In 6 hosts, 5 of which had received implants from 13-day donors, the PA developed into small growths (less than 1 mm in diameter) consisting of connective tissue, smooth muscle, and bone. Various degrees of lymphocytic infiltration were evident.

The major finding of this experiment was that 12-day PA grafts can exhibit different growth responses. 6 weeks after implantation in the testes, grafts of 12-day PA were less than $^1/_{10}$ the size of the same age donor tissue growing for a month in the hypothalamus. Distinct histological differences also separated the types of growths found in the 2 sites. Adenomas and undifferentiated cells, characteristic constituents of tumors from brain implants, were not evident in the testicular growths. Nervous tissue commonly developed from PA grafts to both sites.

At this time it is difficult to explain why grafts from 13-day-old donors showed signs of tissue rejection that were not generally evident in the grafts from 11- and 12-day-old donors. The reason may simply lie in the fact that outbred animals were used in this experiment and there were possibly major histocompatibility differences between the hosts and the 13-day-old embryonic donors. Whether the observed tissue rejection was a result of histocompatibility factors or of more fundamental differences between 12- and 13-day PA could be tested by studies on isogeneic animals.

## A procedure for the rapid freezing of whole embryos

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*Summary.* A procedure for the rapid freezing of whole chick embryos for histochemical treatment is described. The problems of deformation during preparation for quenching and orientation for sectioning have been largely overcome by placing embryos inside lengths of chicken trachea. The subsequenct disorientation of tissues that follows cracking and shattering due to the rapid freezing of whole embryos is avoided. The method permitted a more precise identification of the position and time of appearance of formaldehyde-induced fluorescence and myosin antibody immunofluorescence in serially sectioned embryos.

Studies of the early vertebrate embryo by histological and histochemical methods often require an examination of the position and the time of appearance of developing tissues. This is normally accomplished through techniques of fixation which retain the whole embryo intact for subsequent serial sectioning in any desired plane, thus enabling an understanding of the morphological changes taking place. However, the retention of the form and orientation of the whole embryo necessary for such an analysis is much more difficult for histochemical techniques requiring quenching with, for example, liquid propane.

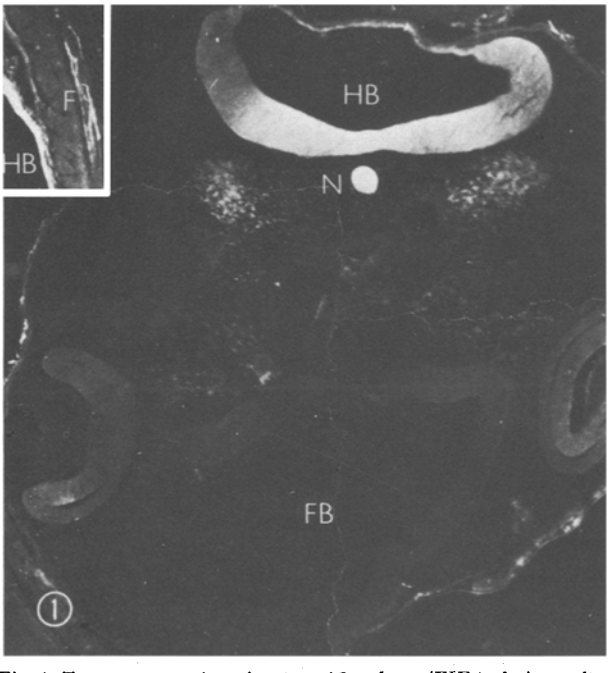


Fig. 1. Transverse section of a stage 16 embryo (FIF technique after α-methylnoradrenaline loading). Note preservation of form of forebrain (FB) and hindbrain (HB). Notochord (N) is intensely fluorescent. Inset: control fluorescent fibres (F) in tracheal housing. ×90.

The normal procedure of placing tissue on small pieces of paper before quenching results in gross deformation of soft embryonic structures when embryos are removed from a supporting liquid medium, making it impossible to cut transverse cryostat sections. An even more serious problem occurs due to cracking of the tissue which often leads to the disintegration of the embryo. Although quenching of small pieces of special interest partially overcomes the latter problem, serial or semiserial sections are generally more informative when examining developing tissues.

We have obtained good serial transverse sections from chick embryos of stage 11–27[2] from both freeze-dried preparations for formaldehyde induced fluorescence (FIF) of catecholamines[3] and fresh frozen preparations for myosin-antibody immunofluorescence[4] by placing the embryo in an older bird's trachea prior to quenching. This housing preserves the form of the embryo and enables precise orientation for later sectioning as well as retaining in a normal location any pieces that might otherwise be lost due to cracking. The trachea also acts as a control through fluorescence of the adrenergic perivascular innervation when using the FIF technique (figure 1) and of the perivascular smooth muscle and tracheal skeletal muscle for myosin-antibody immunofluorescence.

1 We gratefully thank Dr Ute Gröschel-Stewart for preparing sections for immunofluorescence.

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For this procedure tracheas were trimmed to appropriate lengths and cleaned by passing a Pasteur pipette down them. Embryos were then gently drawn into the trachea, caudal end first, by a mouth-controlled Pasteur pipette the tip of which was inserted into the trachea at the opposite end to the embryo. The whole procedure was carried out in a balanced salt solution for FIF or Ames OCT compound (Miles Lab., Indiana, USA) for myosin-antibody immunofluorescence.

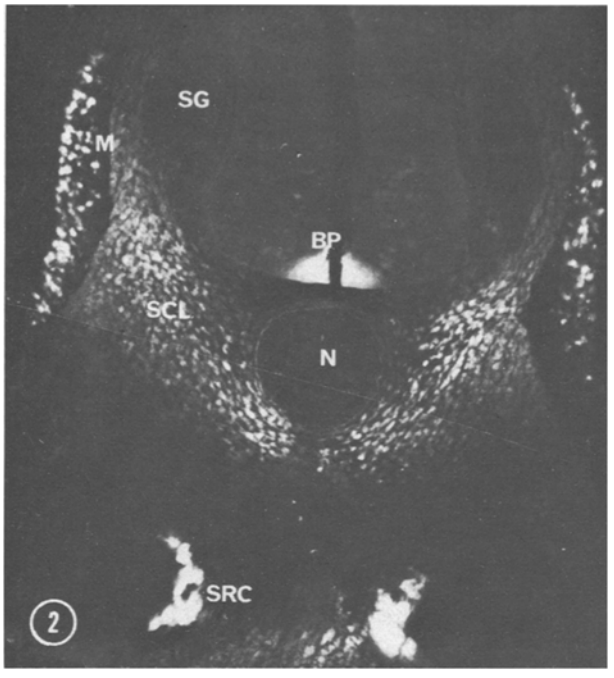


Fig. 2. Transverse section posterior to the forelimbs of a stage 22 embryo (FIF) technique after α-methylnoradrenaline loading). Note fluorescence localized in myotone (M), basal plate (BP), sclerotome (SCL) and suprarenal cortical tissue (SRC). Fluorescence is not present in the sensory ganglion (SG) or notochord (N). ×120.

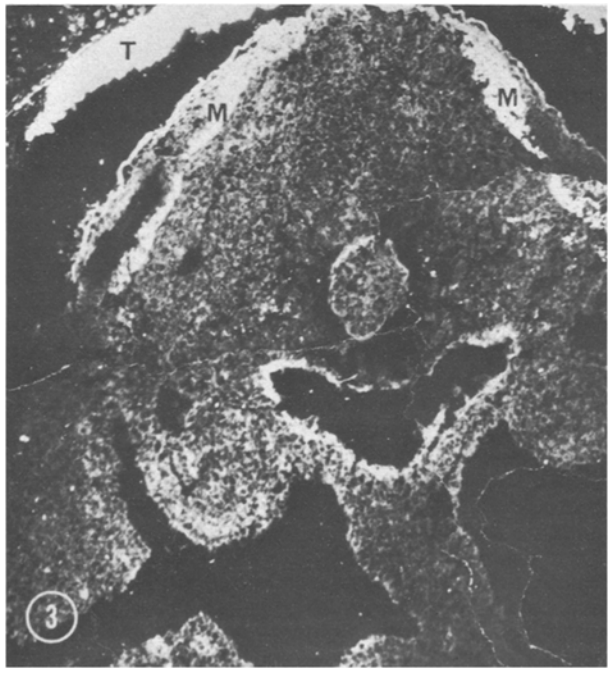


Fig. 3. Transverse cryostat section of a stage 17 embryo (myosin antibody immunofluorescence). Fluorescence is localized in the myotome (M). The tracheal mucosa (T) is autofluorescent. ×130.

Attempts to draw embryos into small tracheas resulted in mechanical damage to limb-buds and heart, whilst too large a tracheal tube sometimes resulted in curling of the tail towards the head, displacement of pieces after cracking and also a tendency for the embryo portion of a section to slide over the tracheal portion during the preparation of slides for FIF microscopy.

Suitable ages for tracheal donors are as follows: 20-day-embryos for embryos of stage 11 or less, 1-day-chicks for stage 12–14 embryos, 1-week-chicks for stage 15–16 embryos, 2-week-chicks for stage 17–18 embryos, 3-week-chicks for stage 19–20 embryos, 5-week-chicks for stage 21–22 embryos, 7-week-chicks for stage 23–24 embryos and 10-week-chicks for stage 25–27 embryos.

A comprehensive FIF histochemical study of early chick embryos by this procedure after loading with α-methyl noradrenaline ($10^{-5}$ g/ml in vitro) revealed fluorescence in the notochord and basal plate of the neural tube (figure 1), confirming the results of Kirby and Gilmore[5] and Lawrence and Burden[6]. Furthermore it showed fluorescence in the myotome, sclerotome, adrenocortical cells (figure 2) and gut mesenchyme, but not in the primitive sympathetic cells until its appearance in untreated embryos[7]. Loading with l-DOPA ($10^{-5}$ g/ml in vitro) produced fluorescence in the notochord and dorsal pancreas confirming the results of Kirby and Gilmore[5] and Andrew[8], respectively. In addition, after loading with l-DOPA fluorescence was present in the primary sympathetic chain as early as stage 18, and lower intensity fluorescence existed in the basal plate, myotome, sclerotome and gut mesenchyme. Dull fluorescence could be observed in some cells of the anterior primary sympathetic chain of untreated control embryos by stage 21. Kirby and Gilmore[9] report difficulty in obtaining properly freeze-dried embryos with their heads left intact and therefore identify the onset of fluorescence in the remainder of the cervical primary sympathetic chain at stage 22. Contrary to the findings of Polak, Rost and Pearse[10], Kirby[11] and Kirby and Gilmore[9], we found no evidence of fluorescence in neural crest cells prior to their localization in the primary chain[7]. Quite likely this difference is due in part to difficulties in identifying embryonic rudiments from embryos deformed during standard preparation.

Immunofluorescence with chick pectoral muscle anti-myosin identified skeletal muscle myosin in stage 13 embryos in a position corresponding to the myotomes of the first 3 somites. The fluorescence was localized in bands, presumably myotubes, extending from the anterior to posterior border of each somite. Up to at least stage 19, fluorescence appeared in all except the most caudal 12–16 somites (figure 3). This correlates well with ultrastructural studies on the appearance of contractile filaments in the chick myotome[12,13].

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