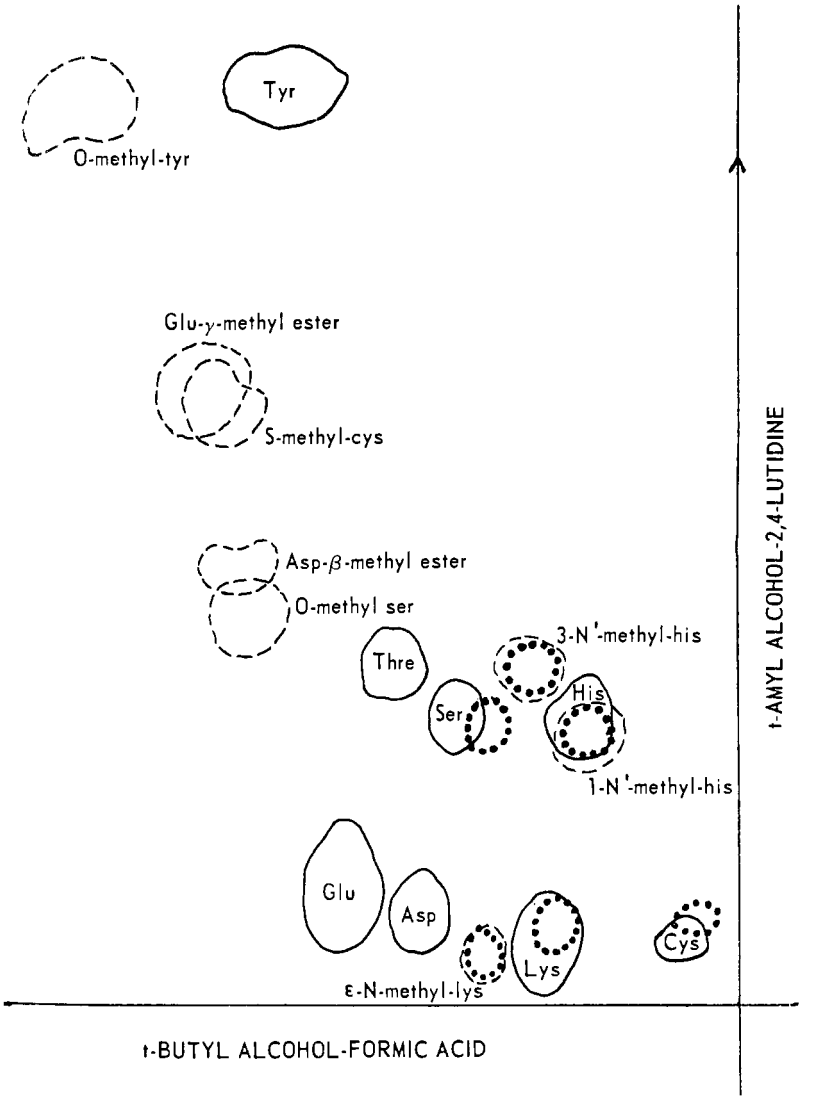

FIGURE 7: Chromatogram and autoradiogram of hydrolysed methylated glycerol-treated muscle fibers. Solid lines enclose spots of naturally occurring amino acids from hydrolysates. Broken lines enclose spots of methyl derivatives of amino acids added as standards. Dotted lines enclose radioactive spots from hydrolysates.

(Figure 5F). Figures 5B,D,F indicate that much of the length lost in A bands of shortened sarcomeres is the result of narrowing or disappearance of the H zone. This holds whether the shortening be of non-methylated or methylated fibers.

Methylation with Acidified Methanol. This means of methylation caused a different sequence of events. When the fibers were put into the methanol they dehydrated extensively and lost one-third of their length. Fibers methylated with DMS did not so shorten. When methanol-methylated fibers were returned to water, they regained the lost length and hydrated as extensively as DMS-hydrated fibers (Figure 6A). When fibers treated in this manner were put into solutions of ATP or PP, they dehydrated and shortened to about the same length as when in acidified methanol. The ATP- or PP-induced shortening was completed within 30 sec or less. The sarcomeres of hydrated methanol-methylated fibers were 3.0 μ long compared to 3.4 μ when DMS was the methylating agent. Application of ATP and PP reversed the hydration and returned the structure to one in which A and I bands and H zones were prominent (Figure 6B,C). I bands were shortened to 0.65 μ and the A bands remained 1.3 μ. These band lengths and sarcomeric appearances compared favorably with those for similar conditions when DMS was used to methylate (Figure 5).

Sites of Methylation. Fibers methylated with ^{14}C-labeled DMS were found to retain one-third of their radioactivity after acid hydrolysis (Table II), thus indicating that other groups as well as carboxyl groups were methylated. On the assumption that the other groups might be associated with histidine and tyrosine residues, or with cystiene residues, fibers were treated

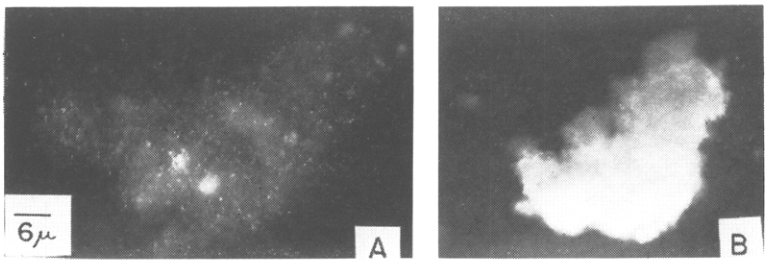

FIGURE 8: Extrusions of methylated myosin B before (A) and after (B) application of 10 mM ATP.

with diazosulfanilic acid to cover the former and with PMB to cover the latter. These treatments did not produce sufficiently large differences between the radioactivity of treated and untreated fibers to indicate methylation of end groups associated with histidine, tyrosine, or cysteine. These failures were probably associated with the relatively small number of these residues in actin and myosin (Kominz *et al.*, 1954).

From these data, it can be concluded that methylation was principally of the free carboxyl groups of glutamic and aspartic acids and to no detectable extent of side chains of tyrosine, histidine, and cysteine. Analyses were then made of nonlabeled fibers by paper chromatography and ^{14}C-labeled fibers by autoradiography to determine whether methylation of these or any other amino acids could be detected by more sensitive methods.

Hydrolysates of fibers methylated in the absence and the presence of each of PMB and Pauly's reagent yielded paper chromatograms identical with those of nonmethylated fibers; *i.e.*, no derivatives were revealed by these procedures. Exposure of the chromatographs of the ^{14}C-labeled hydrolysates to highly sensitive Kodak X-ray film for periods up to 3 weeks revealed five spots with R_F values unlike those of the common amino acids found in muscle (Kominz *et al.*, 1954). The radioactivity may have been from methyl derivatives that were present in inadequate amounts to develop visible spots with ninhydrin. Attempts to identify them were made by chromatographing various methyl derivatives of the amino acids listed above. The results (Figure 7) show that of those tested, only the derivatives of histidine and lysine were near any of the radioactive spots of the autoradiographs. Therefore, the conclusion can be made that the reactive sites of actomyosin are the free carboxyl groups of aspartic and glutamic acids and, tentatively, the histidine and lysine residues.

TABLE II: Loss of ^{14}C from Methylated Muscle Fibers by Acid Hydrolysis.

Expt	Counts per minute per mg of Fiber: Whole Fibers	Counts per minute per mg of Fiber: Hydrolyzed Fibers	% Loss by Hydrolysis
1	107	38	64
2	130	50	61
3	99	47	52
4	123	34	73

Discussion

The ability of complex ions to cause shortening of methylated glycerol-treated muscle fibers is most likely caused by neutralization of a uniformly distributed positive charge along the fiber. Repelling forces between these charges would keep the fiber at full length. The mechanochemical events leading to this condition start with reaction of the methyl radicals with the free carboxyl groups of the protein. This results in the formerly ionizable carboxyl sites becoming nonionizable. The amino groups continue to ionize, yielding $H_3N^+RC\text{-}OOCH_3$, which leaves the fiber cationic.

This type of shortening, which appears to be an electrical charge phenomenon, has been referred to above as "anion induced." This is in contradistinction to "ATP-induced" shortening of glycerol-treated fibers which is considered to be coupled to the ATPase activity of the muscle proteins.

Whether anion-induced shortening is a phenomenon of methylated actomyosin or of collagen of the sarcolemma is not unequivocal from any of the foregoing because only muscle fibers were used. Shortening capability (syneresis) and ATPase activity were therefore ascertained in two-times-precipitated natural actomyosin, methylated and untreated. Shortening and ATPase activity of the untreated protein were definite. The methylated protein possessed no ATPase but extrusions of it were reduced in size about 40% by either ATP (Figure 8) or by PP (not shown). Therefore it is quite certain that the methylated protein in the glycerinated fibers which undergoes contraction is actomysin rather than collagen. This was further verified by direct study of methylated collagen fibers. Neither these fibers nor their unmethylated controls shortened in 0.01 M ATP or PP solution.

The photomicrographs reveal several significant aspects of methylation of glycerol-treated fibers and the subsequent shortening by ATP or PP. Most important is that methylation does not cause a redistribution

of the proteins in the sarcomere. Secondly, anion-induced shortening of methylated muscle appears to involve an over-all sarcomeric shortening (Table I). This type of shortening could be caused by contraction of filaments which extend from Z line to Z line. Such continuous structures could be the result of fusions of the A and I band proteins produced by methylation or of fine filaments extending the length of the sarcomere as described by F. Guba (personal communication). The less shortening of the A band than of the I band is possibly related to the greater mass, or bulk, of the protein in the A than in the I band. Parenthetically, it should be added that some loss of order occurs in both ATP-induced or anion-induced shortening because the Z lines are no longer visible by phase microscopy after shortening.

During hydration neither the fibers nor the sarcomeres lengthen more than 10% when methylated with dimethyl sulfate (Figure 5); swelling is almost entirely confined to increases in thickness. The methylation and hydration occur with no irreversible distortion of the sarcomere and, although the A bands appear to be highly compressed (Figure 5C), application of ATP or PP brings about recovery even to the extent that remanants of H zones can be seen. Also, if the extensive hydration of the I bands (Figures 5C and 6A) is a criterion of methylation, the fine filaments methylated more than the thick filaments. This may point to a difference in extent of methylation of actin and myosin A.

The extreme acid state of the dimethyl sulfate methylating solution came to our attention since the original publications (Bowen and Martin, 1964).[4] According to the results of experiments not shown, fibers treated in sodium acetate buffer overnight and then transferred to 2 mM ATP, 2 mM $MgCl_2$, and 0.04 M Tris buffer (pH 6.0) did not shorten when the sodium acetate buffer was pH 4.5 or less. In sodium acetate buffer of pH 5.0, the shortening of the fibers was 50% of the initial length after 5 min, which compared favorably to that of fibers exposed overnight to water only. The failure of fibers to shorten when treated with sodium acetate buffer at low pH values and the ability of fibers to shorten after treatment in buffered DMS solution which becomes pH 2.0 indicate that methylation is definitely a prerequisite for the type of shortening being reported here.

Treatment of the fibers with 0.02–0.03 M H_2SO_4 greatly reduced the amount of shortening produced by ATP. The fact that any shortening occurred at all is an enigma because sulfate is an anion and must have a totally different effect than that of methyl groups. It is important that sulfation with H_2SO_4, like methylation, produced fibers which shortened upon application of PP and that treatment with HCl produced fibers which underwent slight ATP-induced shortening but none with PP.

Shortening like that described above leads to a hypothesis along the lines of the binding theory of Morales and Botts (1953). Their theory was advanced to account for the ATP-induced contraction of glycerol-treated muscles and of other muscles by neutralization of cationic charges spread uniformly along the fibrils and fibers.

Ernst and Metzger-Török (1962) found that ATP caused contraction of threads of egg albumin, fibrin, and casein. Recently, Puett *et al.* (1966) have reported evidence that differences exist between the binding of anions and cations to proteins. If true, such differentials could leave proteins in a polarized condition, as required by the Morales–Botts hypothesis.

The demonstration that the single muscular twitch is accompanied by the splitting of ATP (Cain *et al.*, 1964) does not deny the possibility that buildup and neutralization of charges play a role in the contraction of muscle. The binding theory would be more nearly a complete explanation of the transduction of energy than the isolated fact that ATP is split during a single twitch. It seems possible from the above that the binding of ATP which is associated with the energy-yielding enzymatic breakdown of this substrate may promote contraction by changes of electric charge on the protein. This could be accomplished by a phenomenon like the contraction and extension of polyelectrolyte gels by alternate application of acid and alkali as described by Katchalsky (1954) and Kuhn and Hargitay (1951).

For attraction and extension to occur in muscle, however, it is necessary for imbalance among charges to be created by some phenomenon which imitates methylation of carboxylic, amino, and possibly sulfhydryl reactive sites. If this were done the results above indicate that the positive charges would predominate and maintain elongation. When balance among charges is created a tendency toward shortening would occur.

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Role of Tyrosyl Groups in Metal Binding Properties of Transferrins*

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ABSTRACT: Chicken ovotransferrin and human serum transferrin, and their iron complexes, were modified chemically with reagents which reacted with the tyrosine moieties of the protein. When the metal-free proteins and the iron complexes were treated with triiodide ion, the metal-free proteins lost their chromogenic capacities with added metal ion, but the iron complexes did not. Also the extent of iodination was greatest with the metal-free proteins. The metal binding sites were also protected in the copper complexes. Acetylation of the hydroxyl groups of both transferrins was done with *N*-acetylimidazole; both metal-free transferrins lost their chromogenic capacity, while the iron complexes retained this capacity. Approximately six more *O*-acyl groups were formed on modification of the metal-free transferrins than on modification of the iron complexes. When the acetylated transferrins were *O*-deacylated with hydroxylamine, metal binding capacity was restored. Thus, chemical modifications of transferrins gave strong support for an essential role of tyrosyl groups in the metal binding property of the transferrins.

The transferrins[1] are naturally occurring chelating agents which form highly associated colored complexes with metal ions (Feeney and Komatsu, 1966). Ovotransferrins[2] are present in avian egg whites. Lactotransferrins are present in the milk of mammals. Serum transferrins are present in the bloods of vertebrates.

Because the metal-free transferrins are unstable under the usual conditions used for chemical modification, definitive modification of the metal-free transferrins without loss of their metal binding properties apparently was not accomplished in earlier studies (Fraenkel-Conrat and Feeney, 1950; Azari and Feeney, 1958, 1961). Several chemical modifications, including iodination, destroyed the chromogenic capacities of the metal-free proteins but not those of the iron complexes (Azari and Feeney, 1961; Jones and Perkins, 1965). Buttkus *et al.* (1965) have recently modified chicken ovotransferrin, human serum transferrin, and human lactotransferrin by treatment with acetic anhydride, succinic anhydride, and KCNO without loss of chromogenic capacity with either copper or iron. They considered this evidence that the more readily available amino groups are not essential for binding. A shift in the absorption maximum of the iron complex to shorter wavelength occurred, however, indicating at least an indirect effect of the modifications. Buttkus *et al.* (1965) concluded that the groups most probably involved in the chelation are the nitrogens of the histidyl residues and the hydroxyls of the tyrosyl residues.

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[1] Human serum transferrin is sometimes named siderophilin or β_1-metal binding globulin.

[2] Ovotransferrin is also named conalbumin.