A CHOLINERGIC COMPONENT IN THE INNERVATION OF THE LONGITUDINAL SMOOTH MUSCLE OF THE GUINEA PIG VAS DEFERENS

The Fine Structural Localization of Acetylcholinesterase

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ABSTRACT

Acetylcholinesterase (AChE) has been detected on the plasma membrane of about 25% of the axons in the longitudinal smooth muscle tissue of guinea pig vas deferens. These axons are presumably cholinergic. No enzyme was detected in the remaining 75% of axons. These axons are presumably adrenergic. The plasma membrane of the Schwann cells associated with the cholinergic axons also stained for AChE. Some axon bundles contained only cholinergic or adrenergic axons while others contained both types of axon. When a cholinergic axon approached within 1100 A of a smooth muscle cell, there was a patch of AChE activity on the muscle membrane adjacent to the axon. It is suggested that these approaches are the points of effective transmission from cholinergic axons to smooth muscle cells. Butyrylcholinesterase activity was detected on the plasma membranes of all axons and smooth muscle cells in this tissue.

INTRODUCTION

Stimulation of the postganglionic nerves to the guinea pig vas deferens results in depolarization of smooth muscle cell membranes and contraction of the longitudinal smooth muscle cells (Hukovic, 1961; Burnstock and Holman, 1961).

These muscle cells are arranged in anastomosing bundles of variable diameter. Each muscle bundle contains nerve fibers running approximately parallel to the bundle axis, either as single axons without Schwann cell covering, or more commonly as groups of axons more or less embedded in a Schwann cell process. The axons lie close to muscle cells at intervals, forming *en passage* approaches; they appear to end in a terminal varicosity lying within 500 A of surrounding muscle cell membranes (Merrillees, Burnstock, and Holman, 1963; Merrillees, 1968).

These axons are predominantly noradrenergic (Hukovic, 1961; Burnstock and Holman, 1962, 1964; Sjöstrand, 1965). Choline acetylase has, however, been reported in extracts of the vas deferens (Ohlin and Stromblad, 1963), and acetylcholinesterase (AChE) is present in nerves within the smooth muscle tissue (Jacobowitz and Koelle, 1965). In addition, treatment with anticholinesterases and cholinergic blocking agents modifies the response to stimulation of the postganglionic nerves, indicating that acetylcholine (ACh), as well as noradrenaline (NA), plays a part in transmission in this organ (Birmingham

and Wilson, 1963; Sjöstrand, 1965; Birmingham, 1966; Bell, 1967).

The role of the cholinergic system in the innervation of this tissue is not clear. It has been suggested that ACh is involved in release of NA from adrenergic nerves in the guinea pig and other mammals (Burn and Rand, 1959, 1965; Koelle, 1962). If this were so, it would be expected that AChE would be associated with all the axons in the vas deferens. On the other hand, a separate synergistic cholinergic and adrenergic innervation has been proposed (Birmingham and Wilson, 1963; Birmingham, 1966; Bell and McLean, 1967; Bell, 1967). The association of AChE with a limited number of axons would support the latter view.

The Karnovsky method of demonstrating AChE under the electron microscope (Karnovsky, 1964) has been used to localize the enzyme in the axons and smooth muscle of amphibian bladder (Robinson and Bell, 1967), and the present paper describes the application of this method to the longitudinal smooth muscle of guinea pig vas deferens. A preliminary description of some of this work has already been published (Burnstock and Robinson, 1967).

METHODS

Guinea pigs were stunned by a blow to the head and bled. The lower body wall was opened and one testis was gently pulled from the body cavity. The vas deferens was cut free from supporting connective tissue and laid under slight tension across a plastic trough filled with ice-cold fixative. The fixative was replaced every 3–5 min. After 20 min the vas deferens was cut free and placed in a bath of fixative at 0–4°C for a further 2 hr.

The fixative used was 4% paraformaldehyde dissolved in Krebs solution as modified by Hukovic (1961). It was prepared just before use by dissolving 4 g of dry paraformaldehyde in 50 ml of warm, distilled water made alkaline with a few drops of sodium hydroxide solution (as in Pease, 1964), then adding this to 50 ml of ice-cold, double-strength Krebs stock solution. Oxygen containing 5% CO_2 was bubbled through the mixture in an ice bath until the pH was 7.1–7.2, and the temperature was less than 4°C. Calcium and magnesium salts were then added to complete the Krebs solution.

After 2 hr of fixation, the vas deferens was cut into 3–5-mm pieces, and the Krebs–formaldehyde solution was replaced drop for drop by distilled water over a period of about 1 hr, either by hand or with a mechanical device. The tissue was then left to wash overnight in distilled water in an ice bath. (Krebs solution or simple phosphate buffers were tried instead of distilled water in early experiments, but they did not improve the tissue preservation, and unless they themselves were completely washed out, they interfered with the subsequent staining reaction).

Cross-sections were then cut at about 150–200-μ thickness by hand, or at 25–100-μ thickness with a modified McIlwain tissue chopper (McIlwain and Buddle, 1953; Smith and Farquhar, 1965).

All preincubation and incubation was at 0–4°C. Incubation for AChE was carried out by preincubating the tissue slice in the butyrylcholinesterase-inhibitor iso-OMPA (Koch-Light, Colnbrook, Bucks, England) (Bayliss and Todrick, 1956) at a concentration of 10^{-5} M for 1 hr, and then incubating in the Karnovsky medium (Karnovsky, 1964) containing acetylthiocholine for 6–30 min at pH 5.0. Incubation for butyrylcholinesterase (BuChE) was carried out by preincubating the tissue with the AChE-inhibitor BW 284C51 [Burroughs-Wellcome, (Aust.), Sydney, Australia] (Bayliss and Todrick, 1956) at a concentration of 5×10^{-6} M for 1 hr, and then incubating in the Karnovsky medium containing butyrylthiocholine and BW 284C51 (5×10^{-6} M) for 20–60 min at pH 6.0.

Controls were performed in which the incubation for AChE followed preincubation in BW 284C51, and incubation for BuChE followed preincubation in iso-OMPA. All tissue incubated for longer than 20 min was compared with tissue incubated in substrate-free medium for the same period. Some tissue was incubated in substrate-free medium lacking ferricyanide, and some in substrate-free medium after preincubation for 4 hr in 0.1 M iodoacetate at pH 7.2.

The staining reaction was stopped by the addition of drops of 5% osmium tetroxide (dissolved in distilled water) to the incubation medium until the final concentration of osmium tetroxide was about 1%. This mixture was then replaced by 1% osmium tetroxide in distilled water, and the tissue was left in this solution for a further 1 hr.

The tissue slices were rapidly dehydrated in a graded series of acetone and water mixtures, beginning at 30%, and then embedded in Araldite. Sections were cut with glass knives, collected on uncoated copper grids, lightly treated with lead citrate (Reynolds, 1963), and examined with an Hitachi HU 11B microscope.

Staining density was not uniform throughout the depths of those slices that were thicker than about 30 μ. Pieces 30 μ or thinner were fragile and difficult to handle, and major cutting damage was frequently seen under the electron microscope. Tissue slices of 50–100-μ thickness, on the other hand, showed fewer cutting artefacts, and the staining pattern of the cells in the outer 15–20 μ of these thicker slices was identical with the staining seen throughout the

thinner pieces. All the micrographs presented in this report illustrate sections taken from tissue within 20 μ of the cut surfaces of 50–100 μ slices.

All counting of stained and unstained axons was performed on the screen of the microscope. Sections used for this purpose were taken from thin tissue slices that had proved to be evenly stained throughout the slice. This was done to minimize the bias toward a low proportion of stained axons that might have resulted from inadequate penetration of the incubating medium.

All measurements of closest point between axons and smooth muscle cells were made to the nearest ½ mm on micrographs printed at 30,000–40,000 magnification. No allowance was made for any change in dimension that may have occurred during processing of the tissue. The micrographs used in this portion of the study were all taken from tissue which had been incubated for 15 min or longer, as this time had previously been shown to be sufficient to ensure that all the enzyme present had produced clearly visible stain particles.

RESULTS

With the medium containing acetylthiocholine, stain was detected in tissue slices incubated at pH 5.0 for 6 min or longer. This staining reaction was prevented by prior incubation of the tissue in BW 284C51 at a concentration of 5×10^{-6} M, but it was not affected by similar treatment with iso-OMPA at a concentration of 10^{-5} M. This staining is, therefore, interpreted as resulting from the action of AChE.

With the medium containing butyrylthiocholine, stain resulted from 20–50 min incubation at pH 6.0. The reaction was unaffected by BW 284C51 but was inhibited by iso-OMPA under the conditions described above. This staining is interpreted as resulting from the action of BuChE

No stain was detected in tissue slices incubated in substrate-free medium for less than about 50 min, but longer incubation times caused a reaction that is referred to in this report as "nonspecific" staining. Nonspecific stain was absent when ferricyanide was omitted from the incubation medium, and was partly inhibited by prior incubation in 0.1 M iodoacetate at pH 7.2.

Acetylcholinesterase Localization

AChE was localized in two distinct sites in the longitudinal muscle tissue of the vas deferens.

AXONS: Most of the tissue slices used in this study were incubated for AChE for 8–10 min at pH 5.0. Under these conditions between 15 and 20% of all the axons were stained. A slightly increased proportion of axons was stained after longer incubation times, but the maximum proportion seen was 25% (after 20-min incubation). Further increase in incubation time did not affect the proportion of stained axons present. Incubation for less than 8 min resulted in light, inconsistent staining. Fig. 4 illustrates unstained axons.

Axons most commonly occurred in bundles of two or three, embedded in a Schwann cell process; the stain was usually deposited on and between the plasma membranes of the axon and Schwann cell (Figs. 1–3, 5–7). Axons stained in this way are referred to as "heavily stained." Sometimes the stain particles were smaller and clearly attached only to one or the other of the membranes, and not between them (Figs. 1, 2, and 6). Axons stained in this way are called "lightly stained." Occasionally, the stain particles were too small to be clearly visible under routine conditions (Fig. 1), and some difficulty was experienced in classification. If the detection of very light staining was important from the standpoint of this study, then sections were examined without lead citrate counterstaining (Fig. 7). Since no difficulty in detection of stain was experienced in tissue incubated for 15 min or longer, and since the proportion of lightly stained axons decreased with increased incubation times, the between-membrane stain on the heavily stained axons was presumably due to an accretion of stain particles on the membranes. This explanation is supported by the appearance of discrete particles of stain lying freely in the intercellular spaces after excessive incubation of the tissue.

Occasionally, axons showing light and heavy staining occurred in the same bundle (Fig. 1). The lightly stained axons in these bundles probably had a lower enzyme activity than the heavily stained axons. When all the axons in a bundle were lightly stained (Fig. 2), however, local irregularities in the incubating conditions may have suppressed the staining reaction. For this reason, no attempt has been made to compare the enzyme activity of axons in different bundles. Difficulties in the classification of very lightly stained axons only occurred in a small number of cases (presumably those axons with lower than average enzyme activity) in tissue incubated for short periods of time. This problem did not affect either the data obtained on the total proportion of stained axons in the tissue (25%), or the data ob-

tained for Fig. 8, as these counts and measurements were made on tissue that had been incubated for sufficient time to allow all the enzyme present to produce a clearly visible deposit of stain (i.e., longer than 15 min). In all cases, the density of staining on the axon membrane appeared to be equal to the density of staining on the adjacent Schwann cell membrane.

Single axons were not commonly seen in this tissue. However, a small number of bare, stained axons were noted. In view of the evidence that single, bare axons are close to terminating (Merrillees, 1968), it is most likely that the stain can be detected all the way to the end of a fiber.

Serial sectioning was not attempted, but, because axons sectioned through varicosities (diameter approx. 1–2 μ) and through intervaricosity regions (diameter approx. 0.1–0.2 μ) were stained (as in Fig. 1), it is, therefore, considered likely that the enzyme was present on the plasma membranes of the entire terminal varicose region of the staining axons.

MUSCLE CELLS: When a stained axon was found close to a smooth muscle cell, stain was often observed on the muscle cell membrane adjacent to the axon. This postsynaptic stain was only present on a patch of muscle cell membrane close to the axon, and was absent from the rest of the muscle cell surface (Figs. 2, 5–7). In tissue incubated for longer than 10 min, the area of muscle cell membrane associated with stain did not appear to be any more extensive than that observed after the shorter times. *Caveolae intracellulares* and occasional submembrane vesicles in the region of the stained muscle membrane were also stained by AChE activity (Figs. 2, 6, and 7).

When very lightly stained sections from tissue incubated for 6–7 min were examined, the distribution of stained axons was patchy, probably owing to uneven penetration of the incubation medium. However, where a stained axon lay close to a smooth muscle cell, both presynaptic and postsynaptic deposits were visible (Fig. 7), indicating equality of enzyme activity at these two sites.

A detailed study has been made of 60 stained axons approaching to within 0.4 μ of a muscle cell membrane in sections from tissue incubated for 15 min or longer. In each case, the closest point between the axon membrane and the muscle membrane was measured on micrographs of between 30,000 and 40,000 magnification, and it was noted (*a*) whether postsynaptic stain was present and (*b*) whether Schwann cell processes intervened between axon and muscle cell. The results are shown in Fig. 8. Two clear trends emerged: firstly, when an axon approached close to a muscle cell the muscle membrane was stained; secondly, the axons farther away were usually covered with Schwann cell processes. Wherever the separation was less than 1100 A, the postsynaptic membrane was stained. In addition, the separation was greater than 1100 A in only 18% of all those approaches with post-synaptic stain. On the other hand it is difficult, with such a small sample, to be definite about the possibility of a relationship between the distance of separation and the presence of a Schwann cell covering, and a larger sample must be investigated in order to reach a conclusion on this point.

Butyrylcholinesterase Localization

The BuChE activity of this tissue was considerably lower than the AChE activity. It required at least a 20-min incubation at pH 6 to detect BuChE, and about a 30-min incubation to obtain adequate and reproducible staining. Because of the low level of activity, it was difficult to obtain clear BuChE staining uncontaminated by nonspecific deposits.

The membranes of *all* axons and Schwann cells stained lightly for BuChE. All axons showed a uniform deposit of stain, and no differences between axons could be distinguished on the basis of staining for this enzyme.

The plasma membranes of all muscle cells also stained lightly for BuChE. The stain was distributed in patches on each muscle cell and was particularly noticeable in the *caveolae intracellulares* (Fig. 9).

The highest BuChE activity in the vas deferens was detected in the pinocytotic vesicles of capillary endothelium (Fig. 10). Most vesicles near the plasma membrane and some vesicles deeper in the cytoplasm of the endothelial cell were stained.

Nonspecific Deposits

Incubation of tissue slices in substrate-free medium for periods of longer than 50 min resulted in a staining reaction which was independent of the activity of cholinesterase in the tissue. The reaction was limited to two main sites: the plasma membranes of all the cells and the mitochondria of smooth muscle cells.

The nonspecific stain was deposited as isolated 500-A particles on the plasma membranes of all the cells seen in this tissue. The longer incubation times resulted in a spread of the stain until all the membranes were covered with an amorphous electron-opaque deposit (Fig. 11). The deposit superficially resembled the BuChE stain, but the nonspecific deposit could be distinguished from the latter by its slow development, the large size of its particles, and its lack of sensitivity to iso-OMPA.

The mitochondrial stain appeared to be finer than the plasma-membrane deposit and was mainly seen in the cristae and between the inner and outer mitochondrial membranes (Fig. 11). Although the mitochondrial stain was always present when the nonspecific stain was seen (i.e., after 50-min incubation), on rare occasions it was also seen after 10–20-min incubation. It was not possible to trace this effect to variations in the handling or incubating of the tissue. Reducing enzymes have recently been demonstrated at similar mitochondrial sites (Ogawa, Saito, and Mayahara, 1968), and this type of nonspecific deposit may be due to the action of these enzymes.

DISCUSSION

Histochemical Methods

The use of the electron microscope in enzyme methods, has been followed by a great increase in resolution, which accentuates the problem of accurate localization of the reaction product.

These problems arise at two stages in the histochemical process: firstly, the problem of fixation of the enzyme in the correct relation to the other tissue elements; and secondly, the problem of depositing stain close to this fixed enzyme.

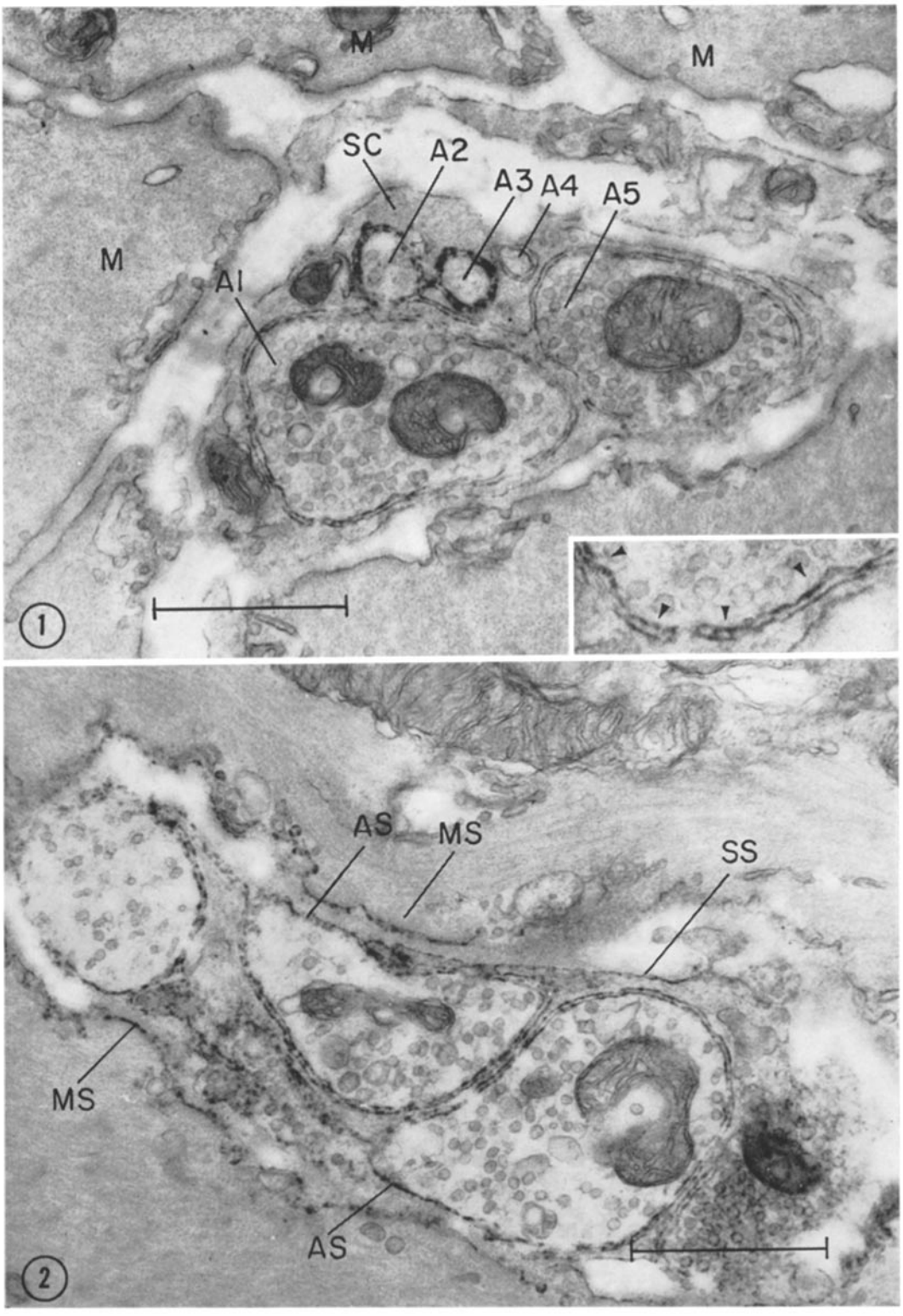
In the first case, it has been shown that, in smooth muscle tissue, enzyme diffusion can be avoided if the tissue is fixed *in situ*, and that the enzyme diffusion can be recognized as a distinctive cloud of stain particles around each axon (Robinson and Bell, 1967). Because fixation was carried out *in situ* for 20 min in this study, and because the characteristic cloud of stain particles was not seen, enzyme diffusion during fixation can be ruled out as a source of artefact.

In the second case, accurate localization of the enzyme sites by deposited stain is largely dependent on the nature of the histochemical method used. The number and speed of the reactions, the solubility of the reactants, and the minimum size of the final deposit are significant in this respect, and, in the case of the Karnovsky method, are dealt with in the basic papers (Karnovsky and Roots, 1964; Karnovsky, 1964). However, two points have arisen in the present study which require discussion.

Firstly, false localization could result if either of the intermediate products of the staining reaction

FIGURE 1 A small bundle of axons, embedded in Schwann cell process, (*SC*) passing close to a number of smooth muscle cells (*M*). Two axons (*A2* and *A3*) are cut through intervaricosity regions, and are clearly stained. *A1* is cut through a varicosity and is more lightly stained. A portion of its membrane and the membrane of the associated Schwann cell are shown at higher magnification in the inset to demonstrate the attached stain particles (arrowheads). It is not possible to be certain whether axons *A4* and *A5* are stained. Dense patches are visible on their membranes but there is not sufficient detail present to be certain. When positive identification of staining was required, the short incubation times which lead to only marginal staining in some axons were avoided. Incubation for acetylcholinesterase, 8 min. × 30,000. Inset: × 60,000. In all figures, the bar represents 1 μ.

FIGURE 2 An axon bundle in which all the axons are stained to the same extent. Stain is deposited on the axon membranes (*AS*), on the muscle membranes adjacent to the axons (*MS*), and on the Schwann cell membranes (*SS*). Note the staining of muscle *caveolae intracellulares*. "Lightly stained" axons such as these are less common in tissue incubated for longer periods, and are absent from tissue incubated for longer than 20 min. When all the axons in a bundle are more lightly stained than other axons in the same section, it is not possible to be certain whether this is due to lower than average enzyme activity in the axons or to locally inadequate incubation conditions. Incubation for acetylcholinesterase, 10 min. × 30,000.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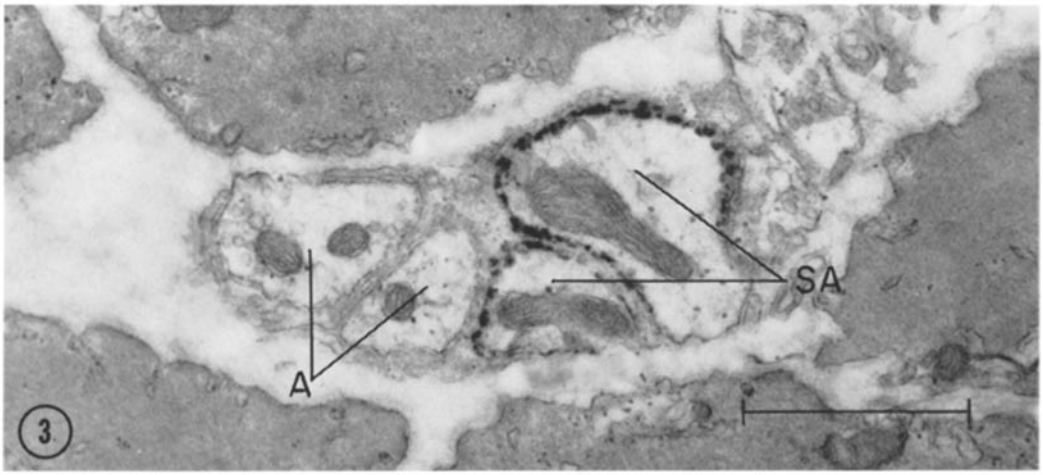


FIGURE 3 Two stained axons (*SA*) in a bundle with two unstained axons (*A*). Incubation for acetylcholinesterase, 10 min. × 30,000.

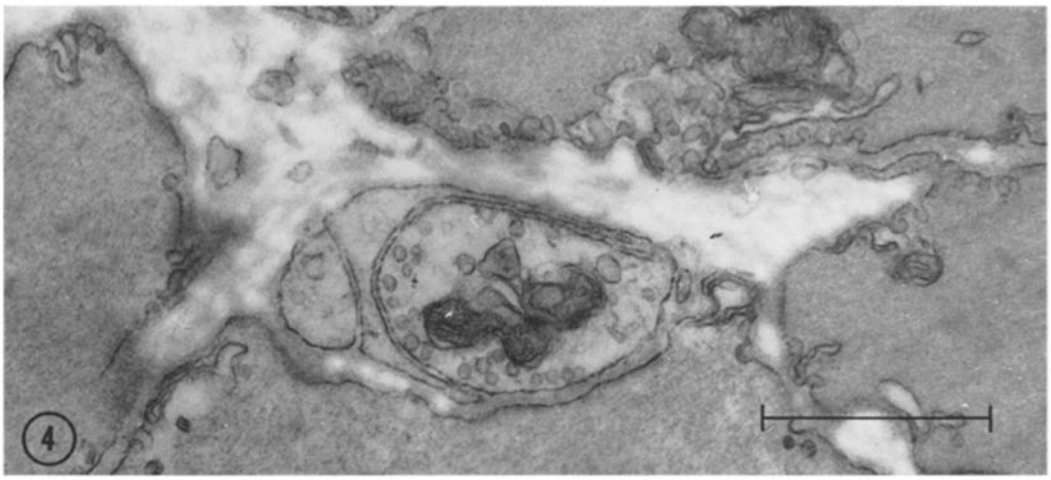
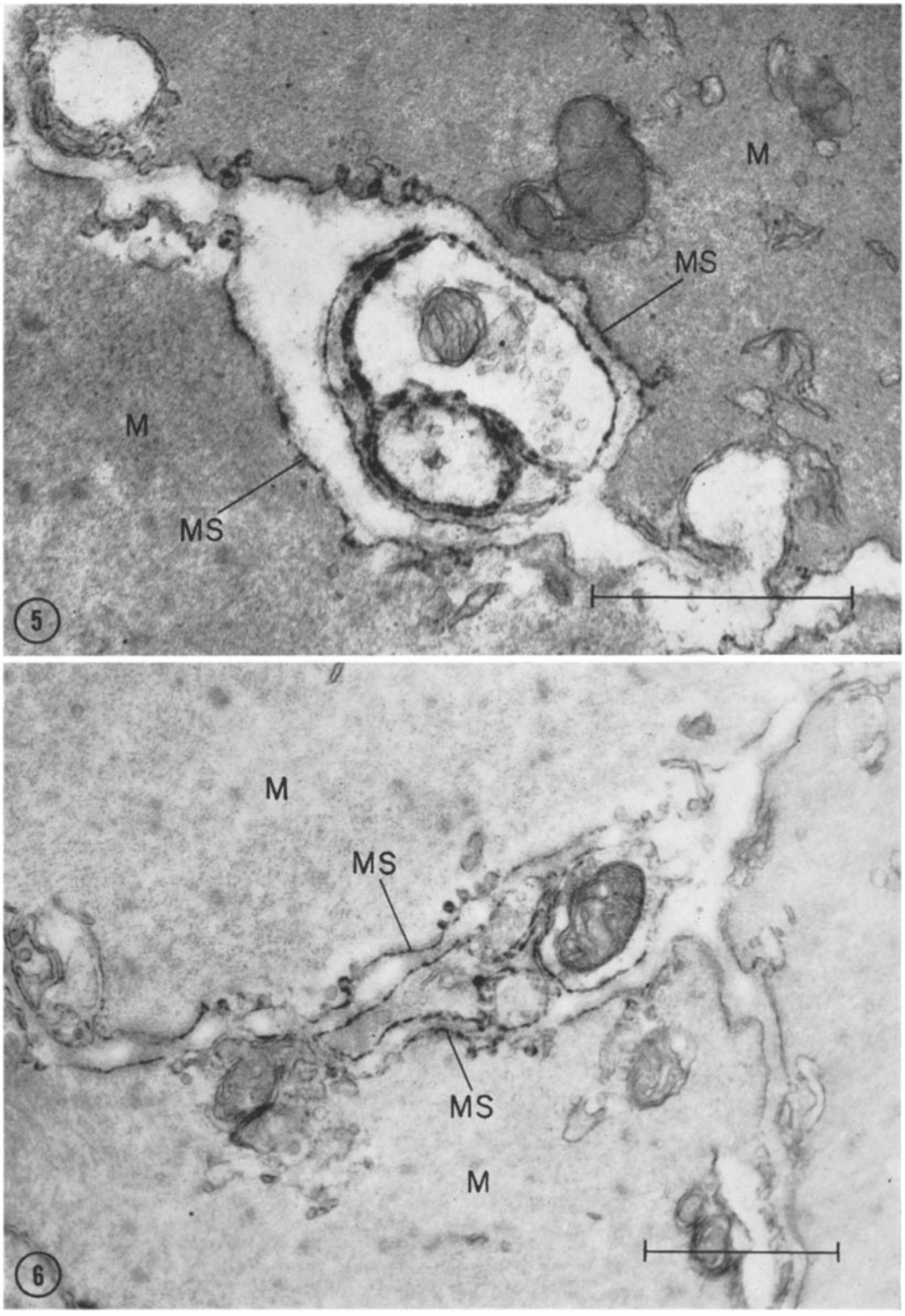


FIGURE 4 Unstained axons passing close to a smooth muscle cell. Note the uneven density of the membranes of the axons, of the Schwann cell and of the muscle cells. This appearance is partly due to the plane of section through the membranes and partly due to what appears to be uneven staining by either osmium tetroxide or lead citrate (or both) following formaldehyde fixation. All axons in unincubated tissue or tissue preincubated with AChE inhibitor have this appearance, as do about 75% of the axons in tissue incubated for AChE. Incubation for acetylcholinesterase, 10 min. × 30,000.

FIGURE 5 A pair of stained axons, one of which is lying close to a smooth muscle cell (*M*). Stain (*MS*) is visible on the muscle membrane adjacent to the stained axon and on the membrane separated from the axons by the Schwann cell. Incubation for acetylcholinesterase, 8 min. × 40,000.

FIGURE 6 A small bundle of stained axons passing close to smooth muscle cells (*M*). Stain (*MS*) is deposited on the muscle cell membranes in the vicinity of the axon bundle. Note the staining of *caveolae intracellulares*. Incubation for acetylcholinesterase, 8 min. × 30,000.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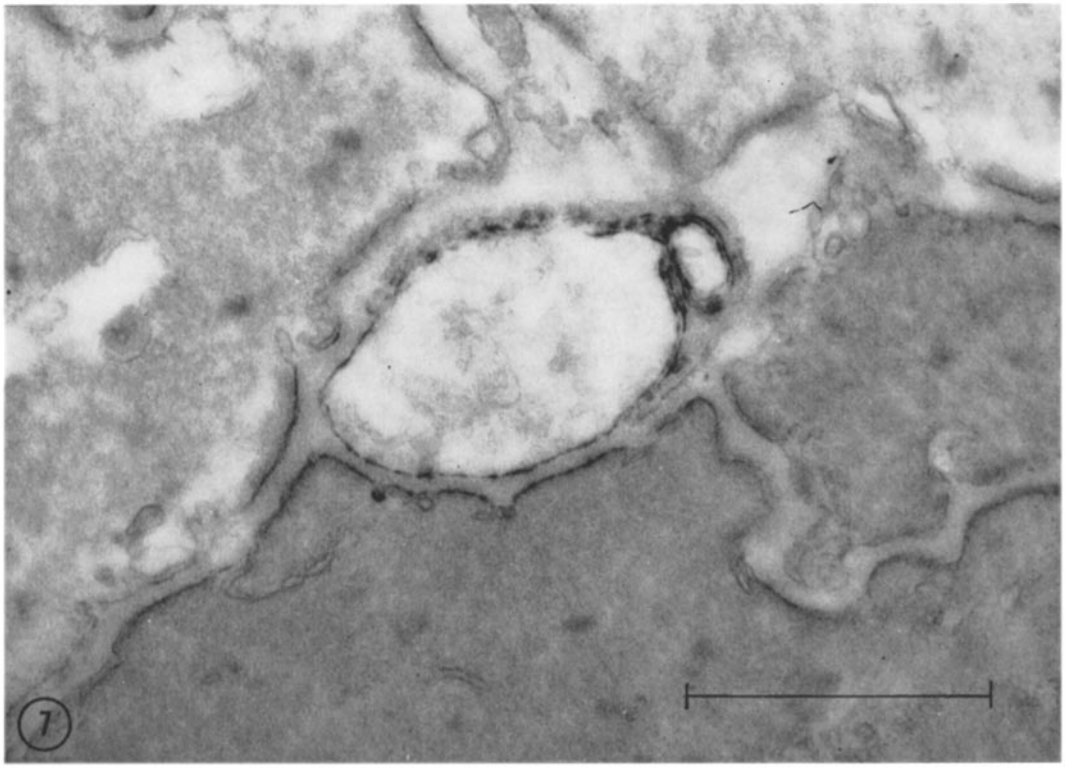

FIGURE 7 A micrograph taken from a section which was not treated with lead citrate before microscopic examination. The section was cut from tissue incubated for 6 min. This is the minimum length of time required to build up visible deposits of stain under these conditions. The stain is visible on the axon membrane and adjacent muscle cell membrane in approximately equal densities, indicating equality of enzyme activity at these two sites. Note the staining of *caveolae intracellulares*. Incubation for acetylcholinesterase, 6 min. × 40,000.

(thiocholine or ferrocyanide) moved from its site of formation before it took part in the next reaction, or if the final reaction product (copper ferrocyanide) moved after its formation. The actual enzyme site (presumably, but not necessarily, stained) could then be referred to as a "primary". or "true" site, and the reaction product located away from the enzyme could be referred to as a "secondary" or "false" site. The process which results in the formation of secondary sites is often loosely referred to as "diffusion."

If, in the present case, it is assumed that the axon plasma membrane is a primary site, then it is possible that the muscle plasma membrane and the Schwann plasma membrane are secondary sites. This is thought to be unlikely because:

(*a*) These localities are membrane surfaces, and precise localization has been demonstrated *on* the membranes without any evidence of staining *between* them (Figs. 2, 5–7), and the intemerdiate products of the Karnovsky method have been shown to have no affinity for membranes (Karnovsky, 1964).

(*b*) The muscle or Schwann cell plasma membranes always showed staining of density equal to that of the adjacent axon membrane, even under minimal incubating conditions (Fig. 7), and, in the case of the muscle membrane stain, even if the distance between axon and muscle membrane was as great as 0.2 μ (Fig. 8).

(*c*) Discrete particles of the final reaction product were produced by extended incubation. These particles appeared to lie freely between the cells and did not appear to associate with any tissue elements.

The *second* point which requires discussion is the

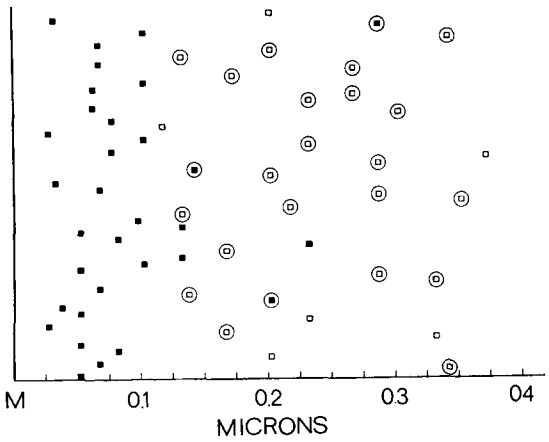

FIGURE 8 Distribution of all the stained axons which approached within 0.4 μ of a muscle cell membrane in sections stained for 15 min or longer. The vertical line at the left of the figure (*M*) represents muscle membrane, and the horizontal scale indicates the distance in microns between the axon membrane and the muscle cell membrane at the closest point between them.

Each square represents one stained axon. The full squares (■) indicate that the membrane of both muscle cell and axon were stained; the empty squares (□) indicate that only the axon was stained. A ring around the square indicates that a Schwann cell was present between axon and smooth muscle cell. The muscle membrane was stained in every approach in which the distance at the closest point was less than 1100 A, but in only a few approaches with a distance greater than this.

presence of the nonspecific deposits. The Karnovsky method depends on the reduction of ferricyanide by the released thiocholine, and, because other reducing groups are common in tissue protein (Herriott, 1947), stain may be deposited also at these other reducing-group sites in the fixed tissue. An adequate control is incubation without substrate, or with the enzyme inhibited, and it has been reported that nonenzymatic reduction did not interfere at all with the localization of AChE in the heart (Karnovsky, 1964). The stain resulting from this nonenzymatic reaction did not interfere with the specific staining produced by short incubation times in the vas deferens but it might have masked low levels of specific staining after extended incubation times. Reduction of ferricyanide by tissue protein has been attributed to the activity of sulfhydryl groups (Mirsky and Anson, 1936 *a*), and to the reducing properties of tyrosine and tryptophan (Mirsky and Anson, 1936 *b*). Reduction of ferricyanide by sulfhydryl groups is potentiated by copper ions at low pH and by prior protein denaturation (Anson, 1942). These conditions are present during incubation in Karnovsky medium.

Preincubation in substrate-free medium was not applied in the present study because of the possibility of the nonspecific reduction of ferricyanide. However, as acetylthiocholine has been shown to diffuse slowly through fixed tissue (Davis and Koelle, 1967), preincubation would probably have had no beneficial effect on localization.

Functional Interpretation

The presence of a cholinergic system in this tissue has been interpreted in three ways. One point of view is that separate adrenergic and cholinergic axons innervate the smooth muscle cells (Birmingham and Wilson, 1963; Birmingham, 1966; Bell, 1967). Another view favors the proposal that the cholinergic system is present in every adrenergic axon and is an essential part of either conduction along the axon (Nachmansohn, 1959) or release of noradrenaline (Burn and Rand, 1959, 1965; Koelle, 1962). This view does not, of course, preclude the possibility of pure cholinergic axons as well as the mixed axons, but much of the evidence for the dual role of ACh collapses if the co-existence of pure cholinergic and mixed axons is admitted. The third view is a compromise which, while allowing only one transmitter to each axon, postulates that the membrane of adrenergic axons is sensitive to ACh, and that ACh released from a cholinergic axon may cause the release of NA from an adjacent adrenergic axon (Koelle, 1963).

The views mentioned above have conflicting requirements for the localization of the enzyme. The first view (separate systems) would require two classes of axon, one group associated with AChE and the other not. The second and third views require that all axons are associated with the enzyme but that some may have a higher level than others.

Jacobowitz and Koelle (1965) have reported the results obtained when the fluorescent localization of NA and the Koelle method for AChE were applied consecutively to individual frozen sections of the guinea pig vas deferens. These two techniques can demonstrate single varicose fibers when used alone under favorable conditions; however, their combination resulted in some loss of detail

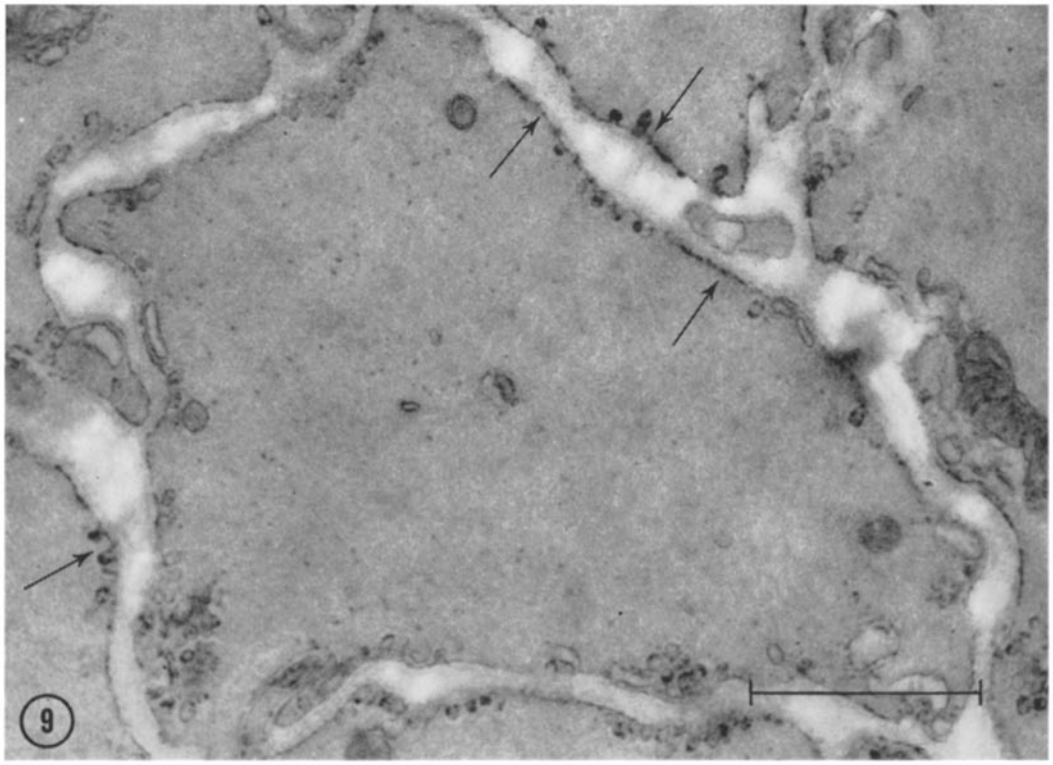

FIGURE 9 Stain due to butyrylcholinesterase activity deposited on smooth muscle cell membranes (arrows). The membranes of all axons and muscle cells stained for BuChE. Incubation for butyrylcholinesterase, 30 min. × 30,000.

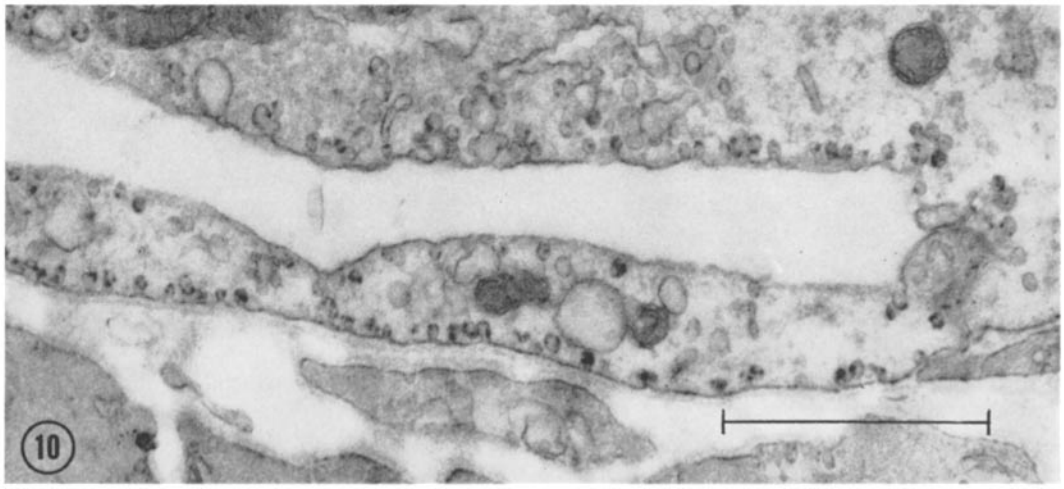

FIGURE 10 Portion of a capillary endothelial cell stained for BuChE. The plasma membrane and some of the vesicles in the cytoplasm are stained. The incubation time was too short to demonstrate the enzyme in smooth muscle cell membranes. Incubation for butyrylcholinesterase, 20 min. × 35,000.

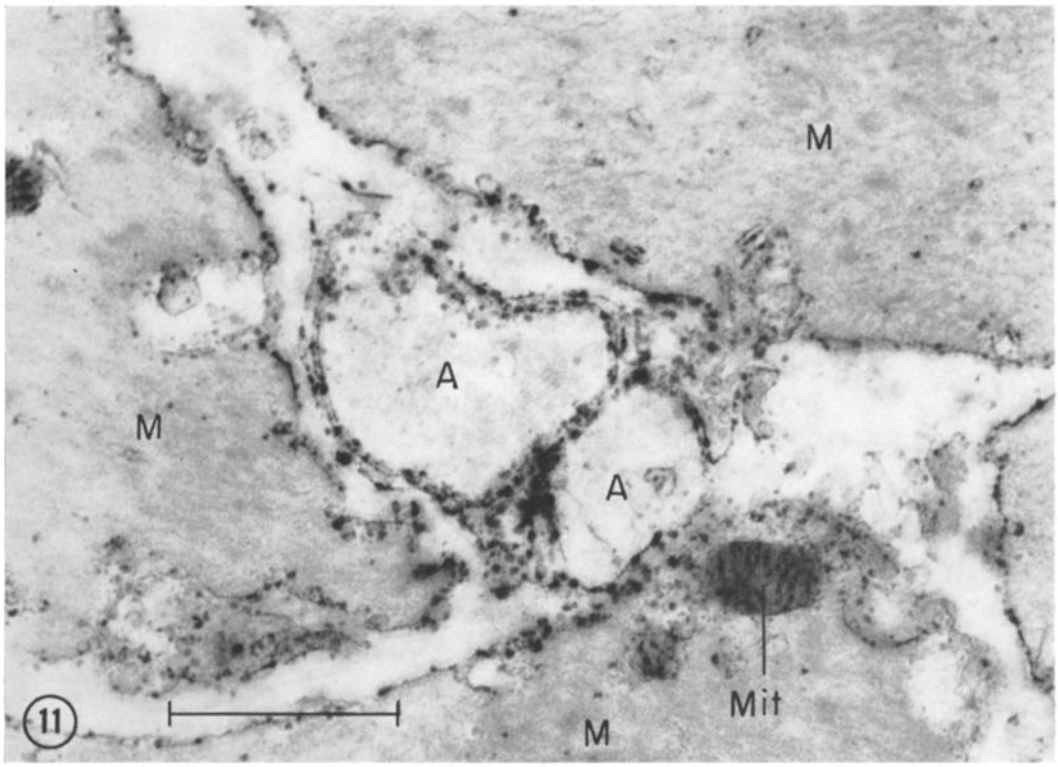

FIGURE 11 The result of extended incubation in substrate-free medium. Note the large grains of stain attached to the cell membranes of axons (*A*) and muscle cells (*M*) and the stain associated with the cristae of the mitochondria (*Mit*). Incubation in substrate-free medium for 50 min. × 30,000.

and only axon bundles could be detected. Many bundles showed the reaction for both NA and AChE, and this was interpreted as not disproving the suggestion that NA and AChE could occur in the same axon. The problem of resolution can be overcome by studying the NA fluorescence and AChE reaction in the cell bodies which lie in the hypogastric nerve (forming a "hypogastric ganglion") and which give rise to the axons innervating the smooth muscle cells of the vas deferens (Bell and McLean, 1967). The cells of this ganglion have been classified by these workers into three groups:

(*1*) The majority showed variable NA fluorescence. Most of these showed no AChE activity, while a few showed low levels of activity (all probably adrenergic).

(*2*) A lesser number showed high levels of AChE activity and no fluorescence (cholinergic).

(*3*) There appeared to be a few which showed neither fluorescence nor enzyme activity (unknown identity).

Although Giacobini (1957) has demonstrated that the AChE levels of cell bodies and the proximal portions of the attached axon are related, it is not clear whether this relationship holds for the terminal sections of the axons as well. The present study was undertaken to overcome the restrictions of the methods used to date, by examining the AChE activity of individual axons within the smooth muscle tissue.

Unfortunately, NA cannot be demonstrated with the electron microscope with the same certainty as it can be with the fluorescence microscope, although there is now good correlation between the occurrence of small granular vesicles and NA in the axons of most mammals (for a review see Burnstock and Robinson, 1967). These vesicles were not seen in the noradrenergic axons in the longitudinal smooth muscle of the guinea pig vas deferens by Merrillees, Burnstock, and Holman (1963) or by Merrillees (1968) after osmium tetroxide fixation, nor in the present

study after formalin fixation. However, because of the strong evidence in favor of a predominant noradrenergic innervation in this tissue (see above), it has been assumed in the present work that the majority of the axons seen in the longitudinal smooth musculature of the vas deferens are noradrenergic.

Comparison of levels of enzyme activity at different sites is difficult with the light microscope and almost impossible with the electron microscope, owing to the physical displacement of stain particles in overincubated tissue and the possibility of uneven incubating conditions. For these reasons, levels of enzyme activity have been estimated in relation to the incubation time required to produce stain after constant fixation procedures, and no attempt has been made to compare levels of stain at distant sites in the same section. Consequently, the 20% of axons that were stained after 10-min incubation times probably represented the proportion of axons with the highest enzyme activity, and the further 5% that stained after the 20-min incubation represented the proportion of axons with moderate activity. Further increase of the incubation time failed to increase the proportion of stained axons. The 5% which stained only after 20 min may have represented axons with either a lower enzyme activity, or axons with high activity that were not stained at the shorter incubation times due to uneven incubation conditions.

Therefore, there appears to be a correlation between the heavily stained (cholinergic) cell bodies in the hypogastric ganglion (Bell and McLean, 1967) and the most reactive axons reported in this study, with perhaps a further correlation between the extra 5% of axons which stain after longer incubation times and the lightly stained cell bodies seen in the hypogastric ganglion. The remaining 75% of the axons are probably related to the fluorescent (adrenergic) and nonfluorescent ganglion cells which do not stain for AChE.

AChE has been demonstrated on the presynaptic membrane and on, or in association with, the postsynaptic membrane at most of the cholinergic synapses recently studied histochemically with the electron microscope[1] (Bloom and Barnett, 1966; Brzin, Tennyson, and Duffy, 1966; Davis and Koelle, 1967; Robinson and Bell, 1967; Hirano and Ogawa, 1967). Therefore, the distribution of AChE at the close approaches of less than 1100 A between stained axons and smooth muscle cells in the vas deferens resembles the basic pattern for cholinergic synapses; and, in view of the evidence associating high levels of AChE and cholinergic function in the guinea pig (Bell and McLean, 1967), these close approaches probably represent cholinergic junctions.

A possible functional explanation of the constant association of postsynaptic stain with closest-points of less than 1100 A, and the almost constant lack of postsynaptic stain at contacts of greater than this distance (Fig. 8) can be found in the work of Bennett and Merrillees (1966) on transmission in the guinea pig vas deferens. After combining data from microelectrode and electron microscope serial sampling studies, they concluded: "if transmitter is released only from varicosities, then only varicosities within about 1000 A of the muscle membrane will have an appreciable effect during transmission." If this suggestion is correct, only those cholinergic junctions which have an appreciable effect during transmission show AChE stain on the postsynaptic membrane, and those approaches with a separation too great to allow appreciable transmission rarely show postsynaptic stain.

The data in Fig. 8 suggest a further possible correlation with the theoretical calculations. No Schwann cell process intervened between axon and muscle cell at the postsynaptically stained (probably functional) approaches with a separation of less than 1100 A, but Schwann cell processes intervened in 80% of all the approaches with a separation of greater than 1100 A and less than 4000 A. However, in view of the exceptions and the small size of the sample, no definite conclusions can be reached on a possible relationship at this stage.

The lamina propria of the guinea pig vas deferens is innervated by axons containing AChE (Jakobowitz and Koelle, 1965), and some of the stained axons detected in the smooth muscle tissue in the present work may be preterminal portions of these fibers. Nevertheless, the description of cholinergic synapses reported here and the evidence for cholinergic junctions from transmission studies, taken together, form a good case for separate cholinergic axons innervating the smooth muscle in the vas deferens.

The lack of detectable stain in 75% of the axons seen in this study, while not conclusive, sug-

[1] Bell, C. Unpublished data.

gested that AChE, and presumably also ACh, are absent from the adrenergic axons of the guinea pig. This view is supported by a report of a number of unstained axons innervating the guinea pig heart (Hirano and Ogawa, 1967) and by a similar conclusion about the adrenergic axons of the cat based on combined electron microscope radioautographic and AChE studies of the innervation of the nictitating membrane and pancreatic arterioles (Esterhuizen, Graham, Lever, and Spriggs, 1968; and Graham, Lever, and Spriggs, 1968).

I would like to thank Prof. G. Burnstock and Dr. N. Merrillees for their encouragement and advice. I am also grateful to Drs. C. Bell and M. Bennett for valuable discussions.

This work was supported by the National Health and Medical Research Council of Australia, the Australian Research Grants Commission, and the National Heart Foundation of Australia.

Received for publication 26 September 1968, and in revised form 17 December 1968.

REFERENCES

ANSON, M. L. 1942. Some factors which influence the oxidation of sulfhydryl groups. *J. Gen. Physiol.* **25**:355.

BAYLISS, B. J., and A. TODRICK. 1956. The use of selective acetylcholinesterase inhibitors in the estimation of pseudocholinesterase activity in rat brain. *Biochem. J.* **62**:62.

BELL, C. 1967. An electrophysiological study of the effects of atropine and physiostigmine on transmission to the guinea-pig vas deferens. *J. Physiol. (London).* **189**:31.

BELL, C., and J. R. MCLEAN. 1967. Localization of norepinephrine and acetylcholinesterase in separate neurons supplying the guinea-pig vas deferens. *J. Pharmacol. Exp. Ther.* **157**:69.

BENNETT, M. R., and N. C. R. MERRILLEES. 1966. An analysis of the transmission of excitation from autonomic nerves to smooth muscle. *J. Physiol. (London).* **185**:520.

BIRMINGHAM, A. T. 1966. The potentiation by anticholinesterase drugs of the responses of the guinea-pig isolated vas deferens to alternate preganglionic and post-ganglionic stimulation. *Brit. J. Pharmacol. Chemother.* **27**:145.

BIRMINGHAM, A. T., and A. B. WILSON. 1963. Preganglionic and post-ganglionic stimulation of the guinea-pig vas deferens preparation. *Brit. J. Pharmacol. Chemother.* **21**:569.

BLOOM, F. E., and R. J. BARRNETT. 1966. Fine structural localization of acetylcholinesterase in electroplaque of the electric eel. *J. Cell Biol.* **29**:475.

BRZIN, M., V. M. TENNYSON, and P. E. DUFFY. 1966. Acetylcholinesterase in frog sympathetic and dorsal root ganglia. *J. Cell Biol.* **31**:215.

BURN, J. H., and M. J. RAND. 1959. Sympathetic post-ganglionic mechanism. *Nature (London).* **184**: 163.

BURN, J. H., and M. J. RAND. 1965. Acetylcholine in adrenergic transmission. *Ann. Rev. Pharmacol.* **5**:163.

BURNSTOCK, G., and M. E. HOLMAN. 1961. The transmission of excitation from autonomic nerve to smooth muscle. *J. Physiol. (London).* **155**:115.

BURNSTOCK, G., and M. E. HOLMAN. 1962. Effect of denervation and of reserpine treatment on transmission at sympathetic nerve endings. *J. Physiol. (London).* **160**:461.

BURNSTOCK, G., and M. E. HOLMAN. 1964. An electrophysiological investigation of the action of some autonomic blocking drugs on transmission in the guinea-pig vas deferens. *Brit. J. Pharmacol. Chemother.* **23**:600.

BURNSTOCK, G., and P. M. ROBINSON. 1967. Localization of catecholamines and acetylcholinesterase in autonomic nerves. *Circ. Res.* **20** (*suppl. 3*):43.

DAVIS, R., and G. B. KOELLE. 1967. Electron microscopic localization of acetylcholinesterase and nonspecific cholinesterase at the neuromuscular junction by the gold–thiocholine and gold–thiolacetic acid methods. *J. Cell Biol.* **34**:157.

ESTERHUIZEN, A. C., J. D. P. GRAHAM, J. D. LEVER, and T. L. B. Spriggs. 1968. Catecholamines and acetylcholinesterase distribution in relation to noradrenaline release. An enzyme histochemical and autoradiographic study on the innervation of the cat nictitating muscle. *Brit. J. Pharmacol. Chemother.* **32**:46.

GIACOBINI, E. 1957. Quantitative determination of cholinesterase in individual sympathetic cells. *J. Neurochem.* **1**:234.

GRAHAM, J. D. P., J. D. LEVER, and T. L. B. SPRIGGS. 1968. An examination of adrenergic axons around pancreatic arterioles of the cat for the presence of acetylcholinesterase by high resolution autoradiographic and histochemical methods. *Brit. J. Pharmacol. Chemother.* **33**:15.

HERRIOTT, R. M. 1947. Reactions of native proteins with chemical reagents. *Adv. Prot. Chem.* **3**:169.

HIRANO, H., and K. OGAWA. 1967. Ultrastructural localization of cholinesterase activity in nerve endings in the guinea-pig heart. *J. Electron Microsc.* **16**:3131.

HUKOVIC, S. 1961. Responses of the isolated sympathetic nerve–ductus deferens preparation of the guinea-pig. *Brit. J. Pharmacol. Chemother.* **16**:188.

JACOBOWITZ, D., and G. B. KOELLE. 1965. Histo-

chemical correlations of acetylcholinesterase and catecholamine in post-ganglionic autonomic nerves of cat, rabbit and guinea-pig. *J. Pharmacol. Exp. Ther.* **148**:225.

KARNOVSKY, M. J. 1964. The localization of cholinesterase activity in rat cardiac muscle by electron microscopy. *J. Cell Biol.* **23**:217.

KARNOVSKY, M. J., and L. ROOTS. 1964. A "direct-coloring" thiocholine method for cholinesterases. *J. Histochem. Cytochem.* **12**:219.

KOELLE, G. B. 1962. A new general concept of the neurohumoral functions of acetylcholine and acetylcholinesterase. *J. Pharm. Pharmacol.* **14**:65.

KOELLE, G. B. 1963. Cytological distribution and physiological functions of cholinesterase. *In* Handbuch der Experimentallen Pharmakologie. G. B. Koelle, editor. Springer-Verlag, Berlin. Suppl. **15**:187.

MCILWAIN, H., and H. L. BUDDLE. 1953. Techniques in tissue metabolism. 1. Tissue chopper. *Biochem. J.* **53**:412.

MERRILLEES, N. C. R. 1968. The nervous environment of individual smooth muscle cells of the guinea-pig vas deferens. *J. Cell Biol.* **37**:794.

MERRILLEES, N. C. R., G. BURNSTOCK, and M. E. HOLMAN. 1963. Correlation of fine structure and physiology of the innervation of smooth muscle in the guinea-pig vas deferens. *J. Cell Biol.* **19**:529.

MIRSKY, A. E., and M. L. ANSON. 1936 *a*. Sulphydryl and disulfide groups of protein. *J. Gen. Physiol.* **19**:439.

MIRSKY, A. E., and M. L. ANSON. 1936 *b*. The reducing groups of protein. *J. Gen. Physiol.* **19**:451.

NACHMANSOHN, D. 1959. Chemical and molecular basis of nerve activity. Academic Press Inc., New York. 101.

OGAWA, K., T. SAITO, and H. MAYAHARA. 1968. The site of ferricyanide reduction by reductases within mitochondria as studied by electron microscopy. *J. Histochem. Cytochem.* **16**:49.

OHLIN, P., and B. C. R. STROMBLAD. 1963. Observations on the isolated vas deferens. *Brit. J. Pharmacol. Chemother.* **20**:299.

PEASE, D. C. 1964. Histological Techniques for Electron Microscopy. 2nd edition. Academic Press Inc., New York. 53.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.

ROBINSON, P. M., and C. BELL. 1967. The localization of acetylcholinesterase at the autonomic neuromuscular junction. *J. Cell Biol.* **33**:93.

SJÖSTRAND, N. O. 1965. The adrenergic innervation of the vas deferens and accessory male genital glands. *Acta Physiol. Scand.* **65** (Suppl. 257):1.

SMITH, R. E., and M. G. FARQUHAR. 1965. Preparation of non-frozen sections for electron microscope cytochemistry. *Sci. Instrum. (News).* **10**:13.