THE FINE STRUCTURAL LOCALIZATION OF ACETYLCHOLINESTERASE AT THE MYONEURAL JUNCTION

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ABSTRACT

A study of the cytochemical localization of acetylcholinesterase activity, combining histochemistry with electron microscopy, showed that the final product of the reaction, which was deposited at or near enzyme sites, occurred at four places in the myoneural junction. These included: plasma membrane of the muscle covering the junctional folds, the primary and secondary synaptic clefts, parts of the plasma membrane covering the axon terminal, and vesicular structures in the terminal axoplasm. No reaction occurred in the presence of 10^{-4} eserine or DFP, whereas 10^{-5} DFP inhibited the reaction at all sites except in the vesicles of the terminal axon. These findings are discussed with reference to the histochemical method used and to the occurrence of esterolytic activity in the vesicles, as well as to some of the current hypotheses concerning the relationship of the site of acetylcholinesterase and synaptic transmission.

Over the past century, the physiological and morphological relationships between muscle and nerve cells have received serious and continuing attention. Since its first description by morphologists (1–3), the structure of the junction between nerve and muscle, the neuromuscular or myoneural junction, has been a subject of controversy (see reviews 4–6). Recently, however, electron microscopic investigations (6–14) have largely clarified the complex morphological details of the junction. From a physiological point of view, junctional transmission has come to be regarded by some as an extension or slight modification of the events which occur during activity in peripheral nerve. More pharmacologically oriented investigations have, over a period of many years, quite clearly established the central role played by acetylcholine (ACh) in junctional transmission. Briefly, the hypothesis established by these investigations states that ACh is produced under the influence of the nerve impulse, and subsequently causes the end-plate potential. Almost simultaneously ACh is removed by the action of the enzyme acetylcholinesterase (AChE).

The usefulness of this theory is best demonstrated by its continuing ability to stimulate experimental work which has provided growth and substantiation of these concepts. However, central to this hypothesis are the role and the site of activity of AChE. The question of the site has led to numerous histochemical observations with a variety of techniques that are reviewed elsewhere (5, 15, 16). Although these studies, made with the light microscope, have verified the presence of high AChE concentrations at the myoneural junction, the resolutions obtained were not sufficiently great to permit the acquisition of crucial information on the intracellular distribution of enzyme activity. With the introduction of histochemical methods for the electron microscope, this situation has changed. Among the numerous strides made in this still young area was the adaptation of azo dye histochemistry to electron microscopy (17). This study localized

cholinesterase activity at neuromuscular junctions with naphthyl acetate and hexazonium pararosaniline. Several other less informative papers combining histochemistry and electron microscopy in a study of this structure have appeared very recently (18–20).

The present paper is concerned with the localization of AChE activity in diaphragmatic neuromuscular junctions by a metal salt method that employs thiolacetic acid, first introduced by Wilson (21), as a substrate, and lead nitrate as the capturing reagent. Preliminary results of this study were reported earlier (22).

MATERIALS AND METHODS

A variety of rodent and lizard muscles were tested histochemically before the final selection of tissue. During the routine histochemical procedures, the appearance of staining at the neuromuscular junctions was immediately visible with a dissecting microscope. However, in the control procedures during which enzyme activity was inhibited, it became desirable to select accurately for examination bits of tissue containing unreacted junction. Therefore, the diaphragm of the rat was finally used because the sites of the neuromuscular junctions could be easily predicted. All the junctions in this muscle occur in a strip of tissue, less than 1 cm wide, which runs over the dome of each leaf in a direction perpendicular to the long axis of the muscle fibers. This strip is quite clearly marked by branches of the phrenic nerve and several accompanying small blood vessels.

It became apparent in the early stages of the study that the fresh neuromuscular junctions contained too much enzymatic activity to permit the accurate localization of end product at the submicroscopic level. Although the results appeared satisfactory at the light microscope level, examination with the electron microscope of sections from fresh blocks that had been incubated for only 3 to 5 minutes at room temperature revealed an accumulation of final product so intense as to obscure fine structural details. In addition, there was obvious diffuseness of deposition of final product in some of these specimens, as though the products of enzymatic activity had been produced so rapidly that they diffused before reacting with the reagent to form the final product. Three forms of preincubation fixation of tissues were successful in eliminating the above difficulties: (*a*) cold 10 per cent formalin in 0.1 M Sörensen's phosphate buffer (pH 7.4) containing 0.4 M sucrose, (*b*) 0.6 per cent potassium permanganate, and (*c*) 0.25 per cent osmium tetroxide buffered to pH 7.4 in an acetate-veronal mixture. Formalin prefixation was discarded for two reasons: enzymatic activity still remained quite high, and although the final preservation of tissue was satisfactory, it was less good than after other preparative procedures. Potassium permanganate was discarded because of its well known poor fixation of muscle fibers. It is mentioned here only to point out that in this study, as well as in other unpublished ones, some enzymatic activities associated with membranous structures are retained. The best results were obtained with preincubation fixation in 0.25 per cent osmium tetroxide containing 0.4 M sucrose for 5 to 7 minutes at 1°C.

Although it was ascertained in previous studies (21, 23) that the pH optimum of AChE for thiolacetic acid was between 5.1 and 5.5, this was not used because of the rapid rate of hydrolysis of substrate and the deleterious effect of the acid pH on fine structure. Instead, 0.25 ml of the substrate was carefully titrated to pH 7.2–7.4 with NaOH (1.0 N and 0.1 N), and enough cacodylate buffer (0.05 M) at the same pH was added to bring the volume to 20 ml. At room temperature slow spontaneous hydrolysis of substrate was noted above pH 6.8 and rather rapid hydrolysis occurred above pH 7.6. In the cold (1°C) no spontaneous hydrolysis was noted at pH's slightly above neutrality for the duration of incubation used. In addition, the lower temperature of incubation slowed down enzymatic activity sufficiently to produce excellent cytological localization of final product as was obtained in the studies on the M band enzyme (23). The same procedure used at room temperature for a shorter period of time produced much less satisfactory results.

The final ingredient of the incubation mixture was 48 mg of lead nitrate (0.006 M) in 5 ml of cacodylate buffer, which was added slowly to the previous ingredients with constant stirring. Occasionally a light cloudiness, which was not a function of pH, appeared in the incubation mixture on the addition of the reagent. This sometimes disappeared on further stirring or was filtered off. The cloudiness was probably due to the formation of a lead-thiolacetate complex, and the loss of lead ions from the incubation mixture was discounted since the amount of lead nitrate used was in excess of that previously required (23), as well as in excess of that determined in preliminary experiments to give clear-cut cytological results.

In summary, the final incubation mixture consisted of (*a*) 0.25 ml of thiolacetic acid titrated to pH 7.2–7.6 with NaOH (final concentration, 0.12 M), (*b*) enough 0.05 M cacodylate buffer to make 25 ml, (*c*) 48 mg lead nitrate.

In the final experiments the incubation was carried out in the cold (1°C) until the first sign of a staining reaction was seen with a dissecting microscope. This usually took 40 to 50 minutes. Prolongation of the incubation another 5 to 10 minutes

resulted in an accumulation of end product such as to obscure the fine structural details at the sites of enzymatic activity. After incubation, the tissues were washed briefly in 13 per cent sucrose and in 1 per cent osmium tetroxide to stop the reaction, and cut into small blocks, approximately 1 mm^3, which contained the neuromuscular junctions. These blocks were then fixed in 1 per cent osmium tetroxide for 1 hour at 1°C to obtain more adequate morphological preservation. Tissues were dehydrated in increasing concentrations of ethanol and were embedded in a mixture of *n*-butyl and methyl methacrylate (3:2). Thick sections (1 μ) were cut with a Porter-Blum microtome and viewed with a phase microscope. If the thick sections showed delicately stained neuromuscular junctions with the phase microscope, adjacent thin sections were made for viewing with an RCA EMU-3D electron microscope.

Control studies consisted of similarly prepared tissues incubated in the absence of substrate, tissues prefixed in 1 per cent osmium tetroxide for a sufficiently long time (10 minutes) to completely inhibit enzyme activity and subjected to regular incubation, and tissues subjected to either of two well known AChE inhibitors, eserine salicylate (1×10^{-6} M to 1×10^{-4} M) and diisopropylfluorophosphate (DFP) (1×10^{-6} M to 1×10^{-4} M). In the case of these inhibitors, the prefixed tissues were preincubated in 13 per cent sucrose containing the inhibitor for 15 minutes and then were incubated in the regular medium containing the same concentration of inhibitor. This procedure was necessary in the case of eserine, a competitive inhibitor, and was used routinely to decrease the complications frequently met when histochemical tests are subjected to inhibition procedures. In addition, whenever inhibition studies were performed a regular incubation with the opposite leaf of the diaphragm was run in parallel. The incubation of the inhibited preparation was terminated when the neuromuscular junctions of the routine preparations were intensely stained. After incubation, the processing of the inhibited tissues was the same as previously described.

RESULTS

Fine Structure of the Neuromuscular Junction

The myoneural junctions of the rat diaphragm are similar in submicroscopic detail to those previously described (6–14). To appreciate the results of the histochemical experiments a brief summary of the fine details is necessary. Especially prominent is the manner in which the surface of the muscle is thrown into the complex folds of the subneural apparatus. In the region of the synaptic troughs (depressions in the muscle surface that accommodate the axon terminal (8)), the muscle plasma membrane is thrown into deep infoldings, some of which are branched or anastomose with the neighboring infoldings. These junctional folds surround the terminal parts of the axon and measure 0.5 to 1.0 μ in length and approximately 1000 A in diameter (Fig. 1).

In the space (synaptic cleft (24)), approximately 600 A wide, between the plasma membrane of the muscle and that of the axon (primary cleft), as well as in the space created by the deep infoldings (secondary cleft) described above, a moderately dense homogeneous material occurred. This filling material was densest at the center of the space and decreased in density in the area immediately adjacent to the plasma membranes. Occasionally the homogeneity of this material was interrupted by small vesicles or granular material of slightly increased density (Fig. 2).

A rather thick area of sarcoplasm occurred in the region of the myoneural junction and separated the junctional folds from the subjacent myofibrils. This area of sarcoplasm contained numerous mitochondria and several large nuclei with prominent nucleoli. The mitochondria were typical of those found in the peripheral sarcoplasm and usually presented irregularly oval or round profiles. In addition, the sarcoplasm contained patches of moderately dense granules and occasional small, smooth, membrane-bounded vesicles and profiles of sarcoplasmic reticulum.

The axolemma followed at a 1000 A interval the general outline of the junctional troughs without penetrating the subneural infoldings. Two organelles characterized the axoplasm: large numbers of mitochondria that contained longitudinal or somewhat curled cristae, and numerous vesicles (Fig. 2). The mitochondria and the vesicles were each concentrated in certain well defined areas. The vesicles, 200 to 400 A in diameter, were round or oval, were bounded by a thin membrane, and contained a homogeneous material of low density. Occasionally, however, elongate profiles of membrane-bounded tubules having the same diameter as the vesicles occurred adjacent to the populations of vesicles. The vesicles were most numerous in the most peripheral part of the axoplasm, most frequently occupying the area subjacent to the plasma membrane. Occasionally, there were areas in the peripheral axoplasm in which the density of vesicles was greater

than that in adjacent regions. Some of the vesicles were disposed immediately under the plasma membrane, but no continuity between the surface membrane and the vesicular membrane was apparent. Neurofibrillae were not found in the terminal part of the axoplasm (Figs. 1 and 2).

Histochemical Localization

The product of the histochemical reaction (PbS) was deposited as a fine, black precipitate. With phase microscopy this appeared sharply localized to the myoneural junction in what is now the well known histochemical pattern. Electron microscopy of parallel thin sections revealed that enzyme activity was localized with satisfactory resolution and adequate preservation of morphological detail. The structures that demonstrated enzyme activity were (*a*) the muscle plasma membrane covering the junctional folds, (*b*) the material in the primary and secondary clefts, (*c*) the plasma membrane covering the axon terminal, and, finally, (*d*) vesicles and granules within the terminal axon (Figs. 3 to 9 and 11). No particular sequential pattern was obtained at these sites by varying the incubation time, although in a few specimens, interrupted early in the incubation, the sites in the terminal axon appeared less reactive than was usually the case. Staining reaction in the clefts was particularly enhanced by prolonged incubation.

The staining of the plasma membrane covering the junctional folds of the muscle was a constant feature. In fact, in some specimens the incubation was timed or the junction selected as to position in the block where the membrane staining was a most prominent feature (Fig. 5). The membrane activity occurred as a dense, thin coating which was frequently continuous over the muscle membrane for relatively long stretches (Figs. 3 to 8). Occasionally, the deposits were interrupted and occurred as closely spaced, small dots or dashes.

The final product in the primary and secondary synaptic clefts was granular and varied considerably in density. In the over-reacted specimens, the granular material was so closely packed that this dense product filled the spaces and obscured the membranous reaction. It was more usual, however, owing to selection of short incubations or of junctions occurring in the interior of the blocks, to find granular material of varying sizes and density scattered in this space. Most of the granules were more than 100 A in diameter and some of them were several times this size, and the variation in density appeared to be due to the amount of final product (PbS) precipitated at these sites (Figs. 3 to 7, 9, and 11). Under-reacted specimens contained less final product in the synaptic clefts along with stained membranes of the junctional folds (Figs. 5 and 8). Sarcoplasm of muscle immediately adjacent to reactive plasma membrane and adjacent to reactive material in the clefts never contained final product.

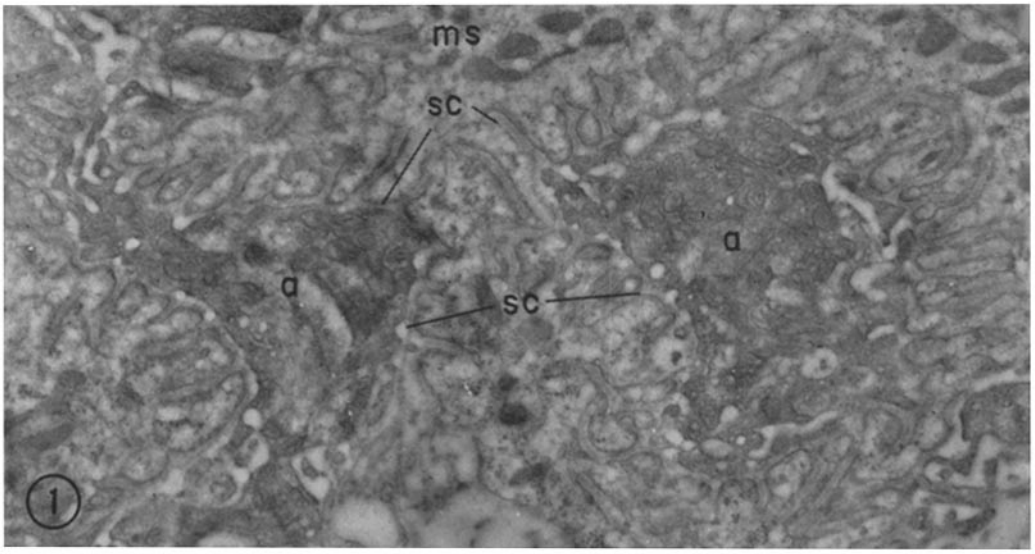
The final areas of enzyme activity to be described occurred in the terminal part of the axon. Although the deposits in this region were sharply localized and occurred regularly (43 of the 44 junctions examined in the final stages of the study), there was variation in number and in the mor-

FIGURE 1

Low power micrograph of myoneural junction taken from a block of muscle prefixed in 0.25 per cent osmium tetroxide for 10 minutes to destroy enzymatic activity, incubated in the regular medium for 50 minutes, and refixed in 1 per cent osmium tetroxide for 1 hour. Two terminal axons (*a*) are completely surrounded by junctional folds and sarcoplasm (*ms*). The primary and secondary synaptic clefts (*sc*) are clearly depicted. × 19,000.

FIGURE 2

Enlargement of parallel section through left part of Fig. 1 showing the fine structural details of the motor ending. The terminal part of the axon (*a*) contains mitochondria (*m*) that have longitudinal or somewhat curled cristae, and small vesicles (*v*). The primary and secondary synaptic clefts (*sc*) contain a homogeneous material of slight to moderate density. The muscle sarcoplasm (*ms*) contains a few mitochondria and dense granular material. × 41,000.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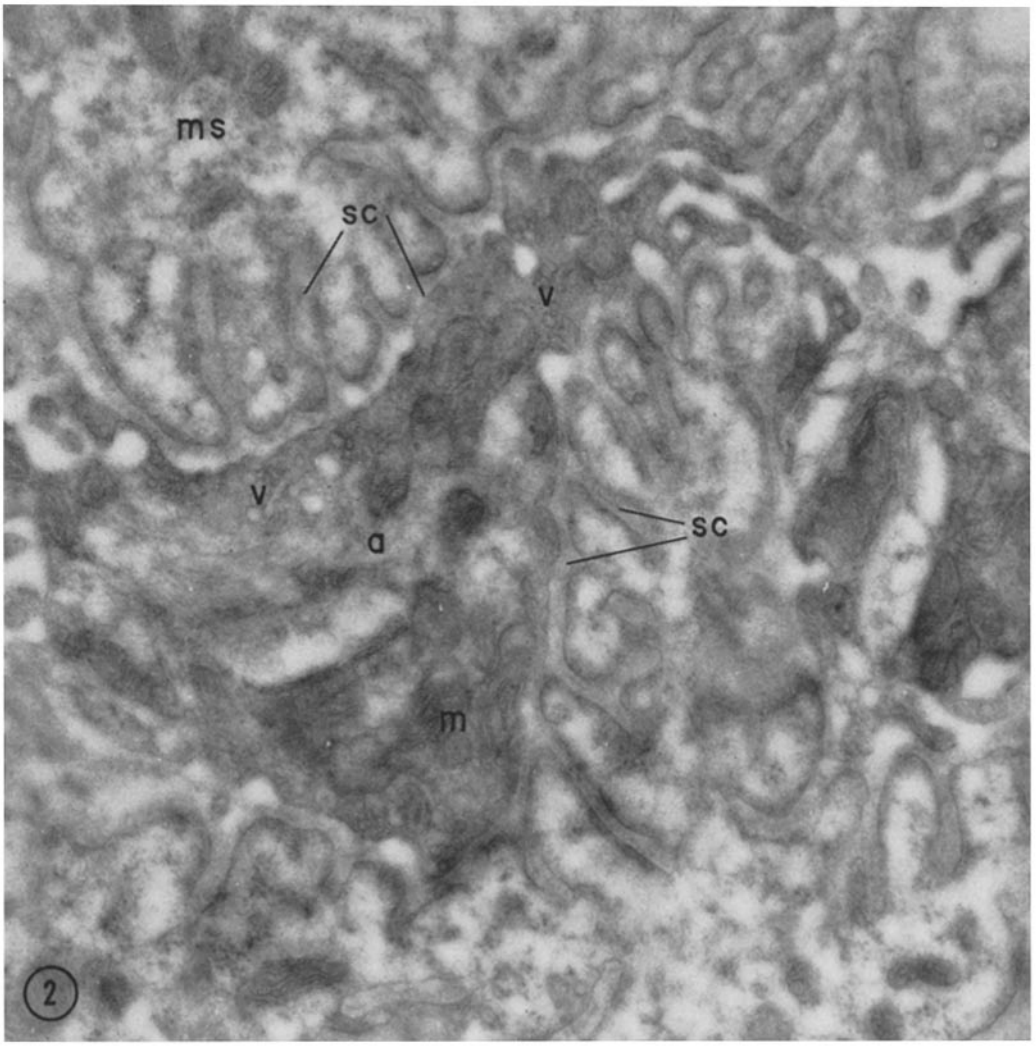
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phological form of the deposits. The plasma membrane at the surface of the axon facing the synaptic gutter regularly showed activity. However, the entire membrane did not exhibit activity; only irregular short lengths of membrane were coated with final product. These were interrupted by short segments of unstained membrane or gaps in which no surface membrane could be made out (Figs. 3, 4, 6, 7, and 11). At these latter spaces, direct continuity of the contents of axoplasm and synaptic clefts appeared to exist.

In most specimens the synaptic vesicles showed activity (Figs. 6 to 9). In some cases this was expressed by staining of the membrane which surrounded the vesicle (Figs. 6 to 9), while in others only a part of the membrane was stained (Figs. 3 and 7) and in still others the entire content of the vesicle showed activity (Figs. 6 and 8). Occasional linear segments of membranous material or short profiles of tubules were reactive. In some of the incubated specimens, however, much of the final product appeared in morphological forms unrelated to any of the known structures found in the terminal axoplasm. These consisted of granules of various sizes and densities, short dense rods, or short segments of arcs, and were localized in the most peripheral parts of the axoplasm of the nerve terminals (Figs. 3, 4, and 7). These deposits were sometimes concentrated in certain regions of the terminal axoplasm while other adjacent areas contained fewer reactive sites. It should be pointed out that while some sort of reaction was present in almost all the terminal axons examined, the amount of reaction was moderate or slight in a few of the specimens (Fig. 5). Some of the granular deposits in the terminal axoplasm appeared quite similar to those in the synaptic clefts (Figs. 3, 4, and 11) but, by and large, the two populations of granules were different in character.

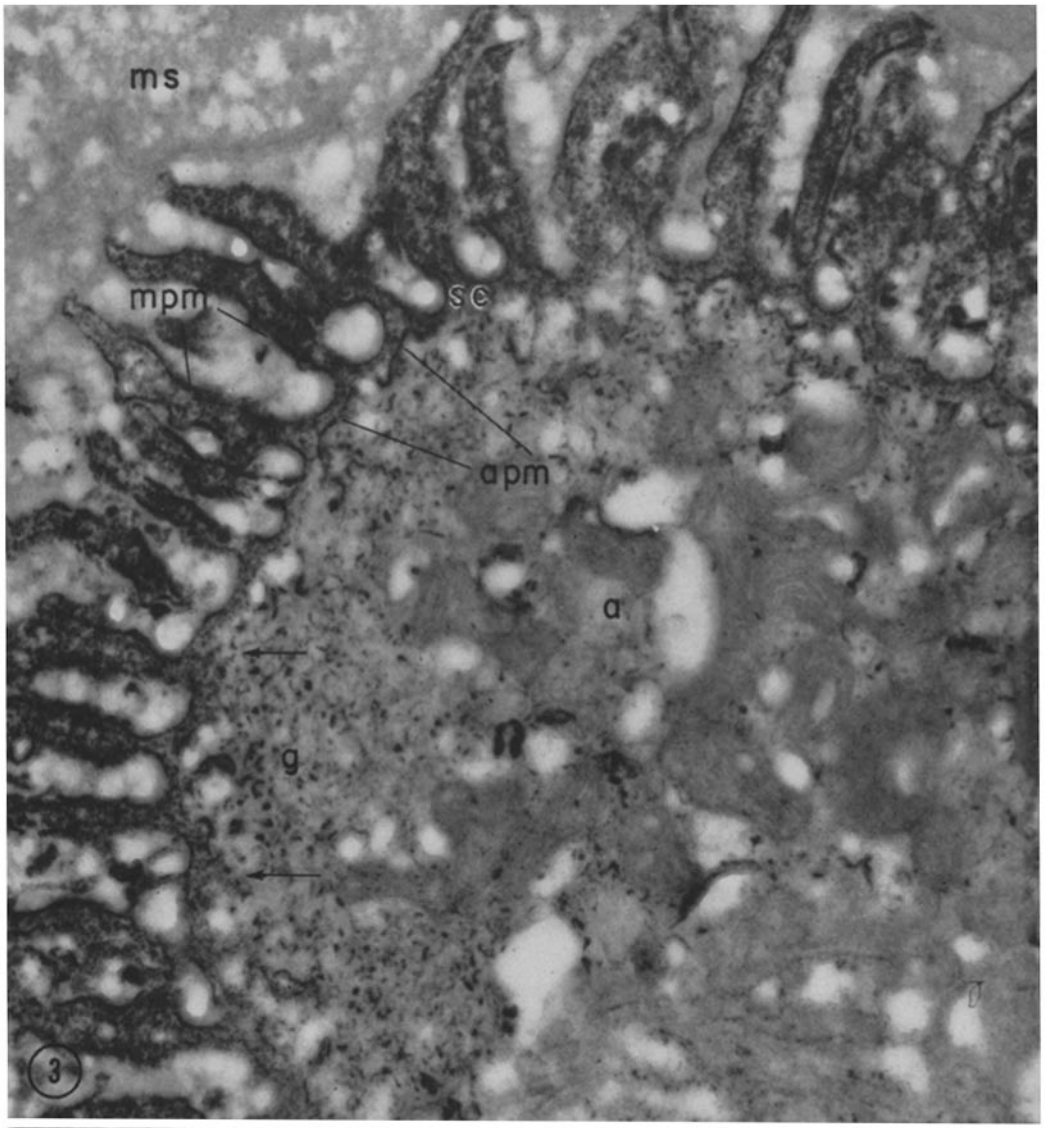
It was difficult to create a morphological order of the active sites of the terminal axoplasm. It should be recognized that vesicular and granular patterns of deposition may be the result of many factors, including sectioning. Furthermore, fine details of localization can be seriously affected by damage suffered by the specimen during incubation and subsequent preparation. Such accidents may account for the discontinuities observed in the plasma membrane of the nerve endings and for the unusual patterns of PbS deposition in the axoplasm. However, these deposits may represent the staining of intact and disrupted vesicles and

FIGURE 3

This micrograph indicates the distribution of reactive product (PbS) deposited as a result of AChE activity. The sites that show activity include the plasma membrane of the muscle (*mpm*) lining the junctional folds. This deposition sometimes occurs as interrupted granules and sometimes as a thin, dense staining of the membrane. Granular material of several sizes and densities occurs within the primary and secondary synaptic clefts (*sc*). Interrupted parts of the plasma membrane of the axon (*apm*) are also reactive. However, in other sites this membrane appears lacking or shows no activity. Finally, numerous vesicles and granules (*g*) in the terminal part of the axon (*a*) show activity. In some instances the membrane around the vesicle appears reactive, and in others, the contents. In addition, short linear or curved segments of membranes show activity, as well as amorphous appearing granules. At several sites (arrows) the active material in the terminal axon appears directly continuous with that in the synaptic cleft. Several tubular structures in the axon (lower right) show slight activity. Mitochondria in the terminal axon show activity (due to a different enzyme) but the sarcoplasm (*ms*) is free of activity. × 41,000.

FIGURE 4

Micrograph of part of myoneural junction showing activity at the plasma membranes of the muscle and the axon (*a*), within the synaptic clefts (*sc*), and in the terminal part of the axon. Much of the active granular material (*g*) appears in a form unrelated to the fine structural elements known to exist in the axon terminal. *ms*, sarcoplasm. × 41,000.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tubules and their contents. This conglomeration of product could be looked upon as a near continuum of tightly packed, active membranous and granular structures extending from the terminal axoplasm through the active but disrupted axon plasma membrane into the synaptic clefts and including the muscle plasma membrane. The presence of granular end product, some of similar nature, in both the terminal axoplasm and the synaptic cleft suggests the discharge of enzymatically active material from the terminal part of the axon during incubation. This finding may be correlated with the disrupted plasma membrane of the terminal part of the axon and not with the fusion of vesicles with the plasma membrane. Although in many cases stained vesicular structures were seen in close relationship to the membrane, morphological evidence of their fusion was not seen. However, it is also possible that the granules in the synaptic cleft represent only the precipitation of final product on the homogeneous filling material which was normally present.

Mitochondria in both the terminal axoplasm (Fig. 11) and the peripheral sarcoplasm at the junction usually showed no activity. As previously reported (23), mitochondria between the myofibrils showed activity but this was due to an enzyme that differed in character from that of the myoneural junction. Axoplasm in cross-sections of nerve, a few microns from a myoneural junction, also showed no activity. In practically all cases, myofibrils of muscle fibers surrounding the junction were contracted.

Control Studies

Pieces of diaphragm containing end-plates that were fixed for 10 minutes in 0.25 per cent osmium tetroxide prior to incubation followed by post-incubation fixation for an hour showed no reaction. The junctions in these specimens were indistinguishable from those that had been fixed without incubation. Omission of the substrate resulted in no reaction, indicating that lead did not combine with those structures to give a nonr specific reaction. Even when the pH of an incubation solution was raised rapidly to 8.5 to cause spontaneous hydrolysis of substrate and artifactitious precipitation of final product in an eserine-inhibited specimen, the localization of the lead sulfide was diffuse and bore no relationship to structure.

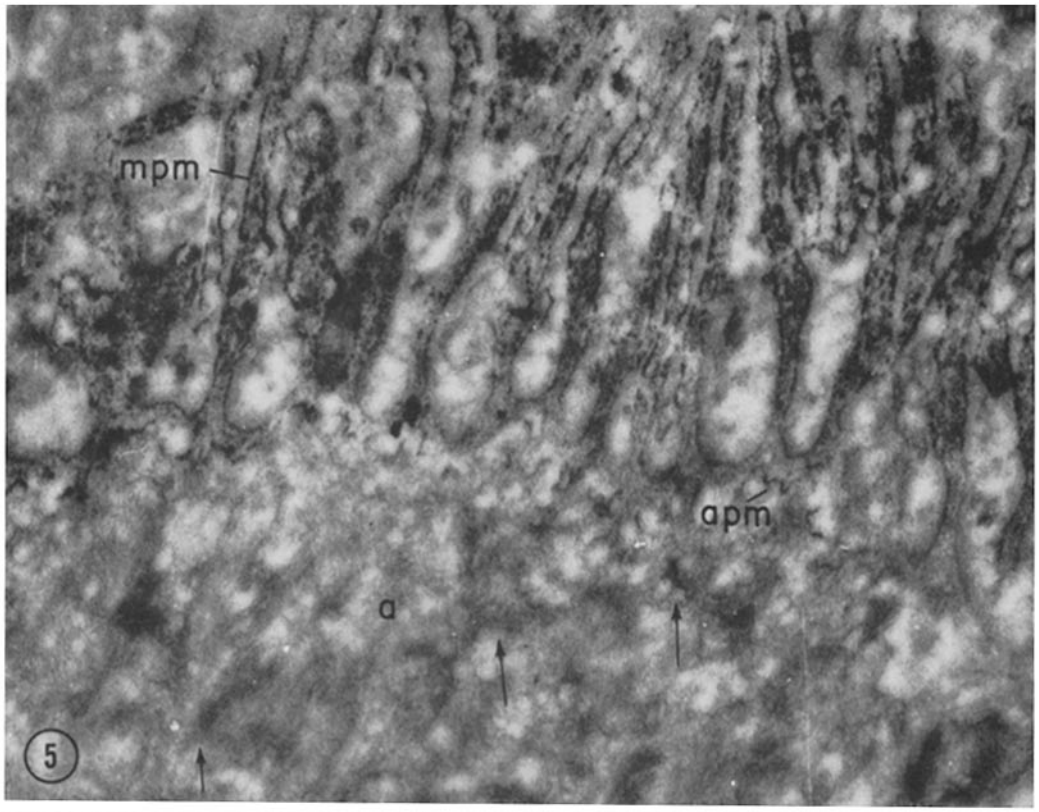
Eserine (10^{-6} M) and DFP (10^{-6} M) did not cause inhibition of the reaction, but these concentrations of inhibitors caused a delay in the appearance of the final reaction. It should be noted that especially with fresh or formalin-fixed material these inhibitory procedures improved the cytological localization of final product. This point reemphasizes a guiding principle in this work; that is, the most accurate localization occurred when enzymatic activity was not biochemically maximal. In other words, the rate at which the product of enzyme reaction occurs must not

FIGURE 5

Micrograph of myoneural junction in which the incubations were stopped somewhat earlier than in the other illustrations. In comparison with Fig. 3 or 4, there is less reactive granular material in the synaptic clefts but the plasma membrane (*mpm*) lining the junctional folds appears strongly reactive. Only small bits of the plasma membrane of the axon (*apm*) appear reactive and there is a suggestion of a beginning of a reaction on membranous material in the terminal axon (*a*) (arrows). × 57,000.

FIGURES 6 AND 7

Micrographs of myoneural junctions subjected to a regular incubation. In both junctions the granular material in the synaptic clefts (*sc*) and the membranes of both the axon and the muscle are active. Most prominent is the reaction of vesicular structures (*v*) in the axon (*a*). In some instances in both figures the membrane around the vesicle is reactive; in others, the contents are active. In Fig. 6, the membranes lining tubular elements show activity. In Fig. 7, many small reactive granules (*g*) are present as well as short, curved segments of reactive membranes. × 41,000.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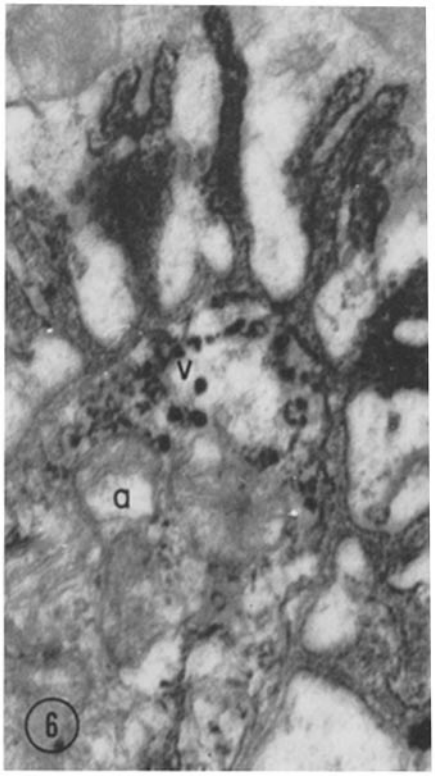
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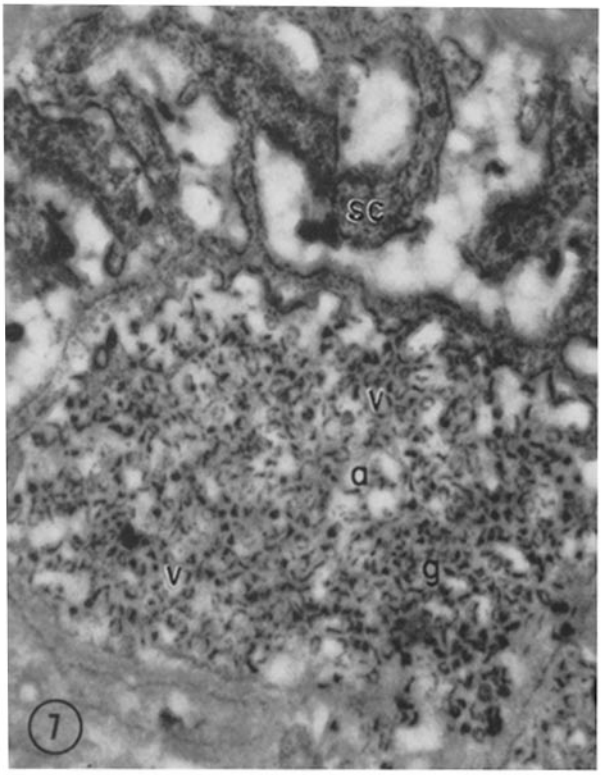
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exceed the rate at which the final product can be formed. Eserine (10^{-4} M) (Fig. 10) or DFP (10^{-4} M) (Fig. 14) caused complete inhibition of the end-plate reaction at all sites. It should be pointed out that the material in the synaptic clefts and that in the synaptic vesicles of those inhibited specimens appeared similar to that found in the unincubated specimens. Vesicles were not disrupted and granular material was not found in the terminal axoplasm or in the synaptic cleft.

A curious but exciting result was obtained when DFP was used as an inhibitor at 10^{-5} M, namely, selective inhibition of the reaction on the membranes of the junctional folds and the terminal axon. Although there was decreased amount of granular product in the synaptic clefts, the synaptic vesicles were not disrupted and they appeared intensely reactive (Figs. 12 and 13). The findings in some of these specimens reemphasized the possible relationship between the reactive vesicles of the axoplasm and the reactive granules in the synaptic clefts. Although the use of inhibitors introduces additional complications in the interpretation of histochemical reaction, this result is mentioned here as a suggestion that several enzymes of marked similarity and only slight differences are present on the two sides of the neuromuscular junction.

DISCUSSION

The results presented here show the accuracy with which histochemical techniques can be expected to give information at a cytological level. They provide critical localization of the reaction together with reasonable preservation of morphological detail. Analysis of some of the general principles and the prerequisites for obtaining these results are given and are in agreement with those reported earlier (25).

Comparison of the present results with those of Lehrer and Ornstein (17) indicate several differences that are worthy of comment. The obvious differences in the contrast of the final product in the two investigations (PbS and azo dye) suggest that it is preferable to use in electron microscopy a histochemical technique that includes a metal salt as final product. This does not mean that the application of azo dye histochemistry to electron microscopy does not have a reasonable future. On the contrary, further development of new diazonium salts should provide improvement of this potentially important avenue of research. The lack of contrast obtained with the present azo dye method (17), however, required that the specimens be over-incubated for reasonable deposition of final product. This conclusion is warranted since the heavy concentration of final product in the synaptic cleft demonstrated by Lehrer and Ornstein (17), who incubated 4 to 6 hours at 0°C, is most closely paralleled in the present studies only by those specimens which were clearly over-incubated. In the present experiments the best cytological localization occurred only when the incubation was stopped at the very first sign of product deposition.

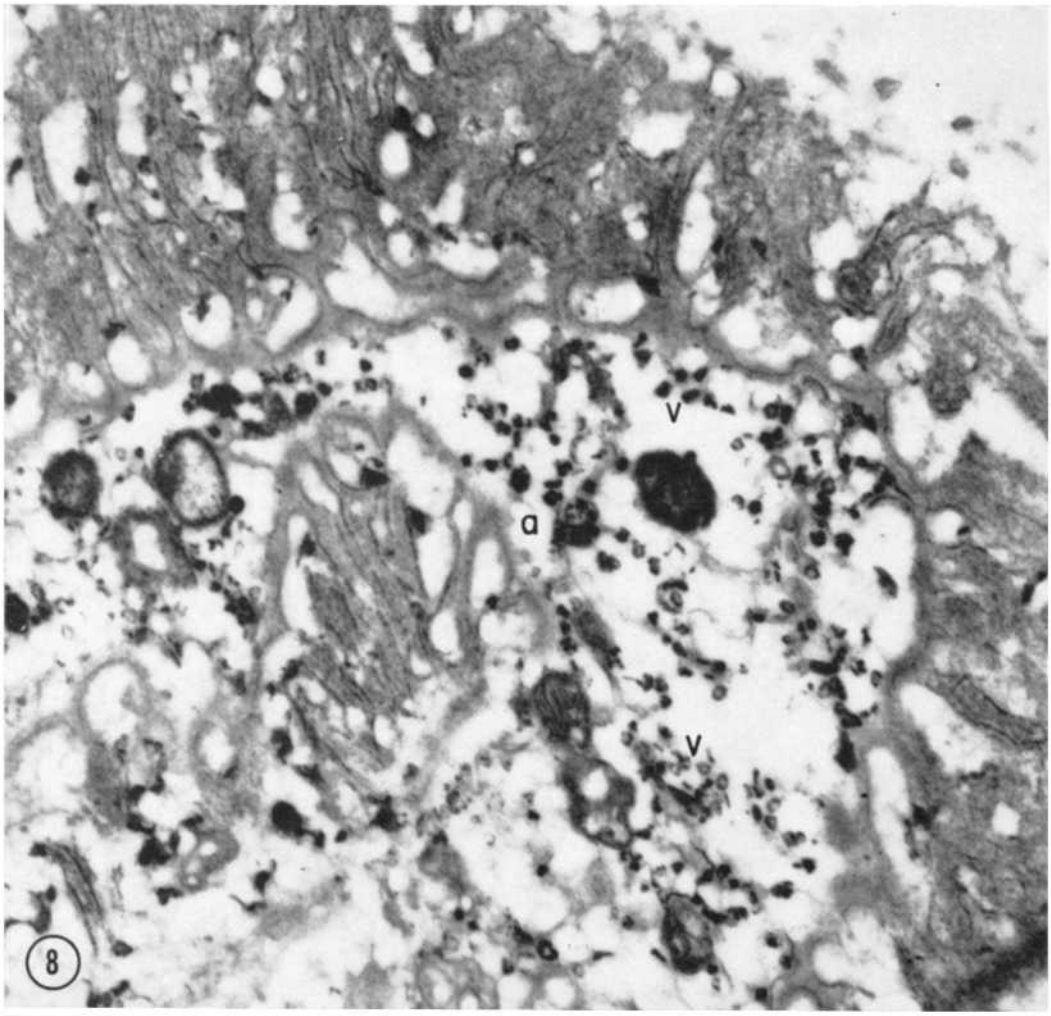
The second difference concerns the reactivity of structures in the terminal part of the axon. Since these structures were very small, the degree to which contrast was provided by the azo dye method may have been insufficient for the visu-

FIGURE 8

This micrograph shows another example of the range of findings in reactive myoneural junctions. In this example the muscle plasma membrane lining the junctional folds is reactive but the granular material in the synaptic clefts is weakly reactive, if at all. On the other hand, reactive intact vesicles (*v*) are plentiful in the axon (*a*). In some vesicles the contents are reactive, and in others, the limiting membrane. None of the vesicles appear disrupted in comparison with Fig. 7. Several larger organelles in the axon appear reactive and some of these may be mitochondria. × 46,000.

FIGURE 9

Micrograph of myoneural junction showing enzyme activity mainly localized to granular material in the secondary synaptic clefts and in vesicular structures (*v*) of the axon terminal (*a*). The surface membrane of the axon, although irregular in contour and apparently disrupted at several sites, is histochemically active. × 41,000.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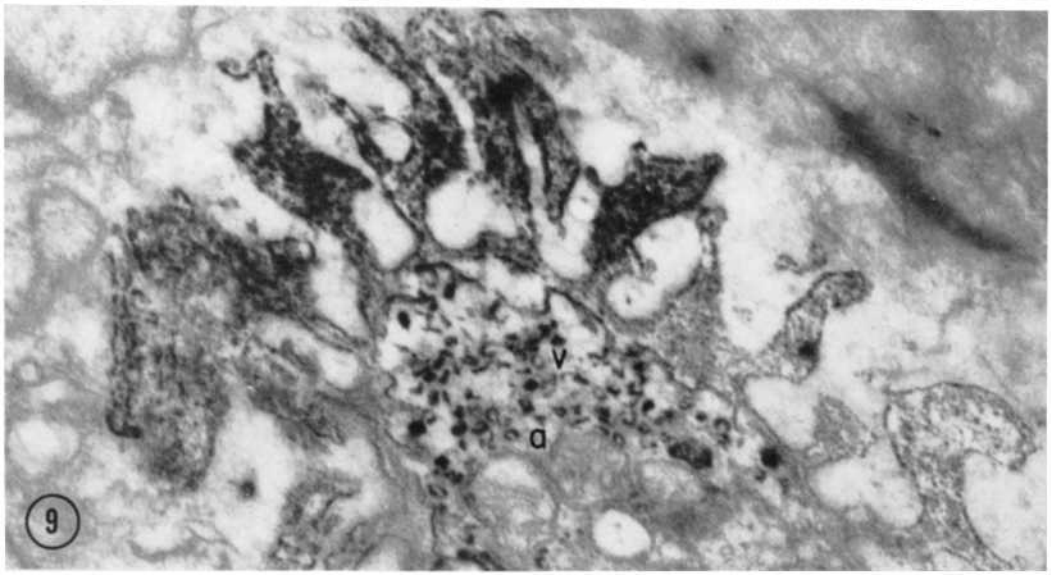
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alization of activity in these sites. However, there are other possible explanations for this difference; for example, another enzyme system may exist, in the terminal axon, that splits thiolacetic acid and not naphthyl acetate. This possibility is suggested by two of the present observations. First, in the usual incubation, a somewhat variable reaction occurred in the axon terminal in comparison with that of the membrane of the junctional folds and the granules of the synaptic clefts. Secondly, the activity of the vesicles after DFP (10^{-5} M) differed from that of the other usually active structures.

Theoretical considerations of the role of thiolacetic acid as a substrate suggest that PbS could be produced as a result of another enzyme system, carboxylic acid esterases aside. If, for example, an acyl transferase or deacylase system were active, as in the choline acetylase system, thiolacetic acid could possibly serve as acetyl donor, or even improbably as a substitute for acetyl CoA, and the same final product would be formed. As a result of this speculation, a series of preliminary experiments were performed on unfixed junctions in which the AChE activity was inhibited with eserine and the substrate and lead were used within various combinations of CoA, ATP, choline chloride, cysteine and Mg^{++}, and cacodylate or tris buffer, pH 7. These experiments were completely unsuccessful and no reaction developed. These negative results and those of the inhibition experiments force the conclusion that the substrate was hydrolyzed by an enzyme or enzymes which had an AChE-like function. The differences noted in the reaction between the two sides of the junction, when less than a completely inhibiting dose of DFP was used, might be due to increased complexity of the histochemical incubation in the presence of an inhibitor, or more likely to slight differences in the enzyme at different sites (junctional folds *vs.* synaptic vesicles).

It is therefore tempting to conclude that the active sites of AChE are located on the plasma membrane of both the terminal axon and the junctional folds of the muscle, in the space between them, and on vesicular and tubular structures in the terminal part of the axon. This visual finding supports the inferences drawn from biochemical data that AChE is present on both sides of the junction (26–30). On the other hand, review of the histochemical results, derived from four different methods studied with the light microscope, suggests that the activity is located at the level of the subneural lamellae (5). Only several authors (31–33) of many suggest that enzyme activity may also be obtained in the nerve terminals at synapses. This situation may be due to the lack of adequate resolution with light microscopy, a suggestion which is supported by the fact that light micrographs of the present method gave results that are essentially identical with those of previous methods. Only when the electron microscope was used with material incubated under the conditions enumerated was the reaction of terminal axoplasm clearly apparent in parallel thin sections.

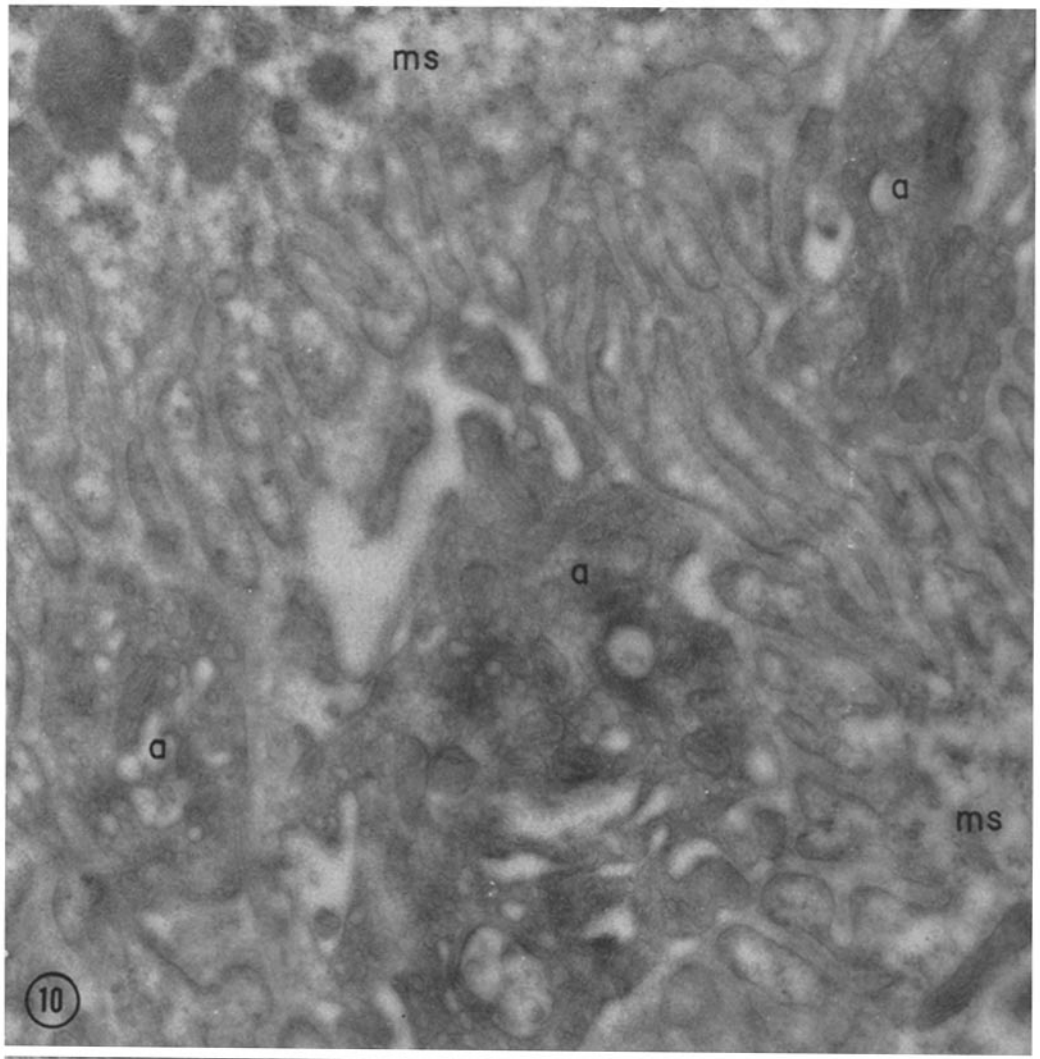
Another factor to be accounted for is the distinct difference in the morphological appearance of the myoneural junction in the control tissue

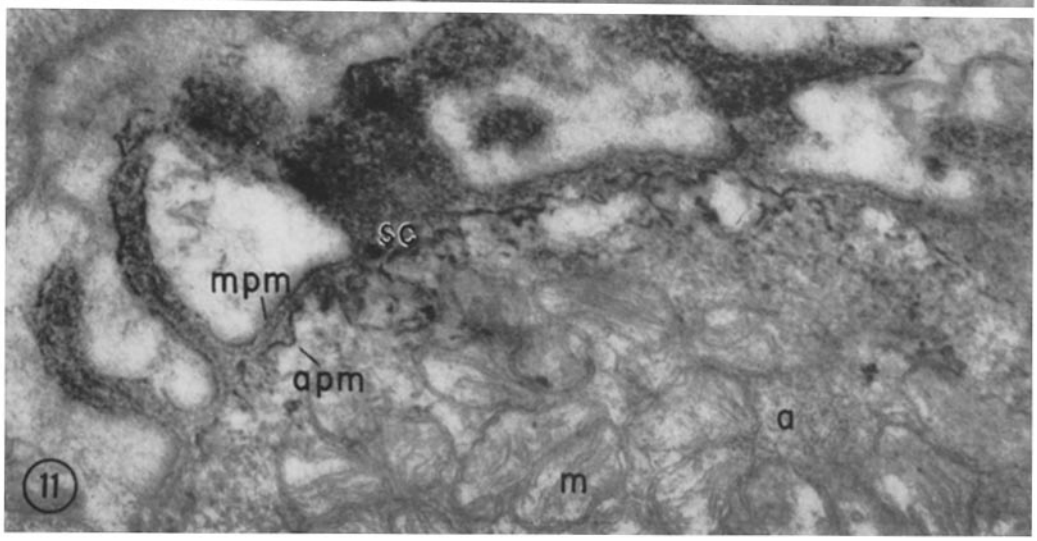
FIGURE 10

Micrograph of myoneural junction that was incubated in the presence of eserine (1×10^{-4} M). This concentration of inhibitor eliminated all enzyme action present at the junction. Preservation of the fine structure was adequate with this procedure, and only spotty extraction and vacuolization occurred in the terminal part of the axon (*a*). *ms*, sarcoplasm. × 41,000.

FIGURE 11

Micrograph of the regular reaction (eserine omitted) run in parallel with that of Fig. 10. Final product of the enzyme reactions is localized to plasma membranes of the muscle (*mpm*) and axon (*apm*), granular material in the synaptic clefts (*sc*), and granules and bits of membranes in the axon. Mitochondria (*m*) in the axon (*a*) show no activity. × 41,000.


ms
a
a
a
ms
10


sc
mpm
apm
a
m
11

(unreacted or completely inhibited) and the experimental tissue (histochemically reactive) in the present work. A similar difference appeared when the results of the present experiment were compared with those of Lehrer and Ornstein (17). Not much change in fine structure occurred as a result of incubation of formalin-fixed myoneural junctions with naphthyl acetate and a diazonium salt (17), whereas major changes occurred in junctions briefly fixed in osmium tetroxide and incubated with thiolacetic acid. Though the simplest explanation of the present physical damage would appear to relate active enzyme activity to little or no fixation by osmium tetroxide, this does not explain the relative absence of damage in the specimens where enzyme activity was inhibited by eserine or DFP. This suggests the possibility that junctional AChE activity may be associated with morphological change.

The results presented here do not support the speculation of De Robertis and Bennett (34), who proposed that the vesicles in the presynaptic axoplasm might be associated with a transmitter substance. They suggested that the vesicles might flow toward the synaptic membrane and discharge their contents into the synaptic cleft. The ACh or other synaptic mediators originally contained within the vesicle would be destroyed at the postsynaptic membrane. This view was criticized by Nachmansohn (30) because it did not fit some of the known biochemical events and proposed physicochemical mechanisms.

Numerous authors have found synaptic vesicles concentrated in the presynaptic axoplasm in invertebrate and vertebrate synapses (see reviews 24, 35, 36). There can be no doubt from the large number of observations that such accumulations of vesicles exist at a variety of synapses. Although De Robertis (35, 36) has claimed a decrease in the number and size of the vesicles on electrical stimulation, there is no other evidence in the literature that the vesicles actually can or do unite with the presynaptic membrane, a necessary prerequisite for the discharge of their presumed contents. Furthermore, Robertson (37) doubted the functional significance of the synaptic vesicles as a transmitter storage device, and several authors (13, 14) found no change in the synaptic vesicles upon electrical stimulation of neuromuscular junctions.

The internal structure of the presynaptic axonal terminal has also attracted the attention of the neurophysiologists, who have speculated on its possible significance (14, 38–42). These speculations are, by and large, in line with the De Robertis and Bennett hypothesis and relate the quantal discharge of a chemical transmitter such as acetylcholine, to the synaptic vesicles.

Though there is general agreement that acetylcholine plays an essential role in the process of synaptic and neuromuscular transmission, there is a difference of opinion as to its precise mechanism of action. The ester cannot be the only factor that determines the nature of the change. The possibility exists that ACh acts in both the pre- and postsynaptic membranes to permit ion flux, as is hypothesized to occur in conduction (30). The present cytochemical demonstration of the enzyme, AChE, both in the nerve terminal and on the junctional folds in muscle supports the above view as proposed by Nachmansohn and

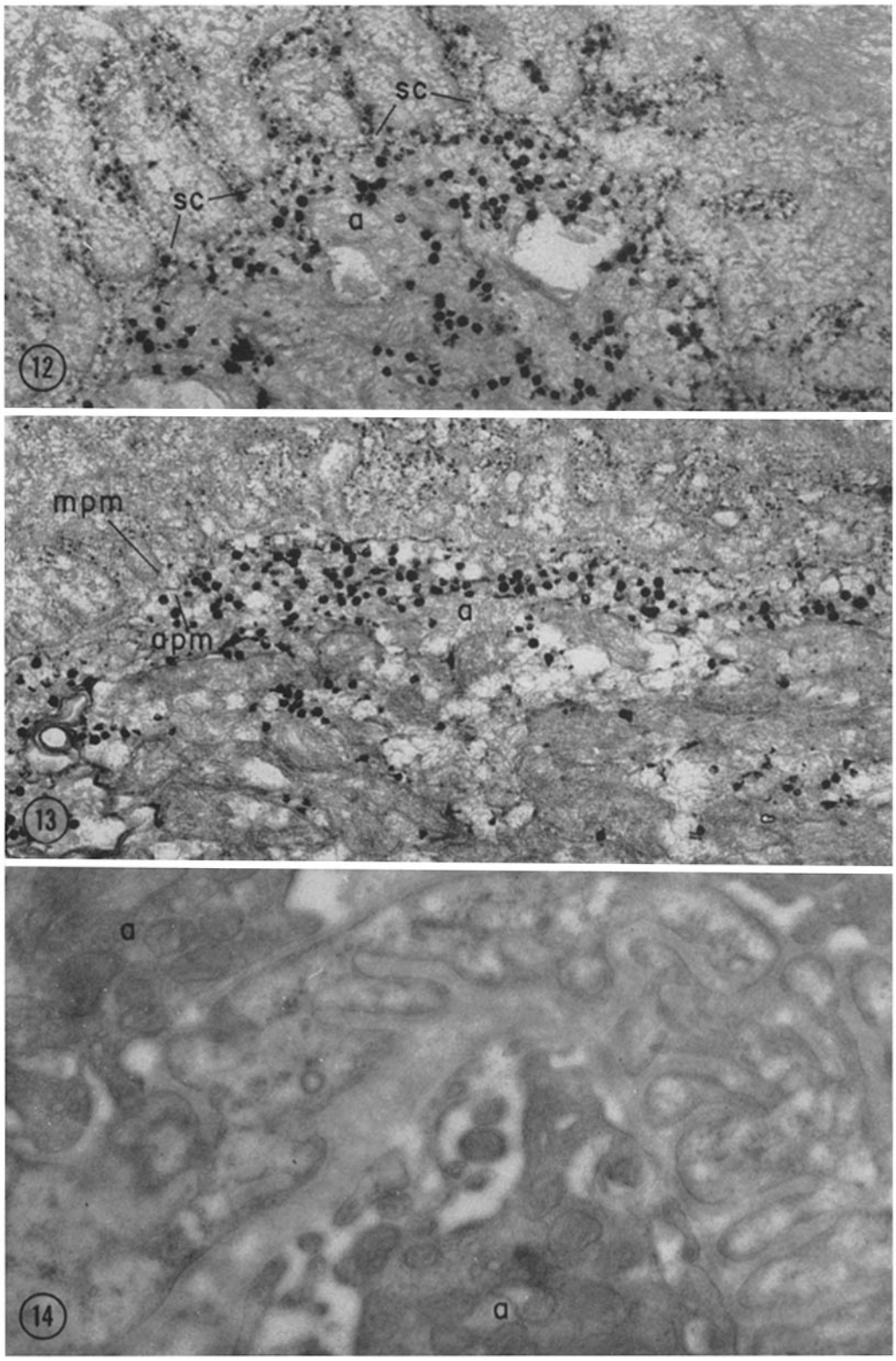
FIGURES 12 TO 14

These micrographs indicate the results of histochemical incubation when DFP was used as an inhibitor.

The junction illustrated in Fig. 12 was from the center of a block subjected to 1×10^{-5} M DFP. Only some of the granular material in the primary and secondary synaptic clefts (*sc*) and the granules and vesicles in the terminal axon (*a*) show activity. The other usual sites of activity are unreactive. This micrograph also indicated that the size and shape of some of the deposits of end product are similar in the axon and in the synaptic clefts. × 56,000.

Fig. 13 shows a junction from the periphery of the same block. Granular deposits in the synaptic clefts are fewer and smaller than those in Fig. 12 but the vesicles in the terminal axon (*a*) remain quite active. Plasma membranes at the surface of the junctional folds (*mpm*) and axon (*apm*) are largely unreactive. × 46,000.

In Fig. 14, all enzymatic activity was inhibited by 1×10^{-4} M DFP. Terminal parts of the axon (*a*) show some distortion due to incubation procedures. × 41,000.


sc
sc
a
12
mpm
apm
a
13
a
a
14

his coworkers. Therefore, the results of the present work favor the interpretation that the process of synaptic transmission requires the presence of receptor protein and enzyme on both sides of the intercellular gap (30, 43). A split system of the formation and release of the substrate in one cell and its reaction and hydrolysis by a receptor in another cell seems rather unlikely. The fast and precise function of synaptic transmission must be based upon the structural, chemical, and energetic organization of the cells, a situation similar to that pertaining to the relationship of electron transport in oxidative reactions to the structure of mitochondrial membranes. This suggests that the near continuum of AChE sites, from terminal axon through the gap to the muscle membrane, may coincide more or less closely with the sites of the production of ACh.

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BIBLIOGRAPHY

1. ROUGET, M., *Compt. rend. Acad. sc.*, 1862, **55,** 548.
2. KRAUSE, W., *Z. rationell. Med.*, 1863, **18,** 136.
3. RANVIER, L., Leçons sur l'histologie du système nerveux, Paris, Savy, 1878.
4. TIEGS, O. W., *Physiol. Revs.*, 1953, **33,** 90
5. COUTEAUX, R., *Internat. Rev. Cytol.*, 1955, **4,** 335.
6. ANDERSSON-CEDERGREN, E., *J. Ultrastruct. Research*, 1959, Suppl. 1.
7. PALADE, G. E., *Anat. Rec.*, 1954, **118,** 335.
8. ROBERTSON, J. D., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 369.
9. REGER, J. F., *Anat. Rec.*, 1955, **122,** 1.
10. REGER, J. F., *Anat. Rec.*, 1958, **130,** 7.
11. REGER, J. F., *J. Ultrastruct. Research*, 1959, **2,** 269.
12. EDWARDS, G. P., RUSKA, H., and DEHARVEN, E., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 167.
13. DE HARVEN, E., and COERS, S., *J. Biophysic. and Biochem. Cytol.*, 1959, **6,** 7.
14. BIRKS, R., HUXLEY, H. E., and KATZ, B., *J. Physiol.*, 1960, **150,** 134.
15. COUTEAUX, R., *Exp. Cell Research*, 1958, Suppl. 5, 294.
16. SCHWARZACHER, H. G., *in* Histochemistry of Cholinesterase, Bibl. Anat., 2, (H. G. Schwarzacher, editor), Basle, S. Karger, 1960.
17. LEHRER, G. M., and ORNSTEIN, L., *J. Biophysic. and Biochem. Cytol.*, 1959, **6,** 399.
18. BIRKS, R. I., and BROWN, L. M., *J. Physiol.*, 1960, **152,** 5P.
19. BROWN, L. M., *in* Histochemistry of Cholinesterase, 1960, Bibl. Anat., 2, (H. G. Schwarzacher, editor), Basle, S. Karger, 1960, 21.
20. ZACHS, S. I., and BLUMBERG, J. M., *J. Histochem. and Cytochem.*, 1961, **9,** 317.
21. WILSON, I. B., *Biochim. et Biophysica Acta*, 1951, **7,** 520.
22. BARRNETT, R. J., *J. Histochem. and Cytochem.*, 1958, **6,** 101.
23. BARRNETT, R. J., and PALADE, G. E., *J. Biophysic. and Biochem. Cytol.*, 1959, **6,** 163.
24. PALAY, S. L., *Exp. Cell Research*, 1958, Suppl. 5, 275.
25. BARRNETT, R. J., and PALADE, G. E., *J. Histochem. and Cytochem.*, 1958, **6,** 1.
26. COUTEAUX, R., and NACHMANSOHN, D., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43,** 117.
27. NACHMANSOHN, D., and HOFF, E. C., *J. Neurophysiol.*, 1944, **7,** 27.
28. STOERK, H. C., and NORPETH, E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57,** 154.
29. SAWYER, C. H., and HOLLINSHEAD, W. H., *J. Neurophysiol.*, 1945, **8,** 137.
30. NACHMANSOHN, D., Chemical and Molecular Basis of Nerve Activity, New York, Academic Press, Inc., 1959.
31. COUTEAUX, R., and TAXI, J., *Arch. anat. micr. et morphol. exp.*, 1952, **4,** 352.
32. COËRS, C., *Acad. roy. Belgique Cl. sc. Mem.*, 1953, **36,** 447.
33. KOELLE, W. A., and KOELLE, G. B., *Fed. Proc.*, 1958, **17,** 384.
34. DEROBERTIS, E., and BENNETT, H. S., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 47.
35. DEROBERTIS, E., *Exp. Cell Research*, 1958, Suppl. 5, 347.
36. DE ROBERTIS, E., *Internat. Rev. Cytol.*, 1959, **8,** 61.
37. ROBERTSON, J. D., *Exp. Cell Research*, 1955, **8,** 226.
38. FATT, P., and KATZ, B., *J. Physiol.*, 1953, **117,** 109.
39. DEL CASTILLO, J., and KATZ, B., *Progr. Biophysics and Biophysic. Chem.*, 1956, **6,** 121.
40. KATZ, B., *Bull. Johns Hopkins Hosp.*, 1958, **102,** 275.
41. ECCLES, J. C., The Physiology of Nerve Cells, Baltimore, The Johns Hopkins Press, 1957.
42. ECCLES, J. C., and JAEGER, J. C., *Proc. Roy. Soc. London, series B*, 1958, **148,** 38.
43. NACHMANSOHN, D., *Am. J. Physic. Med.*, 1959, **38,** 190.