THE LOCALIZATION OF ACETYLCHOLINESTERASE AT THE AUTONOMIC NEUROMUSCULAR JUNCTION

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ABSTRACT

Acetylcholinesterase has been localized at the autonomic neuromuscular junction in the bladder of the toad (*Bufo marinus*) by the Karnovsky method. High levels of enzyme activity have been demonstrated in association with the membranes of cholinergic axons and the adjacent membranes of the accompanying Schwann cells. The synaptic vesicles stained in occasional cholinergic axons. After longer incubation times, the membrane of smooth muscle cells close to cholinergic axons also stained. Axons with only moderate acetylcholinesterase activity or with no activity at all were seen in the same bundles as cholinergic axons, but identification of the transmitter in these axons was not possible.

INTRODUCTION

The localization of acetylcholinesterase (AChE) at the skeletal neuromuscular junction has been studied by light microscopy (Couteaux, 1958) and electron microscopy (Zacks and Blumberg, 1961; Barrnett, 1962, 1966; Miledi, 1964; Lewis, 1965; Davis and Koelle, 1965), and its role in the cholinergic transmission process at that site is well established (Fatt and Katz, 1951; Takeuchi and Takeuchi, 1959).

Autonomic axons do not synapse with smooth muscle cells at specific end plate regions, but rather form "*en passage*" synapses at points of close apposition between axon varicosities and muscle cell membranes (Hillarp, 1946; Merrillees[1]). The transmission process at this type of junction has been studied most thoroughly in the guinea-pig vas deferens (Burnstock and Holman, 1961) where the predominant transmitter is noradrenaline (Burnstock and Holman, 1964; Sjöstrand, 1965). On the other hand, pharmacological evidence indicates that the bladder of the toad (*Bufo marinus*) is innervated almost entirely by cholinergic nerves (Burnstock et al. 1963; Bell and Burnstock, 1965). In support of this evidence, histochemical staining for cholinesterase has revealed high levels of AChE associated with almost all the axons, and lower levels of diffuse activity in the muscle bands (Bell, 1967). Fluorescent histochemical methods have revealed only a few catecholamine-containing axons supplying the muscle (McLean and Burnstock, 1966). The toad bladder, therefore, provided a suitable subject for the study of localization of AChE at the junction of cholinergic axons with smooth muscle cells and of the distribution of axons with differing cholinesterase content within the axon bundles.

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[1] Merrillees, N. C. R. 1966. The nervous environment of individual smooth muscle cells reconstructed from serial sampling with the electron microscope. Data in preparation.

METHODS

Toads were killed by pithing, and the body cavity opened. In initial experiments a piece of bladder then was removed from the animal and immediately fixed under moderate tension in ice-cold phosphate-buffered 4% formaldehyde at pH 7.3-7.5 containing 0.44 M sucrose. In subsequent series the primary formalin fixation was completed in situ by dropping cold fixative onto the lightly stretched bladder within the body cavity. The following steps were carried out at 0-4°C in an ice bath. After about 20 min of fixation the bladder was cut into pieces a few millimeters square and washed in 0.44 M sucrose for 1-3 hr. Since the bladder wall is very thin, these pieces were incubated whole in the incubation medium of Karnovsky (1964), buffered to pH 6.0 with phthalate, briefly washed again in 0.44 M sucrose, and postfixed in phosphate-buffered osmium tetroxide containing 0.44 M sucrose. Tissue pieces usually were incubated for 10, 15, 20, 30, and 40 min in each experiment because the activity of the enzyme differed from animal to animal. Sections of blocks containing tissue from each incubation duration were examined and the minimum duration of incubation (M.D.I.) necessary to show evidence of staining was noted for each animal. This provided a basis for comparison of the results of different experimental procedures carried out on different animals. Individual incubation times, therefore, are referred to the M.D.I. for that particular animal. In some experiments the specific AChE inhibitor BW284C51 (Austin and Berry, 1953; Bell and Burnstock, 1965) was present in both the post-formalin washing and incubation media at a concentration of 5×10^{-6} M. Controls were provided by tissue which was incubated in substrate-free medium and tissue which was not incubated at all. After rapid dehydration in acetone the tissue was embedded in Araldite (CIBA, Melbourne) or an Araldite/Epon mixture (Mollenhauer, 1964), cut with glass knives on a Huxley microtome, and viewed with a Siemens Elmiskop I.

RESULTS

Details of the fine structures of the axons, presynaptic organelles, and axon-muscle relationships in the toad bladder will be reported elsewhere. Briefly, bundles of nonmyelinated axons spread throughout the bladder between the bands of smooth muscle cells and give rise to smaller bundles which enter the muscle tissue. After repeated branching, the bundles are reduced to one or two axons which commonly are found passing within 1000 A of a muscle cell. These "terminal" portions of the axons may be free of Schwann cell. Vesicles are scarce in the axons in the large bundles, but are more common in the small bundles and single axons lying within the muscle bands. Autonomic axons in the toad lung and bladder contain vesicles with a wide range of diameters (from about 150 to 2000 A), the larger ones containing a dense core after routine osmium-tetroxide fixation (Robinson, 1965). After primary formalin fixation vesicles of the same sizes are seen, but none have a dense core.

It has been reported previously that the ratio of pseudo ChE to AChE in the toad bladder is very low (Bell and Burnstock, 1965). This also has been reported for frog and toad central nervous tissue (Shen et al. 1955; Chacko and Cerf, 1960). In the present study all staining was absent after prior incubation with the specific AChE inhibitor BW284C51. Therefore it has been assumed that all observed activity was due to AChE.

Acetylcholinesterase Localization

The majority of axons of the toad bladder were associated with a high level of AChE activity. This activity was localized on the membrane of the axon and was also present on the adjacent part of the membrane of the accompanying Schwann cell (Figs. 1 and 7). Increasing the time of incubation in the staining medium resulted in an increased deposit of stain between the two membranes, frequently obliterating the cleft between the cells (Fig. 1 and 2). A small number of axons did not stain for AChE. These axons sometimes ran in the same bundles as those associated with AChE (Figs. 4 and 7), and sometimes formed the whole of the bundle (Fig. 3). No AChE activity was associated with the membrane of the Schwann cell adjacent to axons which did not contain the enzyme.

Occasionally, axons with only moderate activity in the membrane were found. These axons could be recognised reliably only when they occurred in bundles which also contained axons associated with high levels of activity as in Fig. 4.

At the level of resolution possible with the light microscope the axons of the toad bladder appear to be stained continuously along their length (Bell, 1967). All reacting axons which were traced through a series of sequential sections in the present study showed evidence of staining in each section (Fig. 7). Consequently, although extended serial sectioning has not been attempted, the evidence indicates that AChE is not restricted to localized regions along the terminal part of each axon.
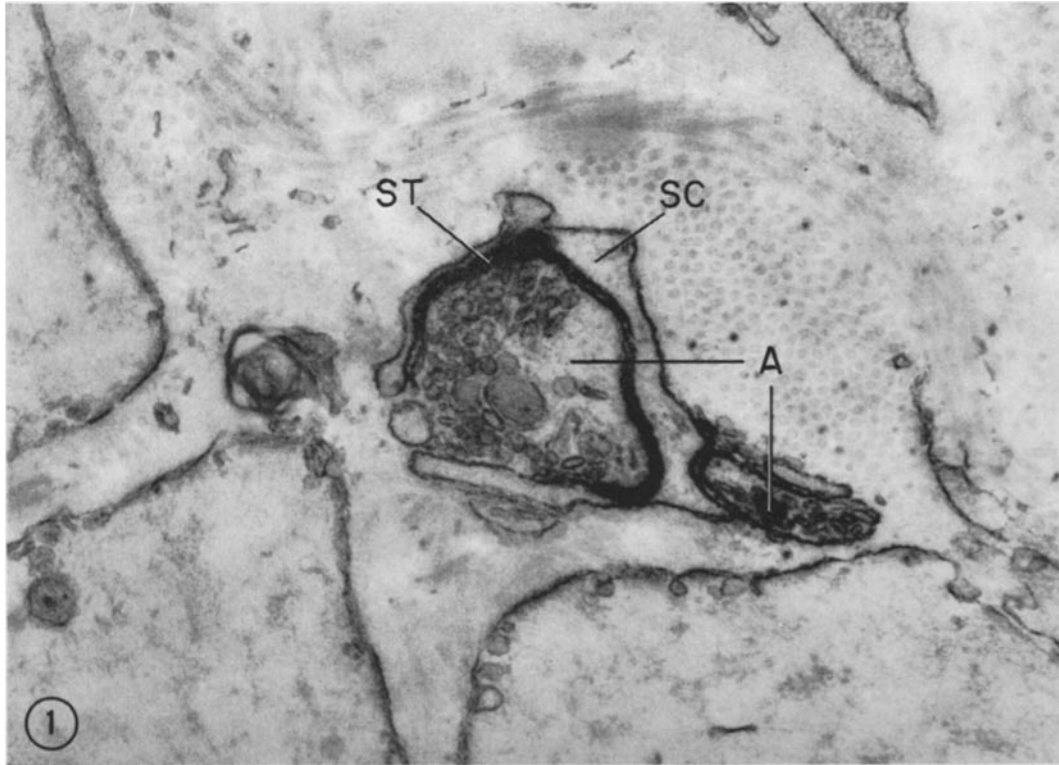
FIGURE 1   A pair of axons (A) and a Schwann cell process (SC). A heavy deposit of stain (ST) can be seen between the membranes of the larger axon and the Schwann cell, and a lighter deposit on the membrane of the smaller axons. This section is from tissue fixed in situ, but there are a few fine grains around the bundle indicating a little enzyme diffusion. Incubation duration 30 min. (2 × M.D.I.) × 35,000.

Because of the small number of larger vesicles seen in this tissue, it has not been possible to establish any definite relationship between the vesicle size and the association of AChE with the axon. However, it is quite clear that vesicles of all sizes occur in the axons associated with high levels of AChE (Fig. 7). In a small number of staining axons the vesicles also stained for AChE (Figs. 5 and 7).

In addition to the enzyme sites associated with the axon, areas of smooth muscle membrane close to axons which stained for AChE also showed evidence of activity (Fig. 5). This postjunctional site was seen only in tissue pieces which had been incubated for three times as long as the period necessary to show the first evidence of axon-bound enzyme in that animal.

Striking differences in enzyme distribution were seen when the details of fixation procedure were changed. In sections of tissue dissected from the animal and immediately placed in fixative, the reaction product was distinctly granular, and each stained axon was surrounded by a cloud of stain (Fig. 2). In bundles with a mixed population of axons, the cloud was localized around stained axons only (Fig. 7). This appearance strongly suggested some type of diffusion from the axon. Shortened incubation times resulted in a reduced cloud, but it was always present when there was some reaction in the axon. On the other hand, when the tissue was fixed in situ, evidence of diffusion was completely absent from around the majority of axons (Figs. 1 and 4). This indicated that diffusion of enzyme was occurring from the axon after the tissue was excised but before fixation had begun at that site.

DISCUSSION

Within the framework of the Karnovsky method (Karnovsky and Roots, 1964; Karnovsky, 1964)
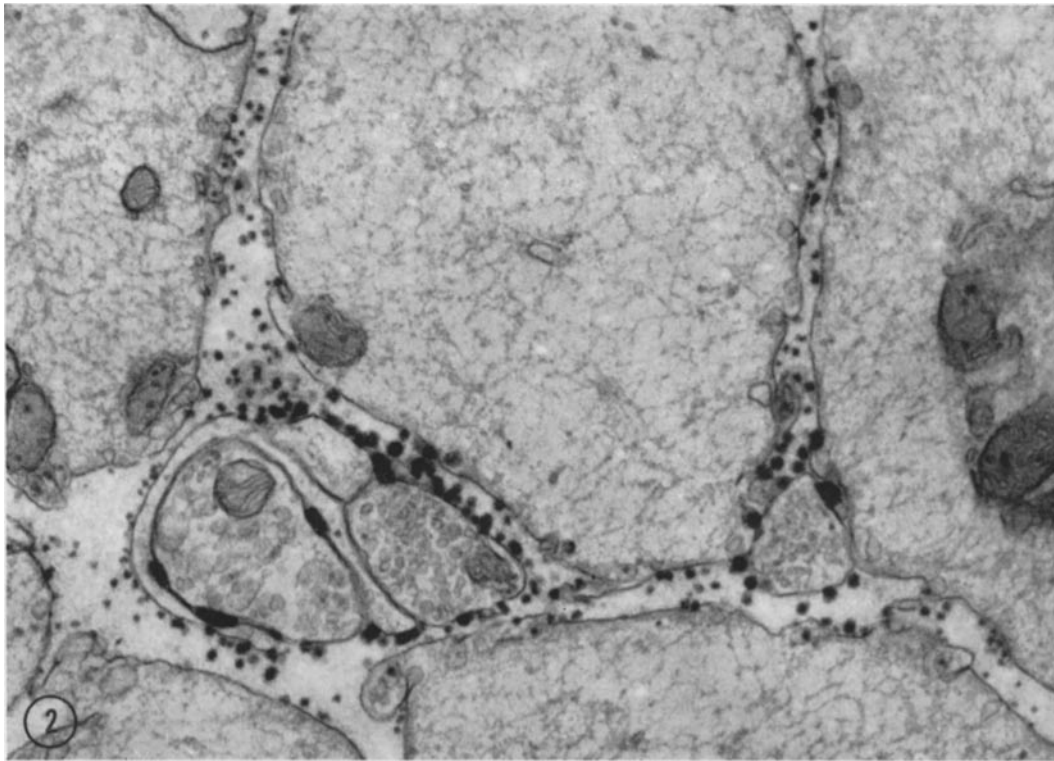
FIGURE 2 A small bundle of axons in a piece of tissue fixed after removal from the animal. Considerably less stain is seen on the membranes of the axons and Schwann cell than is shown in Fig. 1, and there is clear evidence of diffusion. Incubation duration 20 min. (2 × M.D.I.) × 35,000.

there are a number of variables which have an important bearing on the results reported here.

Firstly, the primary formalin fixation may inactivate the enzyme. To reduce this effect the duration of fixation was kept as short as possible. Adequate preservation of the fine structure occurred with 10–15 min fixation, and routine times never exceeded 20 min.

Secondly, diffusion can be considered a major source of artefact in enzyme histochemistry, particularly at the level of resolution possible with the electron microscope. Diffusion may occur before or after fixation.

Lison (1948) was the first to point out that soluble fractions of an enzyme may diffuse through tissue during histochemical preparation. Since then, it has been demonstrated clearly that most of the enzymes that have been studied by histochemical methods consist of a soluble or "lyo" fraction and an insoluble or "desmo" fraction (Nachlas et al., 1956; Hannibal and Nachlas,

1959), and that lyo-enzyme will diffuse from the primary site unless it is precipitated by high concentration of salts (Koelle, 1950, 1951) or by fixation (Nachlas et al. 1956; Eränkö et al., 1964). The work of Koelle (1950, 1951) also showed that the diffusing lyo component of AChE becomes attached to cell and nuclear membranes. It seems reasonable to assume that the lyo fraction is bound in life and begins to diffuse on cell death. In most tissues cell death will begin as soon as the fixative reaches the cell membrane, so that fixation and cell death occur simultaneously and enzyme diffusion does not occur. In smooth muscle, however, axons run through the tissue for long distances, and every axon is cut and isolated from its cell body when the piece of tissue is removed from the animal. The enzyme diffusion present, when excised tissue was fixed, indicates that this cutting is sufficiently traumatic to allow release of enzyme from binding sites. Because no additional sites were demonstrated after fixation in situ, the

FIGURE 3 A bundle of axons showing no evidence of enzyme activity. Other axons in the same section almost were obliterated by very heavy deposits of stain. Incubation duration 40 min. (4 × M.D.I.) × 35,000.

lyo-enzyme must have diffused from sites identical with the desmo-enzyme sites previously seen.

Diffusion at later stages in the histochemical process also may be a source of error. Although Karnovsky (1964) stated that no diffusion was present in the heart using his method, some diffusion occurred in the toad bladder after the longer incubation times in the present study. This diffusion is seen in its typical form in Fig. 5 where it appears as many fine spots of stain near heavily stained sites. It is possible, therefore, to interpret, for instance, the stain in Schwann membrane, muscle membrane, and synaptic vesicle as artefacts caused by diffusion during the incubation period. Although the Schwann membrane staining was associated exclusively with enzyme-containing axons, it appeared in some of the most lightly stained specimens in which no diffuse reaction product was visible between the two membranes. It is unlikely, therefore, that the staining in the Schwann membrane was due to

diffusion during incubation. However, the other sites, which appear only after longer incubation, are more open to question.

A postjunctional localization of AChE at the motor end plate has been reported by Barrnett (1962) and Davis and Koelle (1965). At this site AChE is known to hydrolyse acetylcholine released by the axon, terminating its action on the muscle membrane (Fatt and Katz, 1951; Takeuchi and Takeuchi, 1959). The demonstration that treatment of the isolated toad bladder with the anticholinesterase, neostigmine, produces a marked potentiation of the response to nerve stimulation (Bell and Burnstock, 1965) supports a postjunctional role for the enzyme in this organ. Bennett and Merrillees (1966), during an examination of transmission from autonomic nerves to the smooth muscle cells of the guinea pig vas deferens, concluded that only a transmitter released close to a muscle cell (in this case 1000 A) was capable of exerting an effect. If a similar relationship holds
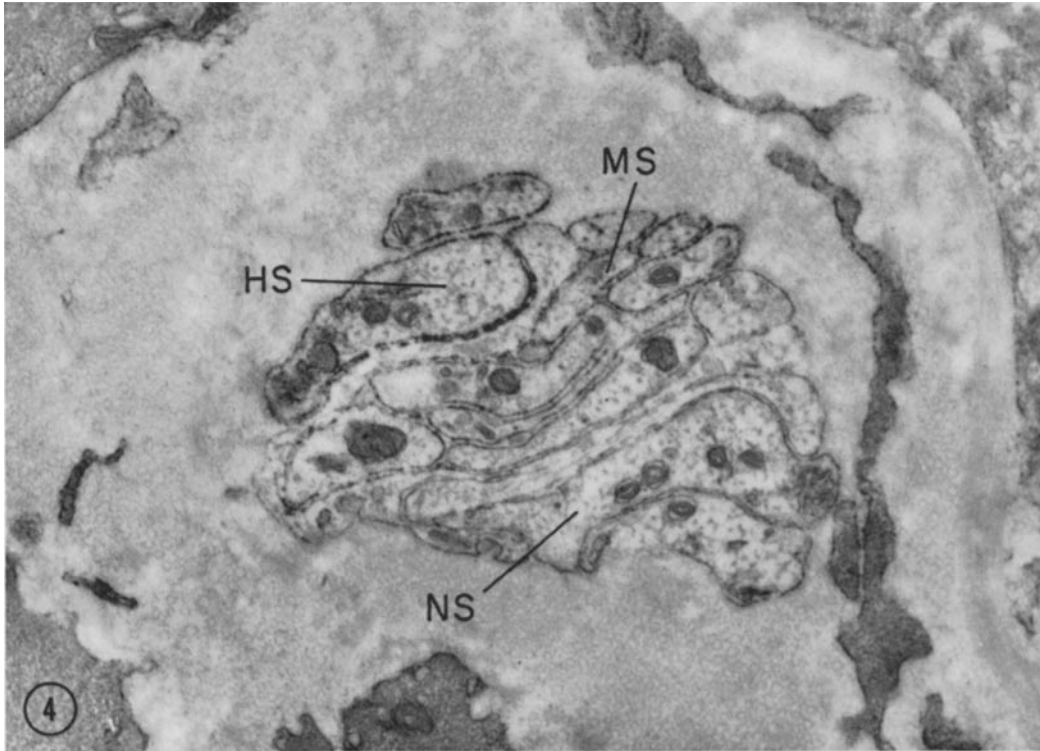
FIGURE 4 A bundle of axons with different enzyme activities. This is typical of the larger bundles and shows heavily stained (HS), moderately stained (MS), and nonstaining (NS) axons running together. In this case, as in many others with moderate incubation duration, it is difficult to separate a weak positive reaction and a negative reaction. However, some axons still show an apparently negative reaction after the longest incubation times used in this study. Incubation duration 30 min. (2 × M.D.I.) × 20,000.

true for the toad bladder, it might be expected that an enzyme concerned with transmitter inactivation would be concentrated at areas of close contact between axons and muscle cells.

The staining of synaptic vesicles has been reported at the motor end plate by Barrnett (1962, 1966) using thiolacetic acid as substrate, and by Miledi (1964) using acetylthiocholine. However, other studies using these substrates have not reported vesicle staining (Lewis, 1965; Davis and Koelle, 1965). In the present study of the toad bladder, vesicle staining only occurred in a small proportion of the heavily stained axons. The effect cannot be correlated with any morphological artefact, such as a broken axon membrane which is sometimes present at these sites (Fig. 5), because these artefacts occur in other axons without vesicle staining and, of course, it is not clear whether this damage occurred before or after incubation. For these reasons, and in view of the different appearance of the vesicle stain, no

definite opinion can be expressed about the significance of this apparent enzyme site at the present time.

A knowledge of the amount of AChE associated with an axon may be of assistance in the identification of its transmitter (Koelle 1955; Sjöqvist 1963a and b). Cholinergic neurons from motor nuclei and spinal cord contain high levels of AChE (Koelle, 1951; Giacobini, 1959), and it has been inferred that the rare neurons with high AChE activity in the cat sympathetic ganglion are also cholinergic (Sjöqvist, 1963a and b; Hamberger et al., 1963). However, in the rat superior cervical ganglion (Härkönen, 1964), neurons which contain AChE also may contain catechol amine. Furthermore, quantitative analysis of single neurons isolated from sympathetic ganglia of many species has revealed a wide range of individual AChE activities. Although only a small number showed high levels of AChE comparable with those existing in known cholinergic neurons,
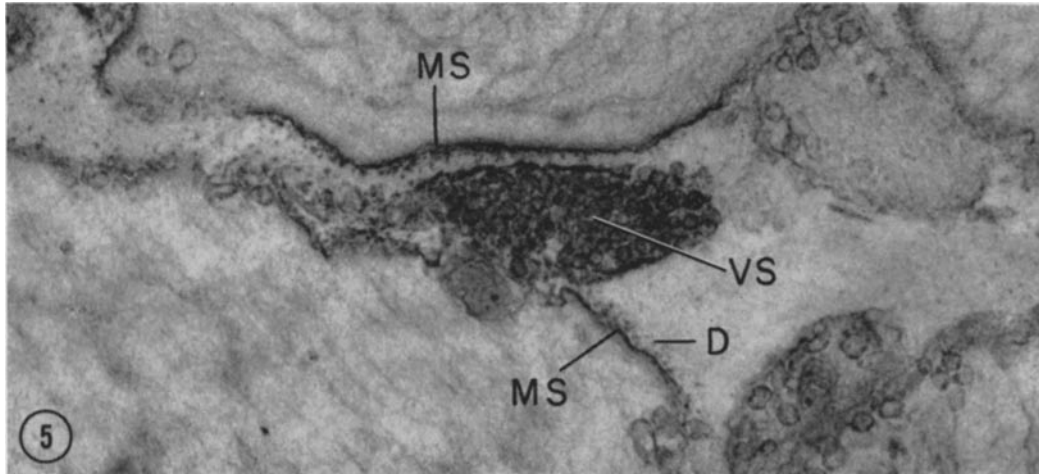
FIGURE 5 An axon in close proximity to two smooth muscle cells. There is clear evidence of vesicle staining (*VS*) and muscle membrane staining (*MS*). There is also some evidence of diffusion (*D*). As this specimen was fixed in situ, enzyme diffusion can be discounted, and reaction product diffusion during the rather long incubation period is the most likely explanation. The small size of the grains of precipitate and their proximity to the muscle membrane and axon membrane sites are quite characteristic of specimens incubated for relatively long periods. The evidence of diffusion from the muscle membrane and the even distribution of stain throughout the vesicles suggests that these are genuine enzyme sites and not artefacts due to diffusion from the axon membrane. Incubation duration 30 min. (3 × M.D.I.) × 35,000.

the majority of neurons tested showed some activity (Giacobini, 1959).

It seems likely, therefore, that sympathetic adrenergic axons may be associated with AChE as well as catechol amine in a number of species. This means that the histochemical demonstration of AChE in an axon does not identify automatically that axon as cholinergic in the accepted sense. Burn and Rand (1959, 1962) and Koelle (1961) have proposed hypotheses in which acetylcholine is involved in the transmission process at sympathetic nerve terminals, and the presence of catechol amine and AChE in the same neuron has been regarded as evidence in favor of these hypotheses (Härkönen, 1964).

In the present study of the toad bladder, three classes of axon have been distinguished: heavily stained, moderately stained, and nonstaining. The great majority of the axons fall into the first category, and only a small number in the other two classes have been seen. It may be that the relative numbers of axons in each class will depend on the duration of incubation, as these classes in all probability represent a continuous gradation of AChE content (Giacobini, 1959). However, proof of this point would require a detailed statistical analysis which has not been attempted. Pharmacological evidence (Burnstock et al., 1963; Bell and Burnstock, 1965) indicates that the innervation of this organ is predominantly cholinergic, and catechol amine–containing axons are rare (McLean and Burnstock, 1966). Therefore, it is fairly certain that the heavily stained axons which constitute the bulk of the population are cholinergic. The moderately stained axons are rare and may represent cholinergic axons with low levels of AChE, *or* adrenergic axons of the type suggested by Burn and Rand (1959). It would be expected that some of the axons in the tissue are sensory, but, since no morphological criteria for the identification of sensory axons in single sections have been established yet, their proportion is unknown. Since sensory neurons may show low levels of AChE activity (Koelle, 1955), sensory axons could be represented by either the moderately staining or nonstaining class of axon in this study.

FIGURE 6   A very large bundle of axons in a piece of tissue fixed after removal from the animal. Stained and nonstained axons are segregated into separate groups, suggesting that axons of similar origin tend to remain together. Incubation duration 30 min. (2 × M.D.I.) × 10,000.

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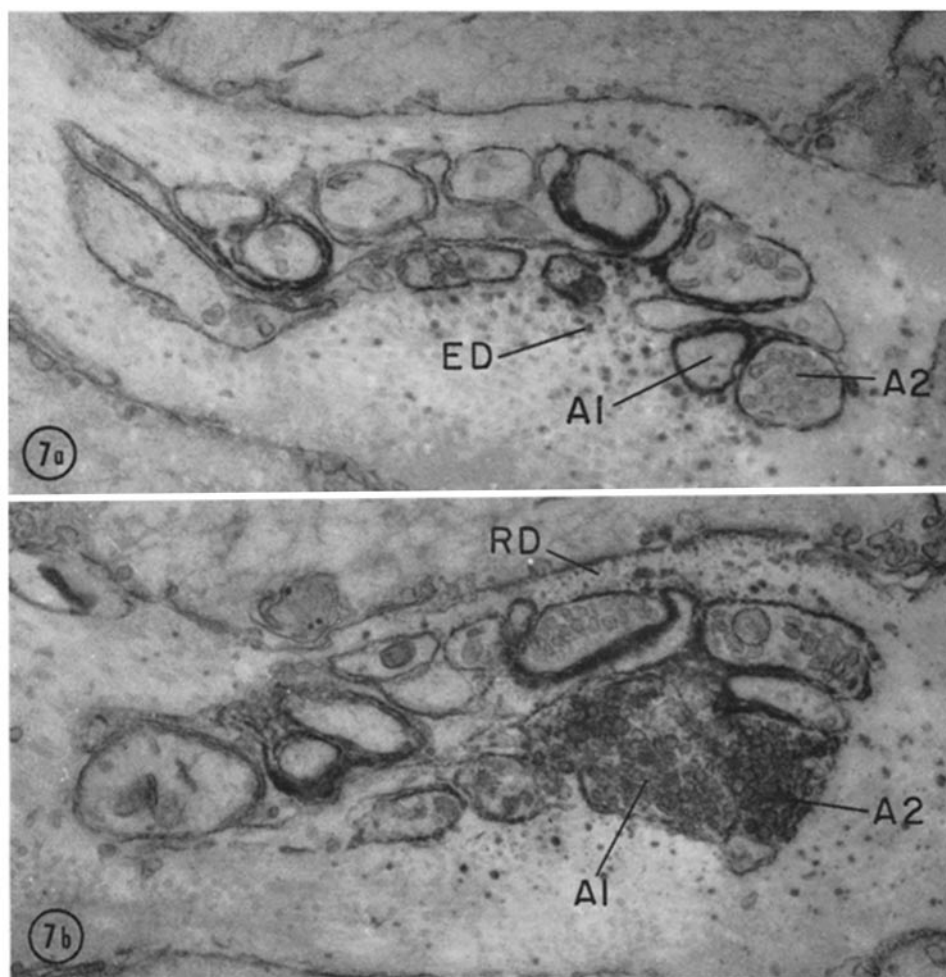BARRNETT, R. J. 1966. Ultrastructural histochemistry

FIGURE 7 Two micrographs from a set of serial sections which illustrate the continuity of enzyme activity along an axon. Two stained axons (*A1* and *A2*) are seen sectioned through intervaricose regions in Fig. 7 *a*, and through varicosities in Fig. 7 *b*, about 2 μ away. The other axons, which also show continuity of staining reaction, are sectioned through intervaricose regions throughout the series. Although this tissue was fixed in situ, there is evidence of both enzyme diffusion (*ED*) and reaction product diffusion (*RD*) resulting from the long incubation. As reaction product diffusion will occur from primary and diffused enzyme sites, the combined diffusion pattern becomes quite complex and difficult to interpret. Incubation duration 30 min. (2 × M.D.I.) × 35,000. 7 *a*. Where the axons have been favorably sectioned, the separate deposit of stain on the axon membrane and Schwann membrane can be seen clearly. 7 *b*. Note the heavy vesicle staining in axons A1 and A2 in the varicosity region. The membranes separating these axons are not shown clearly in this section due to oblique sectioning, but reappear in later sections of the series.

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