

On the mechanism of the uptake of horseradish peroxidase into the retrograde transport system of ligated postganglionic sympathetic nerves *in vitro*

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INTRODUCTION

There are at least two routes by which horseradish peroxidase enters damaged axons: by diffusion through the damaged axonal membrane, and by pinocytosis within coated vesicles (Anderson, Mitchell & Mayor, 1979). The diffuse entry of horseradish peroxidase (HRP) into damaged axons *in vivo* occurs mainly during the first 60 minutes after nerve injury (Kristensson & Olsson, 1976), whereas the pinocytotic uptake of HRP by damaged axons *in vitro* continues for at least 24 hours after axotomy (Anderson *et al.* 1979).

The diffuse entry of HRP into damaged axons *in vivo* has been correlated with its subsequent retrograde transport (Kristensson & Olsson, 1976). However, *in vitro* studies using HRP-polylysine conjugate have emphasised the importance of the uptake of macromolecules in a membrane-bound form for retrograde transport (Anderson, Medlen, Mitchell & Mayor, 1981).

The purpose of the present study was to examine some of the characteristics of the uptake and retrograde transport of HRP by damaged postganglionic sympathetic axons *in vitro*. This involved a study of, firstly, the effect of delay between the time of nerve injury and the time of application of HRP and, secondly, the effects of low temperatures on the uptake of HRP in a form suitable for retrograde transport. Since low temperatures prevent pinocytotic uptake but do not prevent diffuse entry, this offers an opportunity to assess the importance of pinocytotic uptake for the subsequent retrograde labelling of the perikarya.

MATERIALS AND METHODS

Materials

Sigma type II HRP (Lot No. 78C-9950) was used in all experiments. Eagle's Minimal Essential Medium (MEM) was obtained from Gibco. 1 ml of 200 mM L-glutamine and 10000 units each of penicillin and streptomycin were added per 100 ml MEM.

A twin chamber apparatus, similar to that described by Banks, Mayor, Mitchell & Tomlinson (1971) but with smaller chambers, was used in all experiments.

Ganglion/nerve preparation

Male Hartley guinea-pigs weighing approximately 250 gm were anaesthetised with intraperitoneal Sagatal (May & Baker: 40 mg/kg body weight) and the hypogastric

nerves were ligated with fine (5/0) silk approximately 2.5 cm distal to the inferior mesenteric ganglion. The ganglion/ligated nerve preparation was then removed from the animal and suspended in a twin chamber apparatus so that the ganglion was positioned in one compartment with the hypogastric nerves extending through a silicone grease barrier to the ligation in the other compartment. In all experiments MEM, previously gassed with 95 % O₂ and 5 % CO₂, was immediately added to the compartment containing the ganglion. Both compartments were gassed with 95 % O₂ and 5% CO₂ whenever the apparatus was incubated at 37 °C.

Delay experiments

A solution of 20 mg HRP in 5 ml of previously gassed MEM at 37 °C was added to the compartment containing the ligated nerve, either as soon as the preparation was placed in the apparatus or after a delay of 3 hours or 17 hours. In experiments when there was a delay between setting up the preparation and addition of HRP, the compartment containing the ligated nerve contained MEM without HRP. The apparatus was kept in a water bath at 37 °C throughout the experiment. The ligated nerves were exposed to HRP for 24 hours in all experiments and the preparation was then fixed.

Cold experiments

Nerves treated with HRP at 0 °C, washed, and returned to 37 °C

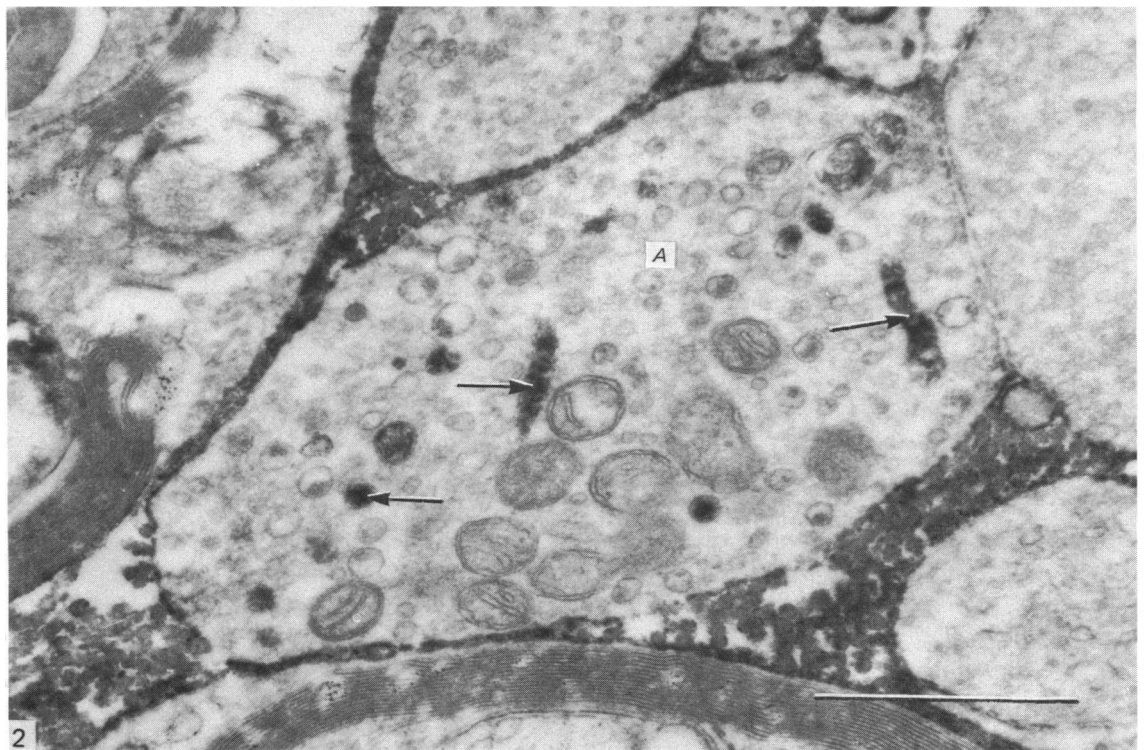
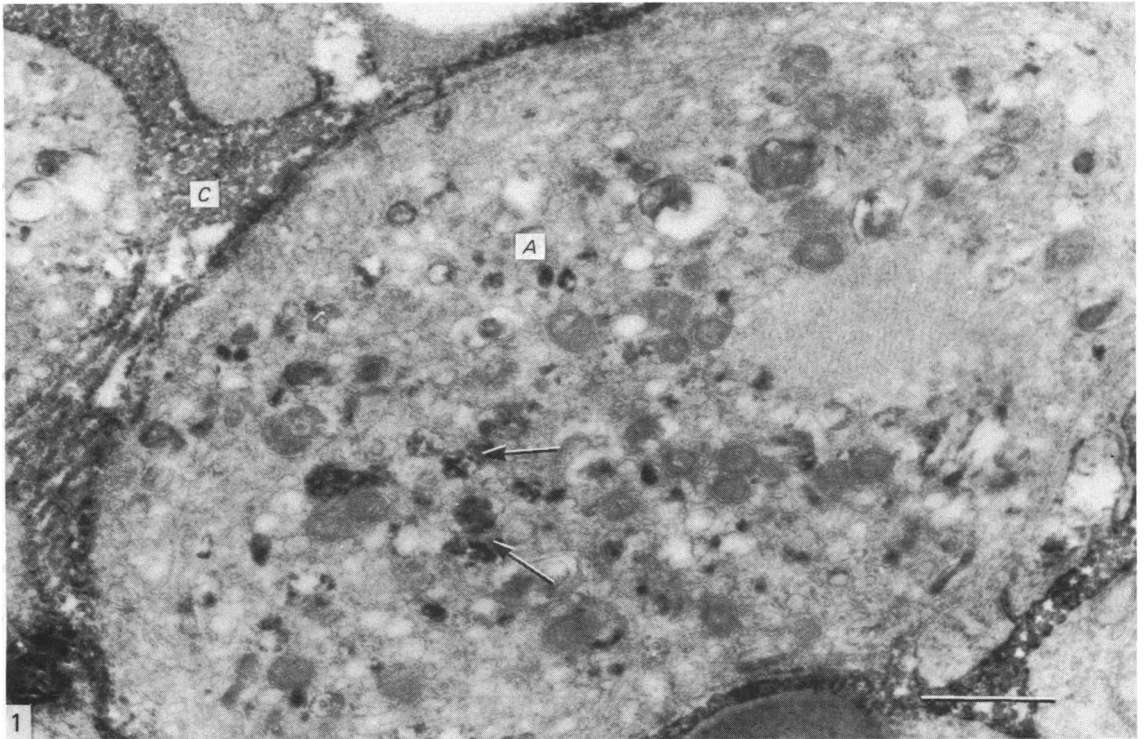
The incubation chamber was cooled in crushed melting ice before setting up the experiment. MEM was gassed at 37 °C and then cooled in crushed melting ice. The ganglion/nerve preparation was kept in cooled MEM for 30 seconds before placing it in the incubation chamber. 20 mg HRP was dissolved in 0.1 ml MEM, and cooled to 0 °C. One drop of the solution was applied directly to the site of ligation of the nerve. MEM at 0 °C was added to the compartment containing the ganglion and the apparatus was kept at 0 °C for 10 minutes. 5 ml of MEM at 0 °C, containing the remainder of the 20 mg of HRP, was added to the compartment containing the ligated nerve and the apparatus kept at 0 °C for a further 50 minutes. The ligated nerves were then given three washes, each of 5 minutes duration, in MEM at 0 °C without HRP. The apparatus was then returned to a water bath maintained at 37 °C. Preparations were fixed after either 15 minutes or 24 hours incubation at 37 °C.

Nerves treated with HRP at 0 °C and returned to 37 °C without washing

A similar series of experiments was carried out except that the HRP was not removed from the compartment containing the ligated nerve following incubation at 0 °C. These experiments were designed as controls to show whether exposure of the ganglion/nerve preparation to low temperatures produced lasting damage to the retrograde transport mechanism.

Fig. 1. Electron micrograph showing a grossly swollen non-myelinated axon (A) immediately proximal to the ligation, from an experiment in which HRP was added to the compartment containing ligated nerve 17 hours after nerve injury. The peroxidase-containing organelles are irregular in shape, and include multivesicular bodies (arrows). C, collagen fibrils. Bar = 0.5 µm. Unstained.

Fig. 2. Electron micrograph showing a less grossly swollen non-myelinated axon (A) containing tubular and vesicular profiles labelled with HRP reaction product (arrows). HRP added immediately after nerve injury. Bar = 0.5 µm. Stained with lead citrate.



Nerves treated with HRP at 37 °C for 60 minutes, washed, and then incubated at 37 °C

In a further series of experiments, 20 mg HRP were dissolved in 0.1 ml MEM at 37 °C. One drop of the solution was placed on the site of ligation of the nerve. MEM at 37 °C was added to the compartment containing the ganglion. After 10 minutes incubation at 37 °C, 5 ml MEM at 37 °C, containing the remainder of the 20 mg of HRP, were added to the compartment containing the ligated nerve and incubation was continued at 37 °C. After 50 minutes further incubation, the ligated nerves were given three washes, each of 5 minutes duration, in MEM at 37 °C without HRP. The apparatus was then maintained at 37 °C for a further 24 hours.

Fixation, histochemistry and microscopy

The ganglion/nerve preparations were fixed at room temperature for 3 hours in a solution containing 1.25% paraformaldehyde and 3.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. The preparations were then washed overnight in the phosphate buffer at 4 °C. The inferior mesenteric ganglion was sliced, using a Sorvall tissue chopper set at 50 μm . These slices, and the millimetre length of hypogastric nerve immediately proximal to the ligation, were rinsed in distilled water for 5 minutes and reacted for peroxidase activity, using the technique of Graham & Karnovsky (1966). The tissues were subsequently osmicated and processed into Araldite. Unstained 1 μm sections were examined using phase contrast microscopy and stained and unstained thin sections were used for electron microscopy.

The percentage of HRP labelled neurons in the ganglion was estimated by the following method: four, randomly chosen, 50 μm slices of each ganglion were sectioned at 1 μm . The numbers of labelled and unlabelled nucleated neurons were counted in sections obtained at a separation of 20 μm .

RESULTS

Delay experiments

The ultrastructural appearances of the axons in the millimetre of nerve immediately proximal to the ligation after 24 hours incubation with HRP *in vitro* have been described previously (Anderson *et al.* 1979). In the present study, the appearances were very similar, irrespective of any delay between the time of nerve injury and the time of exposure to HRP. In all experiments, the most grossly swollen non-myelinated axons were found within the half millimetre of nerve immediately proximal to the ligation. Such axons frequently contained many irregular, membrane-bound organelles labelled with peroxidase reaction product (Fig. 1). Slightly more proximally, the non-myelinated axons were less grossly swollen and contained more regular membrane-bound organelles labelled with peroxidase reaction product. These organelles usually took the form of large, rounded vesicles or elongated cisternae (Figs. 2, 3). In all experiments, coated presumptive pinocytotic vesicles were seen at the surface of swollen axons (Fig. 3, insert), indicating that the pinocytotic uptake of HRP can still occur even 41 hours (in the case of experiments with a 17 hours delay) after nerve injury.

Many strongly labelled neuronal perikarya were found within the inferior mesenteric ganglion irrespective of any delay between the time of nerve injury and the time of application of HRP (Fig. 4; Table 1). However, a much larger series of experiments would be necessary to detect whether or not any small variations in the uptake process occur during the first hours after axonal injury.

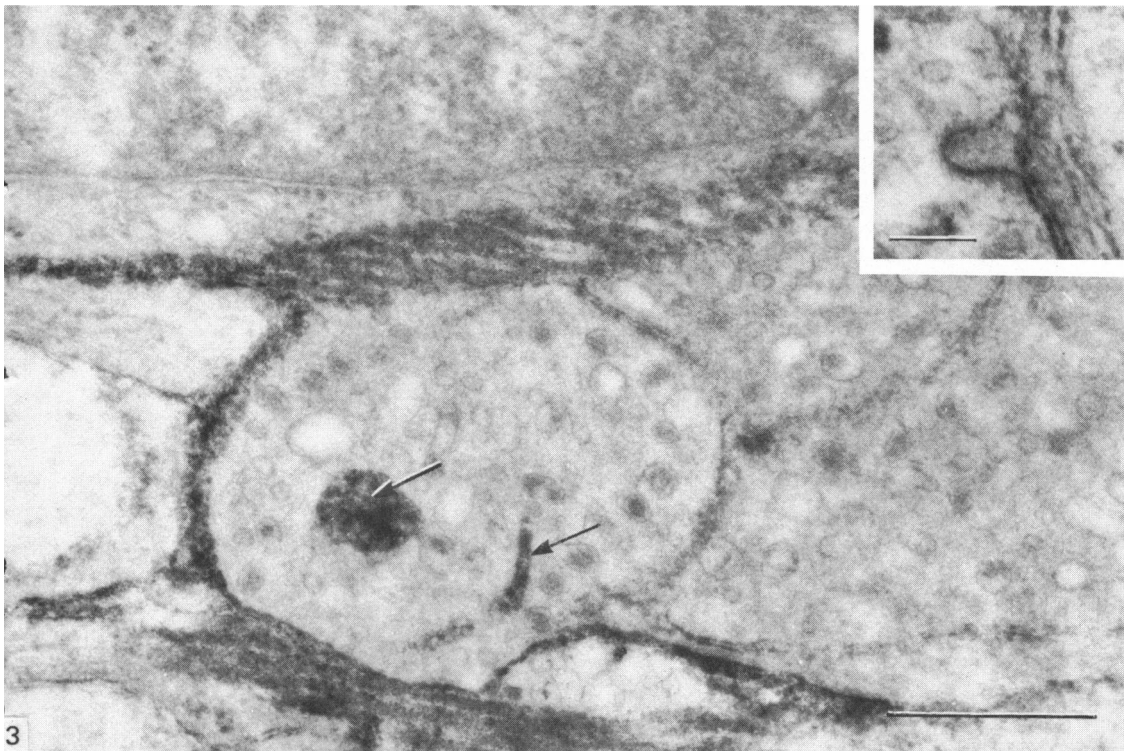


Fig. 3. Electron micrograph showing a moderately swollen non-myelinated axon from an experiment in which HRP was added 17 hours after nerve injury. The axon contains a large rounded vesicle and an elongated cisterna (arrows) labelled with peroxidase reaction product. These organelles are similar to those in which HRP is transported along the axons. Bar = 0.5 μm . Unstained. Insert: Electron micrograph showing a coated pinocytotic vesicle at the surface of a swollen axon from the same experiment. Bar = 0.1 μm . Stained with lead citrate.

Cold experiments

Nerves treated with HRP at 0 °C, washed, and returned to 37 °C

In experiments where the preparations were fixed 15 minutes after return to 37 °C, some HRP was localised clinging to the cell surfaces and collagen fibrils despite the three washings of the nerves (Fig. 5). The non-myelinated axons immediately proximal to the ligation were not greatly swollen, but many contained dense, diffuse peroxidase reaction product (Fig. 5). No membrane-bound organelles labelled with HRP reaction product were found within the axoplasm. However, occasional coated presumptive pinocytotic vesicles were found at the surface of axons and Schwann cells, indicating the resumption of pinocytosis following the return of the nerves to 37 °C. Thus, cold treatment enabled the axons to be loaded with diffuse HRP but not with membrane-bound HRP.

After 24 hours incubation at 37 °C, the HRP had disappeared from the extracellular space and axons. When the ganglia from such experiments were examined no HRP labelled nerve cell bodies were found. Thus, the diffuse HRP that had entered the axons during the cold treatment was inadequate to ensure the retrograde labelling of their perikarya.

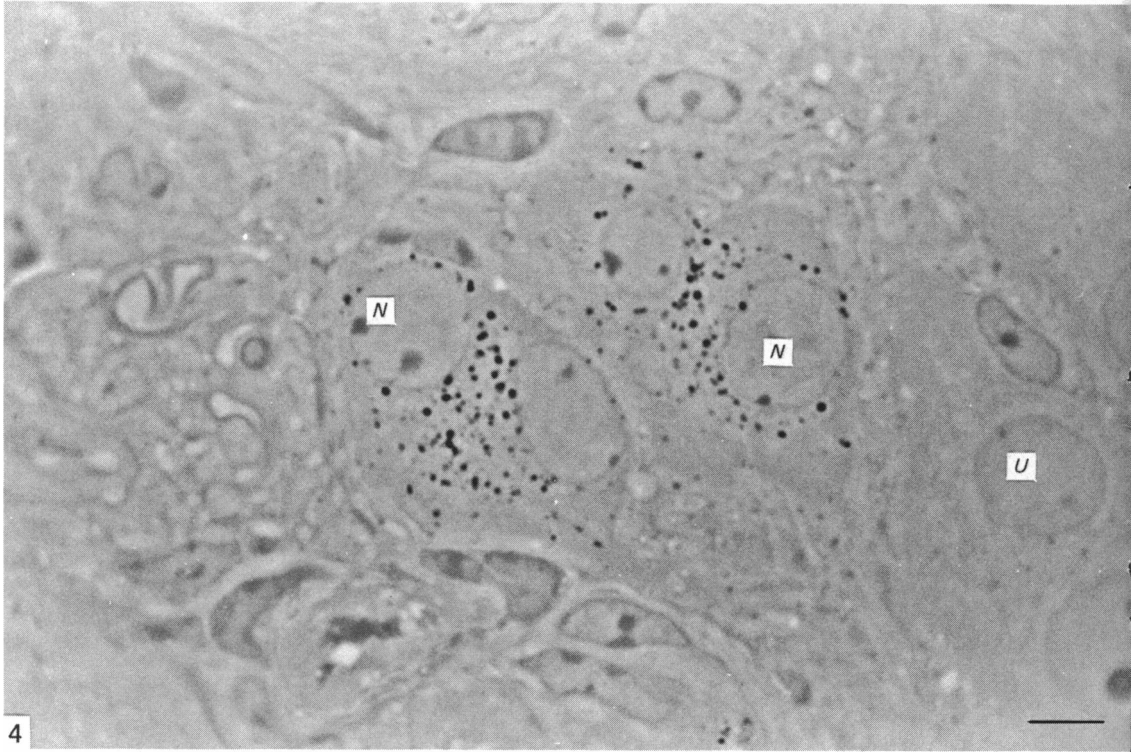


Fig. 4. Phase contrast micrograph of a $1\ \mu\text{m}$ section of an inferior mesenteric ganglion of a guinea-pig from an experiment in which peroxidase was added to the ligated nerve compartment 17 hours after nerve injury. Two perikarya retrogradely labelled with HRP may be seen. *N*, nuclei of labelled neurons; *U*, nucleus of unlabelled neuron. Bar = $5\ \mu\text{m}$. Unstained.

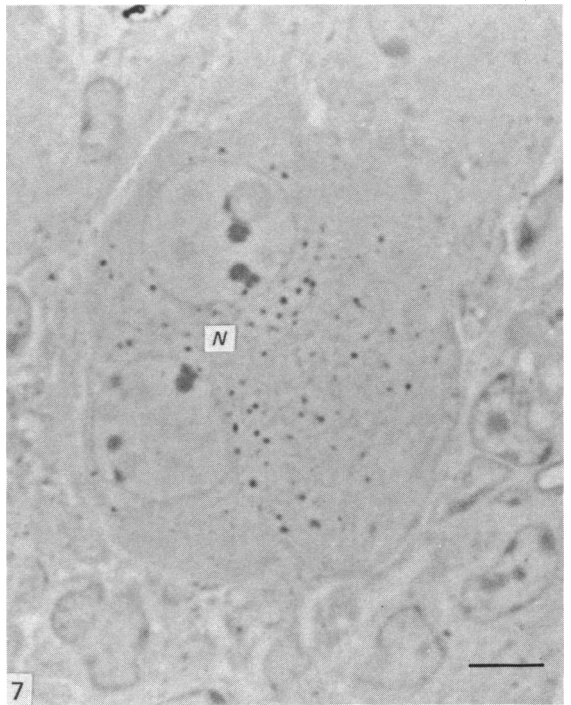
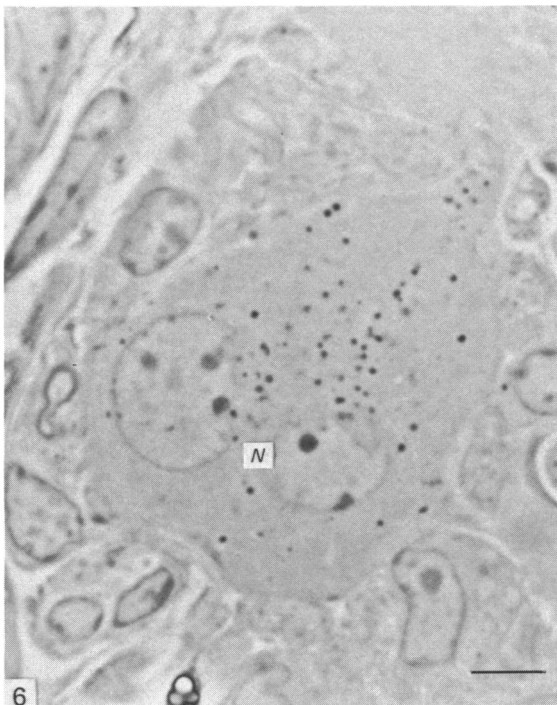
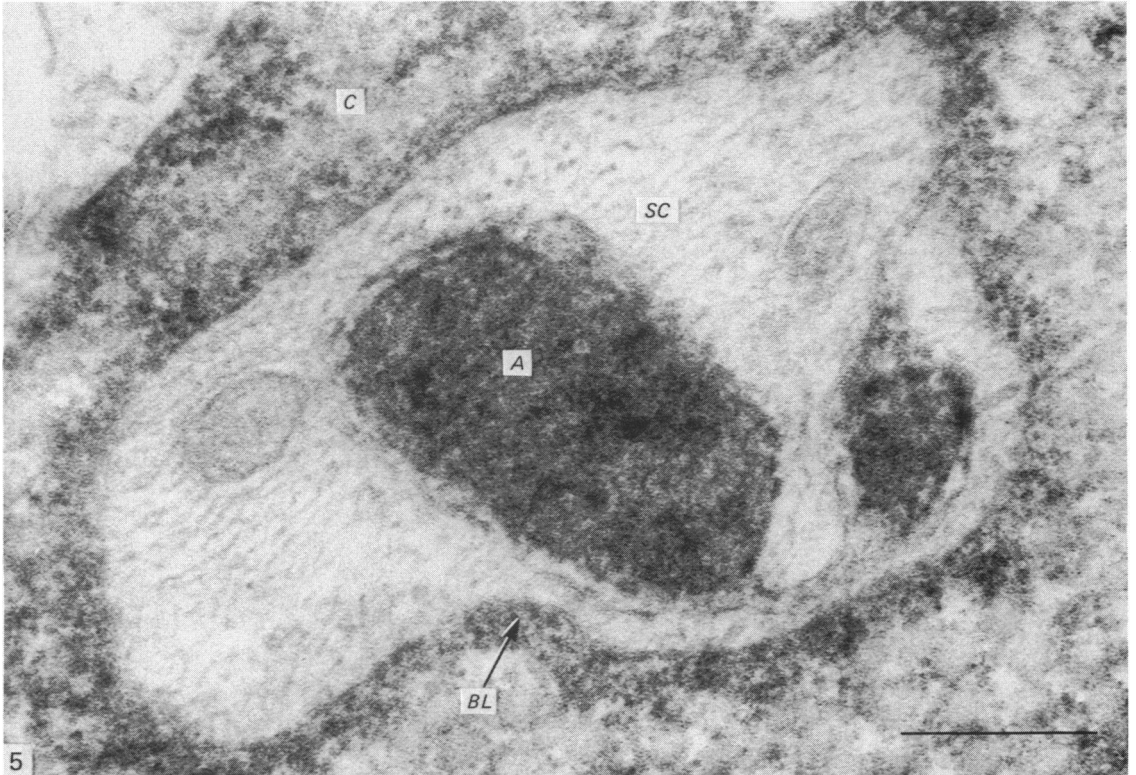
Table 1. *The effect on the percentage of neurons retrogradely labelled with HRP of delay between the time of nerve injury and the time of initial exposure to the enzyme*

Animal	Time, after ligation, of HRP application	% Inferior mesenteric ganglion neurons labelled
GP 192	Immediate	18
GP 227	Immediate	17
GP 229	Immediate	20
GP 196	3 hours	19
GP 197	17 hours	16
GP 225	17 hours	22
GP 231	17 hours	19

Fig. 5. Electron micrograph showing a non-myelinated axon (*A*) filled with diffuse HRP following exposure to the enzyme at $0\ ^\circ\text{C}$ and subsequent washing in HRP-free medium. *BL*, basement membrane material surrounding Schwann cell; *C*, collagen fibrils; *SC*, Schwann cell. Bar = $0.2\ \mu\text{m}$. Stained with lead citrate.

Fig. 6. Phase contrast micrograph of a $1\ \mu\text{m}$ section of an inferior mesenteric ganglion of a guinea-pig from an experiment in which the ligated nerves were exposed to HRP for one hour at $0\ ^\circ\text{C}$ and then incubated for a further 24 hours at $37\ ^\circ\text{C}$ in the presence of the enzyme. The neuronal perikaryon (*N*) is strongly labelled with HRP. Bar = $5\ \mu\text{m}$. Unstained.

Fig. 7. Phase contrast micrograph of a $1\ \mu\text{m}$ section of an inferior mesenteric ganglion of a guinea-pig showing a neuronal perikaryon (*N*) retrogradely labelled with HRP 24 hours after exposing the ligated hypogastric nerves to the enzyme for a one hour period at $37\ ^\circ\text{C}$. Bar = $5\ \mu\text{m}$. Unstained.



Nerves treated with HRP at 0 °C and returned to 37 °C without washing

These nerves were exposed to HRP for 24 hours at 37 °C. Many strongly labelled neuronal perikarya were found within the ganglia from such experiments (Fig. 6), indicating that cold treatment had not produced lasting damage to the retrograde transport mechanism.

Nerves treated with HRP at 37 °C for 60 minutes, washed and then incubated at 37 °C

Weakly labelled neuronal perikarya were found within the inferior mesenteric ganglia from these experiments (Fig. 7). This indicates that even 60 minutes treatment of the nerves with HRP, under conditions which allow both the diffuse entry and the pinocytotic uptake of the enzyme, was sufficient to ensure retrograde labelling of the perikarya.

DISCUSSION

The present study shows that the entry of HRP into damaged postganglionic sympathetic axons *in vitro* is not limited to a short period after nerve injury. This is in direct contrast to the results of Kristensson & Olsson (1976) and Olsson, Forsberg & Kristensson (1978) in studies using damaged mouse sciatic and facial nerves *in vivo*. However, the study by Sparrow & Kiernan (1979), of the retrograde transport of HRP by damaged axons in the rat hypoglossal nerve *in vivo*, gave results comparable to those in the present investigation, i.e. HRP applied either at the time of crushing or after a 12 hours delay produced similar numbers of labelled perikarya in the hypoglossal nucleus. The differences between these various studies cannot, therefore, be produced solely by differences between the *in vivo* and *in vitro* conditions. It may be germane that the present study and those by Kristensson & Olsson (1976) and Sparrow & Kiernan (1979) each utilise a slightly different type of nerve injury and a different mode of HRP application. Furthermore, it is not unlikely that different types of axon (e.g. non-myelinated and thickly myelinated) may vary in the kinetics of their uptake of HRP in a form suitable for retrograde transport.

The continuing potential for the uptake and retrograde transport of HRP shown by the damaged non-myelinated axons in the present study suggests that the pinocytotic uptake of the enzyme may be a more important means of entry to the retrograde transport mechanism than the sudden diffuse passage of HRP through the damaged axonal membrane. The results of treatment of the damaged axons with HRP at 0 °C further substantiate this view. Thus, the apparently large quantities of enzyme which entered the axons in a diffuse form at 0 °C, in the absence of pinocytosis, were inadequate to ensure retrograde labelling of the perikarya. In contrast, 60 minutes treatment with HRP at 37 °C, which allows both the diffuse entry of the enzyme into the axons and also its pinocytotic uptake, did produce retrograde labelling of the perikarya. Although the results of these experiments clearly cannot rule out any involvement of the diffuse entry of HRP into damaged non-myelinated axons in the subsequent retrograde labelling of the perikarya, they are much more easily compatible with an important role for pinocytosis in the process.

It is, of course, possible that the mechanism of uptake of HRP by damaged large myelinated axons in, for example, the sciatic nerve may be quite different from the mechanism of uptake by damaged non-myelinated axons in an abdominal autonomic

plexus. However, pinocytosis by means of coated vesicles is used for the highly selective uptake of physiologically important proteins in several cell types (Wild, 1980), and if such an uptake system occurs in many types of damaged axons it will be interesting to identify the substances for which it is designed: trophic factors or substances involved in stimulating the extension of new sprouts are possibilities.

SUMMARY

The mechanism of the uptake of horseradish peroxidase (HRP) by damaged postganglionic sympathetic axons was studied *in vitro*. HRP was applied to the damaged axons at the time of nerve injury or after a 3 hours or 17 hours delay. An interval of 3 hours or 17 hours between nerve injury and exposure to HRP had no effect on the localisation of the HRP in the damaged axons or on its retrograde transport to their perikarya. Evidence was found for the pinocytotic uptake of the enzyme by the damaged axons and its accumulation within those axons in elongated cisternae and larger rounded vesicles.

In further experiments the damaged axons were treated with HRP at 0 °C and then washed in HRP-free medium. The tracer entered the axons in a diffuse form under these conditions but no pinocytotic uptake was observed. However, following 24 hours further incubation at 37 °C, HRP could not be found in the perikarya. Treatment at 0 °C did not produce any lasting damage to the retrograde transport mechanism.

The results of these experiments are compatible with the involvement of pinocytosis in the uptake of HRP in a form suitable for retrograde transport.

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