THE LOCALIZATION OF CHOLINESTERASE ACTIVITY IN RAT CARDIAC MUSCLE BY ELECTRON MICROSCOPY

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ABSTRACT

A method has been developed for localizing sites of cholinesterase activity in rat cardiac muscle by electron microscopy. The method utilizes thiocholine esters as substrates, and is believed to be dependent on the reduction of ferricyanide to ferrocyanide by thiocholine released by enzymatic activity. The ferrocyanide thus formed is captured by copper to form fine, electron-opaque deposits of copper ferrocyanide, which sharply delineate sites of enzymatic activity at the ultrastructural level. Cholinesterase activity in formalin-fixed heart muscle was localized: (*a*) in longitudinal elements of the sarcoplasmic reticulum, but not in the T, or transverse, elements; and (*b*) in the A band, with virtually no activity noted in the M band, or in the H zone. The I band was also negative. No activity was detected in the sarcolemma, or in invaginations of the sarcolemma at the level of the Z band. The perinuclear element of the sarcoplasmic (endoplasmic) reticulum was frequently strongly positive. Activity at all sites was completely abolished by omitting the substrates, or by inhibition with eserine 10^{-4} M and diisopropylfluorophosphate 10^{-5} M. Eserine 10^{-5} M completely inhibited reaction in the sarcoplasmic reticulum, and virtually abolished that in the A band. These observations, together with the use of the relatively specific substrates and suitable controls to eliminate non-enzymatic staining, indicate that cholinesterase activity was being demonstrated. The activity in rat heart against different substrates was that of non-specific cholinesterases, in accordance with biochemical data. The activity in the A band was considered to be probably due to myosincholinesterase. It is proposed that the localization of cholinesterases in myocardium at the ultrastructural level should be taken into account in considering the possible functions of these myocardial enzymes, and it is hoped that knowledge of their localization will open up new avenues of approach in considering their physiological role in myocardium, which at present is not definitely known.

Myocardium of several species (1–3, 49), including the rat (1, 2), is known to have considerable cholinesterase activity, the function of which is not at present understood with any certainty. The localization of cholinesterase activity at the ultrastructural level might afford some clues to the functions of myocardial cholinesterases.

It was previously suggested by Barrnett and Palade (4) that the so called M band enzyme which they detected in rat cardiac muscle, using thiolacetic acid as substrate, was a cholinesterase. We subsequently showed (5) that this suggestion was incorrect, and believe that the cholinesterases of rat cardiac muscle are not demonstrated with the thiolacetic acid technique.

It would be desirable to use more specific substrates for cholinesterases than thiolacetic acid. Thiocholine esters are used in the various modifi-

cations of the Koelle-Friedenwald method (6, 7) for light microscope demonstration of cholinesterase activity. Although useful results are obtained at the histological level, application of these methods to electron microscopy has not met with success, both in our laboratory and elsewhere (8).

Because thiocholine esters do have the great advantage of being relatively specific substrates, which are hydrolysed at fast rates by cholinesterases, we recently developed a method which, when applied to rat cardiac muscle, gave satisfactory results in that fine localization at the ultrastructural level was obtained.

This paper presents the method and the results of its application to rat cardiac muscle.

MATERIALS AND METHODS

ANIMALS: Male and female Sprague-Dawley rats, weighing 50 to 250 gm, were stunned by a blow on the head, and decapitated.

HANDLING OF TISSUE: The beating heart was rapidly excised, and allowed to beat in cold 0.44 M sucrose; most of the contained blood was thereby ejected. Small blocks (about 3 mm^3 in size) of the left ventricle and septum were taken. In some experiments the intact atria were removed as well. The tissues were washed free of blood in cold 0.44 M sucrose, and were then placed in fixative.

FIXATION: Fixation was done in 4 per cent formaldehyde buffered to pH 7.6 with 0.075 M phosphate buffer; the fixative contained 0.44 M sucrose. Fixation was carried out for 2½ to 3½ hours at 2–4°C. The blocks of tissue were then blotted, and placed in 0.44 M sucrose at 2–4°C for about 16 hours, after which they were blotted and frozen on a freezing microtome with compressed CO_2. Sections were cut at a thickness of 50 μ, and were transferred to cold 0.44 M sucrose before being placed in the various preincubation and incubation media.

INCUBATION MEDIUM: The stock solutions were routinely prepared the day before the experiment was run, although they keep for a week or more. Glass-distilled water was used throughout. The stock solutions were: (*a*) sodium hydrogen maleate buffer,[1] pH 6.0, 0.1 M; (*b*) sodium citrate, 100 mM; (*c*) copper sulphate, 30 mM; (*d*) potassium ferricyanide, 5 mM.

The final medium was prepared shortly before use as follows:—(final concentrations of constituents are given in parentheses) 5 mg of thiocholine ester (~2 mM) were dissolved in 6.5 ml of sodium hydrogen maleate buffer (0.065 M). The following were then added in order, with thorough mixing after the addition of each constituent: sodium citrate 0.5 ml (5 mM); copper sulfate 1.0 ml (3 mM); water 1.0 ml; potassium ferricyanide 1.0 ml (0.5 mM); sucrose 1.5 gm (0.44 M).

The substrates used were acetylthiocholine iodide, butyrylthiocholine iodide, and propionylthiocholine chloride. It was not necessary to remove the iodine before using the iodides.

The medium was placed in 10-ml beakers held in crushed ice, and the sections were transferred with glass rods for the incubation. The period of incubation varied from 15 to 40 minutes. The media were stable for at least 1 hour. The incubations were run in the cold to reduce spontaneous hydrolysis and to reduce the rate of enzyme activity.

ELECTRON MICROSCOPY: After incubation was completed, the sections were washed four times in cold 0.44 M sucrose over a period of about 10 minutes. They were then fixed in osmium tetroxide buffered at pH 7.2 with *s*-collidine (10), and embedded in Vestopal W by the method of Kurtz (11).

Thick sections for orientation by phase and light microscopy (in the latter case, stained with toluidine blue) were cut on a Porter-Blum (Servall) microtome, and thin sections on a LKB Ultratome. In sections of 1 μ thickness, the localization of reaction product was not detectable by either phase or light microscopy.

It was shown that alkaline lead staining solutions did not affect the final reaction product, and consequently thin sections were stained with lead according to method A of Karnovsky (12) to enhance contrast.

The thin sections were examined in a RCA3-F electron microscope; photographs were taken at original magnifications of 3,600 to 18,000 and were subsequently enlarged.

CONTROLS: To assess the effect of the standard fixation of 2½ to 3½ hours on enzyme activity, both unfixed, fresh-frozen sections and sections fixed for either 1 hour or 24 hours were also examined.

In some experiments, to remove cholinesterases derived from the blood, the heart was perfused by the method of Langendorff until the myocardium was blanched and the perfusate was clear. The composition of the perfusing fluid has previously been given (1).

To evaluate the possibility of non-specific binding of components of the medium, or of one of the products of hydrolysis, namely thiocholine, to tissue components, the following tests were made:—

(*a*) sections were incubated in the medium lacking substrate.

[1] Sodium hydrogen maleate buffer, 0.1 M, pH 6.0: 26.9 ml 0.2 M NaOH plus 50 ml 0.2 M NaH maleate, made to 200 ml with water. 0.2 M NaH maleate is prepared by dissolving in water 23.2 gm maleic acid (or 19.6 gm maleic anhydride) and 8 gm NaOH and making the volume to 1000 ml. For other pH values, see (43, 44).

(*b*) tissue blocks or sections were preincubated at 4°C for 16 hours in 0.1 M *N*-ethylmaleimide in 0.1 M phosphate buffer, pH 7.6, and were then incubated in the complete medium.

(*c*) sections were incubated in substrate only, followed by treatment with the medium lacking substrate.

(*d*) sections were placed in the medium lacking substrate, and the ferricyanide was reduced by adding a reducing agent; *e.g.* cysteine.

(*e*) sections were pretreated or post-treated with cysteine, before or after being placed in the medium lacking substrate.

(*f*) sections were incubated in a solution containing copper ferrocyanide.

To evaluate the role of copper sulfate and potassium ferricyanide in the reaction, sections were (*g*) incubated in the medium lacking copper, (*h*) incubated in the medium lacking ferricyanide. Other experiments on the effects of varying the concentrations of components of the medium, of varying the pH, and the type of buffer, were carried out at the light microscope level in a variety of tissues, and are reported elsewhere (9).

SPECIFIC INHIBITION: The specific reversible inhibitor of cholinesterases, eserine sulfate 10^{-5} M or 10^{-4} M was used. It was freshly prepared as a stock solution 10^{-3} M, and an aliquot replaced a portion of the 1 ml of water in the standard medium. It was found to be important that the eserine was added after the copper citrate complex was formed or else precipitation occurred. Diisopropylfluorophosphate (DFP) 10^{-5} M was similarly used.

To ensure that the inhibitor penetrated the tissue adequately, sections were preincubated in a solution of the inhibitor in 0.44 M sucrose, pH 6.0, for 15 minutes at 4°C before being transferred to the medium which also contained the inhibitor. As controls for the preincubation in inhibitors, other sections were preincubated for 15 minutes at 4°C in 0.44 M sucrose, pH 6.0, before being transferred to the standard medium.

Eserine sulfate 10^{-4} M was found to completely inhibit cholinesterases of rat heart, both in homogenates (1) and in sections prepared for light microscopy (5). Eserine sulfate 10^{-5} M gave 90 per cent inhibition in homogenates (1) and apparently complete inhibition in sections. The homogenates were run with acetylcholine pS2, and the sections with thiocholine methods.

RESULTS

By light microscopy the myocardial fibers showed diffuse brown staining, attributable to cholinesterase activity, but without any specific localization to a finer order of structure being detectable. This diffuse staining of myocardial fibers is in accordance with the experience of others applying either the Koelle-Friedenwald thiocholine, or non-specific esterase methods to heart muscle. By electron microscopy, however, extremely fine, electron-opaque deposits were sharply localized by our methods to two principal sites, namely, the longitudinal elements of the sarcoplasmic reticulum, and the A band. Before proceeding to a more detailed description of these findings, several pertinent questions as to the specificity and accuracy in localization of the method must be answered.

Did non-specific (non-enzymatic) localization occur? (*a*) Control sections, incubated in the medium lacking substrate, showed no reaction. (*b*) Pre-incubation of sections in *N*-ethylmaleimide did not eliminate the reaction (Fig. 2) although sometimes there was slight inhibition of activity, more usually in the A band. Control (*c*) showed no deposits. Controls (*d*) (*e*) and (*f*) showed some deposits haphazardly and irregularly scattered throughout the tissue, without any particular localization.

These findings excluded non-specific binding of the copper or ferricyanide or of copper ferrocyanide by tissue components, or reduction of the ferricyanide by reducing (*e.g.* SH) groups in the tissue to give ferrocyanide, which would then be precipitated as copper ferrocyanide.

Non-specific binding of the liberated thiocholine to the tissue was excluded by preincubation in substrate alone, followed by treatment with the medium lacking substrate: no reaction was obtained.

In the absence of ferricyanide, large, needle-like, haphazardly scattered crystals were seen, indicating diffusion of the reaction product. The presence of ferricyanide was implicated as being necessary for sharp localization.

In the absence of copper, no reaction was obtained, excluding the possibility that part of the reaction product observed was an insoluble disulfide derived from thiocholine by the oxidizing action of the ferricyanide.

Preliminary experiments showed that the reaction product was not soluble in the *s*-collidine buffer of pH 7.2 used in the postfixation in osmium tetroxide. Osmium tetroxide was not apparently reduced (blackened) by copper ferrocyanide. The reaction product was also insoluble in the dehydrating alcohols, styrene, and in Vestopal W.

Interference by eserine or DFP with the reduc-

tion of ferricyanide and formation of copper ferrocyanide was discounted by suitable control experiments.

Was the reaction enzymatic, and due to cholinesterases? The absence of reaction product when substrate was omitted indicates the enzymatic nature of the reaction. The use of thiocholine esters as relatively specific substrates, and the inhibition of activity at all sites by eserine sulfate (Fig. 11) and DFP in low concentrations, indicated that cholinesterase activities were, in all probability, being demonstrated. Eserine 10^{-4} M and DFP 10^{-5} M completely inhibited at all sites. Eserine 10^{-5} M completely inhibited activity in the sarcoplasmic reticulum, and almost completely in the A band.

Removal of blood by perfusion had no effect on the enzymatic activity or localization.

What was the effect of fixation? It was found that fixation for 2½ to 3½ hours as detailed above gave the optimal results, in terms of adequate enzyme activity and preservation of structure. To exclude differential inhibition of some sites of activity and not of others, unfixed, frozen sections were also examined. Unfixed, fresh-frozen sections gave much poorer preservation of structure than did fixed sections. Enzymatic activity in the sarcoplasmic reticulum was higher than in fixed sections, as judged by the rate of deposition of reaction product. When unfixed sections were compared with fixed sections, no additional sites of activity attributable to cholinesterases were noted. Curiously, however, the A band did not react whereas the longitudinal elements of the sarcoplasmic reticulum reacted strongly. However, additional sites of non-enzymatic deposition of electron-opaque deposits were seen, namely, on the membranes of swollen (and only swollen) mitochondria, and in collagen. Reaction at these sites occurred in the absence of substrate, and was not inhibited by eserine in the presence of substrate. It was therefore concluded that fixation was essential for the adequate preservation of structure, for the demonstration of A band activity, and to eliminate non-enzymatic reaction in collagen and swollen mitochondria.

After fixation for 24 hours, followed by washing in 0.44 M sucrose for 2 hours, enzymatic activity was considerably diminished, but adequate preparations could be obtained by prolonging the incubation. Fixation for 1 hour gave inadequate structural preservation, and some degree of non-enzymatic reaction in collagen and in swollen mitochondria still occurred.

Although the effects of varying the length of the postfixation wash were not systematically studied, the impression was gained that thorough washing gave better enzymatic activities. Therefore, routinely, washing in cold 0.44 M sucrose was done for about 16 hours (overnight). The presence of sucrose 0.44 M in fixatives, incubation media, and washes gave better preservation of structure, in agreement with Barrnett and Palade (4).

Did diffusion of the enzyme or reaction product occur? No discoloration of the media, which might have indicated enzyme diffusion from the tissue, was observed either with unfixed, fresh-frozen sections or with fixed sections. Rat cardiac cholinesterases are extremely firmly bound to tissue components (13). Even with incubations as long as 40 minutes, no evidence of diffusion of reaction product was seen in the electron microscope, despite extremely heavy focal concentrations of reaction product in the tissue. This was true for both fixed and unfixed sections.

It was concluded (*a*) that the method, as carried out under the conditions prescribed, dependably demonstrated sites of cholinesterase activities in rat cardiac muscle, and (*b*) that the localization was precise at the ultrastructural level.

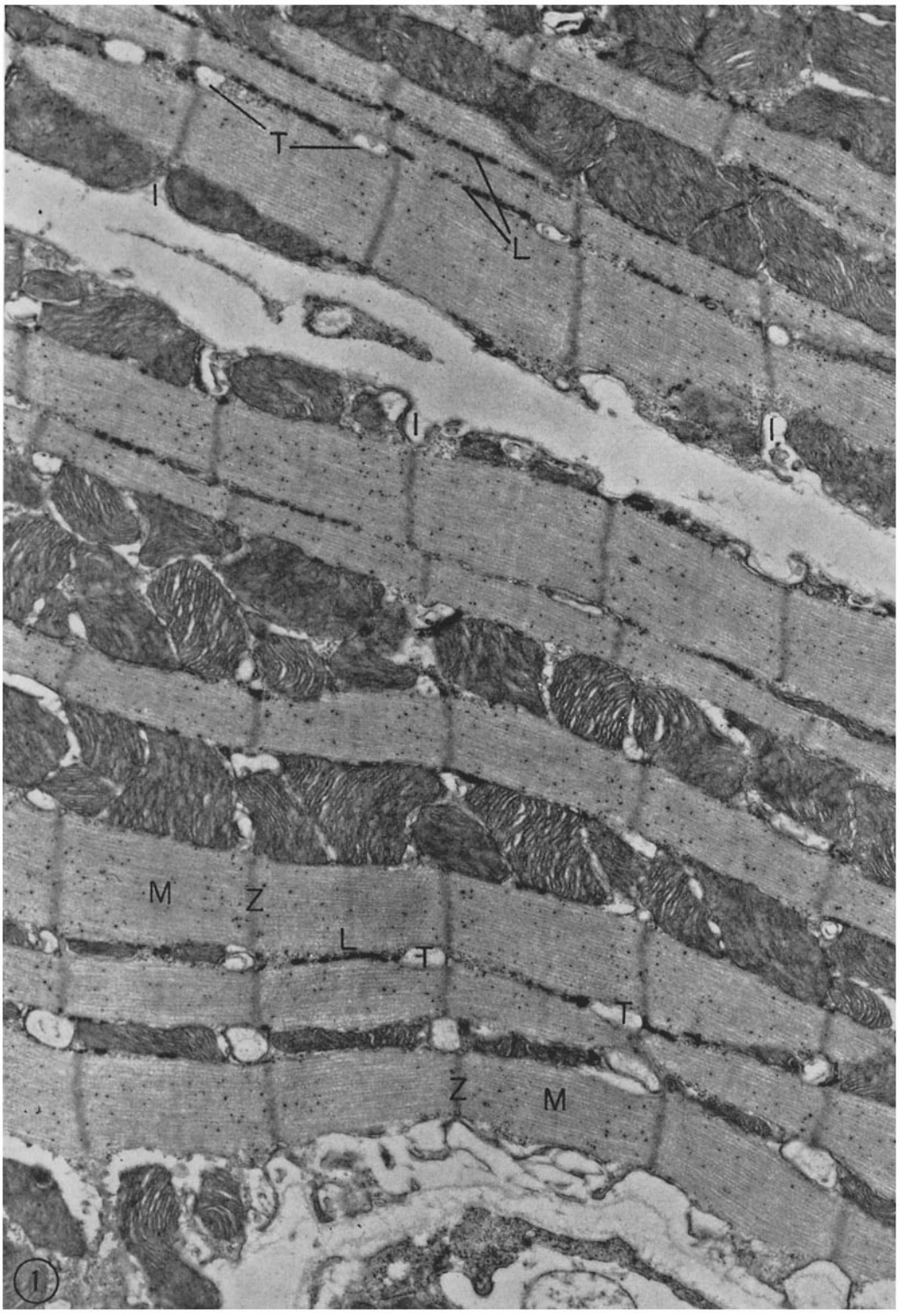
We may now proceed to a more detailed consideration of the sites of cholinesterase activity which were detected. The observations refer to ventricular muscle, unless otherwise mentioned.

Localization of Cholinesterase Activity

As mentioned above, two main sites of cholinesterase activity were observed, (*a*) in elements of the sarcoplasmic reticulum and (*b*) in the A band.

SARCOPLASMIC RETICULUM: Strong activity was observed in longitudinal elements (14) of

FIGURE 1. Longitudinal section of rat ventricular muscle, cholinesterase activity. Substrate butyrylthiocholine, incubation 40 minutes. Black reaction product indicates sites of cholinesterase activity present in longitudinal elements (*L*) of the sarcoplasmic reticulum. Transverse elements (*T*) at or near level of Z lines are negative. Invaginations (*I*) of sarcolemma at Z line level are negative. The A bands are lightly reacted, and show more reaction laterally; *i.e.*, towards Z lines (*Z*). M bands (*M*) are negative. × 22,000.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the sarcoplasmic reticulum, which was thus strikingly delineated, both in longitudinal (Figs. 1, 2, 5) and in transverse or oblique (Figs. 3 and 6) sections. The reaction product appeared to be deposited initially on or near the inner surface of the lining membranes of the longitudinal elements of the sarcoplasmic reticulum, and, with marked reaction, the reaction product filled the intramembranous space (Fig. 3). Although in general the reaction was widespread throughout the longitudinal elements of the sarcoplasmic reticulum, some areas had both positive and negative elements (Figs. 1 and 2). Whether this was due to differential inhibition by fixation, or was truly representative of qualitative differences, is not known. It is again emphasized that generally nearly all longitudinal elements were strongly positive. In our material, larger, oval, or sometimes almost circular, vesicles were seen situated in close proximity to the Z lines, or opposite the I band, to one side of the Z band (Figs. 1 and 4). These structures were uniformly negative in marked contrast to adjacent longitudinal elements of the sarcoplasmic reticulum on either side of them, which were frequently strongly positive (Figs. 1 and 4). The former, larger elements, were interpreted as being either transverse (T) elements (14, 15) of the sarcoplasmic reticulum which are frequently large in rat cardiac muscle (14), or portions of deep invaginations, at or near the Z line, of the sarcolemma (14, 15). Indeed, in cardiac muscle the invaginations of the sarcolemma at the Z line level are thought to be continuous with the transverse elements (15). The adjacent, positively-staining longitudinal elements sometimes curved in crescentic or U-shaped fashion around the larger, negatively-staining transverse elements (Fig. 3). Porter and Palade have described similar appearances in rat cardiac muscle (14). It was admittedly sometimes difficult in our material, particularly in the atria, to identify positively definitive and typical dyads and triads (14), composed of terminal cisternae of the longitudinal system lying on either side of the transverse element at or near the Z line level (14, 15), probably because structural preservation was at times not quite good enough.

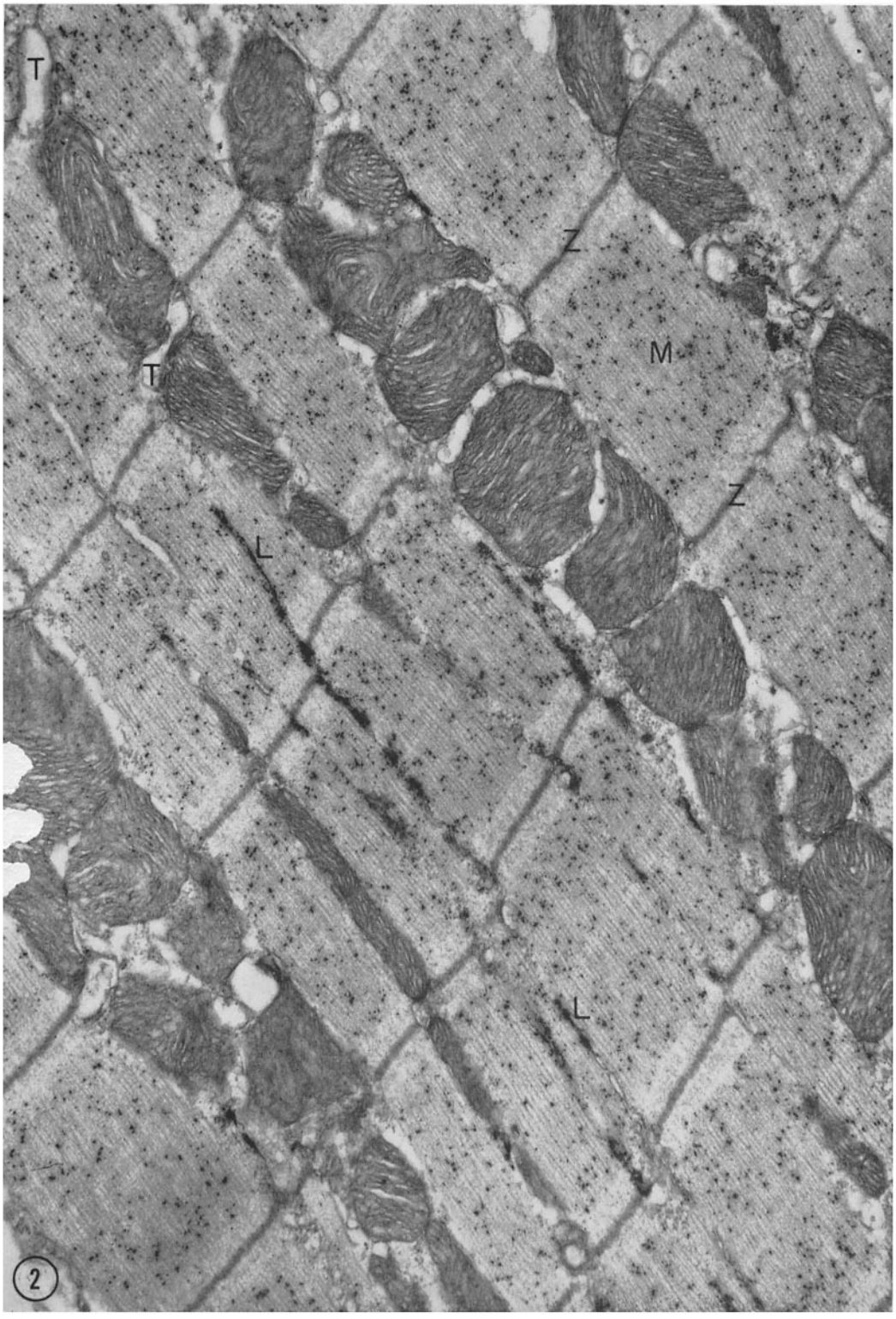
In strongly reacted preparations, the perinuclear cisterna of the sarcoplasmic (endoplasmic) reticulum was also strongly reactive (Figs. 6 and 7).

The sarcolemma was uniformly unreactive against all substrates (Figs. 1, 6, and 8). Shallow invaginations or "scallopings" of the sarcolemma at the level of the Z band were also negative (Figs. 1 and 8). Narrower and deeper invaginations of the sarcolemma at the Z line level, as observed by others (for Bibliography, see reference 15), were not seen. There is evidence in myocardium that invaginations of the latter type are continuous with the transverse elements of the sarcoplasmic reticulum (for Bibliography, see reference 15). Vesicles and flattened saccules were noted immediately beneath the sarcolemma and the sarcolemmal invaginations: these elements, possibly, homologous with longitudinal elements of the sarcoplasmic reticulum, were positive (Figs. 6 and 8). They were not particularly distributed at the level of the Z bands.

REACTION IN THE A BAND: With somewhat longer incubation times than were necessary for eliciting reaction in the sarcoplasmic reticulum, activity was readily detected in the A band (Figs. 1, 2, and 9). The H zone and the M band were both virtually negative. The Z line and I bands were also negative. The deposits were scattered rather diffusely in the A band, but gave the impression at times of being more numerous toward the A–I junction (Figs. 1, 2, and 9), especially in lightly reacted preparations (Fig. 1). The deposits were situated on, or in close proximity to, the myofilaments, but the details of their relationship to thick or thin filaments has not as yet been resolved. In hypercontracted muscle, as would be expected with obliteration of the I zones, the deposits were concentrated on, or close to, the contraction bands (Fig. 8).

ATRIAL MUSCLE: In a few experiments atrial muscle was also examined. Structural preservation was not so good as that obtained in ventricular

FIGURE 2 Longitudinal section of rat ventricular muscle, cholinesterase activity. Sections pretreated with *N*-ethylmaleimide. Substrate propionylthiocholine, incubation 40 minutes. Longitudinal elements (*L*) of the sarcoplasmic reticulum are strongly positive. Transverse elements, or portions, (*T*), of deep invaginations of the sarcolemma at the Z line level (*Z*) are negative. There is strong reaction in the A bands (*cf.* Fig. 1). The M bands (*M*) and adjacent H zones are virtually negative. The Z lines (*Z*) are adjacent I bands are negative. × 34,000.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muscle. It was difficult to find definitive triads or dyads. It appeared, however, that the distribution of cholinesterase activity was similar to that observed in ventricular muscle (Fig. 5). No additional sites of activity were found when acetylthiocholine, as against butyryl- or propionylthiocholine, was used as substrate.

ACTIVITY AGAINST DIFFERENT SUBSTRATES: Adequate reaction at all sites was obtained with all three substrates utilized, the intensity of reaction being greatest with propionyl-, moderate with butyryl-, and least with acetylthiocholine. This would indicate non-specific (butryl- or propionyl-) cholinesterases which is in accordance with biochemical observations (1, 2, 13, 26). The A band reacted especially well with propionylthiocholine. No differences in localization in ventricular muscle were obtained with acetyl- as against butyryl- or propionylthiocholine.

OTHER SITES: The only other sites of cholinesterase activity detected in rat myocardium, besides the sarcoplasmic reticulum and the A band, were the vesicles of the cardiac capillary endothelium.

The intercalated discs have previously been reported to be positive for cholinesterase by light microscope histochemistry, using thiolacetic acid as substrate (16). The discs were uniformly negative in our preparations (Figs. 7 and 10). However, vesicules and tubules in proximity to the disc, presumably longitudinal elements of the sarcoplasmic reticulum, were positive (Fig. 10), and may have led to erroneous interpretation at the level of light microscopy.

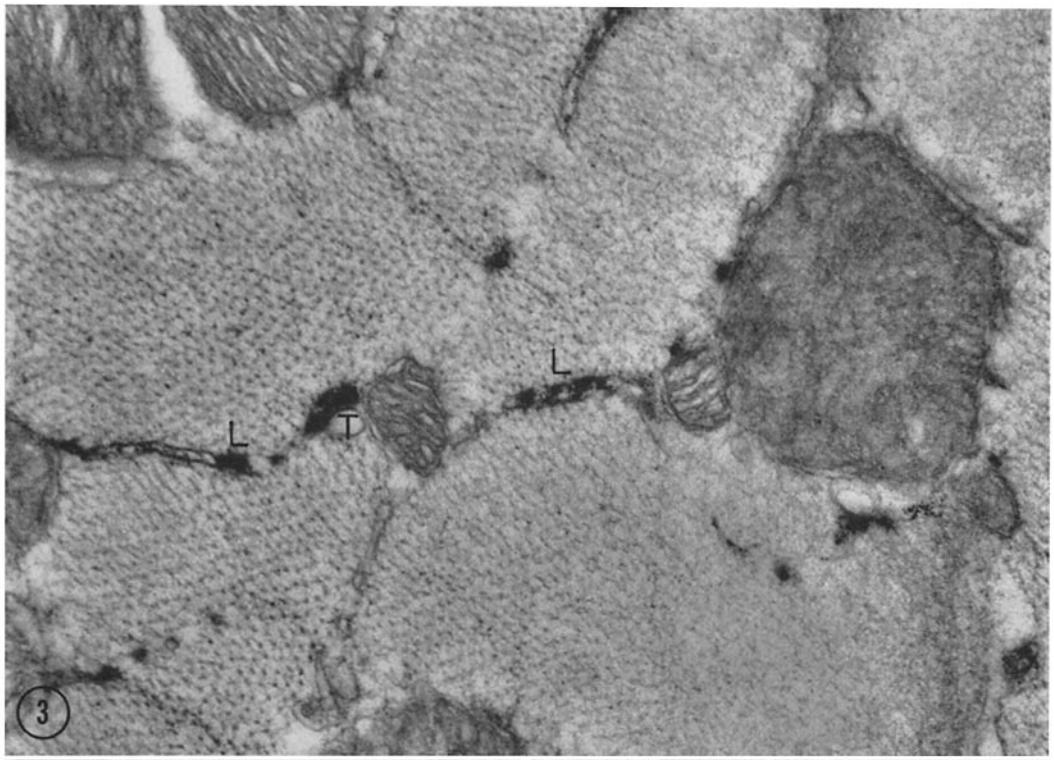
DISCUSSION

BASIS OF THE REACTION: It is suggested that the reaction proceeds as follows: Thiocholine, liberated by enzymatic hydrolysis of the thiocholine ester, preferentially reduces ferricyanide to ferrocyanide. The ferrocyanide reacts with copper to give the brown, insoluble, ultramicroscopic and electron-opaque precipitate of copper ferrocyanide (Hatchett's brown). The redox potential of the couple $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ relative to the normal hydrogen electrode is -0.44 volt (17), and that of Cu^{+}/Cu^{2+} is -0.17 volt (17), and therefore it is suggested that there is little likelihood of copper salt being reduced rather than the ferricyanide, and that the reaction product is, therefore, cupric ferrocyanide rather than cuprous ferricyanide. The latter, however, is also brownish in color. To prevent the formation and precipitation of copper ferricyanide, the copper is complexed with citrate. Because ferricyanide may be inhibitory (18), the concentration is kept extremely low (0.5 mM). Observations, reported elsewhere (9), suggest that the sequence of events is as described above. The effects of varying the concentrations of the different components of the medium, and also of varying the pH are also described. Suffice it to say in this context that the medium, as used in the present experiments at pH 6.0, appeared to be optimal for studying rat cardiac muscle at the ultrastructural level, and most other tissues by light microscopy, except where activity was extremely high, such as at the motor end-plate, where diffusion of reaction product occurs unless the pH is lowered. At lower pH levels the concentration of citrate is increased

FIGURE 3. Transverse section of rat ventricular muscle, cholinesterase activity. Sections treated as in Fig. 1. Reaction product is present within the space enclosed by the membranes of the longitudinal elements (*L*) of the sarcoplasmic reticulum. Presumptive transverse element (*T*) is negative, and adjacent, positively-staining longitudinal element curves in U fashion over it. In left half of figure, some of thicker filaments in cross-section show increased density, which may represent myosincholinesterase activity in the A bands. See Fig. 1, where in longitudinal section the A band activity is seen to be rather sparse. × 50,000.

FIGURE 4. Rat ventricular muscle, cholinesterase activity. Sections treated as in Fig. 1. Transverse elements of the sarcoplasmic reticulum (*T*), at level of Z lines (*Z*), are negative, whereas adjacent longitudinal elements (*L*) are strongly positive. There is sparse reaction in the A bands. × 33,000.

FIGURE 5. Rat atrial muscle, cholinesterase activity. Substrate acetylthiocholine, incubation 40 minutes. Network of longitudinal elements (*L*) of the sarcoplasmic reticulum contains intraluminal reaction product. Network abuts on level of upper Z line. *Z*, Z lines. × 41,000.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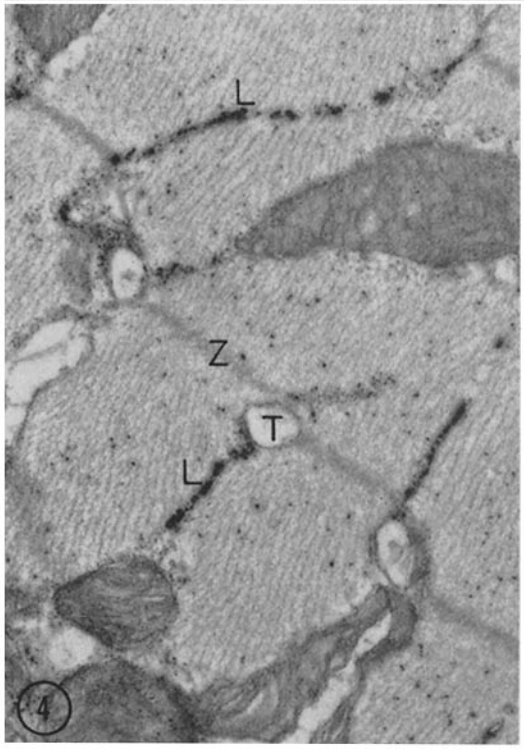
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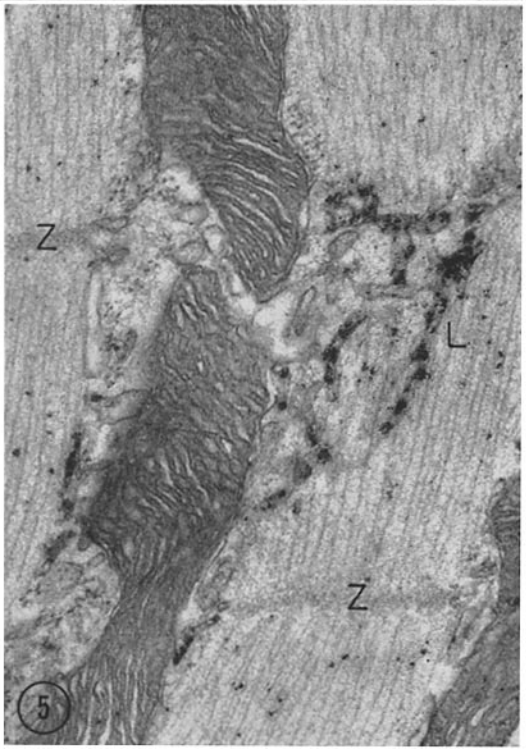
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to maintain a stable medium and, conversely, above pH 6.5 copper ferrocyanide does not form readily in the system unless the concentration of citrate is lowered below 5 mM. At pH 7.2, in the presence of citrate 3 mM, which is the minimum concentration which stabilizes the medium at this pH, the same localizations in rat cardiac muscle were found as at lower pH levels. Some degree of diffusion of end product, as evidenced by nuclear staining, occurred. At pH levels lower than 6.0 the rate of enzymatic reaction was slower, and preservation of tissue structure less good than at 6.0. Therefore, the histochemical pH optimum was considered to be 6.0. (For definition of this term, see Barrnett and Palade, reference 4). The biochemical optimal pH of myosincholinesterase is 8.5 (28).

SPECIFICITY OF THE METHOD: It is felt that the method as applied here to rat cardiac muscle delineates the sites of activity of cholinesterase at the electron microscope level. Failure to observe non-specific and non-enzymatic staining under the conditions prescribed, taken together with the inhibition of reaction by eserine 10^{-5} M and DFP 10^{-5} M, supports this view.

It is possible that non-specific thioesterases may hydrolyse thiocholine esters. Such enzymes have been observed in the brains of various species (45). Eserine in low concentration inhibits cholinesterases and distinguishes them from such thiolesterases, which are not inhibited (45). Some non-specific esterases (Type A) are inhibited by eserine in fairly high concentration, but they do not split choline esters (46).

Specificity of thiocholine esters as substrates for cholinesterases is generally accepted by histochemists (7), with the exception noted above. From our point of view, therefore, an enzyme which hydrolyses thiocholine esters at a rapid rate, and which is also inhibited by low concentrations of eserine (used as a highly selective inhibitor for all cholinesterases) is considered to be a cholinesterase, as in this study.

It is also realized that within the cholinesterase group, thiocholine esters are not so specific as are choline esters for distinguishing between acetylcholinesterases (specific cholinesterases) and butyryl- or propionylcholinesterases (non-specific cholinesterases). Nevertheless, specific cholinesterases split acetylthiocholine at high rates, and butyrylthiocholine very slowly, whereas non-specific cholinesterases split all thiocholine substrates considerably, and therefore some distinction can be made.

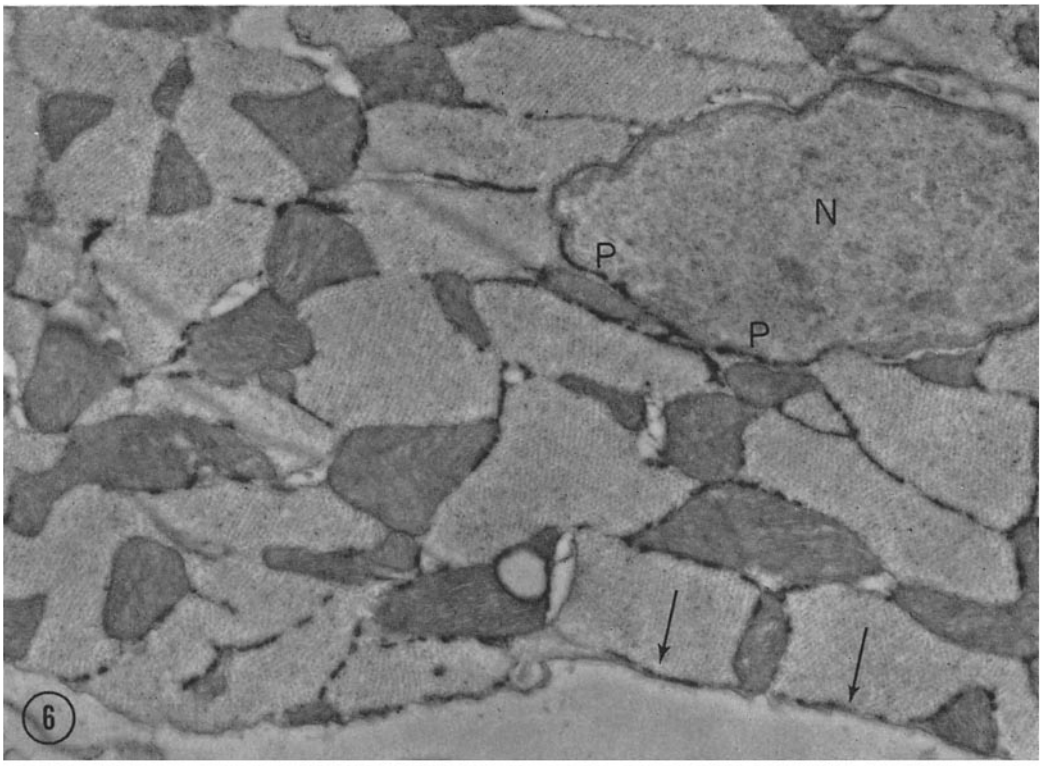
Thiocholine is a thio-ester and it is of interest that enzyme(s) in the sarcoplasmic reticulum of striated muscle have been shown to split the thioesters thiolacetate and thiolbutyrate, (47) but apparently no inhibition with eserine or organophosphates was obtained, distinguishing this activity from cholinesterase activity. Further, cholinesterases did not appear to split thiolbutyrate (48), although it is well known that cholinesterases in some sites do split thiolacetate (21–24).

It is fully realized that failure to demonstrate activity in any site cannot be taken to imply absence of the enzyme. The conditions under which the experiments were run may well have adverse and inhibitory effects on activity at particular sites. However, the failure to observe any additional sites of activity in fresh-frozen sections lends some confidence to our view that no major site of activity has been missed, at least in so far as the effects of fixation are concerned. Reaction in the myofilaments in fixed, but not in unfixed material, is puzzling, and may be due to some form of unmasking of the enzyme by fixation, or to failure of the substrate to penetrate myofilaments in unfixed material.

COMPARISON WITH OTHER ELECTRON

FIGURE 6. Oblique section of rat ventricular muscle, cholinesterase activity. Substrate butyrylthiocholine, incubation 30 minutes. Network formed by longitudinal elements of the sarcoplasmic reticulum is clearly delineated. There is strong reaction in the perinuclear cisterna of the reticulum at *P*. *N*, nucleus. At arrows, vesicles and tubules beneath the sarcolemma are positive. The sarcolemma itself is negative. × 22,000.

FIGURE 7. Longitudinal section of rat ventricular muscle. Sections treated as in Fig. 6. Around the nucleus (*N*) there is strong reaction in the perinuclear cisterna (*P*) of the reticulum. The portion of intercalated disc (*D*) is negative. There is slight activity in the A bands. The M bands and H zones are virtually negative. The Z lines and I bands are negative. × 22,000.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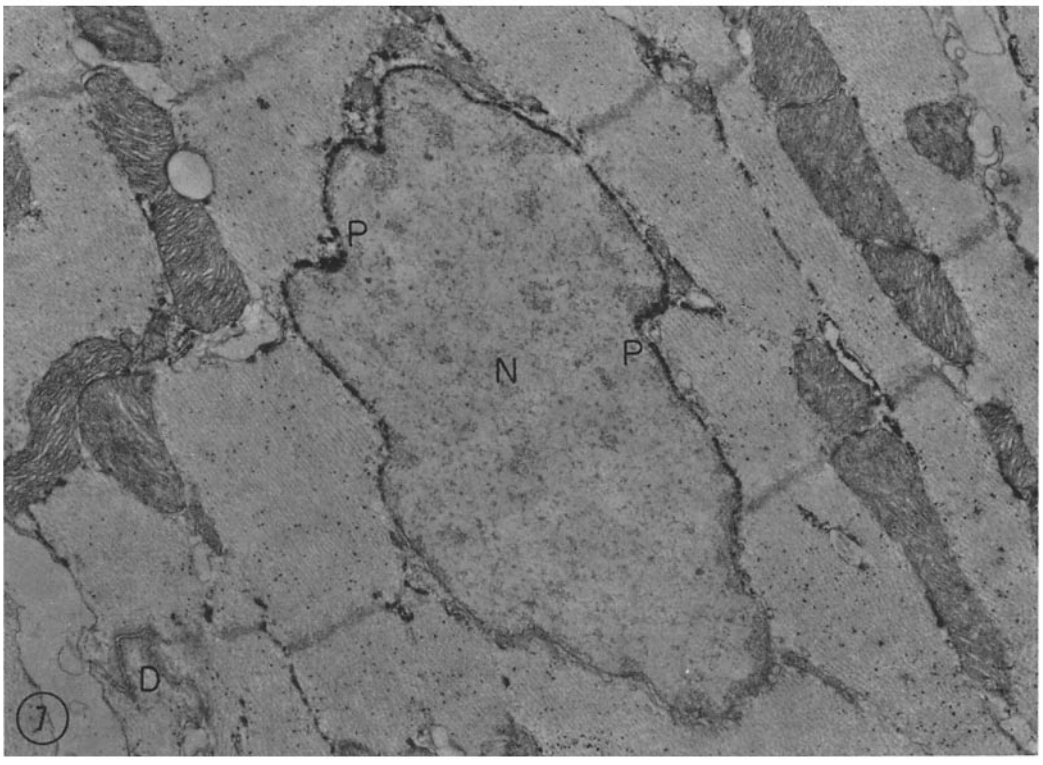
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MICROSCOPIC METHODS FOR CHOLINESTERASE: Although the method works very well when applied to rat cardiac muscle under the conditions prescribed, it is not claimed at the present time that it is a useful method for studying cholinesterase activity in other tissues at the ultrastructural level, for we have not as yet had the opportunity of applying it extensively in tissues other than cardiac muscle. However, the method has been successfully applied, as a "direct-coloring" method, to many tissues for light microscope histochemistry, where it proved to have some advantages over the Koelle-Friedenwald methods. These advantages include: color directly developed at the sites of enzymatic activity, (in comparison with the Koelle-Friedenwald methods where the initial reaction product, copper thiocholine, is colorless and has to be visualized by conversion to brown copper sulfide); absence of large crystals and needle-like formations even after long incubations; and more intense color at sites of low activity. The application of the method to light microscopic histochemistry is discussed in detail elsewhere (9).

Methods in which the released thiocholine is precipitated as copper thiocholine have not been successfully applied in electron microscopy. Our experience confirms that of Brown (8), in that the reaction product, copper thiocholine, is diffusible, partially soluble, and the crystals formed are too large for fine localization. Substitution of silver for the copper, as was done by the above-mentioned author, gave what appeared to be non-specific precipitation, and complete inhibition of enzymatic activity in heart muscle.

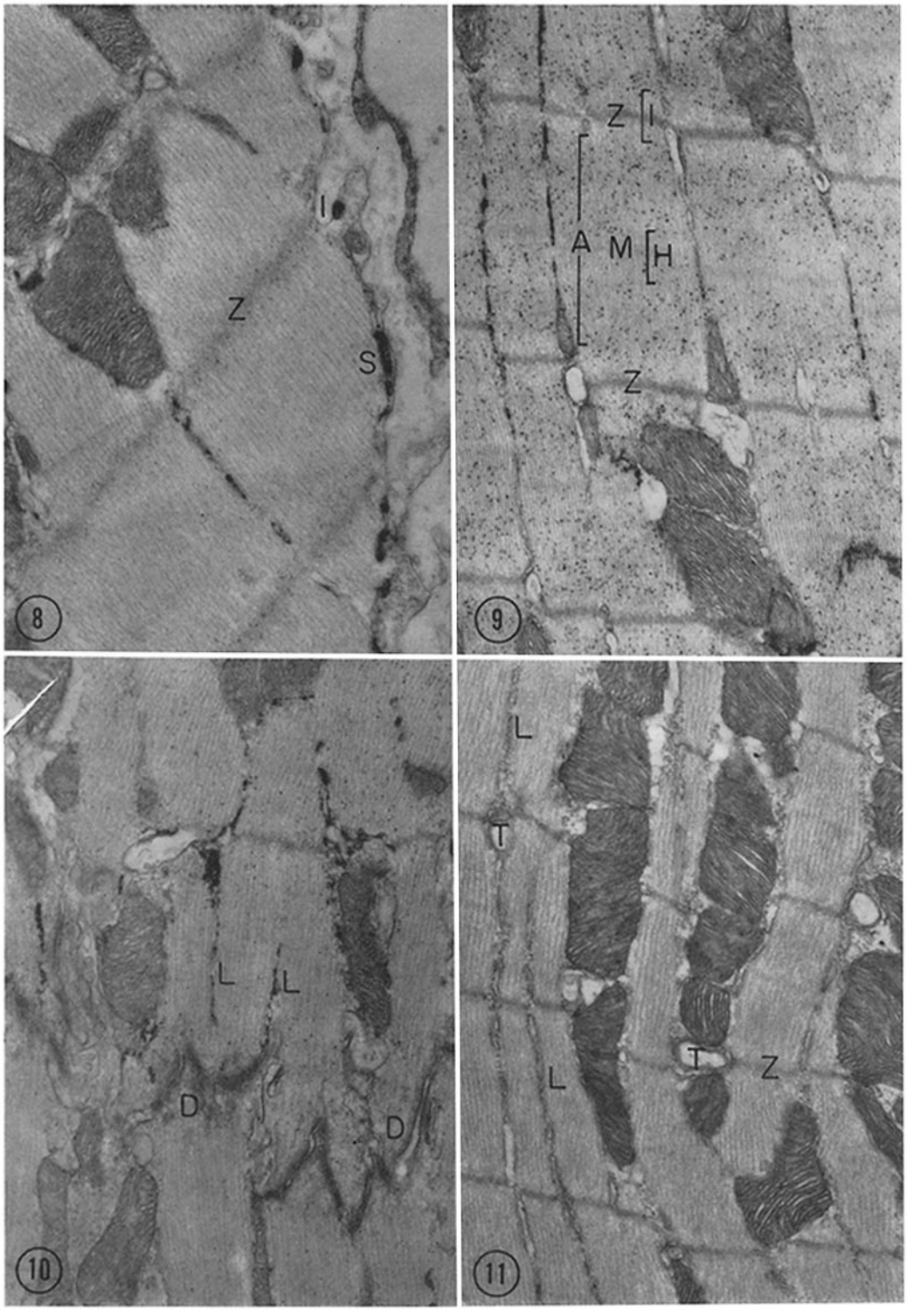
Non-specific esterase methods have been applied in electron microscopy, cholinesterase activity being detected by the use of specific inhibitors. Lehrer and Ornstein (19) successfully applied the α-naphthol acetate "hexazonium pararosanilin" method to motor end-plates, and Wachtel *et al.* (20) applied the same method to electric organs. As pointed out by Barrnett (21), long incubation times are required to visualize the end product, which is relatively non-electron opaque, and when this method was applied to cardiac muscle we were unable to demonstrate any sites of activity with the electron microscope despite long incubations, and good color development as seen in the light microscope. The sites involved are, of course, small and focal, as compared with the motor end-plate. Barrnett (21) and Zacks and Blumberg (22) applied the thiolacetic acid method to motor end-plates, and Torack and Barrnett (23) applied the same method to brainstem. Thiolacetic acid is not a specific substrate for cholinesterases, non-specific esterases being demonstrated as well (24), but, in conjunction with specific inhibitors, good results for cholinesterases were obtained at these sites. However, as shown previously (5), we could not demonstrate

FIGURE 8 Rat ventricular muscle, cholinesterase activity. Substrate butyrylthiocholine, incubation 40 minutes. The sarcolemma and invagination (*I*) of the sarcolemma at the Z line (*Z*) are negative. Vesicles and tubules immediately beneath the sarcolemma are positive (*S*). "Peppering" at the contraction bands is thought to be due to hypercontraction of the muscle, and consequent movement of reaction product in lateral edges of A bands toward Z lines and into contraction bands. × 22,000.

FIGURE 9. Longitudinal section of rat ventricular muscle, cholinesterase activity. Substrate butyrylthiocholine, incubation 30 minutes. Note strong reaction clearly restricted to the A band (*A*). Z line (*Z*), I band (*I*), H zone (*H*), and M band (*M*) are all negative. × 22,000.

FIGURE 10. Rat ventricular muscle, cholinesterase activity. Sections treated as in Fig. 9. Intercalated disc (*D*) is negative. Adjacent longitudinal elements (*L*) of the sarcoplasmic reticulum are positive. There is some activity in the A bands. × 22,000.

FIGURE 11. Longitudinal section of rat ventricular muscle, cholinesterase activity inhibited with eserine sulfate 10^{-4} M. Sections pretreated with eserine 10^{-4} M for 15 minutes, then incubated for 40 minutes in medium containing butyrylthiocholine as substrate and eserine 10^{-4} M. Note complete absence of reaction product in longitudinal elements of the sarcoplasmic reticulum (*L*), and A bands. Compare with Figs. 1, 4, and 9. *T*, transverse elements of the sarcoplasmic reticulum or portions of deep invaginations of sarcolemma at the level of the Z lines (*Z*). × 22,000.


I
Z
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11

cholinesterase activity in rat cardiac muscle with this method. The suggestion of Barrnett and Palade (4), that the demonstrable reaction in the M band was due to a cholinesterase, was not confirmed (5). Indeed, in the present study, utilizing thiocholine esters as specific substrates for cholinesterases, little or no activity was detectable in the M band.

REACTION IN THE A BAND: The reaction in the A band is of some interest, for myosin has been shown to have cholinesterase activity (25). Myosincholinesterase from cardiac muscle differs from that from striated muscle in its acivity towards different substrates (26). The former, for example, splits butyrylcholine at higher rates than acetylcholine, whereas the reverse applies to the latter. Myosincholinesterase also splits thiocholine esters (26). Propionylthiocholine gave the most intense reactions in our experiments. It has been shown that the cholinesterase of rat cardiac muscle is largely of the non-specific type (1, 2), propionylcholine being split at the highest rates (13). Eserine 10^{-5} M to 10^{-6} M caused 50 per cent inhibition of myosincholinesterase of cardiac muscle in isolated preparations of actin-free myosin (20). We achieved complete inhibition with eserine 10^{-4} M in fixed, intact muscle, and virtually complete inhibition with 10^{-5} M eserine.

More specifically, myosincholinesterase activity has been associated with the light, or L fraction of meromyosin (27, 28). According to Marshall *et al.* (29), who used antigen-antibody fluorescent techniques, L meromyosin is localized throughout the A band, except for the M band region, but is more concentrated in the lateral parts of the A band, that is, toward the A–I junction. In our preparations, in lightly reacted muscle, the reaction product in the A band was apparently more concentrated towards the lateral edges; in heavily reacted preparations the whole A band was positive, except for the H zone, but one had the impression that even in these cases the reaction product was more heavily concentrated toward the lateral edges.

Although we have not as yet definitively associated the reaction observed with thick filaments, or with myosin by extraction experiments, we believe that the localization seen in the A band is indicative of the activity of myosincholinesterase. The absence of reaction in the I band substantiates this view, and also implies that the activity either is associated with thick filaments, or is present in areas where both thick and thin filaments co-exist, as in the A band, excluding the H zone.

REACTION IN THE SARCOPLASMIC RETICULUM: The presence of activity in the longitudinal elements of the sarcoplasmic reticulum, but not in the transverse elements, invaginations of the sarcolemma, or in the sarcolemma itself, is of interest in relation to the current theories of conductance of the stimulus for contraction (for recent reviews, see references 15, 30). As Huxley and Taylor showed (31), a localized contraction wave was elicited when a weak stimulus was applied through a microelectrode placed on the sarcolemma of single muscle fibers at or near the level of the Z bands. The wave of contraction spread and stopped at the adjacent M bands. The discovery of the invaginations of the sarcoplasmic reticulum provides an ultrastructural basis for conduction of excitation to myofibrils located deep within the myofibril, at a distance from the sarcolemma (14, 15, 30). The significance of localized cholinesterase activity, not in the T elements, but in the longitudinal elements of the sarcoplasmic reticulum, is at present unknown, but should be taken into account in elucidation of the role, if any, of acetylcholine and cholinesterases in impulse conduction in cardiac muscle at this level.

SIGNIFICANCE OF LOCALIZATION: Apart from the well known fact that cardiac muscle is under the control the the postganglionic cholinergic fibers of the parasympathetic system, and apart from the possibility that conduction in the heart is based on a type of "cholinergic" mechanism, acetylcholine-cholinesterase systems have been postulated to play other important roles in the physiology of cardiac muscle. It is impossible to deal with the complex, and sometimes conflicting evidence here. The reader is referred to recent reviews (32, 33, 50).

In brief, it has been shown that the myocardial fibers themselves have high choline acetylase activity. The evidence suggests that acetylcholine, of myogenic, not neurogenic, origin, is continuously formed in the myocardium (34–38). This locally formed acetylcholine is thought to be involved in the maintenance of muscle tone, motility, and rhythmicity, rather than in being involved in vagal control (36, 38). The pacemaker is thought to be under the control of this locally formed acetylcholine (38). The activity of myocardial cholinesterase may be of importance in controlling the acetylcholine level of cardiac tissue, acting as a

general tissue enzyme to prevent excessive accumulation of acetylcholine at the pacemakers and in the conducting system (36, 38), and acting to maintain or increase the resting membrane potential of the muscle cells, by regulation of membrane-permeability to potassium, which is enhanced by acetylcholine (39). To quote from a recent review (40): "It could be suggested that in the heart . . . . . a degree of 'alertness', or basic functional level, is maintained by a constant production of non-neurogenic acetylcholine, the degree of 'alertness' or the level of acetylcholine accumulation, being controlled by the activity of butyrylcholinesterase. Variations from the basic functional level of a tissue could normally be initiated by the liberation of neurogenic acetylcholine, the accumulation of which would be controlled by acetylcholinesterase."

It should be pointed out that the type of cardiac cholinesterases (1) and the responses of the myocardium to acetylcholine (41, 42) may differ in various species and at various sites in the heart. The frog heart, for instance, may lack butyrylcholinesterase, and have only acetylcholinesterase activity (1). The localization of activity may well be different in these various instances, and a comparative study is therefore being undertaken.

It is hoped that the demonstration of cholinesterase activity in the A hand and in the longitudinal elements of the sarcoplasmic reticulum will open up new avenues of approach to elucidating the functions of myogenic acetylcholine and of myocardial cholinesterases. In the absence of supporting evidence, it is at present inadvisable to speculate too far. Cholinesterase activities, however, may well prove to be strategically located to subserve the possible functions described above.

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