1 I would like to thank Maureen Gardner for assistance with scanning electron microscopy, and Dr O.P. Flint for reading the manuscript and suggesting several improvements.
2 A.S.G. Curtis and T.E.J. Büültjens, in: Locomotion of Tissue Cells, p. 171. Ciba Found. Symp. 14, new series, 1973.
3 M.H. Gail and C.W. Boone, Expl Cell Res. *70*, 33 (1972).
4 E.A.G. Chernoff and J. Overton, Devl. Biol. *57*, 33 (1977).
5 H.J. Haas and N.T. Spratt, Anat. Rec. *184*, 227 (1976).
6 D.A.T. New, J. Embryol. exp. Morph. *3*, 326 (1955).
7 J.R. Downie, in preparation.
8 D.A.T. New, J. Embryol. exp. Morph. *7*, 146 (1959).
9 J.R. Downie and S.M. Pegrum, J. Embryol. exp. Morph. *26*, 623 (1971).
10 H.-V. Koecke, Embryologia *4*, 55 (1958).
11 R. Bellairs, A. Boyde and J.E.M. Heaysman, Wilhelm Roux Arch. EntwMech. Org. *163*, 113 (1969).
12 A.L. Romanoff and A.J. Romanoff, in: The Avian Egg. Wiley & Sons Inc., New York, 1949.
13 C.G. Sibley, Peabody Mus. Nat. Hist., Bulletin 32, 1970.
14 J.R. Downie, J. Embryol. exp. Morph. *35*, 559 (1976).
15 I have cultured several kinds of chick epithelia on vitelline membrane; Oliver Flint has had success with embryonic mouse tissues (both unpublished observations).
16 C. Jensen, J. Embryol. exp. Morph. *21*, 467 (1969).
17 R. Bellairs, D.R. Bromham and C.C. Wylie, J. Embryol. exp. Morph. *17*, 195 (1967).

Axoplasmic transport in regenerating limbs of *Ambystoma maculatum* larvae[1]

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Summary. Axoplasmic transport of ^{3}H-leucine labelled molecules from spinal cord segments into regenerating larval salamander limbs was observed. However, labelled molecules were not observed in cells of the regeneration blastema.

Although limb regeneration in urodele amphibians is nerve-dependent, the precise nature of the neural influence is not known. It is thought, however, that nerve cell bodies produce a trophic substance, likely a peptide or protein, which is transported intracellularly, by axons, to the regeneration areas. Presumably, this substance(s) then initiates and/or controls cellular activities[2,3]. Indeed, it has been established that nerves play a major role in the regenerative process by stimulating mesenchymatous cell proliferation[4–7]. Nerve-to-nerve cell and nerve-to-muscle cell transport has been reported in other systems[8,9], but whether or not this type of intercellular transport occurs during urodele limb regeneration is unknown. The present investigation was undertaken to determine whether or not some substance(s), synthesized in the spinal cord of larval *Ambystoma*, is transported via axons to the regeneration area of the forelimb and, possibly, into the blastema cells.

Materials and methods. Our methods involved labelling spinal cord segments, which innervated regenerating forelimbs, and studying the subsequent distribution of the label. *Ambystoma maculatum* larvae (20–25 mm) were collected locally, kept at 20 (±1) °C in individual dishes and fed *Tubifex* worms twice weekly. Donor larvae were anaesthetized in 0.2 g/l tricaine methanesulfonate and injected i.p. with 15 µCi (30 µl volume) of ^{3}H-L-leucine, ^{3}H-uridine, or ^{3}H-D-glucose using a No. 34 gauge needle (Hamilton). 24 h later these larvae were reanaesthetized. Next, their ^{3}H-labelled spinal cords were surgically removed in one piece from the cervical to pelvic regions, placed in amphibian Ringer's solution and cut into 2 or 3 segments. Using fine forceps as a probe, a tunnel was made mid-way in the dorsal tail-fin of an anaesthetized unlabelled host larva (figure 1), and a labelled donor spinal cord segment was transplanted (allografted) through the posterior tunnel opening. Next, the host animal's right forelimb was disarticulated at the shoulder and the skin removed from the upper-arm. Then, the denuded part of the limb was inserted into the anterior tunnel opening so that the head of the humerus abutted the labelled donor spinal cord segment[7,10] (figure 1).

Within 2 days the limb transplants (autografts) became vascularized and subsequently well-innervated by nerves from the allografted spinal cord segments (figure 1). Spasmodic movements of the autografted limbs indicated when innervation had occurred[7,10]. Animals were than anaesthetized and the transplanted limbs amputated through the radius-ulna or carpal regions (figure 1). After 2–14 days of regeneration, graft-bearing tails were fixed in G-Bouin's[11], embedded in paraffin, and serially sectioned at 5 µm. Routine liquid emulsion (Kodak NTB-2) autoradiography was employed, followed by staining with haematoxylin and orange-G-eosin[12]. Free, unincorporated ^{3}H-leucine was apparently washed-out during fixation and dehydration of tissues[13].

Results and discussion. In all 23 cases, the transplanted spinal cord segments were ^{3}H-labelled uniformly and heavily with 200–400 grains/1000 µm^2. No other tissues were labelled above background levels. In the 8 cases of the ^{3}H-leucine series, the nerve trunks that grew-out from the spinal cord transplants and innervated the unlabelled regenerating limbs were also heavily labelled (figures 2 and 3). Furthermore, labelled nerve fibres can also be traced out into the regeneration area (figures 4 and 5). In the 9 ^{3}H-uridine and 6 ^{3}H-glucose cases, however, there was no isotope found in the nerve trunks or nerves innervating the

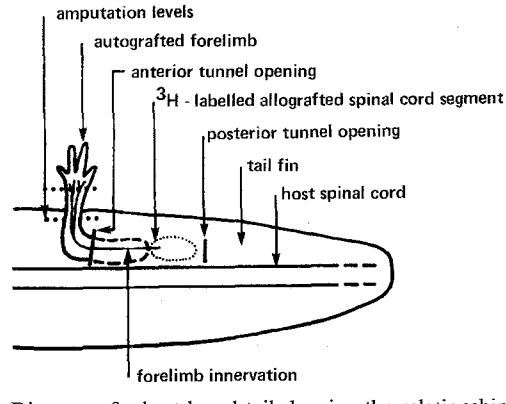
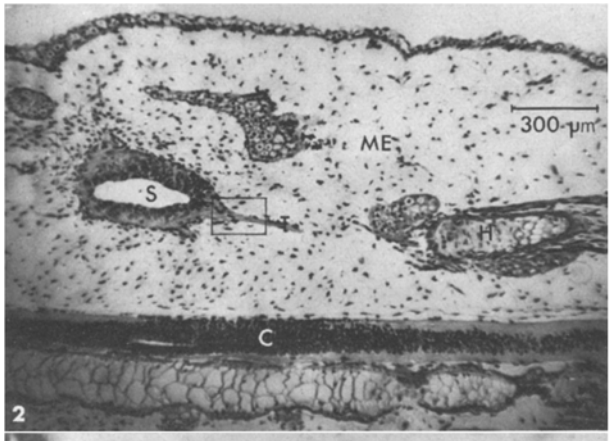
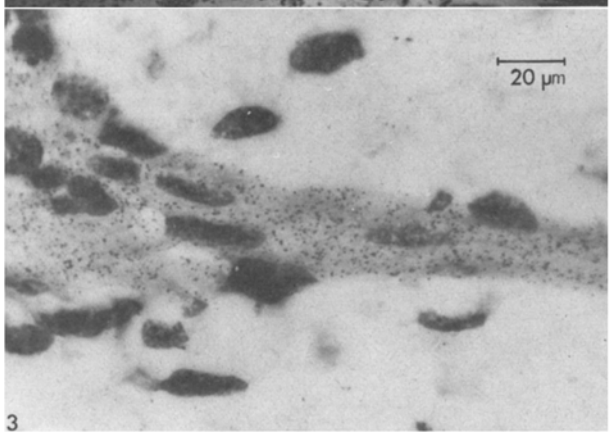
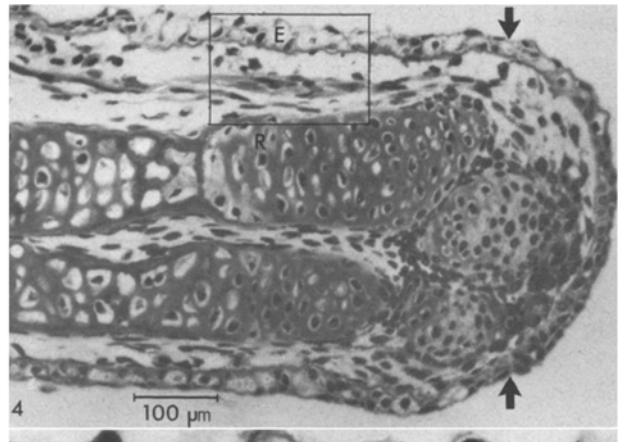


Fig. 1. Diagram of a host larval tail showing the relationship of an autografted forelimb and a ^{3}H-labelled, allografted, spinal cord segment in the tunnel of the dorsal tail fin of a non-labelled host larva. Amputation levels are indicated.







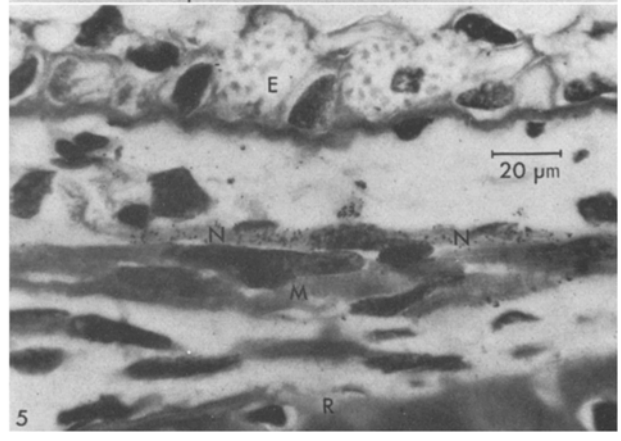


Fig. 2. Photomicrograph of a longitudinal section through the dorsal tail fin of a host larva showing a ^{3}H-leucine labelled, allografted, spinal cord segment (S), and the nerve trunk (T) which grew out from it through the mesenchyme (ME) of the host tail fin towards the humerus (H) of the unlabelled autografted limb. The spinal cord (C) of the host animal is parallel to the bottom of the photomicrograph. Anterior is to the right. Fig. 3. High-power photomicrograph of the framed area in figure 2 showing the heavy. ^{3}H-leucine labelling of the spinal cord allograft and its emerging nerve trunk. Fig. 4. Photomicrograph of a longitudinal section through an autografted forelimb which has a small blastema after 4 days of regeneration. The amputation level is indicated by arrows. Nerve fibres, well-labelled with ^{3}H-leucine, lie within the framed area between and parallel to the epidermis (E) and the radius cartilage (R) of the forearm. These nerve fibres are directly connected to the labelled allografted spinal cord segment and can be traced back, through serial sections, to it via its emerging nerve trunk. Fig. 5. High-power photomicrograph of the framed area in figure 4 showing nerve fibres (N), well-labelled with ^{3}H-leucine, adjacent to a muscle bundle (M). These labelled nerve fibres are in the regeneration area and also exhibit labelling more distally in the blastema. However, no label was detected in the blastema cells.

forelimb autografts, even though their spinal cord allografts were heavily labelled at the time of fixation. We conclude, therefore, that intracellular axoplasmic transport occurred only in the cases with ^{3}H-leucine labelled allografts.

The results show that ^{3}H-leucine was incorporated into molecules, presumably peptides or proteins[14,15], which were transported from the spinal cord allografts, via their axons, to the regeneration area of the forelimb autografts. The possibility exists, therefore, that the transported ^{3}H-leucine labelled molecules might have some relation to the 'nervous agent responsible for the trophic action of nerves'[3] in urodele appendage regeneration.

Whether or not molecules that incorporated ^{3}H-leucine were actually transferred to the surface of the blastema cells and/or into the cells remains an open question. Indeed, we did not observe ^{3}H-label in the blastema cells of any of our cases. However, this could be a consequence of undetectable quantities of isotope that may have been intercellularly transported and/or the very short time the label may have remained at the nerve-blastema cell junction[16] and/or in the blastema cells before catabolism and removal from the cells. The present findings on *Ambystoma* larvae confirm previous observations on adult newt forelimb regeneration[13].

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2 M. Singer, in: Regeneration in Animlas and Related Problems, p. 21. Ed. V. Kiortsis and H.A.L. Trampusch. North Holland Publ. Co., Amsterdam 1965.
3 M. Singer, C.E. Maier and W.S. McNutt, J. exp. Zool. *196*, 131 (1976).
4 A.L. Mescher and R.A. Tassava, Devl Biol. *44*, 187 (1975).
5 M. Globus and R.A. Liversage, J. Embryol. exp. Morph. *33*, 803, 813 (1975 a, b).
6 M. Globus and S. Vethamany-Globus, Devl Biol. *56*, 316 (1977).
7 R.A. Liversage and M. Globus, Can. J. Zool. *55*, 1195 (1977).
8 J. Alvarez, Acta physiol. latinoam. *20*, 271 (1970).
9 B. Grafstein, Science *172*, 177 (1971).
10 R.A. Liversage, J. exp. Zool. *151*, 1 (1962).
11 R.A. Liversage, J. exp. Zool. *165*, 57 (1967).
12 S.R. Scadding and R.A. Liversage, Am. J. Anat. *140*, 349 (1974).
13 T.L. Lentz, J. Cell Biol. *52*, 719 (1972).
14 R. Lasek, Brain Res. *7*, 360 (1968).
15 S. Ochs, J. Johnson and M.H. Ng, J. Neurochem. *14*, 317 (1967).
16 M.M. Salpeter, J. Morph. *117*, 201 (1965).