

Enzymatic Activity in the M Band*‡

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PLATES 102 TO 107

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ABSTRACT

Experiments which combined histochemistry and electron microscopy were performed in studying the sites of enzymatic hydrolysis of thiolacetic acid in the presence of lead ions in diaphragmatic and cardiac muscle. It was found that in these striated muscles the electron opaque, final product of the histochemical reaction (PbS) was discretely deposited on the swelling of the thick elemental filaments that occurs at the M band. Additional sites of enzymatic activity occurred in mitochondria and in round sarcoplasmic bodies. A reaction, probably non-enzymatic, also occurred in contraction bands in the area of the Z bands and in the sarcoplasmic reticulum. To ascertain the enzymatic nature of the reaction and to define the enzyme involved, control experiments were carried out and the effect of various esterase inhibitors was assayed.

It is suggested that the M band enzyme is a cholinesterase, but the enzymes in the mitochondria and the sarcoplasmic bodies that hydrolyze the substrate appear to be different. A possible role of the M band enzyme is discussed.

INTRODUCTION

In a recent series of experiments designed to relate the fine structure of cells with their biochemical activity, we studied a histochemical procedure capable of demonstrating at least the sites of acetylcholinesterase (AChE) activity. We used thiolacetic acid (1, 2) as the substrate and lead nitrate as reagent and found that the final product of the histochemical reaction was discretely deposited in the M band of the myofibrils of skeletal and cardiac muscle. In addition, deposits of the same product were encountered in mitochondria and other cytoplasmic organelles. This paper describes our findings and presents the still incon-

clusive results of our attempts to identify this new M band enzyme.

Materials and Methods

The experimental tissues consisted entirely of diaphragmatic muscle and ventricular portion of the cardiac muscle of rats. The diaphragm was used either as small blocks, 1 to 2 mm.³, or as intact leaves or long strips which, after incubation, were cut into small fragments. Heart muscle was used either as cryostat sections, small blocks or centrifuged pellets of briefly blenderized muscle (40 seconds in 0.44 M sucrose).

The histochemical experiments involved the use of thiolacetic acid (CH₃COSH) as substrate. This compound, introduced by Wilson (1) and first used histochemically by Crevier and Bélanger (2), is hydrolyzed enzymatically by AChE at acid pH's to liberate acetic acid and hydrogen sulfide as products (1). In the presence of lead ions lead sulfide is precipitated as the final product of the reaction, presumably at the sites of enzymatic activity. In the histochemical incubation mixture of Crevier and Bélanger (2), a precipitation invariably occurred, thereby reducing to an uncontrollable extent the concentration of substrate and reagent (lead ions) in the medium. For this reason we modified the procedure by titrating 0.25 ml. of 0.24 M thiolacetic acid with 1 N and 0.1 N sodium hydroxide

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(NaOH). It should be noted here that during the titration the mixture displays moderate buffering capacities in the acid ranges up to approximately pH 5, and weaker ones up to approximately pH 6.2. At that point the titration curve rises very steeply, and small quantities of dilute NaOH will produce large changes in pH. The buffer is presumably formed by thiolacetic acid and sodium thiolacetate. The titration was stopped at various levels, from pH 4.5 to 6.8, depending on the experiment, and the volume was raised to 20 ml. by the addition of 0.01 M cacodylate buffer (3) at the same pH as that of the titrated substrate. The final necessary ingredient of the standard incubating mixture was lead nitrate (0.001 M). If the lead were introduced before the buffer, a yellow cloudy precipitate, presumably of lead thiolacetate, slowly formed. No precipitation occurred when lead was the final ingredient added. In all experiments the over-all osmolar concentration of the incubating medium was raised to 0.44 M by the addition of sucrose in order to aid in the preservation of the fine structure of the muscle fibers. The incubation mixture thus prepared proved stable and yielded reproducible results.

The duration of incubation of tissues in the above medium varied according to the circumstances of the experiment, the range being from 3 to 30 minutes, most frequently from 5 to 10 minutes. Since the duration of incubation was influenced by the pH, the following experiments were conducted to determine the histochemical pH optimum.¹ Small strips of the neural portion of diaphragm containing the neuromuscular junctions were incubated in the standard medium at various pH's from 4.5 to 6.8. The pieces were observed with a dissecting microscope ($\times 25$) and, at the first indication of a visible deposition of product at the neuromuscular junctions (subsequently proven to result from enzymatic activity), the reaction was terminated and the pieces immediately fixed in osmium tetroxide as described below. In each instance where a histochemical reaction was present at the neuromuscular junctions, deposition of the final product at the M bands of the myofibrils was detected with the electron microscope in thin sections at the periphery of the tissue blocks. The deposits at the M band were too fine to be visualized with either ordinary light microscopy or with phase-contrast microscopy. The most rapid deposition of product occurred in 3 to 5 minutes at pH 5.5 and below, with no appreciable difference in the time required for deposition between pH 5.5 and 4.5. These histochemical determinations are in agreement with biochemical data (1) on thiolacetic acid hydrolysis by

¹ Histochemical pH optimum refers to that pH at which the most rapid deposition of final product occurs. Although this pH may or may not be related to the enzyme pH optimum, it may relate primarily to the conditions for the rapid precipitation of final product.

AChE as a function of pH. Between pH 6.1 and 6.5, the reaction took between 8 and 18 minutes. At pH 6.8 slow spontaneous hydrolysis of the substrate was noted, but this was absent at the lower pH's (5 to 6.4) in aliquots of incubating medium tested for 30 to 60 minutes. Because of these findings, the majority of our experiments were conducted between pH 5.5 and 6.2 despite the deleterious effect of this low pH upon the fine structure of the muscle as seen with the electron microscope.

Various metal salts including magnesium chloride, calcium chloride, and manganese chloride (all 0.004 M in final concentration) were tested as activators after it was found that preincubation of small blocks of diaphragm in versene (10^{-3} M) moderately inhibited the subsequent histochemical reaction. Each of the activators showed some beneficial effect on the rate of staining, especially the magnesium salt; consequently, in most of the experiments the above salts were added from stock solutions.

In a series of experiments the following enzyme inhibitors were assayed: eserine salicylate (5×10^{-4} to 1×10^{-6} M), tetraethylpyrophosphate (TEPP) (1×10^{-5} to 1×10^{-9} M), diisopropylfluorophosphate (DFP) (1×10^{-4} to 1×10^{-6} M) and sodium taurocholate (3×10^{-3} M). Since the use of inhibitors introduced the additional complication of their penetration to the other variables of the histochemical experiments, all tests utilizing inhibitors were carried out according to the following pattern: the tissue blocks were incubated in 0.44 M sucrose containing the inhibitor for 10 to 15 minutes and subsequently incubated in the standard medium containing the same concentration of inhibitor. The inhibitory effects of heat and of brief fixation in 10 per cent formalin or 1 per cent osmium tetroxide on the histochemical reaction were also assayed.

Since the duration of incubation of the blocks of heart muscle in the media containing inhibitors was an unknown factor, and since we had already found that the staining of the M band coincided in time with the staining of diaphragmatic neuromuscular junctions, we simultaneously incubated a leaf of diaphragm and blocks of heart muscle in the same medium but in different vessels. In each case, the incubation of the heart tissue was concluded when the neuromuscular junction of the diaphragm showed the first indication of histochemical reaction. When, because of enzyme inhibition, no reaction occurred at the neuromuscular junctions, the heart blocks were incubated from 20 to 30 minutes, a duration far in excess of that required for the M bands to react under ordinary conditions. Moreover, when thin sections of inhibited heart muscle were examined with the electron microscope, additional sites of the deposition of final product were found in the mitochondria. These sites reacted somewhat differently to the inhibitors from those in the M bands and thereby provided another control.

At the end of the incubation period, the small blocks

were washed briefly in 0.44 M sucrose and fixed for electron microscopy in 1 per cent osmium tetroxide, buffered with veronal-acetate to pH 7.4 and containing enough sucrose to raise the over-all osmolar concentration of the fixative to 0.44 M. After fixation for 45 minutes, the blocks were washed briefly in sucrose, dehydrated in increasing concentrations of alcohol, and embedded in *n*-butyl methacrylate. Thin sections, cut on a Porter-Blum microtome at various levels in the block, were mounted on carbon-coated grids and examined with an electron microscope (RCA-EMU-2B). Thick sections (1 μ) of the same material were mounted on glass slides and examined with an ordinary light or a phase-contrast microscope.

A few experiments were conducted to assay the AChE activity in diaphragm and heart, by the manometric method (4) using acetylcholine as substrate.

RESULTS

The results with muscle fibers of the ventricular wall and the diaphragm were similar both for the myofibrils and other cell constituents. It is pertinent to describe first the electron microscope findings in myofibrils.

Fine Structure.—Figs. 1 to 11 indicate the disposition of the electron-opaque, final product of the histochemical reaction in relation to the fine structure of striated muscle. Within the reactive areas of the specimen, fine, roughly circular, or stellate deposits of highly dense material, approximately 140 to 200 Å in diameter, were found located on all or nearly all the thick elementary filaments of the myofibril at the level of the M band (Figs. 1 to 5, 7, 9, 11). In most of the experimental specimens, the individual filaments and the usual cross-striation pattern of the fibrils was obscured to a varied extent (Figs. 1, 2, 9, 11), due either to contraction (absence of I and H bands), or extraction during incubation (spotty decrease in the density of A bands), or unfavorable incidence of sectioning. However, in practically all sarcomeres the A bands and contraction bands in the region of the Z bands could be recognized, and the position of the dense deposits related to the M band since they regularly occurred in the middle of the A band equidistant from the two adjacent contraction bands. In other specimens which were incubated for a short time and, in addition, contained only partly contracted myofibrils, the relationship of the dense deposits to the elemental filaments and the cross-striations of the sarcomeres could be better visualized (Figs. 3 to 5, 7). The deposits were almost entirely confined to the swelling of the thick elemental filaments that occurs at

the M band level; the rest of each thick filament throughout the H and A bands of the sarcomere was virtually free of any product (Figs. 3, 5), as were the filaments in the I bands. This relationship of the final product of the histochemical reaction to the elementary filaments is clearly demonstrated by sections cut obliquely through the M band (Figs. 3, 4). Regardless of the state of the myofibril, contracted or uncontracted, the sharpness of the localization of the final product was of a high order. The deposits appeared in hexagonal packing; unreactive filaments were rarely encountered (Figs. 2, 11), and only occasionally there seemed to be more than one reactive site along a given filament at, or close to, the M band (Figs. 1, 2, 5, 7, 9, 11).

In addition to the M band, another site in the sarcomere, namely the area of the Z band, was apparently labelled with the final product. A very fine, minute stippling of electron opaque particles, approximately 80 to 120 Å in diameter, occurred over a broad zone centered on the Z band in some specimens (Figs. 3, 5, 7). These deposits were not only smaller but also less opaque than those at the M band. In addition, they were rather irregularly scattered within the reactive areas which were not restricted to the Z bands but frequently spread over the contraction bands. In some cases the latter appeared reactive in their entirety (Figs. 3, 5). However, many sections containing reactive M bands evinced little or no reaction at the Z bands or at the contraction bands (Figs. 1, 2, 9, 11). Although there was no absolute difference in the reaction at the Z band zones with pH, fewer deposits occurred in the specimens incubated at pH 5.5 than in those incubated at pH 6.4 for the same period of time.

The sarcoplasmic reticulum showed extensive swelling and disruption. Sometimes a few small, opaque deposits, similar to those in the Z band area (Figs. 2, 3, 5), were found on the thickened disorganized membranes, and it was usually the case that the deposition in these parts paralleled that of the Z band area.

The mitochondria which in all incubated specimens appeared swollen, vacuolated, and showed spotty extraction of the matrix also contained dense deposits, roughly the same size and density as those occurring in the M band (Figs. 1, 2, 7, 9, 11). The sites of these deposits, which bore no spatial relationship to the M band, evinced no apparent pattern. Sometimes they occurred in relationship to the outer membrane (Figs. 1, 7, 9);

more often they occurred in the interior of the mitochondria (Figs. 2, 11), but it was not clear whether the fine precipitate was deposited in the matrix or on the cristae. The reaction in mitochondria was similar throughout the range of pH's used for the incubating media in the present experiments. In addition, evidence of intense activity was found in certain small round bodies, containing no internal structure, except for a dense, moderately granular matrix. Such bodies which occur sparsely in the sarcoplasm of heart muscle, and are reminiscent of similar structures found in peribiliary region of hepatic cells or in renal tubular epithelium, were fairly crowded with small dense deposits (Fig. 10).

The distribution of the histochemical reaction described at the M band was restricted to the extreme periphery of the small blocks (1 to 2 mm.³) of diaphragmatic and cardiac muscle. Frequently, only 4 to 6 of the fibers at the periphery of the block showed the reaction. In addition, the reaction was somewhat spotty in its distribution with patches of muscle containing an admixture of reactive and unreactive sites even at the periphery of the block. Fewer deposits occurred at the M band on the elemental filaments in the more central portions of reactive sites than at the periphery. This peripheral distribution was not surprising since the incubations probably were of too short duration (3 to 20 minutes) for the complete penetration of incubating ingredients throughout the block. Longer incubations were not used because of the plan of the experiment (see Methods) and because of the deleterious effects of the acid medium on the preservation of the fine structure of muscle. The problem of penetration could, in part, be overcome by the use of briefly blenderized muscle instead of small blocks. In this instance, although a more widespread reaction occurred at the M bands of the fragmented and separated myofibrils, there were still some fibrils that showed no reaction. No change in reactivity of the M bands occurred if the blenderized pellets of muscle were resuspended and washed in 0.44 M sucrose three times before incubation. The reaction in the area of the Z band in the extreme periphery of the muscle blocks was not a prominent feature (Figs. 1, 2). Only in the blenderized muscle (Figs. 3, 5) was the deposition of a final product heavy. The distribution of the reaction in mitochondria was also restricted to the peripheral portion of the blocks of muscle but was more regular, usually extending over the en-

tire periphery and to a depth somewhat greater than the M band reaction.

The minute, sharply localized deposits of final product on the myofilaments at the level of the M bands were first encountered in the diaphragm in the vicinity of the neuromuscular junctions which were then under study. This was an unexpected finding and led to a series of experiments to determine whether or not the M band was a genuine enzymatic site or a precipitation site. As described below, it was found that there was no relationship between the staining of the neuromuscular junctions and the M bands other than in the development of our experiments.

Experiments with Diaphragm.—M bands of myofibrils, distant from neuromuscular junctions in the same section, were reactive; but, since the topographical information in electron microscopy is virtually reduced to only two dimensions, a junction could have occurred above or below the plane of section. It was but a simple matter to investigate the reactivity of M bands in myofibrils in the aneural portion of the diaphragm, since all the neuromuscular junctions of the rat diaphragm occur in a narrow strip, less than 5 mm. wide, running lengthwise over the dome, perpendicular to the direction of the muscle fibers but parallel and near the main phrenic nerve trunk as it courses through each leaf. The diaphragm was cut into strips so that one strip contained all the end plates and another, distant from the first, was completely free of them as subsequently proven by the results of histochemical staining. When blocks from each strip were incubated in separate vessels, the M bands of myofibrils from both the neural and aneural blocks were stained equally.

Experiments with Heart Muscle.—Because of possible histochemical artifacts in dealing with muscle (diaphragm), which contained sites of high enzymatic activity in its neuromuscular junctions, it was decided to investigate the ventricular portion of heart muscle. Since the substrate was hydrolyzed by a neuromuscular junction enzyme(s), presumably AChE; and, since it was possible that the M band deposits were due to the activity of a related enzyme, it was first decided to investigate the quantity of AChE in the heart muscle. Manometric studies indicated that heart muscle as well as aneural diaphragm contained an appreciable amount of AChE activity (Table I). Secondly, to rule out possible sites of high activity, such as nerve endings, as providing comparable

sites to those of the neuromuscular junctions, serial frozen sections (7μ) of the apex of the heart from which the endocardium and epicardium were stripped were incubated. These showed no activity with the light microscope; but, as indicated in a previous paragraph, M band activity of heart muscle was of a high order in the experiments with the electron microscope.

Control Experiments.—In all of four experiments (1 diaphragm, 3 heart) omissions of the substrate resulted in complete absence of deposition of final product at the M band and in mitochondria (Fig. 6). Similarly, the Z band area and the neuromuscular junctions in diaphragm were unreactive. Prefixation in formalin for 2 hours eliminated the reaction at the M band and in mitochondria, but not at the neuromuscular junctions. Activity in the small, sparsely occurring bodies in the sarcoplasm continued to be intense after formalin fixation (Fig. 10). Prefixation in 1 per cent buffered osmium tetroxide for 10 minutes or heating the tissues to 80°C . for 5 minutes prevented the reaction at all sites.

Experiments with Enzyme Inhibitors.—The M band activity was not inhibited by sodium taurocholate; in fact, activation may be considered since the size of the deposits were routinely larger. Eserine (10^{-5} M), TEPP (10^{-5} M) and DFP (10^{-4} M) caused inhibition of the reaction at the M band. With eserine the inhibition was partial; but with TEPP, and especially DFP (Fig. 8), almost complete inhibition occurred. Weaker concentration of these inhibitors caused less inhibition at the M band. These three inhibitors in high concentration completely eliminated the histochemical reaction at the neuromuscular junction as visualized with the light microscope. No definite statement can be made concerning the reaction in the Z band area and sarcoplasmic reticulum. It should be recalled that the reactivity of these sites in blocks of muscle incubated in the standard medium was little or none; no reaction was found in the inhibition experiments. Sodium taurocholate caused a moderate decrease in the number of deposits in mitochondria; but eserine, TEPP, and DFP did not markedly effect the reaction in these organelles as well as in the small sarcoplasmic bodies. Although the number of deposits within mitochondria decreased somewhat in some instances, especially with DFP, the size of the deposits increased (Fig. 8).

TABLE I
Acetylcholinesterase in Heart Muscle and Diaphragm of Rats

	Ach hydrolysis $\text{CO}_2/\text{gm. tissue/hr.}$	Ach hydrolysis $\text{CO}_2/\text{gm. tissue/hr.}$ DFP 3×10^{-4} M
	$\mu\text{l.}$	$\mu\text{l.}$
Diaphragm (entire leaf).....	620	0
Diaphragm (aneural).....	183	0
Heart (ventricular muscle).....	226	10

Results represent average of two experiments. In each case the wet weight was obtained and an aliquot of blenderized muscle used.

DISCUSSION

On the basis of the evidence presented we feel that the discrete deposition of the final product of the histochemical reaction at the M band of striated muscle reflects enzymatic activity at this site. Not only would the sharp localization obtained in the present experiments suggest a precise reaction at the M portion of the elemental filament of muscle, but the control experiments indicated that this reaction is enzymatic. Absence of substrate prevented any reaction, indicating that the reagent did not combine with the proteins of the M band; brief fixation of the muscle in formalin and osmium tetroxide or brief pretreatment with heat prevented any reaction; finally the addition of well known enzymatic inhibitors (eserine, TEPP, and DFP) to the incubation mixture partially or completely eliminated the histochemical reaction.

Concerning the nature of the enzyme, we are on less certain grounds; in fact, we cannot with any assurance state what the M band enzyme is or what it does. It could simply be a carboxylic acid esterase since these enzymes in other tissues may hydrolyze the substrate (5) at an acid pH. Indeed, an insoluble non-specific esterase has been demonstrated in muscle tissue (6, 7); but the possibility of the M band being the site of this enzyme seems to be ruled out because of the lack of inhibition obtained with sodium taurocholate in the present experiments.

A more likely guess is that the enzyme is a cholinesterase. This is suggested because it is known that purified AChE hydrolyzes the substrate, thiolacetic acid (1); and in the present experiments well known cholinesterase inhibitors

had an adverse effect on the M band enzyme activity, though not as great as on the neuromuscular junction enzyme. Furthermore, the present experiments have confirmed the repeatedly noted fact that aneural parts of skeletal muscle as well as ventricular cardiac muscle contains cholinesterase activity (8-12).

Despite the latter findings, there has been a persisting tendency to explain the cholinesterase activity of muscle as being concentrated at neuromuscular junctions and at other nerve endings. This has been supported by recent histochemical studies which indicate no localization within muscle fibers (13-17). Our experiments may indicate the reason why the localization within muscle fibers has been missed; the deposits of final product were too small to be visualized with the light microscope. The exclusive localization of the enzyme at the neuromuscular junction is difficult to accept since it is known that, besides the aneural parts of skeletal muscle, denervated muscle (9, 18) and embryonic muscles (13, 19, 20) that had not yet developed neuromuscular junctions contain large amounts of cholinesterase.

Attention should be called to the results published by a group of Hungarian biochemists who have investigated the cholinesterase problem in muscle. Beznák (21) first showed that myosin A and B contained cholinesterase activity, and his findings were later confirmed (22, 23) and extended (18, 20, 24, 25). It is now quite clear according to the above references that striated muscle contains two main cholinesterases, acetylcholinesterase that is concentrated at the neuromuscular junctions and "myosincholinesterase" which accounts for approximately half of the total cholinesterase activity of muscle (18).

If the enzyme at the M band is a cholinesterase, it is an attractive hypothesis to equate it with "myosincholinesterase" and thereby account, on a cytochemical basis, for the presence of this enzyme in areas of striated muscle lacking neuromuscular junctions. In this regard our results are in agreement with those of other authors (25), indicating that the neuromuscular junction enzyme and the enzyme in muscle fibers react differently to enzymatic inhibitors. It should be pointed out, however, that the "myosincholinesterase" was associated with the L-meromyosin fractions (22, 24), whereas the proteins at the M band appear to be in the H-meromyosin fractions according to Marshall *et al.* (26).

If we assume that the enzyme at the M band is a cholinesterase, it is reasonable to postulate that it is part of the mechanism by which the conduction of stimuli is controlled within the muscle fibers. This hypothesis may be combined with the morphological interpretation that the sarcoplasmic reticulum, especially its vesicular specializations, possibly act as an intracellular conductor (27). In this instance, the situation would be different from that encountered at the neuromuscular junction because it would require the production of a substrate, whatever it may be, in the intermediate vesicles of the sarcoplasmic reticulum and its diffusion along the myofilaments up to the M band before being hydrolyzed. In this regard, it is noteworthy that in the experiment of Huxley and Taylor (28) a localized contraction wave was obtained when a weak stimulus was applied through a microelectrode on the sarcolemma of single muscle fibers at the level of the intermediate vesicles of the sarcoplasmic reticulum. The wave of contraction produced was limited and stopped at the adjacent M band(s). Obviously it is difficult to arrive at an understanding of the role of the M band enzyme with the present information. However, we feel that the presence and precise localization of this enzyme together with other recent contributions (27-31) should be taken into account in the future interpretation of the mechanisms involved in muscle contraction.

We would like finally to discuss some considerations concerning the histochemical reaction at sites other than the M band. The irregular occurrence of minute, poorly opaque deposits in contraction bands, in the region of the Z bands, and the sarcoplasmic reticulum of striated muscle was not further investigated and for the present is considered spurious. The deposition of opaque final product in mitochondria and in dense sarcoplasmic bodies is under study in the liver and kidney where these structures, as well as the endoplasmic reticulum, have been found to react histochemically with the present incubating mixture (5). Besides other carboxylic acid esterases, a mitochondrial AChE (32) that has an acid pH optimum may be responsible for the reaction we have detected. However, in the present experiments, cholinesterase inhibitors did not eliminate the histochemical reaction in mitochondria. Moreover, theoretical considerations of the substrate suggest that a whole host of enzyme systems that are involved in acyl transfer reactions (33) could

be involved in this histochemical reaction. In many respects the substrate used here is similar to some of those used in the thioester studies of Stadtman (34) in which ester interchange by acyl group transfer reactions occurred spontaneously at alkaline pH's and enzymatically at acid pH's. Reactions such as these could, in part, account for the histochemical staining in the sarcoplasmic organelles.

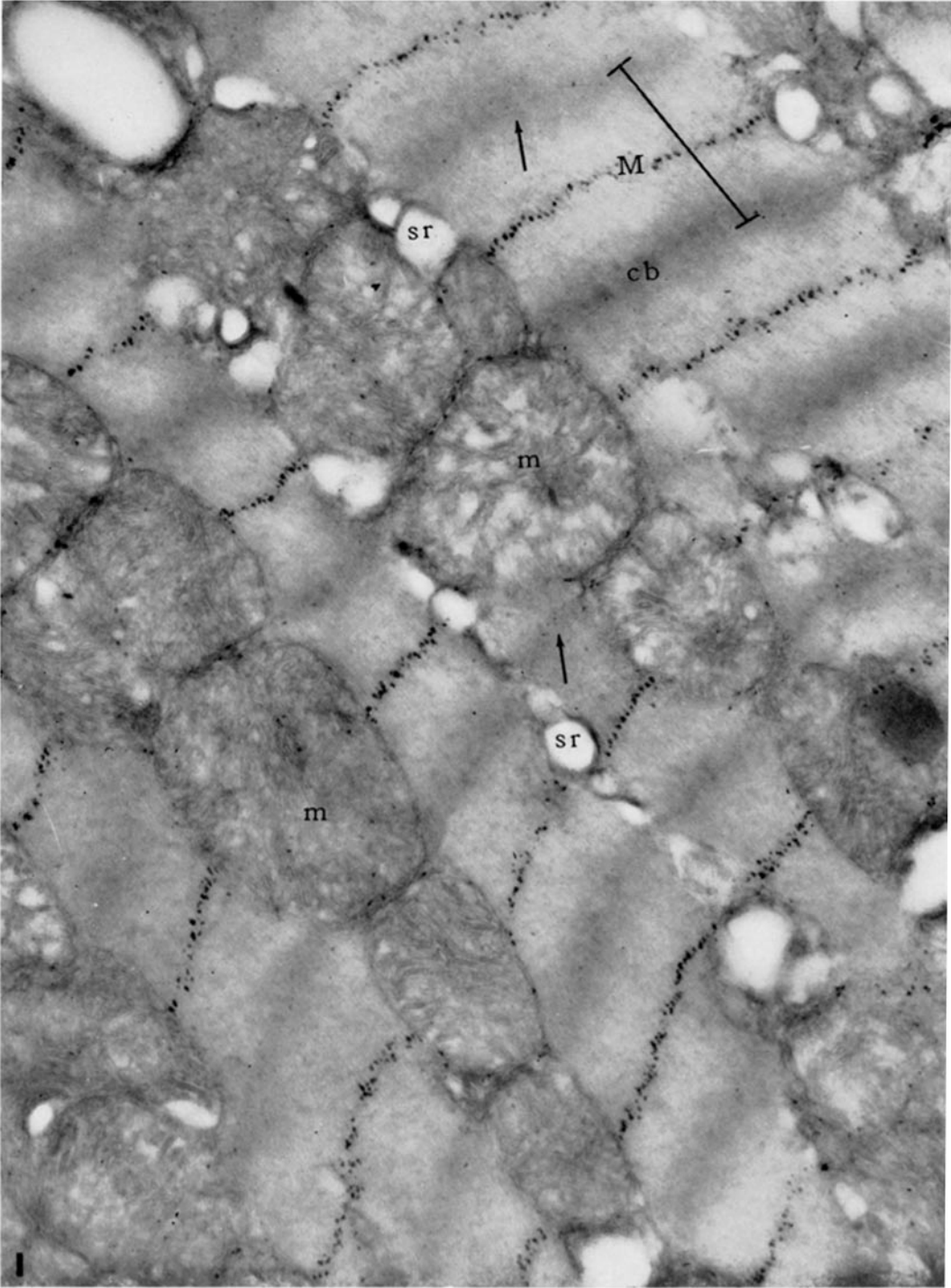
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EXPLANATION OF PLATES

PLATE 102

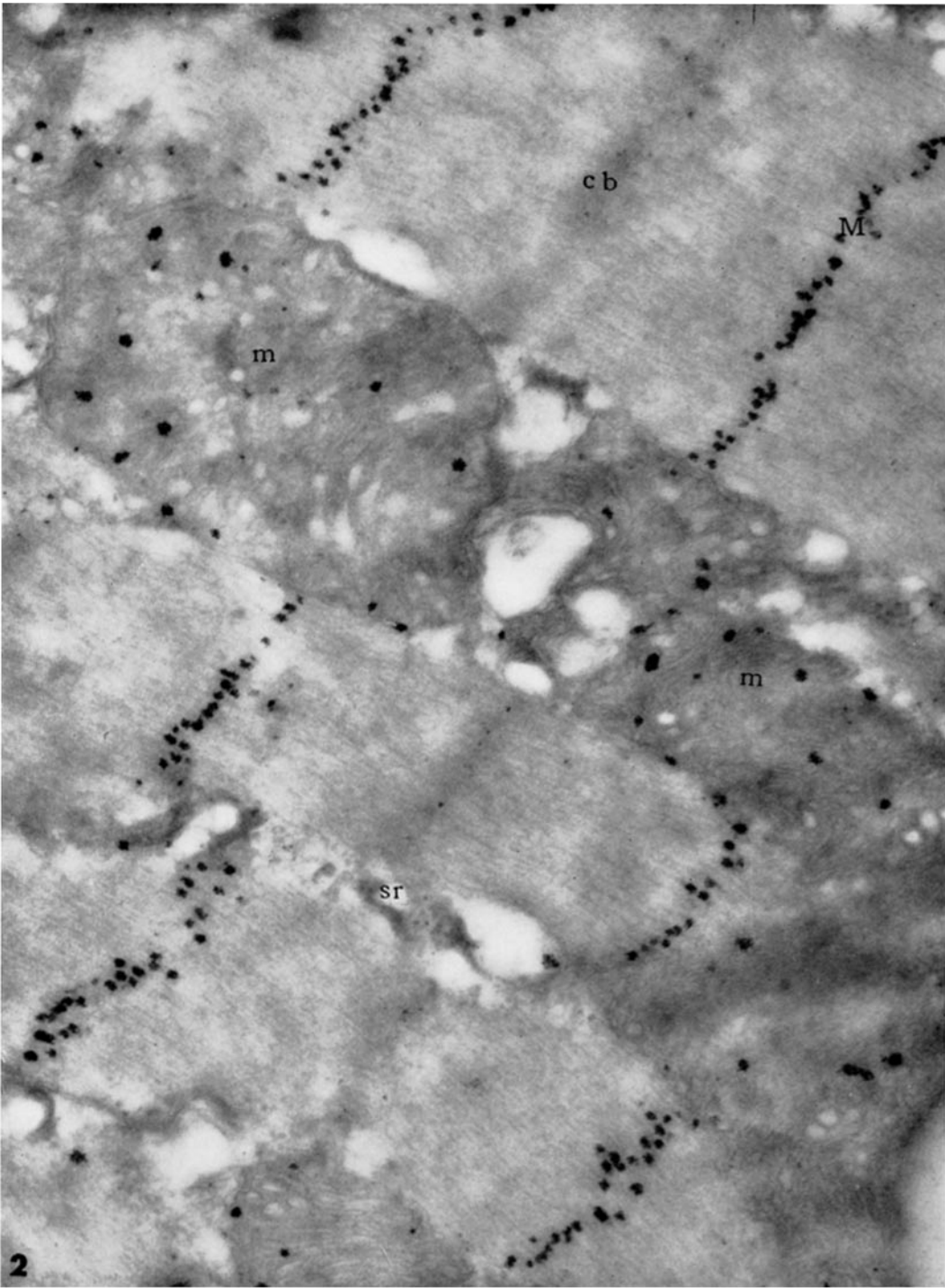
FIG. 1. Section of a block of heart muscle incubated for 5 minutes at pH 5.5 in the standard medium. The muscle fibers are moderately well preserved and show contraction bands (*cb*) superimposed on the Z bands. A sarcomere is indicated by brackets. Most prominent is a linear array of small, rounded, opaque deposits of the final product of the histochemical reaction at the M band (*M*) of nearly all the filaments of the fibers. Only occasionally is a filament not marked by a deposit at the M band, and only rarely do there seem to be two adjacent deposits on one filament. Within each myofibril the deposits at the M band generally are in good register; only occasionally the alignment is disturbed probably due to unequal or slight displacement during sectioning. The contraction bands are irregularly dotted with smaller deposits that are less opaque than those of the M bands (arrows). The mitochondria (*m*) are moderately well preserved but show some vacuolization and spotty extraction of matrix. Dense deposits similar to those at the M bands occur in the interior of the mitochondria and in relation to their surface membranes. The sarcoplasmic reticulum (*sr*) shows extensive swelling and some disruption. $\times 37,000$.



(Barnett and Palade: M band enzyme)

PLATE 103

FIG. 2. Section of a block of diaphragmatic muscle incubated for 12 minutes at pH 6.2 in the standard medium. Muscle fibers show contraction bands (*cb*) which are centered at the Z band, and which contain a few small moderately dense deposits. Very dense deposits are aligned at the level of the M band (*M*). The deposits show an irregular shape and often appear crystalline in nature. The sarcoplasmic reticulum (*sr*) is swollen and disrupted and contains a few small deposits which are similar to those in the contraction bands. The mitochondria (*m*) show spotty extraction of the matrix and contain mainly internal dense deposits. $\times 63,000$.



(Barnett and Palade: M band enzyme)

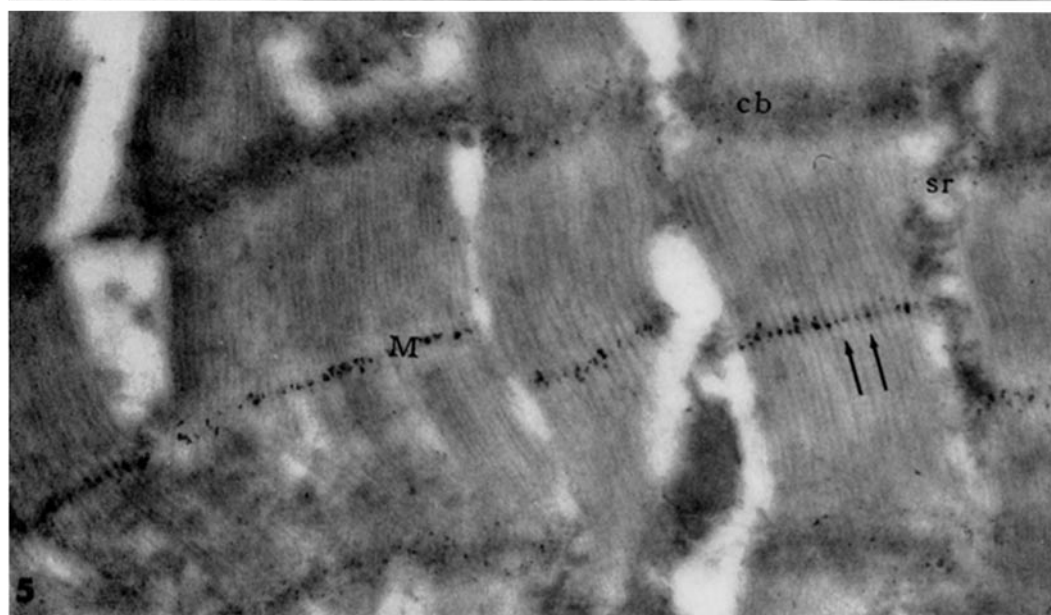
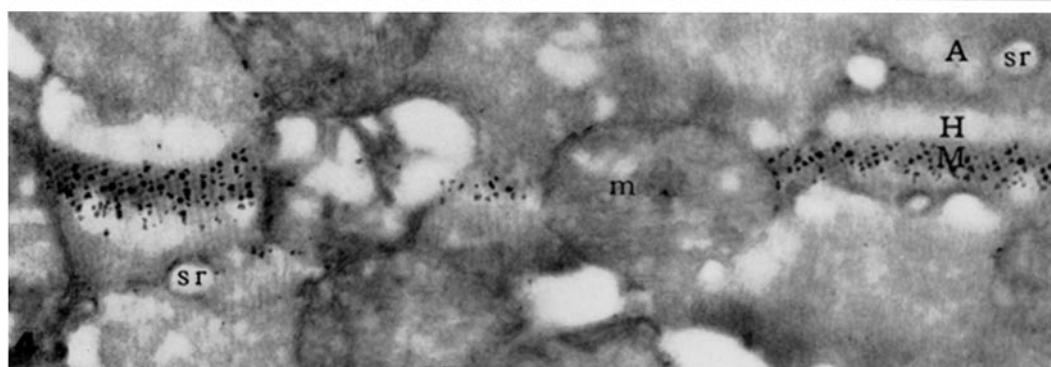
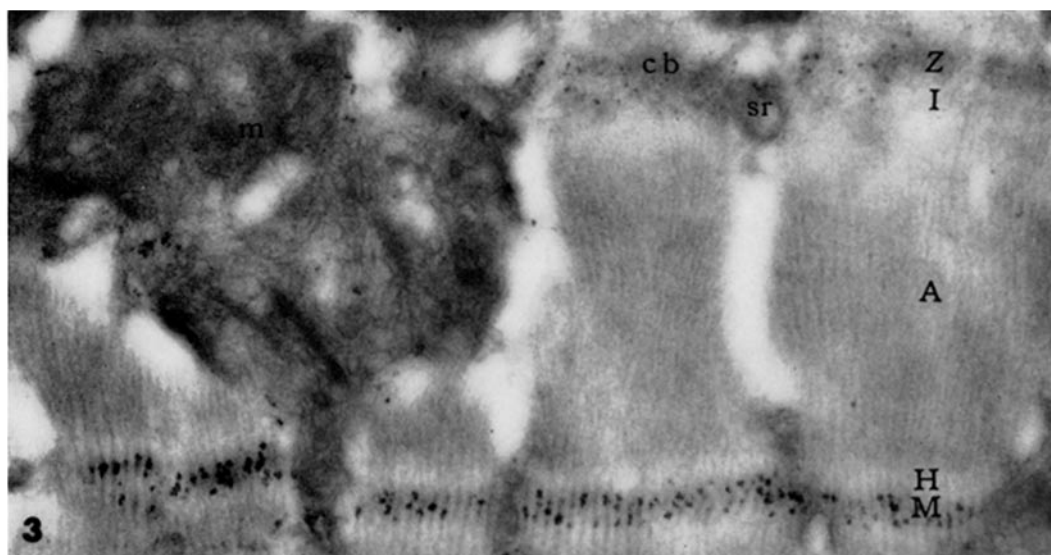
PLATE 104

Figs. 3 to 5 are taken of sections of blenderized heart muscle incubated in the standard medium at pH 6.1 for 8 minutes.

FIG. 3. One half a sarcomere is illustrated, and the Z, I, A, H, and M bands are indicated. The muscle fibers are obliquely sectioned and are partly contracted. The Z band is obscured by a contraction band (*cb*); and this zone, including the sarcoplasmic reticulum (*sr*), is peppered with small deposits of moderate density. Larger, more dense deposits are restricted to the M band (*M*) and the mitochondria (*m*). The A and H bands are free of deposits. $\times 63,000$.

FIG. 4. Oblique, but almost transverse section through the M, H, and A bands of sarcomere. Dense deposits are primarily related to the M band, but a few appear to be in the H band. Sarcoplasmic reticulum (*sr*) and mitochondria (*m*) containing dense deposits are also indicated. $\times 50,000$.

FIG. 5. Longitudinal section through contracted fibers showing the thick elemental filaments of the muscle fibers and the relationship of the dense deposits to the thickening that occurs on the filaments at the M band (arrows). Smaller, less dense deposits occur on the contraction band (*cb*) in the area of the Z band and the sarcoplasmic reticulum (*sr*). $\times 50,000$.

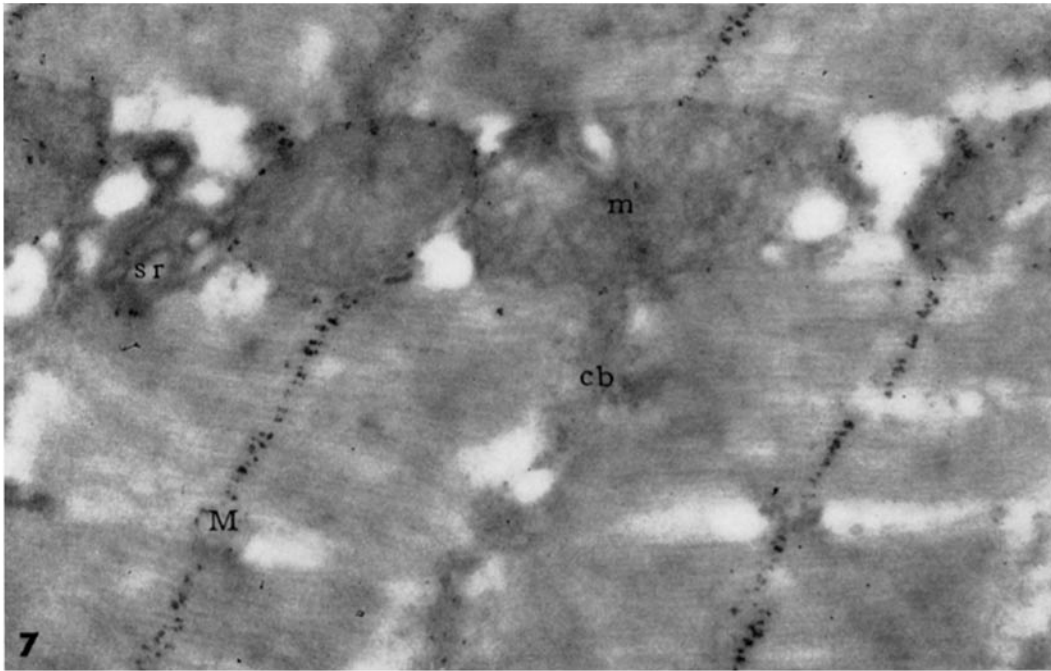
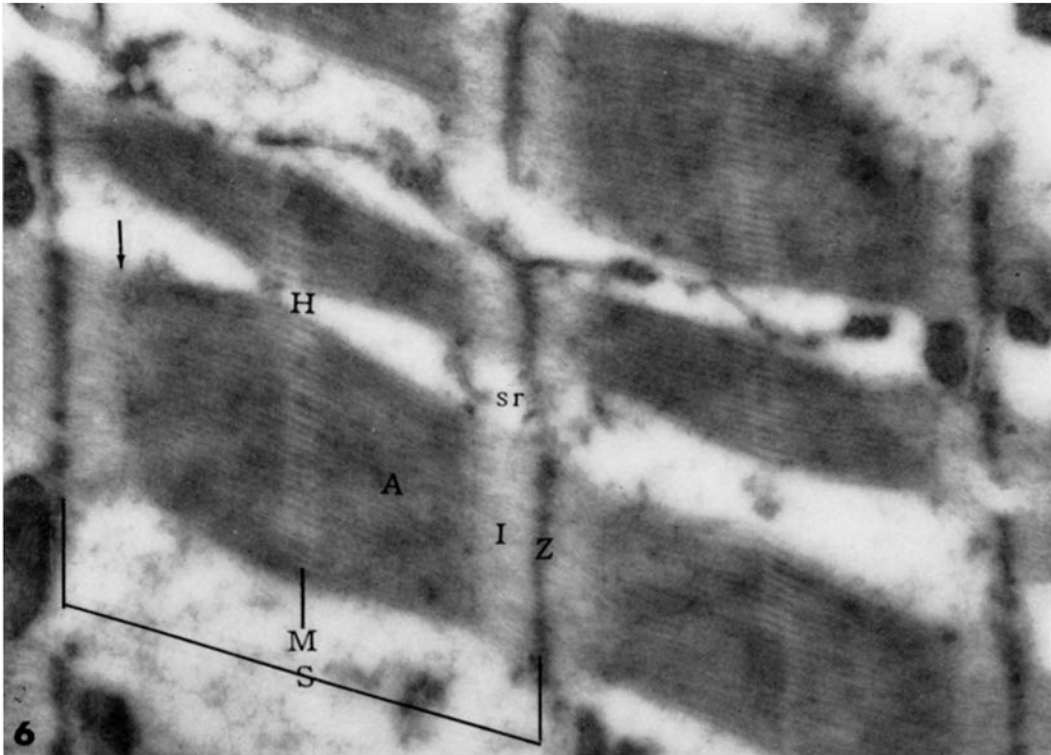


(Barnett and Palade: M band enzyme)

PLATE 105

FIG. 6. Section from control block of heart muscle incubated at pH 6.1 for 20 minutes in a medium lacking substrate but not lead. The M band, indicated in the middle of the H band, has no dense deposits. The A, I, and Z bands are labelled as well as a sarcomere (*S*, bracket). The sarcoplasmic reticulum is unreactive; at the A-I junction (arrow), minute, slightly opaque deposits are barely visible. $\times 63,000$.

FIG. 7. Section of block of heart muscle incubated for 8 minutes in the standard medium at pH 6.1. Dense M band (*M*) deposits are present as well as the less dense deposits in the contraction band (*cb*). The disrupted sarcoplasmic reticulum (*sr*) contains an occasional deposit, and the mitochondria (*m*) show peripheral deposition of final product. $\times 63,000$.

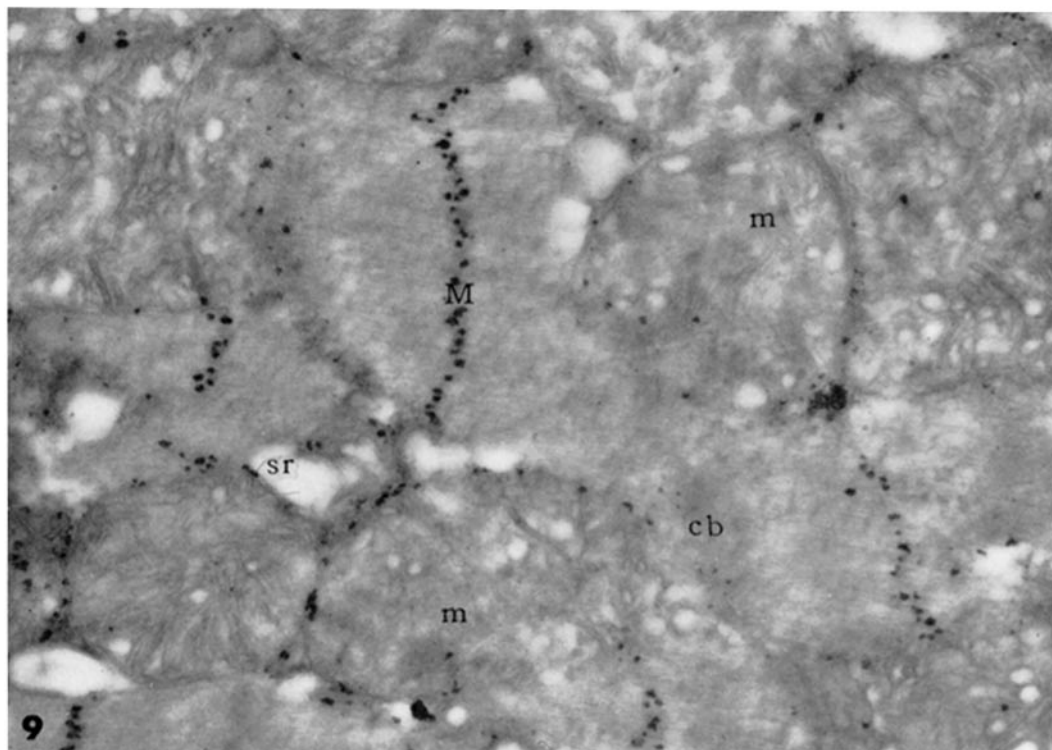
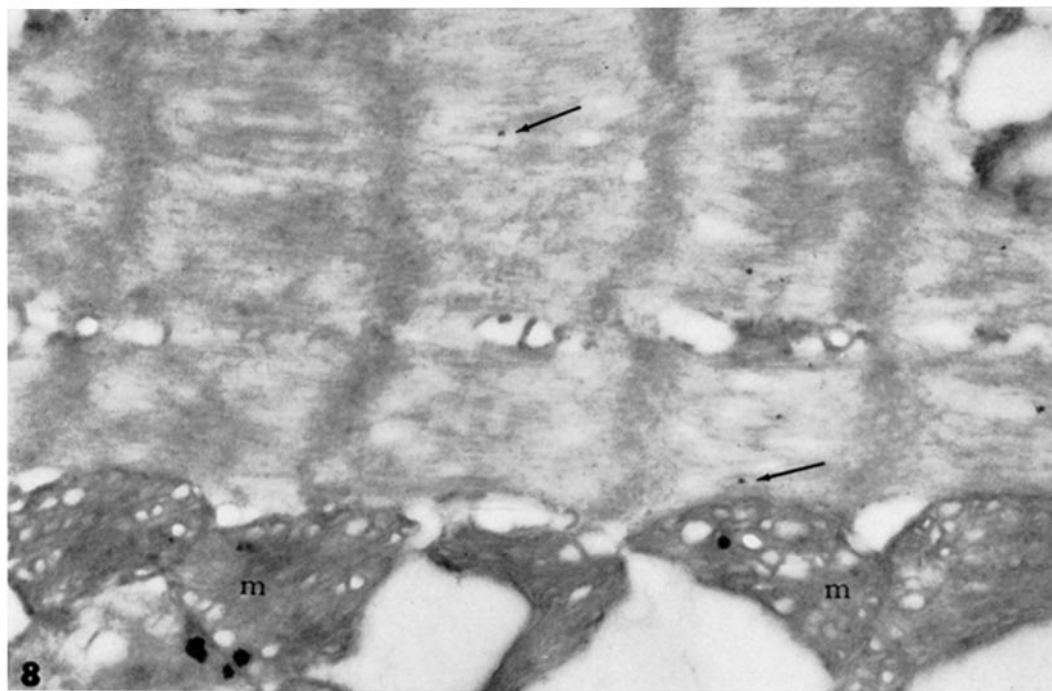


(Barnett and Palade: M band enzyme)

PLATE 106

FIG. 8. Section of block of heart muscle soaked in 0.44 M sucrose containing DFP (10^{-4} M) for 10 minutes, followed by incubation in the standard medium containing DFP for 20 minutes. Although this treatment caused marked alteration of structure in comparison to the standard incubation, sufficient detail is preserved for recognition. The mitochondria (*m*) contained fewer but larger deposits of final product. Sparse, smaller deposits (arrows) are found in occasional fibrils, and their localization is approximately in the middle of the sarcomere. $\times 37,000$.

FIG. 9. Section of block of heart muscle incubated for 15 minutes at pH 6.4 in the standard medium. Alignment of deposits at the M band is not orderly probably because of unequal contraction. Mitochondria (*m*) show many dense deposits, especially on their surfaces; and the sarcoplasmic reticulum (*sr*) and contraction bands (*cb*) contain a few less dense deposits. $\times 50,000$.

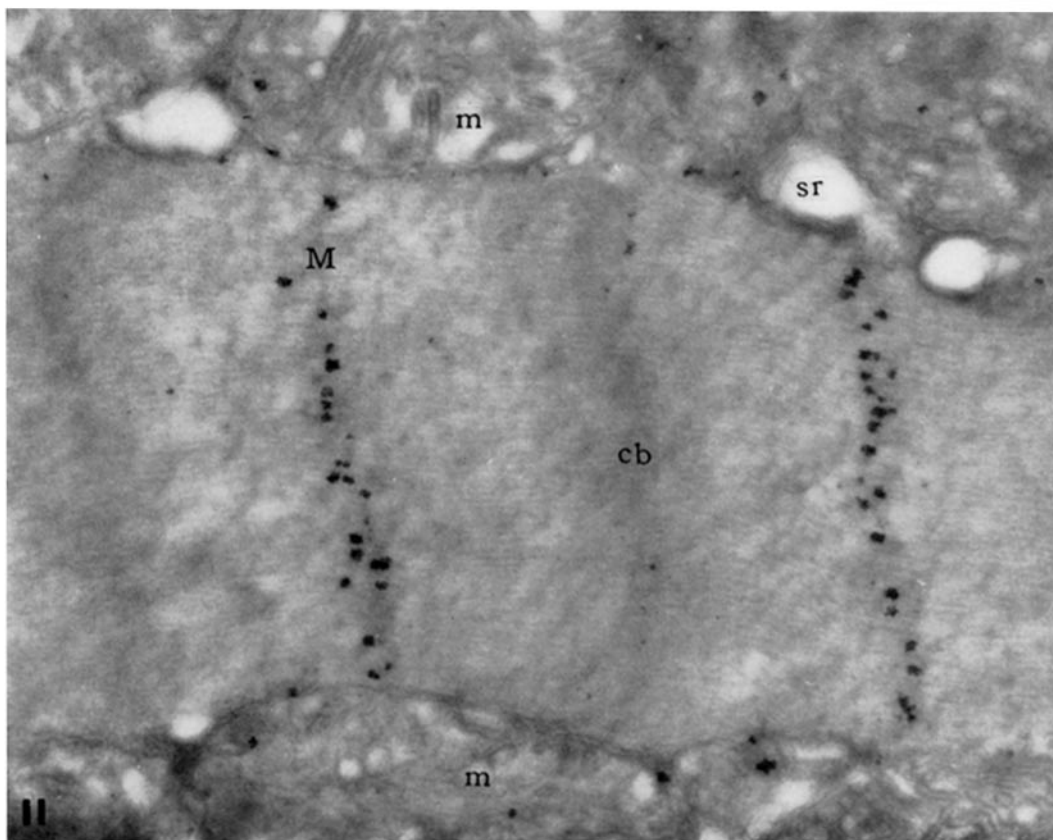
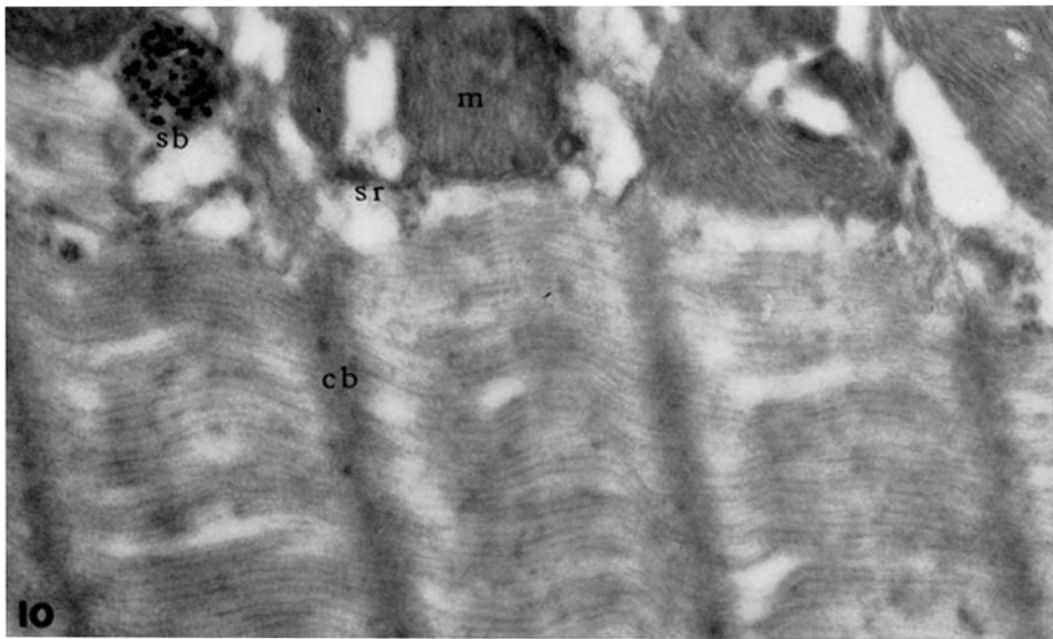


(Barnett and Palade: M band enzyme)

PLATE 107

FIG. 10. Section of block of heart muscle fixed in 10 per cent formalin in 0.44 M sucrose for 1 hour before incubation in the standard medium at pH 6 for 15 minutes. The only sites of activity are the sarcoplasmic bodies (*sb*) which are crowded with dense deposits. Similar reactive structures were found in heart muscle not subjected to any pretreatment and in specimens subjected to the action of enzymatic inhibitors. The M band, contraction bands (*cb*), mitochondria (*m*), and sarcoplasmic reticulum (*sr*) show no activity. $\times 50,000$.

FIG. 11. Section of block of heart muscle incubated in the standard medium at pH 5.6 for 5 minutes. Dense, irregular deposits of final product occur on some of the elemental filaments at the M band (*M*) and in mitochondria (*m*). Sparse, smaller, less dense deposits occur at the contraction bands (*cb*) in the region of the Z band. $\times 75,000$.



(Barnett and Palade: M band enzyme)