FINE STRUCTURAL LOCALIZATION OF ADENOSINETRIPHOSPHATASE ACTIVITY IN HEART MUSCLE MYOFIBRILS

LOIS W. TICE, M.D., and RUSSELL J. BARRNETT, M.D.

From the Department of Anatomy, Yale University School of Medicine, New Haven, Connecticut. Dr. Tice's present address is Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City

ABSTRACT

Activity of myofibrillar adenosinetriphosphatase was demonstrated histochemically at a fine structural level in isolated, unfixed or hydroxyadipaldehyde-fixed cardiac myofibrils in the rat, using a lead precipitation technique and either Ca^{++} or Mg^{++} as activating ion. Activity in relaxed myofibrils was found in the A band, but not the H, I, or Z bands. Deposits of final product frequently exhibited an axial periodicity of near 365 A, and bore a close relationship to filaments within the A band. Several patterns of distribution occurred in contracted myofibrils. In myofibrils which had shortened to the point of disappearance of the I band, final product was distributed throughout the sarcomere, except for the unreactive Z band. A second type of distribution occurred in strongly contracted fibers in which there was intensification of activity in the center of the sarcomere. These findings are discussed in the light of the recent morphological evidence and it is suggested that the distribution of final product is consistent with localization of enzyme activity to the cross-bridges between the thick and thin filaments.

Within the past few years, application of refined morphological techniques to the study of striated muscle has led to new insights into the detailed structure of the myofibril. At a fine structural level two filament types, partially overlapping in the relaxed state, have been resolved in the myofibril: a "thin" filament extending from the Z line to the boundary of the H zone, and a "thick" filament extending from the A-I junction through the A and H zones (1–3). Characteristic properties of the main myofibrillar proteins, including their birefringence (4, 5), mass (6), solubility (1, 6) and antigenicity (7, 8), have been used to localize them microscopically in the sarcomere, and, from these results, it has been inferred that actin is located in the thin filaments while myosin is present in the thick filaments.

The present report is concerned with the histochemical localization of adenosinetriphosphatase (ATPase) activity of the myofibril at a fine structural level. Because of the known association of ATPase activity with the H-meromyosin fragment (9), localization of activity may be taken as an indication of the presence of H-meromyosin, subject to the limitation that only those sites of the protein which are free for enzymatic activity can be so demonstrated. In addition, because of the relation between activity of the enzyme and contraction (10, 11), correlation of the sites of activity in relaxed vs. contracted fibrils should add to the understanding of the events occurring in contraction of the myofibril.

MATERIALS AND METHODS

In preliminary experiments, a variety of striated muscle preparations was surveyed for possible use-

fulness. These included: small (1 mm^3) unfixed blocks, small blocks briefly fixed in 1 per cent buffered osmium tetroxide or 10 per cent formalin, unfixed frozen sections, unfixed muscle briefly blendorized to yield fragments of fiber dimensions, and fresh, washed myofibrils. Although enzymatic activity could be obtained in all preparations of unfixed muscle, isolated myofibrils were selected as the test object of choice. With these, relaxed fibrils could be regularly obtained in control preparations, and ATPase activity and/or contraction could be regularly induced by appropriate incubation with adenosinetriphosphate (ATP) and activating ions. When it later became apparent that enzyme activity would withstand brief fixation in hydroxyadipaldehyde (12), fixed preparations were included to provide a further check on the localization of activity in relaxed and contracted myofibrils.

In the final experiments myofibrils isolated from rat cardiac muscle were prepared using a slight modification of Perry's technique (13). Rat hearts were minced in an ice-cold solution containing 0.04 M sodium borate, 0.025 M KCl and 0.4 M sucrose adjusted to pH 7.1 with dilute HCl. The muscle was then blendorized for 35 seconds in fresh, cold medium, sedimented at 600*g* for 20 minutes and the blendorization step was repeated in fresh medium. After sedimentation, the fibrils were again suspended in fresh medium and resedimented, and this washing step was repeated four to six times. All of these operations were carried out at 1°C. Alternatively, 0.5 cm^3 blocks of cardiac muscle were fixed for 1 hour in ice-cold 6.5 per cent hydroxyadipaldehyde, adjusted to pH 7.2 with 0.05 M cacodylate buffer and containing 0.4 M sucrose, washed, blendorized 60 seconds, and sedimented in buffer. In both of these preparations the myofibril pellets were finally suspended in a 5 ml volume of fresh isolation medium, and a few drops of this thick suspension were added to the incubating medium.

The basic incubating medium was prepared as an aqueous solution containing 0.0025 M ATP, 0.04 M $CaCl_2$ or 0.05 M $MgCl_2$, and 0.005 M $Pb(NO_3)_2$. After the pH had been adjusted to 6.5 with dilute NaOH the turbid solution was filtered and the clear filtrate used for incubation. Analysis of the filtrate for ATP gave values of 3 to 4 $\times 10^{-4}$ M. This medium was arrived at after a series of preliminary experiments in which the concentrations of the substrate, reagent, and activating ions were varied. In addition, the pH of the incubating medium was varied from 6.0 to 7.5 and the effects of addition of a number of dilute (0.05 M) buffers were studied at pH 6.5.

The effects of other variants of the incubating

FIGURE 1

Unfixed isolated myofibrils incubated without substrate in the medium containing Ca^{++} and Pb^{++}. Within the myofibrils Z lines (*Z*) are clearly visible, but the filamentous structure of the adjacent I bands (*I*) has largely been lost. Filaments, clearly seen within the A band (*A*) and H bands (*H*), contain no final product indicating that lead is not non-specifically deposited. Tags of membranous material, presumably derived from sarcoplasmic reticulum (*s r*), adhere to the fibrils. × 24,000.

FIGURE 2

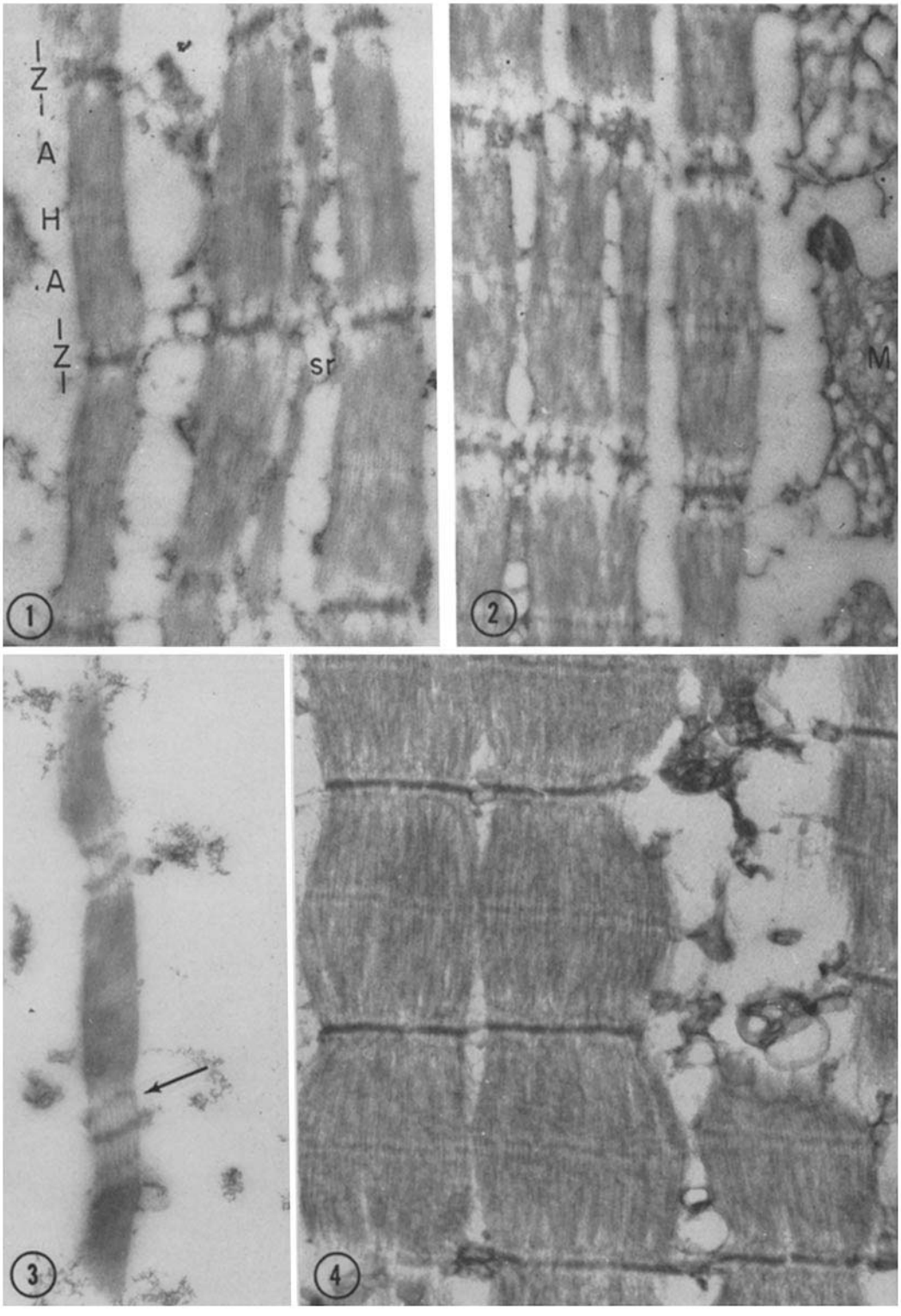
Unfixed isolated myofibrils incubated with AMP and Pb^{++} in the medium containing Ca^{++}. No deposition of final product is seen. At the right of the figure several severely disrupted mitochondria (*M*) appear. Such mitochondria were found within groups of myofibrils but were virtually absent in single isolated myofibrils. Sarcomere length 1.93 μ. × 26,000.

FIGURE 3

Unfixed myofibril incubated in the Ca^{++} and Pb^{++} medium containing thiamine pyrophosphate as substrate resulting in no reaction. The filamentous structure of the I band is relatively well preserved and is bisected by a narrow N line (arrow). Sarcomere length 2.1 μ. × 18,000.

FIGURE 4

Unfixed myofibrils preincubated for 30 minutes with 0.1 M *N*-ethyl-maleimide, followed by incubation in the medium containing ATP, Mg^{++} and Pb^{++}. No reaction product is seen in this slightly shortened group of myofibrils, which appear comparatively well preserved. M band and H zones are clearly depicted but I bands are narrow. Bits and pieces of sarcoplasmic reticulum adhere to the edges of the myofibrils. × 22,000.


I
Z
I
A
H
A
I
Z
I
sr
1
M
2
3
4

medium were examined with both light and electron microscopy using unfixed myofibrils. The fibrils were incubated with either Ca^{++} or Mg^{++}, and Pb^{++}, but no ATP, with ATP and Pb^{++} but no Ca^{++} or Mg^{++}, and with ATP and Ca^{++} or Mg^{++} but without Pb^{++}. The concentrations of Ca^{++} and Mg^{++} added were also varied from 0.005 to 0.05 M. In control experiments, adenosinemonophosphate (AMP), adenosinediphosphate (ADP), inosinediphosphate (IDP), inosinetriphosphate (ITP), β-glycerophosphate and thiamine pyrophosphate were used to replace ATP as substrate at the same or higher concentrations (0.0025 to 0.005 M). The effects of preincubation with *N*-ethyl-maleimide (NEM) (0.1 M) for 1 hour at pH 7.4, or preincubation for the same period of time with Cu^{+++} (10^{-3} M) were assayed for inhibitory effects on the Mg-activated enzyme.

Fibrils prepared after fixation in hydroxyadipaldehyde were incubated in the medium containing ATP, Ca^{++}, and Pb^{++}, in the medium containing ATP, Mg^{++}, and Pb^{++}, without ATP in the presence of Pb^{++}, and Ca^{++}, and with ADP, Ca^{++}, and Pb^{++}. After incubation for 30 to 40 minutes the myofibrils were sedimented at 5000*g* for 30 minutes. The pellet thus formed was cut into small pieces (1 mm^3) and postfixed at 4°C in 1 per cent buffered osmium tetroxide containing sucrose (14) for 1 to 2 hours. The tissue was then dehydrated in increasing concentrations of ethanol and imbedded in methacrylate or in Epon (15). Thin sections were cut on a Porter-Blum microtome, mounted on carbon- and formvar-coated grids, and viewed with an RCA EMU 3F electron microscope.

Light microscope controls were carried out in either of two ways. Histochemically reacted fibrils could be smeared on albuminized slides, dried, washed, and treated with dilute ammonium sulfide to convert precipitated lead phosphate to black lead sulfide. It was frequently more expedient, however, to monitor the histochemical reaction by putting a drop of the suspension of myofibrils and incubating medium on a slide and observing it with an interference microscope. In this way, both the morphological state of the myofibrils and the location and intensity of the reaction could easily be observed prior to electron microscope studies.

RESULTS

The pellets obtained in these preparations consisted largely of single myofibrils and loosely attached groups of myofibrils. In thin sections, fibrils were often seen which were ten to twenty sarcomeres in length. Occasional intercalated discs were encountered. The fibrils, which were some-

FIGURE 5

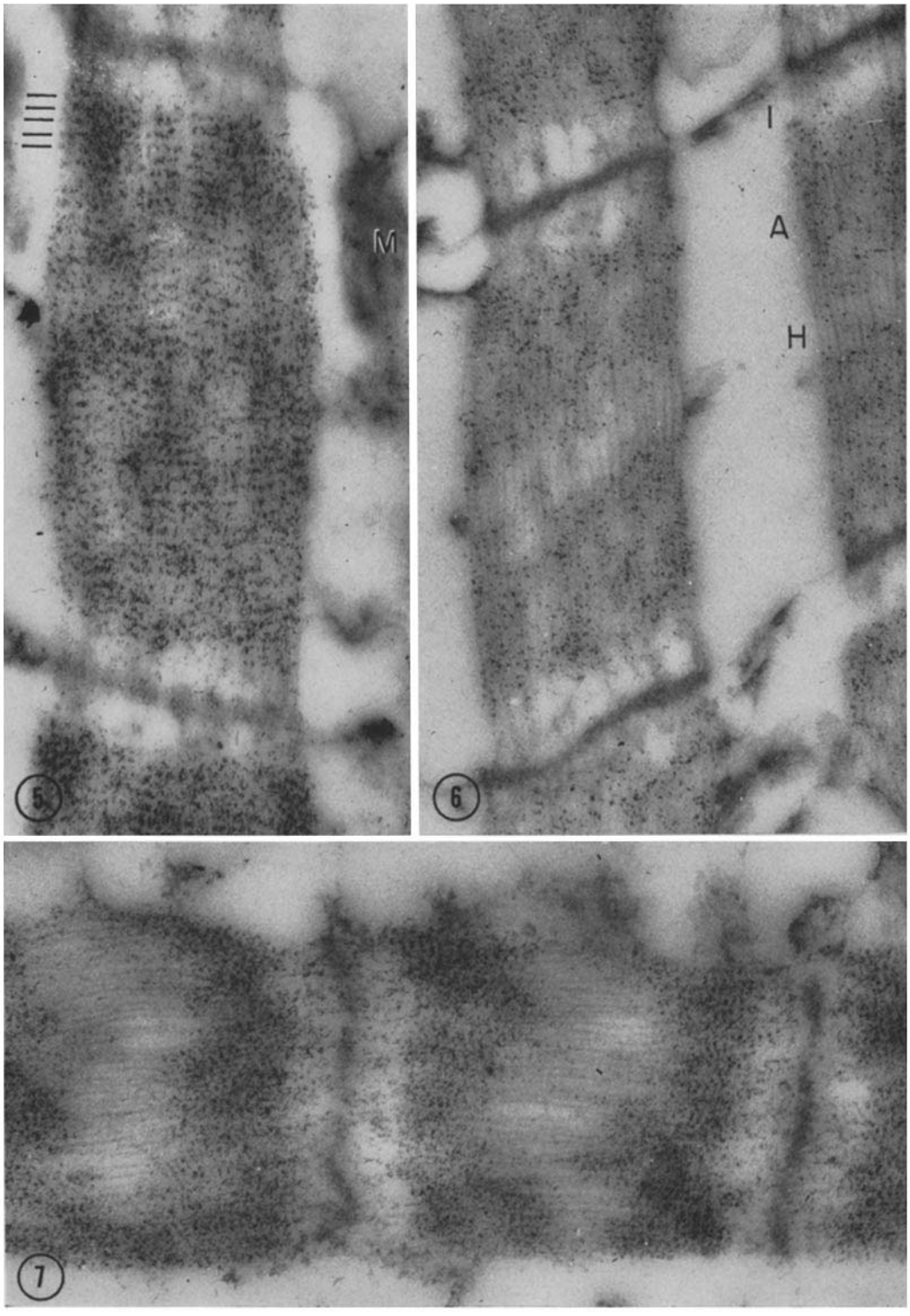
Unfixed myofibril incubated in the medium containing ATP, Pb^{++} and Ca^{++}. Fina product, appearing as multiple, irregular, small deposits of electron-opaque material is seen throughout the A band. No H zone is visible (sarcomere length 1.92 μ). Filaments cannot be seen within the A band and therefore the relation of deposits of final product to the filaments cannot be determined. Definite axial periodicity (390 A) is suggested in the ordering of the deposits (dashes). A few tiny flecks of less dense material appears within the I band and over the Z line. In addition, a few dense deposits of final product are seen in a mitochondrion (*M*) outside the myofibril. × 50,000.

FIGURE 6

Unfixed myofibrils incubated in the medium containing ATP, Pb^{++} and Ca^{++}. In these moderately reactive fibrils deposits of final product appear to be closely related to the filaments within the A band (*A*). A moderately wide, unreactive H zone (*H*) is present (sarcomere length 1.85 μ). Filaments are not clearly visible within the I band (*I*), which contains little or no final product. × 41,000.

FIGURE 7

Unfixed myofibril incubated in the medium containing ATP, Pb^{++} and Ca^{++}. The most striking feature of this rare example of a stretched myofibril (sarcomere length 2.7 μ) is the strikingly widened, unreactive region of the mid-sarcomere (H zone ?). The medial limit of the reactive A band is irregular and a few filaments containing dense product project into the central unreactive zone. Periodicity of product deposition (345 A) is retained under conditions of stretch. × 26,000.


M
5
I
A
H
6
7

times branched, varied in width. Groups of myofibrils often enclosed somewhat disrupted mitochondria as well as swollen and distorted vesicles, presumably derived from sarcoplasmic reticulum. A small quantity of cellular debris was found at the bottom of the pellets, including cell nuclei and occasional segments of nearly intact muscle fibers. Free mitochondria were very rarely seen.

Morphologically, the untreated, isolated, washed myofibrils did not differ from previousy published descriptions. In control preparations Z, I, A, M, and frequently H bands were visible. Occasionally, N bands bisecting the I band were also observed. Relaxed fibrils were 1.9 to 2.2 μ in length in sections cut reasonably perpendicular to the fibril axis. The morphology of contracted fibrils as seen in these preparations will be discussed later.

The presence and degree of contraction of the myofibrils varied both with their mode of preparation and, in the case of unfixed fibrils, with the incubation medium. Myofibrils prepared from fixed muscle were both relaxed and contracted regardless of the incubation procedure. Unfixed myofibrils which had been incubated without ATP, or incubated with ATP and Ca^{++}, were relaxed. After incubation with ATP and Mg^{++} these unfixed preparations contained contracted fibrils almost exclusively (sarcomere length 1.0 to 1.5 μ).

Deposits of final product were seen in the myofibrils after incubation with ATP or ITP in the presence of either Ca^{++} or Mg^{++}. No reaction was encountered in myofibrils incubated with AMP (Fig. 2), IDP, thiamine pyrophosphate (Fig. 3), or β-glycerophosphate as substrates. If the usual substrate, ATP, was replaced by ADP in the presence of either Mg^{++} or Ca^{++}, there were smaller deposits of final product than after incubation with ATP, but no differences were observed in the localization of final product (Figs. 12 and 13).

No final product was seen if myofibrils were incubated without substrate (Fig. 1) or after preincubation with NEM (Fig. 4) or with Cu^{+++}. Reduction of the concentration of either Ca^{++} or Mg^{++} led to a reduction of activity and virtually no activity was observed if activating ions were omitted from the medium.

In fixed or unfixed myofibrils in which an I band was still visible, activity was largely confined to the A band. In many myofibrils activity was so intense that it obscured the fine details of the filaments (Figs. 5 and 9). In less active fibrils where the relationship of final product to filamentous structure could be more easily made out, the small irregularly shaped deposits appeared either superimposed on the filaments or lying just adjacent to them (Figs. 6, 10, and 11).

In addition, definite axial periodicity was often suggested in the distribution of deposits of final product within the A band (Figs. 5, 7, and 8). The presence of such a periodicity appeared unrelated to sarcomere length (Figs. 7 and 8) and the value of the period (365 A, S.D. $\pm$ 20 A) was not significantly correlated with the measured length of the sarcomere. However, in obtaining such a correlation no effort was made to select fibrils uniformly oriented with respect to the cutting axis. In some myofibrils, the regularity of the spacing pattern was marred by missed spaces in which deposits of final product did not appear, while in many others no periodicity was apparent (Fig. 9).

Under circumstances when an H zone would have been expected to be present (sarcomere length greater than 2.0 μ) a zone of diminished or absent reaction appeared within the center of the sarcomere (Fig. 6). Under conditions of stretch (sarcomere length greater than 2.2 μ) this unreactive zone was variably widened (Fig. 7).

However, when the sarcomere length was about 1.9 μ, the histochemically active portion of the sarcomere extended from A-I junction to A-I junction without interruption (Figs. 5, 8, and 9). In these myofibrils, it was impossible to discern whether an H band was present and active or whether in fact no morphological H band existed.

Little final product appeared elsewhere outside the A band (Figs. 7 to 10) although occasional minute flecks of electron opaque material, which might have been final product, were found in the I bands (Figs. 6, 12). These deposits were more frequent in highly reactive unfixed myofibrils than in fixed material, in which activity tended to be somewhat reduced. They were minimal in fibrils exhibiting reduced activity.

In contracted myofibrils, the distribution of product was seen against a strikingly altered, and much more variable, morphological frame of reference. When contraction in unfixed myofibrils had progressed beyond the stage of I band disappearance, it often presented a somewhat unusual morphological picture. This may have been due to extraction of the myofibril during the preparative procedures and to disruption during contraction.

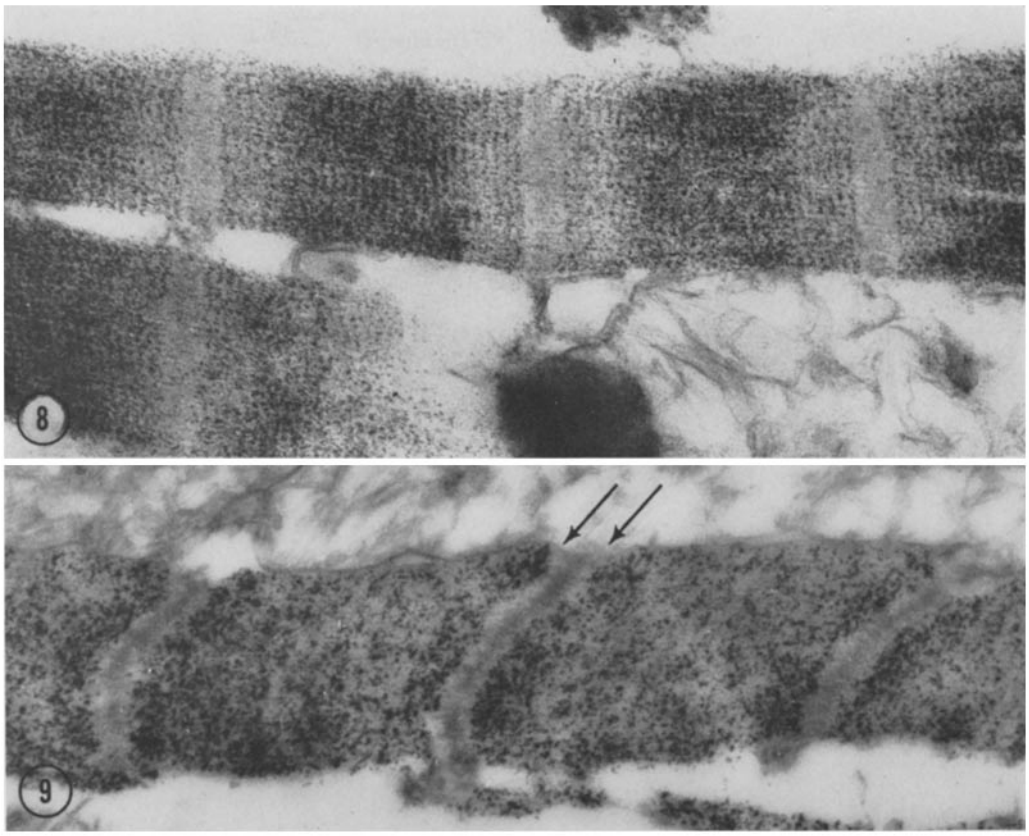


FIGURE 8

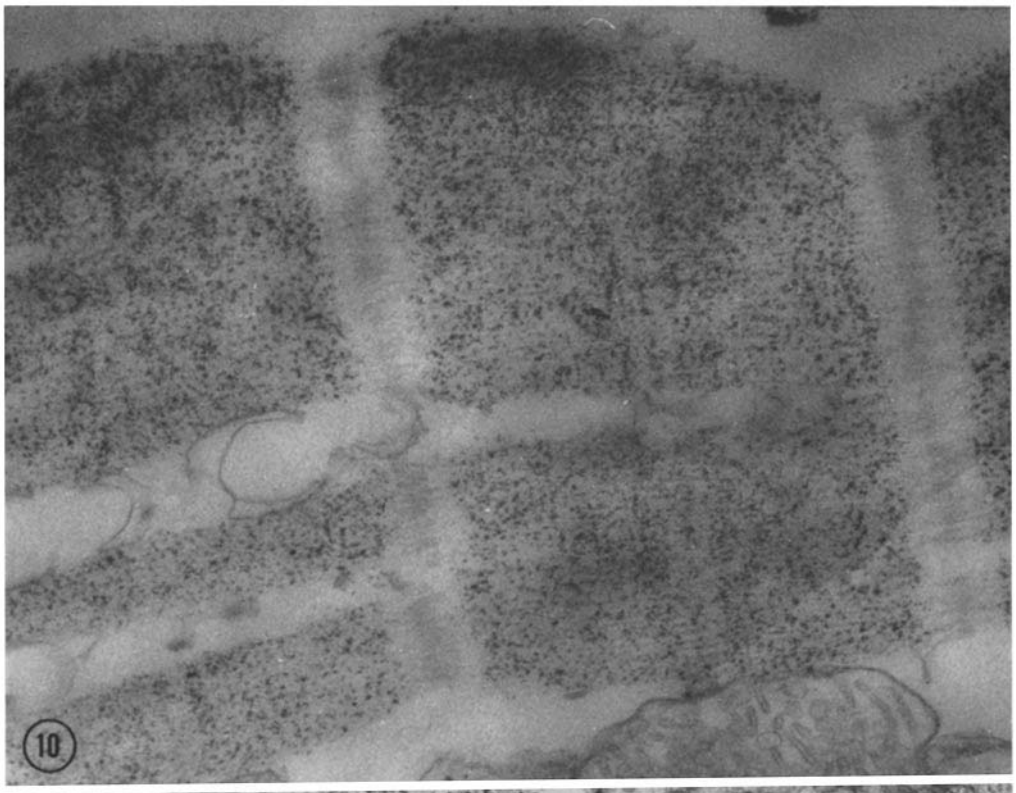
Muscle fixed in 6.5 per cent buffered hydroxyadipaldehyde, blendorized, and incubated in the medium containing ATP, Pb^{++} and Ca^{++}. Sarcomere length 1.64 μ. In this slightly shortened, fixed myofibril dense final product, entirely confined to the A band, is present with an axial periodicity (360 A). The gradient of reactivity from the center of the sarcomere to the A-I junction suggests that the myofibril may have been fixed during some phase of the contraction process. Z bands are indistinct. × 37,000.

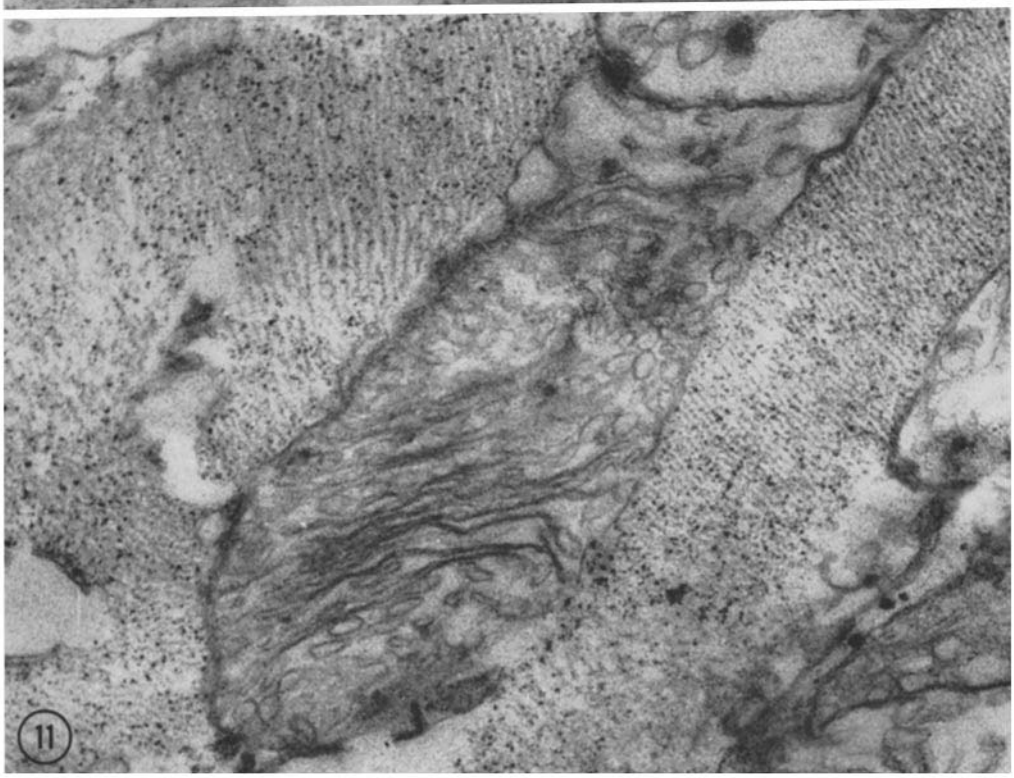
FIGURE 9

Hydroxyadipaldehyde-fixed myofibril obtained under conditions similar to those in Fig. 8. Despite a moderately intense reaction in which the deposits are of large sizes no final product is seen in the narrow I band (arrows) or widened Z band. Sarcomere length 1.7 μ. × 23,000.

The contracted fibrils observed after fixation with hydroxyadipaldehyde resembled the usual picture of contraction much more clearly.

In unfixed, washed myofibrils, incubation in solutions containing ATP, Mg^{++}, and Pb^{++} led to rapid shortening as observed with the interference microscope. Subsequent observation of similar material with the electron microscope revealed that the myofibrils in these preparations fell into two distinct morphological patterns. In one, the arrangement of the filaments between the Z lines was quite homogeneous. M bands were not discernible nor did there appear to be any crumpling or disarrangement of filaments in the middle of the sarcomere (C_m formation). At times, however, disarrangement of filaments in the zone immediately adjacent to the Z lines (C_z formation) was strongly suggested. In many fibrils presenting this morphology, with the exception of the Z line region, which contained little final product, ac-





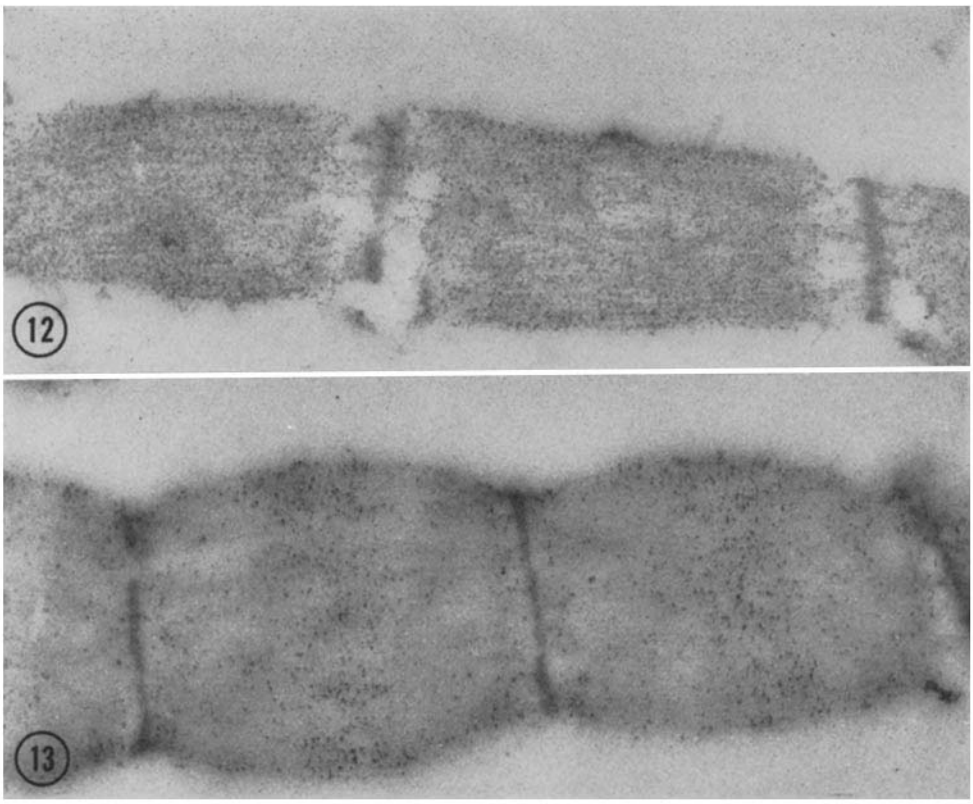


FIGURE 12

Unfixed myofibril incubated in the medium containing ADP, Pb^{++} and Ca^{++}. Small deposits of final product are distributed throughout the A band. No H zone is visible and the I and Z bands are largely unreactive. × 28,000.

FIGURE 13

Unfixed myofibril incubated in the medium containing ADP, Pb^{++} and Mg^{++}. I-bands have virtually disappeared in this myofibril, which contains rather sparse accumulations of final product throughout the A band. The center of the sarcomere appears rather reactive and is separated from a secondary zone of less intense reaction near the Z line by a comparatively unreactive region. Sarcomere length 1.68 μ. × 31,000.

FIGURE 10

Hydroxyadipaldehyde-fixed myofibril incubated in the medium containing ATP+Ca. Within the A band linearly arranged deposits of dense final product appear related to much less dense filamentous structures. A narrow H zone is suggested by a region in the center of sarcomere containing relatively fewer deposits of final product. I bands and Z lines are unreactive. Sarcomere length 1.88 μ. × 38,000.

FIGURE 11

Oblique section through hydroxyadipaldehyde-fixed myofibrils incubated in the medium containing ATP and Ca^{++}. In this section the close relationship of deposits of final product to filamentous structures is particularly clearly shown. Not all the filaments exhibit enzymatic activity. × 39,000.

tivity appeared uniformly distributed throughout the sarcomere (Fig. 14). In a few, activity was more intense near the center of the sarcomere (Figs. 13, 15). In such myofibrils a secondary zone of less intense activity was sometimes seen near the Z line region (Fig. 13) but again little product appeared deposited on the amorphous Z line itself.

In highly active, fresh preparations many myofibrils had a quite different appearance. In these, shortening usually had progressed to sarcomere lengths of 0.9 to 1.3 μ (40 to 65 per cent of rest length). The usual pattern of striation in these fibrils had been replaced by one in which only a widened Z line persisted and in which disruption and/or extraction was apparent (Fig. 16). Little final product was deposited on the center of the widened Z line, but not infrequently a zone of product deposition again was found at its edges. In the middle of the sarcomere a heavy, although apparently random, deposit of final product occurred which obscured the morphological details (Figs. 15, 16). In most fibrils a bulge was also seen in the mid-sarcomere region and this was presumed to correspond to the C_m band (Fig. 16). Relatively few filaments were present running between C_m and C_z, and, again, these were related to occasional deposits of final product.

In preparations of fixed muscle, although many myofibrils were relaxed, a significant number were contracted, and these exhibited histochemically demonstrable activity in the medium containing ATP and Ca^{++} and to a lesser extent in that containing ATP and Mg^{++}. Again in these preparations two categories of contracted myofibrils could be distinguished. In the first of these, which corresponded in its morphology with the first pattern described for unfixed material, M bands or C_m bands were not present. Widened regions of density corresponding in position to the Z line were commonly found. The morphological details of this widened zone were not clear. Activity was found rather uniformly distributed throughout the sarcomere with the exception of the region of the Z line (Fig. 17). Periodicity was not clearly apparent in these specimens, although the linear arrangement of deposits of final product suggested its close relationship to the filaments.

In a second group of myofibrils, Z lines were clearly visible, separated from a histochemically reactive middle zone by a wide, rather unreactive band (Figs. 18, 19). A slight lateral bulge was at times visible in the middle of the sarcomere, which was occupied by a region in which intense activity was apparent (Fig. 18).

Although this picture somewhat resembled that seen in relaxed myofibrils, the sarcomere length averaged 1.3 as opposed to 1.9 to 2.0 μ in the latter. Although wide, unreactive regions appeared on either side of the Z line in the fixed specimens, no unreactive H band was present. Yet, in relaxed fibrils having an I band of corresponding dimensions (Fig. 6), a wide H band would have been expected. The border between the reactive mid-region and the lateral unreactive region was in-

---

Unfixed myofibrils incubated in the medium containing ATP, Pb^{++}, and Mg^{++}.

FIGURE 14

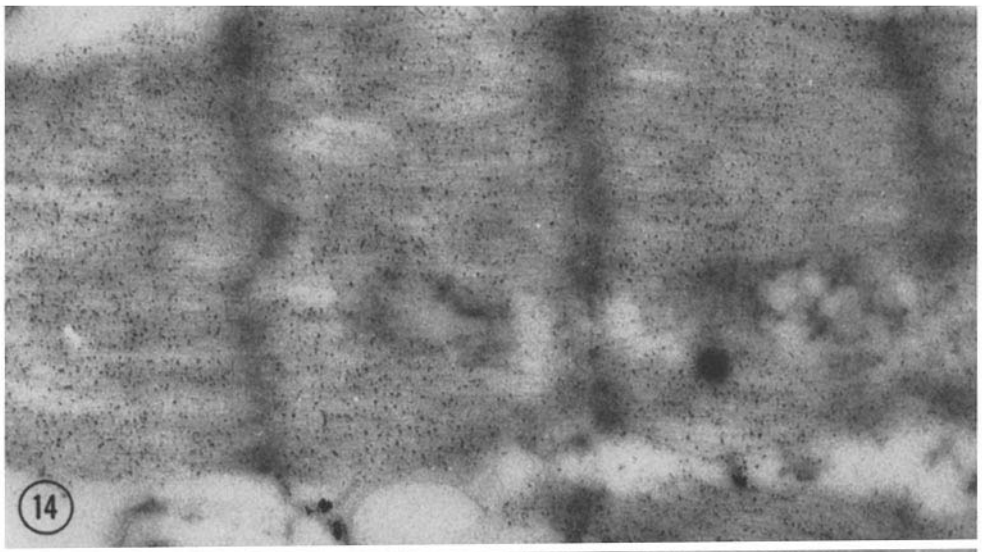
Sarcomere length 1.7 μ. No I and H bands are visible in this myofibril. Widening of the amorphous Z line region suggests that C_z formation is beginning. Final product is seen uniformly distributed throughout the sarcomere but is largely absent from the vicinity of the Z line. × 25,000.

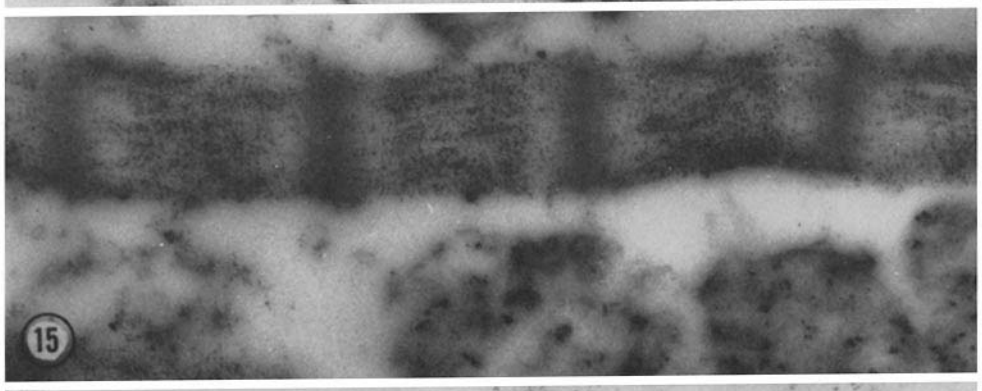
FIGURE 15

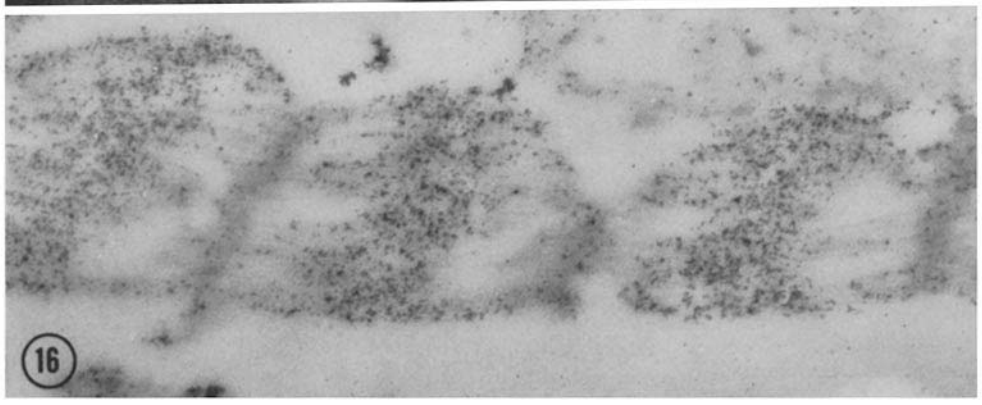
Sarcomere length 1.4 μ. C_z formation is somewhat more pronounced than that shown in Fig. 14. Although deposits of final product may be seen throughout the sarcomere outside the C_z, product deposition is much more intense in the center of the sarcomere. × 23,000.

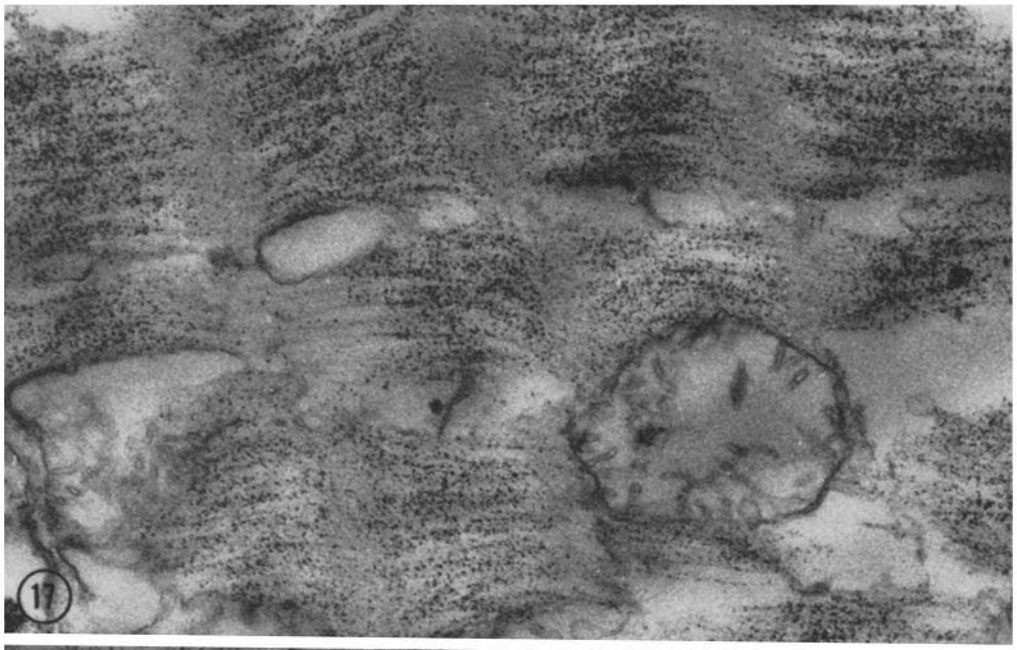
FIGURE 16

Sarcomere length 1.3 μ. This myofibril is a good example of the second type of unfixed, contracted myofibril (see text). Two reactive zones are seen, one, in which the reaction is most intense, in the bulging center of the sarcomere, and the other, much less intense, near the Z line. × 33,000.

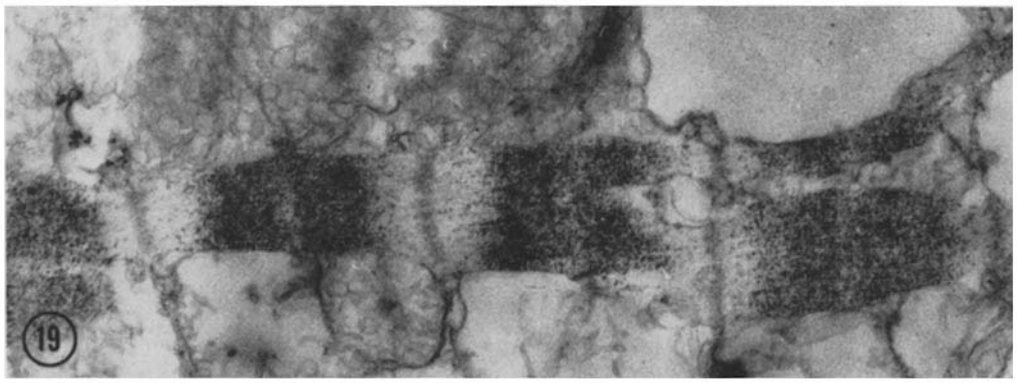

14


15


16


17


18


19

distinct and there was a gradual tapering off of activity from the central highly reactive zone to the unreactive Z band region (Fig. 18).

In addition to the myofibrillar activity described, deposits of final product were found in relation to a few other structures. Mitochondria, when retained within groups of myofibrils, sometimes contained large deposits of final product, particularly after incubation with Mg^{++} as activating ion. Deposits of final product occasionally appeared related to distorted vesicular structures which might have been remnants of sarcoplasmic reticulum.

DISCUSSION

The enzyme studied in these experiments showed histochemical activity to ATP and ITP and, to a lesser extent ADP, but, under these reaction conditions, showed no activity to other organic phosphates. Activity was dependent on the presence of free sulfhydryl groups as well as on the activating ion content of the medium. These findings, together with the correlation demonstrated between activity and contraction in the presence of Mg^{++}, strongly suggest that histochemical activity was due to myosin ATPase. The reaction observed with ADP as substrate may well be due to the persistence of myokinase in the myofibrils (16). Since activity to ADP exhibited localization parallel to that observed with ATP, it is reasonable to assume that ATP, produced as a result of myokinase activity, was that being split. Lack of activity to IDP is consistent with the specificity of myokinase for adenine nucleotides (17). In preliminary experiments we have found partial inhibition of activity in the ATP-Mg^{++} medium when myofibrils had been preincubated with pyridoxal phosphate, which has been reported to resemble the relaxing factor system in its effects (18).

Although sulfhydryl-dependent ATPase activity in myofibrils of striated muscle has been demonstrated histochemically using calcium precipitation techniques (19, 20), application of methods using lead as the precipitating ion has led to conflicting results (19, 21). Failures with these histochemical methods have been attributed to enzyme inhibition by the capture reagent (Pb^{++}), and evidence for such a view has been presented, at least in the case of mitochondrial ATPases (19, 22). We therefore attempted to assess, again in preliminary experiments, the degree to which lead was inhibiting enzyme activity under our reaction conditions. Both with fixed and unfixed muscle, the amount of phosphate released from ATP in the presence of lead was roughly $\frac{1}{6}$ that observed without lead under equivalent conditions, in which the amount of ATP present in the medium without Pb^{++} was corrected to conform to that found by direct assay in the medium with lead after filtration. Moderate inhibition was observed even if the amount of lead added to the incubating medium was reduced to 0.001 M.

Partial inhibition of activity may be due to either or both of two phenomena: decreased activity at all sites, or total loss of activity from some sites. Since lead presumably inhibited activity by combination with some enzyme sulfhydryl groups, and since two enzyme sites have been predicted

---

Hydroxyadipaldehyde-fixed, contracted myofibrils incubated in the medium containing ATP, Pb^{++}, and Ca^{++}.

FIGURE 17

Sarcomere length 1.55 μ. Widening of an unreactive, amorphous region around the Z line is evident. Elsewhere, in the center of the sarcomere, final product is uniformly distributed. × 25,000.

FIGURE 18

Sarcomere length 1.45 μ. Product is predominantly deposited in the center of the sarcomere. Deposits of final product become gradually less frequent at the edges of these reactive regions. × 34,000.

FIGURE 19

Sarcomere length 1.57 μ. This fibril presents essentially the same histochemical picture as Fig. 18, although in this example the gradation of reaction intensity from the center of the sarcomere to the periphery is even more marked. × 24,000.

at each cross-bridge (23), an over-all reduction of activity (as demonstrated chemically) might find expression in a total loss of activity from some of these fine structural locations (as demonstrated histochemically). The latter type of inhibition is consistent with our finding that the pattern of periodic distribution of product was marred by missed spaces lacking final product.

An even more important consequence of partial inhibition of the enzyme, however, was that good histochemical localization of the remaining active sites could be more easily obtained. A prime condition for accurate localization, the availability of sufficient metal ion at the active sites to trap released phosphate, almost simultaneously would be more easily met if the number of phosphate sources (*e.g.* enzyme sites) were small or if they were acting at less than a maximal rate. Consequently, partial inhibition of the enzyme by the capture reagent would be expected to result in somewhat improved localization. Since the accumulated experimental evidence (24) suggests the homogeneity of the myofibrillar ATPase, the possibility of differential inhibition of one of several fibrillar ATPases with Pb^{++} is slight.

The distribution of activity obtained in these experiments may be examined from two points of view. The first of these would attempt to localize activity on the strength of histochemical evidence alone, while the second would use the histochemical evidence only to confirm or deny the predicted location of ATPase sites (3, 23) within the filament hierarchy of the myofibril. Although the first position is somewhat more traditional in histochemistry, it is more appropriate for us to take the option of the second. In doing so, we are aware that the fine structural organization of the myofibril has been disputed by some authors (24–26), but we regard the two-filament model (1–3) as that most amply substantiated by published evidence (27–30). By utilizing evidence obtained with a variety of techniques for the location of actin and myosin within the myofibril (1–8), we gain the distinct histochemical advantage of having an internal check on our localization. From this evidence it is apparent that in any experiment in which adequate localization is obtained, activity must be excluded from the Z and I band regions of the relaxed myofibril. From this criterion it is apparent that although localization of final product in fresh and fixed relaxed myofibrils was substantially the same, that obtained in fixed material was unquestionably better.

The pictorial evidence for a close relation of activity to the filamentous structures within the A band is fairly strong. But, since in these experiments thin filaments were not resolved as discrete structures within the A band, no direct localization of final product to either the thick or the thin filaments was possible. Circumstantial evidence, however, may permit us to decide this point. If activity were related primarily to the thin filaments, we would expect to find it throughout the I band as well as the A band, and "good" localization as defined above would not be obtained. Virtually no I band activity occurred and its appearance did not depend on either the fibril preparation or the incubating medium. If activity were related to the body of the thick filaments, we would expect it to appear throughout the A band region and also in the H band. If an H band was present, it was inactive. Therefore, either the proximity of both thick and thin filaments was required for activity to be manifest, or some structure unique to the A band but absent from the H zone was the active site. The cross-bridges of the thick filaments would meet this requirement.

Several additional points may be submitted as evidence for localization of activity to the cross-bridges. The deposition of product just adjacent to filamentous structures within the A band, as well as the frequent periodic distribution of product, fits well with what would be predicted on the basis of cross-bridge localization. Similarly, the shift, or addition, of active sites toward the M line in early shortening is in good agreement with the morphological changes in cross-bridge sites in this situation (3). Since, under our circumstances of Ca-activation, both myosin and actomyosin ATPase would show activity (31), it is probable that the thick filaments in the H zone would be related to deposits of final product if they had the requisite enzyme sites.

It should be emphasized that other attempts to specifically label parts of this contractile system have yielded quite different results. In fluorescent antibody studies in which antibodies against actin, myosin, and L-, and H-meromyosin were studied in chicken breast muscle (32), localization of H-meromyosin antibodies were found in the H zone as well as in the A band itself, or, in contracted fibrils, in the C_m band. We have no explanation to offer for the discrepancy in results.

Our observations are, of course, subject to the criticism that we can demonstrate only activity and not the presence of potentially enzyme-active sites. However, as Tunik *et al.* pointed out (32), their results may also be criticized on the grounds that both their actin antibody and the H-meromyosin antibody, which contained a cross-reacting component, stained the H zone, which itself might have contained a unique antigen.

In contracted myofibrils the morphological and histochemical changes observed appeared to fall into two patterns. One of these patterns was quite similar in both fixed and unfixed material, and appeared to represent a state of contraction in which both H and I bands had disappeared and where C_z formation had begun. The distribution of product at this stage tended to be uniform throughout the sarcomere with the exception of the region around the Z line, in which product deposition was reduced. If anything, more deposits of product were found in the middle of the sarcomere than elsewhere, although there was no morphological evidence in these fibrils of C_m formation.

The second morphological pattern appeared quite different in unfixed and fixed myofibrils. Although both fibrils were shortened, this was more apparent in the unfixed material, and in both cases there was a lateral bulging of the mid-sarcomere region. In the case of unfixed myofibrils the disruption of filamentous morphology was such as to suggest that abnormal contraction was occurring. In fixed myofibrils which had undergone pronounced shortening, disruption of filamentous morphology was rarely seen. In these fibrils there was a pronounced segregation of activity toward the center of the sarcomere.

It is difficult to interpret these findings without a widely accepted morphological model of the contracted process, particularly since the underlying fine structural details in our preparations were obscured by final product. However, our findings are in agreement with those shown recently by Gilev (33) and allow the interpretation that the change in sites of activity in well contracted fibrils may represent an extension of the processes occurring in early shortening (H band disappearance). As such, the accumulation of final product in the mid-sarcomere region could represent a shift of activity and/or the structures associated with activity toward the middle of the thick filaments.

This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases (A-3688) and from the National Cancer Institute (CRT-5055), National Institutes of Health, Education and Welfare.

Dr. Tice was Fellow of the National Foundation from 1959 to 1961. Present address: Department of Pathology, College of Physicians and Surgeons of Columbia University, N.Y.C.

Received for publication, May 4, 1962.

REFERENCES

1. HUXLEY, H. E., and HANSON, J., 1954, *Nature*, **173**, 973.
2. HUXLEY, A. F., and NIEDERGERKE, R., 1954, *Nature*, **173**, 931.
3. HUXLEY, H. E., 1957, *J. Biophysic. and Biochem. Cytol.*, **3**, 631.
4. DEMPSEY, E. W., WISLOCKI, G. B., and SINGER, M., 1946, *Anat. Rec.*, **96**, 221.
5. HANSON, J., and HUXLEY, H. E., 1955, *Symp. Soc. Exp. Biol.*, **9**, 228.
6. HANSON, J., and HUXLEY, H. E., 1959, *Biochim. et Biophysica Acta*, **23**, 250.
7. FINCK, H., HOLTZER, H., and MARSHALL, J. M., 1956, *J. Biophysic. and Biochem. Cytol.*, **2**, No. 4, suppl., 175.
8. HOLTZER, H., and MARSHALL, J. M., 1957, *J. Biophysic. and Biochem. Cytol.*, **3**, 705.
9. SZENT-GYORGYI, A. G., 1953, *Arch. Biochem. and Biophys.*, **42**, 305.
10. LUNDSGAARD, E., 1930, *Biochem. Z.*, **217**, 162.
11. NEEDHAM, D. M., 1960, Biochemistry of muscular action, *in* The Structure and Function of Muscle, (G. H. Bourne, editor), New York, Academic Press, **2**, 55.
12. SABATINI, D. D., BENSCH, K. G., and BARRNETT, R. J., 1962, *Anat. Rec.*, **142**, 274.
13. PERRY, S. V., 1951, *Biochem. J.*, **48**, 257.
14. CAULFIELD, J. B., 1956, *J. Biophysic. and Biochem. Cytol.*, **3**, 827.
15. LUFT, J. H., 1961, *J. Biophysic. and Biochem. Cytol.*, **9**, 409.
16. PERRY, S. V., 1954, *Biochem. J.*, **57**, 427.
17. COLOWICK, S. P., and KALCKAR, H. M., 1948, *J. Biol. Chem.*, **148**, 127.
18. GERGELY, J., 1959, *Ann. New York Acad Sc.*, **81**, 490.
19. PADYKULA, H. A., and HERNAN, E., 1955, *J. Histochem. and Cytochem.*, **3**, 170.
20. GAUTIER, H., and PADYKULA, H. A., 1962, *J. Histochem. and Cytochem.*, in press.

21. HORI, S. H., and CHANG, J. P., 1961, *J. Histochem. and Cytochem.*, **9**, 625.
22. NOVIKOFF, A. B., HAUSMAN, D. H., and PODBER, E., 1958, *J. Histochem. and Cytochem.*, **6**, 61.
23. HUXLEY, H. E., and HANSON, J., 1960, The molecular basis of contraction in cross-striated muscles, *in* The Structure and Function of Muscle, (G. H. Bourne, editor), New York. Academic Press, **1**, 183.
24. WEBER, H. H., and PORTZEHL, H., 1952, *Adv. Protein Chem.*, **7**, 162.
25. SPIRO, D., 1956, *J. Biophysic. and Biochem. Cytol.*, **2**, No. 4, suppl., 157.
26. HODGE, A. J., 1956, *J. Biophysic. and Biochem. Cytol.*, **2**, No. 4, suppl., 131.
27. SJÖSTRAND, F. S., and ANDERSSON-CEDERGREN, E., 1957, *J. Ultrastruct. Research*, **1**, 74.
28. KARRER, H. E., 1959, *J. Biophysic. and Biochem. Cytol.*, **6**, 383.
29. STENGER, R. J., and SPIRO, D., 1961, *J. Biophysic. and Biochem. Cytol.*, **9**, 325.
30. CARLSEN, F., KNAPPEIS, G. G., and BUCHTHAL, F., 1961, *J. Biophysic. and Biochem. Cytol.*, **11**, 95.
31. HASSELBACH, W., 1952, *Z. Naturforsch.*, **7**, 163.
32. TUNIK, B., and HOLTZER, H., 1961, *J. Biophysic. and Biochem. Cytol.*, **11**, 67.
33. GILËV, V. P., 1962, *J. Cell Biol.*, **12**, 135.