A CYTOCHEMICAL AND ELECTRON MICROSCOPE STUDY OF CHANNELS IN THE SCHWANN CELLS SURROUNDING LOBSTER GIANT AXONS

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INTRODUCTION

It is widely presumed that Schwann cells and glial cells modify the interactions of neurons with the extracellular environment in several ways, in addition to the formation and maintenance of myelin. In particular, it is thought that these cell types control the composition of the medium that gains access to the neuronal surface (see, e.g., references 2, 7, 10, 14, 26, 29, 30) and perhaps also play active roles in transfer of specific molecules to neurons (16, 29).

Among the preparations that should be of use for the experimental study of relations between Schwann cells and axons are the giant neurons of invertebrates such as the lobster and squid. Previous work has led to the suggestion that low molecular weight and colloidal materials can traverse the Schwann cell sheath surrounding giant axons by passage through flat, slitlike channels formed by the overlapping and folding of Schwann cells (1, 4, 6, 10, 23, 30, 31). The present study indicates that in lobster walking limb nerves horseradish peroxidase gains access to the axon surface not only by these routes but also by passage through the networks of anastomosing tubules

found in the Schwann cell cytoplasm. Preliminary reports of the findings have been published (13, 15).

METHODS AND MATERIALS

Nerves were dissected from the walking limbs of lobsters, and bundles of axons were rinsed in crustacean Ringer's solution (0.465 M NaCl, 0.01 M KCl, 0.025 M $CaCl_2$) as previously described (8). Under all conditions used, the isolated nerves were capable of sustained conduction of repeated impulses. Many of the nerves studied were stimulated at 30–50 impulses per second for 20–45 min; such stimulation had no obvious effects on the Schwann cells.

In most cases, the nerves were soaked in horseradish peroxidase (Sigma Biochemicals, St. Louis, Type II; 0.05–0.2% in Ringer's solution at room temperature) for 20–45 min before fixation. Fixation usually was carried out in Karnovsky's (18) phosphate - buffered glutaraldehyde - paraformaldehyde mixture at room temperature for 1–3 hr. The tissue was then rinsed in ice-cold pH 7.4 buffer, either 0.1 M phosphate or 0.1 M cacodylate with 7% sucrose (28). For morphological studies, the tissue was rinsed for approximately 15 min and then was postfixed for 1–2 hr in ice-cold 1% osmium tetroxide in 0.1 M phosphate buffer (20), dehydrated in ethanol, and embedded in Epon (19). In several experiments, *en bloc* staining with uranyl acetate was employed (9).

For demonstration of peroxidase activity, the rinse after aldehyde fixation was prolonged to several hours or overnight. The tissue was then frozen on the head of a freezing microtome, incubated at room temperature in the medium of Graham and Karnovsky (11), and then postfixed in osmium tetroxide and embedded as above.

Cholinesterase activity was demonstrated by the methods of Karnovsky (17) after fixation for 1–2 hr in ice-cold 2–2.5% glutaraldehyde in pH 7.4, 0.1 M phosphate or cacodylate buffer. Rinsing, freezing, and postfixation were done as with the peroxidase-incubated tissue.

As controls for the enzyme incubations, tissue was incubated in substrate-free peroxidase or cholinesterase medium and nerves that had not been soaked in peroxidase were incubated in the full peroxidase medium. In all cases, sites described below as containing reaction product in incubated tissues were found in the controls to show no density likely to be confused with reaction product.

Thin sections for electron microscopy were cut on diamond knives and examined unstained, stained with lead citrate, or stained with uranyl acetate followed by lead (24, 32). Photographs, at initial magnifications of 2–17,000, were taken on an RCA EMU 3F microscope.

RESULTS

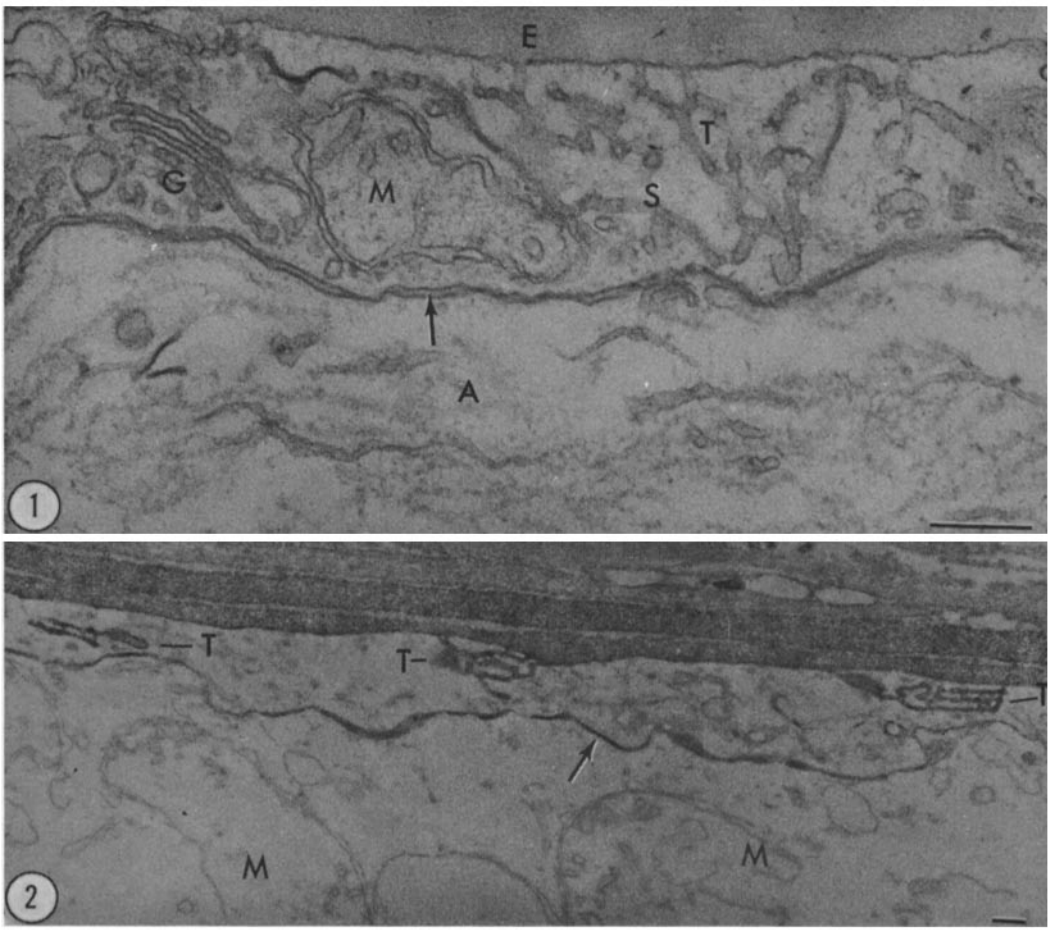
All observations reported are from study of the largest axons in the nerve (usually with diameters of 50–100 μ), and thus are obtained on systems in which a given Schwann cell is associated with a single axon (see references 4, 10, 31).

Reaction product for peroxidase is found primarily in the extracellular spaces in the nerves. It is present between the layers of connective tissue that surround the giant axons, in the axon–Schwann cell space, and in the flat channels crossing the Schwann cell sheath (Figs. 1–4). The flat channels represent the spaces between adjacent Schwann cells and between folded regions of a given cell. Broad (50–75 mμ or more in width) channels of this kind are most often seen in the outer part of the sheath and are continuous with the space outside the sheath (Fig. 5); the narrower channels (Figs. 3, 10) are continuous with the broad ones, with the space outside the sheath, and with the axon–Schwann cell space (see references 4, 8, 10, 31). The narrower channels and the axon–Schwann cell space are approximately 15–20 mμ in width.

In addition, peroxidase in the Schwann cell sheath is demonstrated within networks of anastomosing tubules (Figs. 1, 3, 4) that appear as patches spaced every few microns along the axon (Fig. 2). Although direct continuities with the space outside the sheath are sometimes seen, the tubules most often are found to be continuous with the Schwann cell plasma membranes at the axon–Schwann cell space and at the broad channels (Figs. 6 and 7). The impression gained from extensive study of nonserially sectioned material and limited work with serial sections is that the flat channels and the tubules form a single, elaborately interconnected system by which peroxidase can pass from outside the Schwann cell sheath to the axon–Schwann cell space.

Peroxidase reaction product in the Schwann cells also is found in coated vesicles approximately 100 mμ in diameter (Figs. 5 and 8). Some of the vesicles are seen to be attached to the anastomosing tubules (Fig 9).

When either acetylthiocholine or butyrylthiocholine is used as substrate, reaction product for cholinesterase is present in the spaces between connective tissue cells. With butyrylthiocholine, product also is found in the axon–Schwann cell space and in the anastomosing tubules (Fig. 10).



All figures are electron micrographs of giant axons and the Schwann cells associated with them. Bar length equals 0.25 μ in all micrographs.

FIGURE 1 Portion of a Schwann cell (*S*) and axon (*A*). The arrow indicates the axon–Schwann cell space, and *E*, the extracellular space outside the Schwann cell sheath. In the Schwann cell are seen Golgi apparatus (*G*), a mitochondrion (*M*), and a number of smooth-surfaced tubules (*T*). Stained *en bloc* with uranyl acetate and as a thin section with uranyl and lead. × 53,000.

FIGURE 2 Portion of a fiber fixed after exposure for 20 min to peroxidase and then incubated (45 min) for peroxidase activity. Reaction product is seen in the axon–Schwann cell space (arrow) and in three regions of anastomosing tubules (*T*) present in the Schwann cell. *M* indicates mitochondria in the axon. The thin section was stained lightly with lead. × 18,000.

DISCUSSION

Studies with several types of tracers on crab (1) and squid (30, 31) fibers and on vertebrate unmyelinated fibers (see references 12, 14, 26 for reviews and 29, 33, 34 for consideration of myelinated fibers) have indicated that the axon-Schwann cell space is reasonably accessible to material introduced in the extracellular medium outside the Schwann cell sheath. The present observations demonstrate this to be the case for lobster giant axons as well.

The penetrability of peroxidase into the tubule networks and the dimensions and distribution of the tubules suggest that the networks are a major

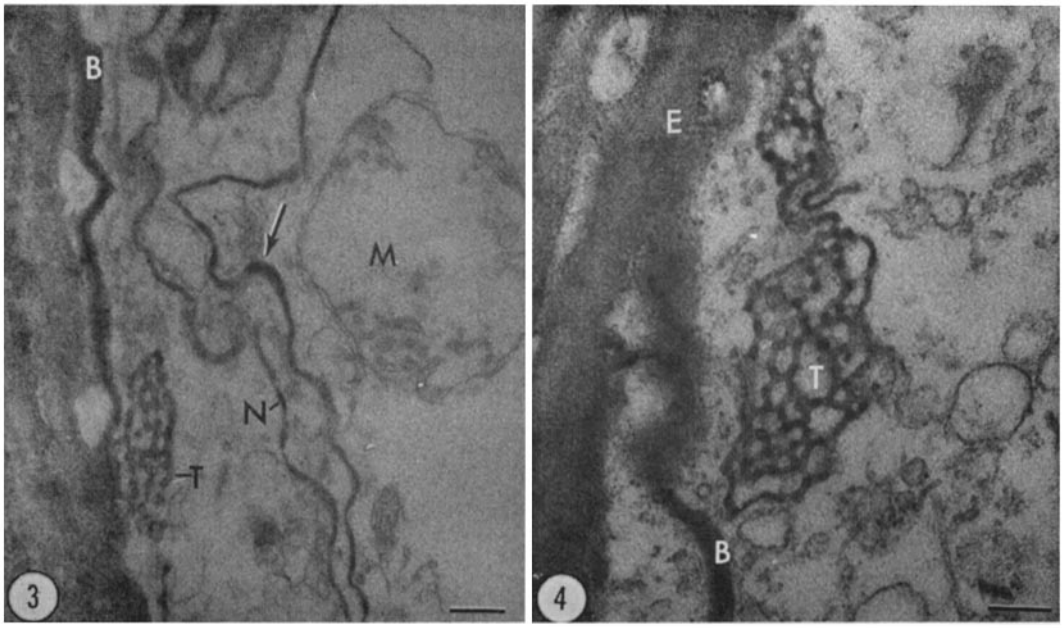
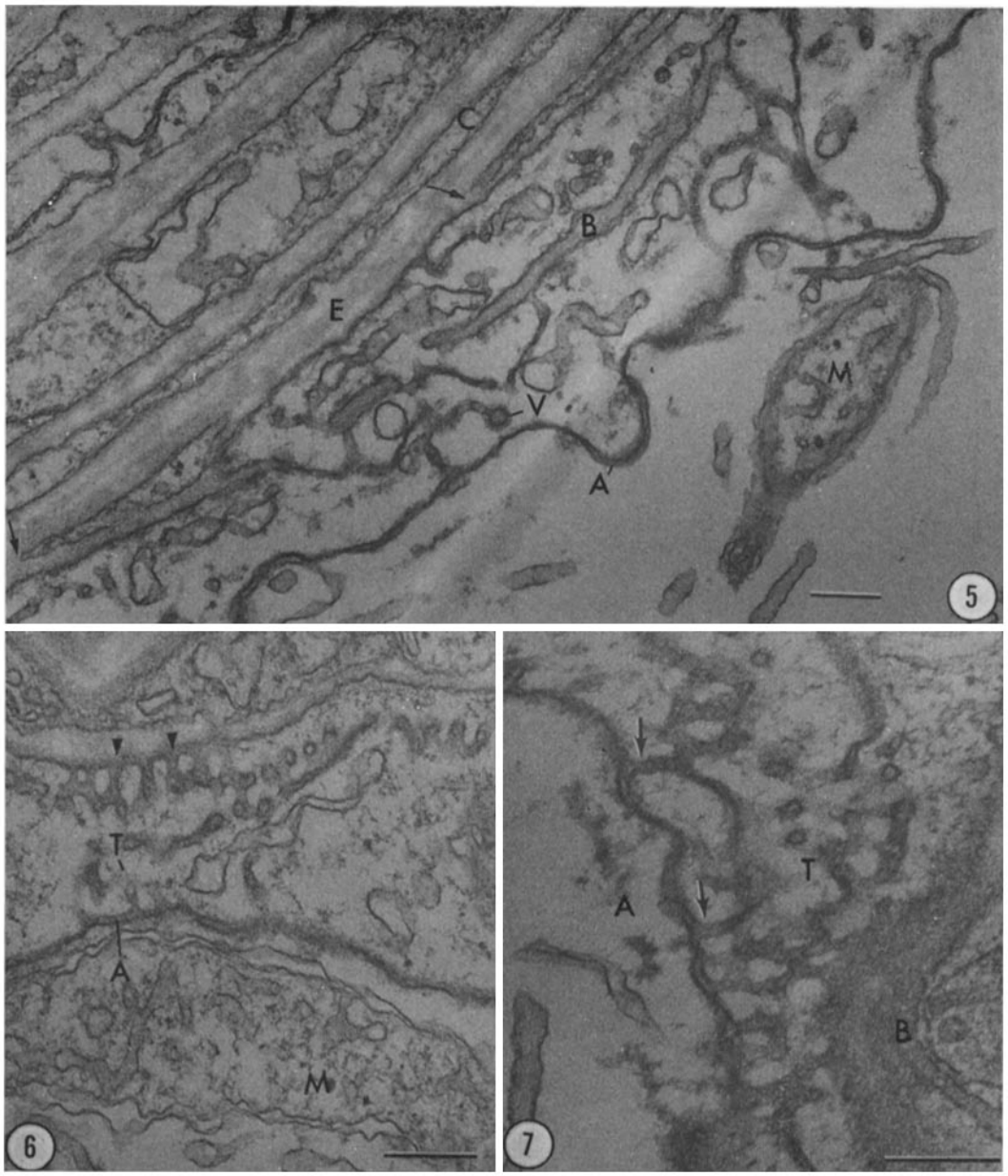


FIGURE 3 Portion of a fiber from the same nerve used for Fig. 2. Peroxidase reaction product is present in the axon–Schwann cell space (arrow) and in a broad channel (*B*) that separates two regions of Schwann cell cytoplasm. Some product also is present in a network of tubules (*T*) and in a narrow Schwann cell channel (*N*). A mitochondrion in the axon is seen at *M*. The thin section was stained lightly with lead. × 27,000.

FIGURE 4 Portion of a Schwann cell from a preparation fixed after 25 min in peroxidase and incubated (45 min) for peroxidase activity. Reaction product is present in a network of anastomosing tubules (*T*). Product also is seen in the extracellular space outside the Schwann cell sheath (*E*) and in the end of a broad channel (*B*). The thin section was stained with lead. × 31,000.

component of the extracellular pathway across the Schwann cell sheath of lobster walking limb axons. Initial peroxidase studies indicate that this is probably also true for the giant axons of the circumesophageal system in the lobster. In addition, although relevant tracer studies have not been reported, it is almost certain that a comparable channel system exists in several crayfish nerves. Networks of tubules have been observed in the Schwann cell cytoplasm (23, 25), and Peracchia and Robertson (discussion of their paper at the 1968 ASCB meetings, reference 22), and Pappas (personal communication) have noted connections between agranular Schwann cell tubules and the plasma membrane.

Presumably, the tubule systems contribute to the relatively rapid passage of ions and water across the Schwann cell sheath (2, 7, 8, 30, 31). It has been proposed (see, e.g., references 30, 31) that the composition of the medium passing to and from the axon surface is under the active control of the Schwann cell. The large surface area of the anastomosing tubule systems would facilitate exchanges between Schwann cell and extracellular fluid that might be involved in such control. The peroxidase-containing coated vesicles seen in the Schwann cells deserve further study in this connection. Vesicles of similar size and appearance have been reported to participate in pinocytosis in many tissues (see e.g. references 9, 12, 27), and pinocytosis can result in a somewhat selective absorption of material from the extracellular environment. The pinocytosis vesicles might also represent a phase in the circulation of membrane between the plasma membrane and intracellular systems although at present, such roles may be more appropriately sought in other systems (e.g. gland cells, neurons [12, 14]) where there are



FIGURES 5–7 are all from the same preparation, exposed for 45 min to peroxidase but not incubated. After fixation the material was stained *en bloc* with uranyl acetate. Subsequently, the thin sections were stained with uranyl acetate and lead. This procedure was found to increase the electron opacity of extracellular spaces and to provide good staining of membranes; these characteristics facilitate tracing of the channels. *M*, mitochondria in the axons.

FIGURE 5 *A*, the axon–Schwann cell space, *E*, the space outside the Schwann cell sheath; *C*, portions of connective tissue cells. Broad channels (*B*) are present within the Schwann cell; arrows, points of continuity with the space outside the sheath. *V*, coated vesicle in the Schwann cell. × 40,000.

FIGURE 6 Arrowheads show connections and near connections between Schwann cell tubules and the plasma membrane bordering a broad channel similar to the channels shown in Fig. 5. The tubules at *T* probably are connected to the axon–Schwann cell space (*A*). × 52,000.

FIGURE 7 *T*, a portion of a Schwann cell tubule system showing connections to the axon–Schwann cell space (arrows) and to a broad channel (*B*) of the type shown in Fig. 5 (*B*). The axon is seen at *A*. × 64,000.

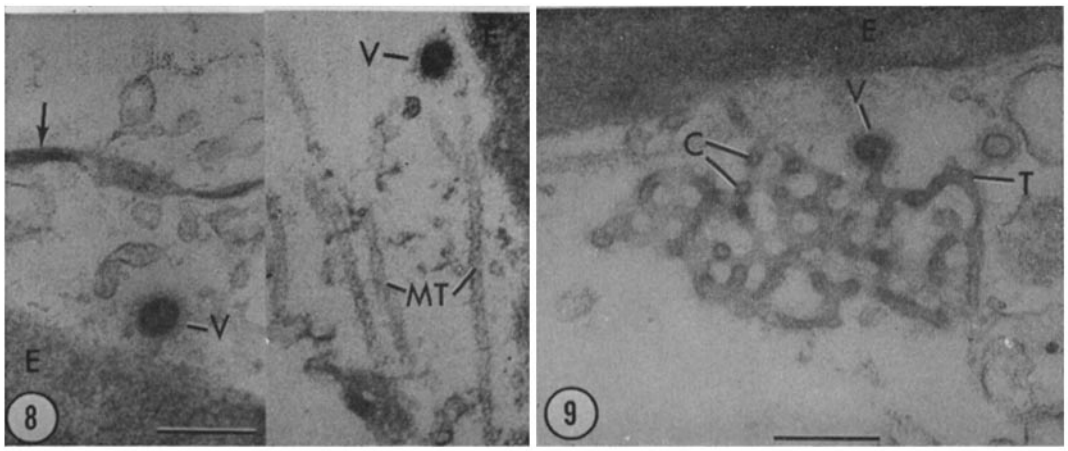


FIGURE 8 Portions of Schwann cells from two preparations of peroxidase-soaked material incubated (45 min) for demonstration of peroxidase activity. Reaction product is present in coated vesicles (*V*), in the axon–Schwann cell space (arrow), and in the extracellular space outside the Schwann cell sheath (*E*). *MT* indicates microtubules. The cell at the left was exposed to peroxidase for 30 min; the other (right) was exposed for 45 min. The thin sections were stained lightly with lead. × 52,000.

FIGURE 9 Portion of a Schwann cell from a nerve soaked in peroxidase for 25 min before fixation and then incubated (45 min) for peroxidase activity. *T*, tubules of an anastomosing network. The circular profiles at *C* presumably are cross-sections of the tubules. Some reaction product is seen in the tubules, but it is more highly concentrated in a coated vesicle (*V*) attached to the tubule system. The space outside the Schwann cell sheath is seen at *E*. The thin section was stained lightly with lead. × 57,000.

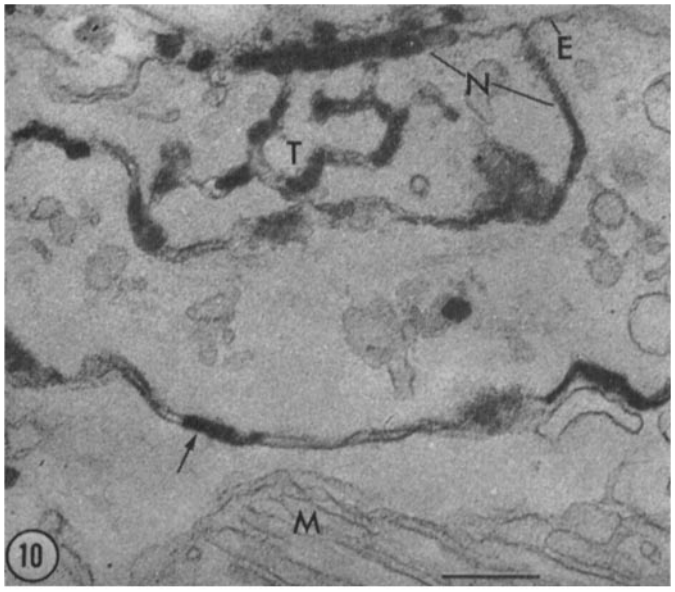


FIGURE 10 Portion of a fiber incubated (130 min) for butyrylcholinesterase activity. Reaction product is seen in Schwann cell tubules (*T*). Some product also is present in other channels (*N*) within the Schwann cell sheath and in the axon–Schwann cell space (arrow). *M*, a mitochondrion in the axon; *E*, the edge of the Schwann cell sheath. The thin section was lightly stained with lead. × 52,000.

known phases of addition of membranes to the plasma membrane (secretion, transmitter release).

Interpretation of the finding of butyrylcholinesterase activity in the networks must await clarification of the role of acetylcholine and related compounds in lobster nerves. We know of no previous study of the cholinesterase activity of the networks. However, from studies of other compartments of lobster nerves (5) and from the extensive work on vertebrate material (see e.g. 3, 21 for discussion and further references), it appears that one or another of the several types of cholinesterase is generally present in the cells surrounding axons and perikarya. The most obvious function the enzymes might serve is in preventing leakage in or out of agents with neurotransmitter-like properties. Further work with inhibitors and with varied fixation and incubation methods will be needed to evaluate the apparent inactivity of the network enzyme toward acetylcholine. For example, since bundles of axons were used in the present study, it is possible that penetration of adequate amounts of substrate to the giant axons was prevented by hydrolysis in the connective tissue.

Another possible artifact to be borne in mind whenever cell surface or extracellular enzymes are being studied is the spread of reaction product from an active site to a nearby inactive site. In the present work, there were no indications of the gradients in distribution of reaction product that might be expected to result from such spreading. Nor, in the limited observations made, were there consistent differences in the time during incubation at which product was seen at different sites.

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