CONTROL OF GROWTH AND DEVELOPMENT OF NEURONES

IN THE CHICK EMBRYO

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DECLARATION

I declare that the work described in this thesis and its composition is entirely my own.

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ABBREVIATIONS

AraC   Cytosine-B-D-Arabinofuranoside

BDNF   Brain Derived Neurotrophic Factor

dCTP   Deoxycytidinetriphosphate

Cyt   Cytosine

2'DC   2'Deoxycytidine

DM   Dorso Medial

DNA   Deoxyribonucleic Acid

DRG   Dorsal Root Ganglia

FCS   Fetal Calf Serum

FITC   Fluoroscein Isothiocyanate

Fn   Fibronectin

^{3}H Thy   Tritiated Thymidine

HRP   Horse Radish Peroxidase

IgI   Immunoglobulin (class G)

LBM   Large Bright pseudoMultipolar

LS   Lumbo Sacral

NC   Neural Crest

NGF   Nerve Growth Factor

NT   Neural Tube

PBS   Phosphate Buffered Saline

PORN   Poly-D-L Ornithine

SDB   Small Dark Bipolar

TD   Tris Saline

VL   Ventro Lateral

ABSTRACT

This thesis is in two major sections

SEGMENTAL SENSORY INNERVATION

During the segmental, sensory innervation of the periphery, neurones situated in the dorsal root ganglia grow out axons at different rates. These vary with segmental level of the ganglia. The most rapid rates of axonal advancement are seen from those ganglia which innervate limb as opposed to non-limb ganglia. How are these differential axonal growth rates controlled?

This problem has been addressed using cell culture techniques. No evidence for a segmentally regulated, intrinsic growth mechanism has been found. Further some common constituents of the peripheral fields of these neurones, were also, on the whole ineffective in regulating neurite growth rates in culture.

I conclude from this that some, as yet, unidentified factor in the periphery is reponsible for the enhanced growth rate of axons into limb tissue.

A number of differences in the neurite growth patterns of neurones isolated from embryos at different stages of development were noted. This suggests that neurite growth is regulated developmentally if not segmentally.

Neurite lengths from cultured neurones were found to be non-normally distributed. They showed a positively skewed, multimodal form. This was not explained by differences in neurite length, branch pattern or the stage of development of the neurones. In the light of this, a quantal hypothesis of neurite growth is proposed, and modelled using Poisson statistics as a theoretical basis.

NEURONAL TOXICITY OF CYTOSINE-B-D-ARABINOFURANOSIDE

Cytosine-B-D-Arabinofuranoside (AraC) is a commonly used anti-mitotic agent in neural cultures. In this study it is also shown to be toxic to cultured neurones and to inhibit neurite growth. This toxicity is dose dependent and inhibited in the presence of 2'DeoxyCytidine (a metabolic precursor of AraC). A relationship between high metabolic demand (neurite growth) and AraC toxicity is discussed in the light of other experimental work.

CONTENTS

SEGMENTAL SENSORY INNERVATION

1 INTRODUCTION

1.1 THE ORIGIN OF SENSORY INNERVATION

The sensory innervation of the periphery is supplied by neurones residing in Dorsal Root Ganglia (DRG), located between brachial and sacral segmental levels. These are paired, segmentally arranged structures located along the length of, and in close proximity to, the Neural Tube (NT). The DRG form from a specialised population of cells, the Neural Crest (NC). These cells first become apparent early in development at the lateral margins of the neural plate. As the neural tube closes they come to lie on its dorsal surface. Following this they migrate around the NT and come to lie laterally to it where they stop and condense to form the DRG anlage (Pannese, 1974).

Neurones from the DRG grow out axons which provide the sensory innervation of the periphery. The body plan of the embryo puts various constraints on this pattern of innervation. Target distances vary and it has been shown that the rates at which axons grow to these targets are proportional to the distances over which the axons have to grow. This study aims to investigate the factors which affect how the pattern of limb and trunk innervation arises. It will be specifically examined whether control is exerted by the neurones or the tissue through which they project.

This introduction is primarily concerned with the events which follow the condensation of the DRG. This next phase of development may be divided into three main stages: i) proliferation and differentiation; ii) degeneration and iii) outgrowth. These processes lead to the establishment of a specific, stereotyped pattern of innervation.

1.1.1 PROLIFERATION AND DIFFERENTIATION

Following DRG condensation, neuroblasts first begin to differentiate at 60hr of incubation (st16: Pannese, 1974; Carr and Simpson, 1978a). Pannese (1974) has divided the subsequent development of these cells into several stages using ultrastructural and morphological criteria. Until the fourth day of incubation the majority of cells are rounded and have a thin rim of cytoplasm surrounding the nucleus and are termed "indifferent cells". These mature into "primitive neuroblasts" which are numerous by 4-6 days of incubation and have bipolar neurites and a spindle shaped morphology, characteristic of young neurones. By the fifth day of incubation "intermediate neuroblasts" are first seen. In these the two neurites have begun to be drawn together and the nucleus is eccentrically situated. The final stage of the differentiation process does not occur until the beginning of the second half of the incubation period (E8) This is the formation of mature "pseudo-unipolar" DRG neurones. This mature morphology was described by

Cajal (1891 and 1904 in Pannese, 1974) and has been demonstrated to be under the control of Schwann cells (Mudge, 1984). The times given for these phases of differentiation are a compromise, as development proceeds at different rates, both within individual ganglia and along the rostro-caudal axis. The earliest cells to differentiate are located in the ventro-lateral (VL) position within the ganglia from 60hr of incubation. The later differentiating populations are located in the dorso-medial (DM) position within the ganglia, and are not found until E5 (Hamburger *et al.*, 1981). Differentiation in the DM population occurs rapidly however, and these neurones constitute a third of the ganglia by E6 - 6.5 and a half by E6.5 - 7. Both populations begin to show the adult, pseudo-unipolar morphology by E8.

Many studies have been carried out to determine the time course of mitotic activity within the DRG. Hamburger and Levi-Montalchini (1949) made counts of the absolute number of mitotic figures present at different stages of development. They found that a peak was reached between E5 - 6, which then decreased until E9, after which time no further mitoses were recorded. The peaks were found to be greater in limb than in non-limb innervating ganglia. Carr and Simpson (1978a) carried out a similar study using the technique of ^{3}H Thy labelling and autoradiography. They calculated an index of the number of mitotic against non-mitotic cells recorded. They found that mitotic activity was elevated through E4.5, (the earliest stage

which they studied) and then decreased until E6.5 in the LV population and E7.5 in the DM populations. They also found a subsequent peak in both populations at E9.5. The discrepancies between the two studies may be attributed to the different expression of results used, total count against an index of mitotic activity.

Experiments concerned with the proliferation response to supernumery limb growth are conflicting, and considered to be outside the scope of this review. In general, the control of cell proliferation is an area of neurobiology which is poorly understood.

1.1.2 DEGENERATION

Naturally occurring cell death is a common feature of the development of many neural populations and is thought to function in the matching of neurone number to target field size. The earliest work into cellular degeneration in DRG was carried out by Hamburger and Levi-Montalchini (1949) who made total counts of degenerating cells in staged embryos. They concluded that degenerative activity was only present in non-limb ganglia and then almost exclusively in the VL population. This they assumed was the major factor governing the size discrepancy seen between limb and non-limb ganglia in the adult. Once again, the more detailed autoradiographic study of Carr and Simpson (1978a) shed more light on the situation. Carr and Simpson showed that

degeneration occurred in the limb and non-limb ganglia in both VL and DM populations. The first degenerative activity was visible at E4.5 rising to a peak at E5.5 in the VL region of limb and non-limb ganglia. By contrast, degeneration in the DM region was still rising in all ganglia at E9.5 (the latest stage used in these studies). Hamburger *et al.* (1981) confirmed and extended these studies, showing a separation of peak degeneration times between the two populations of neurones. This study also shows a marked difference between the timing and quantity of degeneration within the VL population between brachial and thoracic segmental levels. Degeneration is much lower (approximately one sixth) and later E5 compared with E6 in the brachial regions, which explains why these may have been missed in the earlier study. No such difference occurs in the DM population, peak degeneration being at E8. It is interesting to note that many of the casualties in the VL region are primitive neuroblasts and therefore at very early stages in their development. In fact Hughes and Carr (1978a) suggest from labelling studies that some of the degenerating cells may not have completed the proliferative cycle. This is contrary to the accepted view that cell death follows target interactions, which in this case obviously did not occur. It appears, then, that for several stages of development there is a "turnover" of neurones, with cells being born and degenerating simultaneously. This contrasts to the development of motoneurones, when proliferation and degeneration are separated by 2 - 3 days (Hollyday, 1983).

1.1.3 OUTGROWTH

Nansen (1886, in Lundberg, 1969) first made the observation that dorsal root fibres enter the spinal cord and branch. This led to an understanding of the spinal reflex, and the sensory role which DRG neurones play in this. Cajal (1929: in Guth 1960) has illustrated the bipolar processes of DRG neurones entering the dorsal horn of the NT and extending toward the periphery as early as E3. He also states that the majority of cells at this time are primitive neuroblasts and that their growth cones are still within the ganglia. By E3.5 (st21) the peripherally directed process of the earliest differentiated VL neurones have entered the region of the presumptive lumbosacral plexus (Hollyday, 1983; Tosney and Landmesser, 1985a; Bennet *et al.* 1980). These then appear to "wait" while more growth cones arrive to swell the plexus, not leaving the plexus until E4.5 (st23/24). This "waiting" phenomenon has been hypothesised to be caused by the non-permissive nature of early, but not late limb-bud mesenchyme (Landmesser, 1987). Sensory neurone growth cones are among the first to invade the limb proper (Landmesser and Honig, 1986; Hollyday, 1983), with motoneurones at E4.5 (st25: Lance-Jones and Landmesser, 1981a; Tosney and Landmesser, 1985b, c). The invasion of the limb by sensory growth cones however, occurs over a more protracted period, which presumably reflects the extended three day period over which DRG neurones are born (Carr and Simpson, 1978a; Honig, 1982).

Swanson and Lewis (1982) have examined the timetable of innervation of the forelimb. At E5 (st26) axons are shown leaving the brachial plexus and ramifying within the proximal part of the forelimb. By E5 - 5.5 (st26/27) axon tips have reached midway down the developing wing and have formed the two major, dorsal and ventral mixed nerve trunks. At E7 -7.5 (st30/31) the distinctive anastomosis at the level of the future wrist between nerve branches has formed. The four major cutaneous nerve branches have also been formed by this stage (Martin *et al.*, 1989). Through E7.5 - 9 (st 32-35) there is little change in the basic innervation pattern, further elaboration of branching occurs, particularly of cutaneous endings. (See also Roncali, 1970 for a description of forelimb innervation.)

Tosney and Landmesser (1985a) describe a similar time course of events during the innervation of the hind limb. Motor and sensory growth cones enter the plexus region at E3.5 (st21), where they spread and are joined by later entering ones until E4.5 (st23). Distal axon tips then extended through the pelvic girdle pre-cursor and by E6 (st28) the basic adult innervation pattern has been established, and is indistinguishable from that at E10 (st36). It is interesting to note that innervation has proceeded proportionately faster in the hind limb, so enabling the hind limb tip to be reached and the basic pattern of innervation to be laid down 12 - 24 hr in advance of that in the

forelimb.

The centrally directed projection of DRG neurones do not enter the spinal cord grey matter until E6.5 - 7 (st 30 - 31) which is subsequent to the major phase of outgrowth and target innervation in the periphery.

Little information is available concerning the innervation of the thorax. Verna and Saxod (1979) during their studies on chick cutaneous nerve endings, state that dorsal nerve trunks are present by E4 (st26 in his account) and that the first nerve endings have reached the skin by E5-6 (st28/29 again his account). By E6 large cutaneous nerve bundles are present and a pattern of cutaneous innervation similar to that in the adult is established by E14 - 15. Saxod (1978), mentions that the first cutaneous nerve fibres have reached the skin by E4 (st23). It appears that the onset of target innervation occurs earliest in the trunk, where innervation distances are shortest, but takes longer to become fully established than in the limbs.

In summary then, large scale neurone production occurs between E4.5 and 7.5, degeneration between E4.5 and 12 and outgrowth into the periphery between E3.5 and 15. These are the broadest limits and include events in limb and non-limb ganglia in both VL and DM populations. These events are illustrated in Fig.1.1 for ease of comparison.

FIGURE 1.1

Schematic representation of the timing of some of the major
events of limb innervation by DRG neurones. The open bar is the
period over which cell proliferation occurs and the closed bar
is the period over which cell degeneration takes place.

AGE	Stage		
	16		
	17		
E3	18		extension of bipolar neurites
	19		
	20		
	21		Axons enter plexus region
	22		
E4	23		
	24		Axons enter limb
	25		
E5	26		
	27		Axons reach dorsal skin
	28		Axons reach hindlimb tip
E6	29		
	30		Growth cones enter grey matter
E7	31		Axons reach forelimb tip
	32		
E8	33		
	34		
E9	35		
E10	36		
E20	45		

The experiments which will be detailed later were carried out at E4.5, 5.5 and 6.5. From the data it is clear that at each of these stages ganglia will contain proliferating, degenerating and innervating neurones in varying numbers.

1.2 PATTERNING

Now that the time course of DRG sensory neurone development and innervation has been established, we must consider how the pattern of innervation is controlled. That is, is the projection of these neurones specific? If so, is this specificity present from the earliest stages of development, or does it arrive from an initial random projection into the periphery? Finally what factors control the pattern of outgrowth seen, are they intrinsic to the neurones or do they take the form of environmental cues.

1.2.1 SENSORY MODALITIES

In the adult, DRG neurones will innervate a great variety of end-organs in the periphery. These may be broadly divided into two categories; i) exteroceptive (cutaneous) ii) proprioceptive (deep) endings. Can any separation of DRG neurones in these terms be made? As previously mentioned, DRG are made up of an early differentiating VL population and a later differentiating DM population, might these be related to the different sensory modalities seen?

Vistini and Levi-Montalchini (1939 in Hughes and Carr 1978) recorded the earliest exteroceptive responses in the chick at E6, which they stated corresponds to the earliest outgrowth from the DRG, which is mainly from the early differentiating VL neurones. They concluded from this that VL neurones are mainly cutaneous afferents. The earliest proprioceptive reflexes were recorded at E11, which corresponds to DM neurone maturation. They concluded from this that at least some of the DM neurones must be proprioceptive afferents. In a later study, Hamburger and Levi-Montalchini, (1949) also concluded that the large VL neurones were mainly cutaneous afferents.

These conclusions were inferred, and it was not until the pioneering work of Oppenheim and Heaton (1975) using their new retrograde tracer, Horse Radish Peroxidase (HRP) that any definite conclusions could be drawn. They found that at E4.5 - 5 there was no tendency for labelled cells to be found in any particular part of the ganglion. This would appear to suggest that both VL and DM cells were labelled. However, Hamburger et al. (1981) have demonstrated that the earliest DM cells do not appear until E5. These labelled cells would then all appear to be VL in nature. Honig (1982) confirmed these studies using careful retrograde labelling of particular tissues. She showed that at E6.5 (st29-30), VL neurones projected to targets in both skin and muscle, but no labelling of DM neurones was found. This was attributed to the late birthdates of these cells, which

meant that their axons would have not yet entered the limb by this stage. Further experiments at later stages of development showed that DM neurones also project to targets in both skin and muscle. She also demonstrated, in this comprehensive study that these initial projections are not random and later modelled by selective loss of terminals or cell death to give a selective VL-cutaneous, DM-deep pattern of innervation. Rather, these initial projections are stereotyped and similar to the adult pattern which is seen at E10-11 (st 36-37). Finally arguing that the small amount of cell death occurring subsequent to these times (mostly in the DM region) could not result in a later segregation of innervation. It appears from these data that the VL and DM populations show no correlation with projection patterns to different target tissues in the periphery.

1.2.2 SPECIFICITY OF PROJECTION

Motor neurones, located in the lateral motor column of the spinal cord show a stereotyped projection pattern, so much so that their target muscles can be identified by the location of their cell bodies by rostro-caudal and medio-lateral co-ordinates (see Landmesser, 1984 for review). These projections have been demonstrated to be correct from their first establishment. Honig (1982) has investigated the establishment of proprioceptive innervation in the hind limb. She found that each muscle is innervated by neurones situated throughout the

DRG which means that they have different birth dates (birth occurring from VL margin through to DM margin). There is however a strict segmental arrangement of the projection, such that each mixed nerve trunk is made up of axons having cell bodies in two to three contiguous DRG. Individual muscle nerves have a further restricted projection from only one or two ganglia. Finally as already stated, the projection pattern is accurate from its first formation just the same as the situation for motor neurones. These HRP studies were confirmed by electrophysiological recording from nerve roots. In conclusion then, the location of cell bodies within a ganglia cannot be used to predict their target field, but the ganglia as a whole will have a fairly restricted pattern of innervation.

Scott (1982) has made a companion study on the development of dermatomes in the hind limb. A dermatome is the area of skin to which a single ganglion projects. In the trunk, the dermatomes show a simple striped arrangement, which is in register with the innervating DRG. In the limb the situation is slightly more complicated. Scott (1982) determined how this pattern arose. In brief, the projection pattern was found to be stereotyped and correct from its first formation. The specificity of connection does not arise from the modelling of the initial projection or the migration of skin down the leg (both of which had been previously suggested). These studies were carried out using labelling and electrophysiological techniques from the earliest stages at which innervation was found. (E6:st28/29). Each DRG

sends axons out along specific pathways to innervate the skin at a particular point, after which elaboration of nerve branch patterns leads to dermatome establishment.

1.3 CONTROL OF PROJECTION PATTERN

As the majority of connection with the periphery are made prior to the central processes entering the grey matter (Windle and Orr, 1934 in Romanoff, 1960; Honig, 1982), it can be presumed that a neurone's central connections do not determine its peripheral connections. The establishment of a specific innervation pattern, must then either be controlled by factors in the environment of the developing neurone and its processes or be determined by intrinsic properties of the neurones. This question has been tackled by many authors using direct and indirect methods in vivo. The majority of this work concerns the establishment of the innervation of the limbs.

1.3.1 ECTOPIC LIMBS

Hamburger (1939) grafted additional limbs onto host embryos at ectopic sites. These experiments were performed at early stages of development and the contribution of segmental nerves to the grafts was viewed at later stages. He found that in the majority of cases foreign nerves appeared to be "attracted" into

the ectopic limbs. Providing that the site of the graft was relatively close to the neural tube, a plexus at the base of the limb and a morphologically, relatively normal pattern of innervation were formed. In this study no separation between motor and sensory innervation is made. He concludes from these findings that the pattern of innervation is determined by the character of the limb and not by factors intrinsic to the neurones.

Using a different approach to the same question, (Straznichy, 1963) removed brachial   and replaced them with brachial, thoracic or lumbo-sacral segments from clones at the same stage of development. In all cases a relatively normal pattern of innervation was produced, but normal function was only seen after brachial transplants, this being presumably due to inaccuracies in the central projections of the neurites.

Finally, Swanson and Lewis (1982) have shown that a forelimb-bud grafted onto the third brachial arch will induce a relatively normal pattern of innervation from foreign nerves (mainly trigeminal and facial). This and the earlier work stated has led Lewis *et al.* (1983) to put forward the theory that the limb provides "public highways" for nerve growth. "Public" in that they are open to neurones from any source, and "highways" as they constitute a restricted set of permitted routes within the limb. Are axons specified for the route they take within this

system of highways? Lewis (1978), provides evidence that they may not be. he created forelimbs reduplicated in the proximo-distal axis and examined the pattern of their innervation. Here at the first elbow, axons normally branch into three nerve trunks, one of these, the median branch would normally innervate muscle and skin of the hand. However in the reduplicated limb this branch will encounter a second elbow. Lewis found that the median nerve again branched — into three. If axons were chemo-specifically guided into nerve trunks, those which "chose" the median trunk on the first occasion, should also prefer this pathway on the second occasion; the fascicle should not branch again. This result indicates that the axons may not be chemo-specifically guided into pathways within the limb, as they show no consistent preference for a particular pathway.

1.3.2 NEURAL TUBE ROTATIONS

Another type of perturbance experiment involved rotating the neural tube and associated neural crest. The majority of this work has been carried out in the hindlimb and will be briefly summarised. Following "short" neural tube rotations (prior to axon outgrowth at st15/16) when axons are still able to enter their segmentally appropriate plexus they will project to the correct muscles (Lance-Jones and Landmesser, 1980, 1981b). In contrast to this, following "large" neural tube reversals when axons were forced to enter inappropriate plexi two distinct types of behaviour were seen. Some axons innervated appropriate

muscles by taking novel routes through the limb. Others projected to a variety of inappropriate muscles. In these experiments motoneurones alone were studied. Honig, Lance-Jones and Landmesser (1986) studied the distribution of sensory axons following similar perturbations. They found that in the majority of cases, when motoneurones had successfully navigated to their targets (ie. after "short" reversals), sensory neurones from the same segments had also projected correctly.

Further, Scott (1986) has shown that the ventral neural tube also affects the projection of cutaneous axons. Following the rotation of the dorsal half of the neural tube (including the neural crest), DRG cutaneous innervation patterns corresponded to their new position. When the entire NT was rotated, however, axon projections were appropriate to their original segmental position. The converse finding was also true. This led to the proposition that the motoneurones were acting as guides for the sensory neurones.

1.3.3 REMOVAL OF MOTONEURONES, MUSCLE OR SKIN

It should be noted at this point, that early ablation of DRG does not affect the development and projection of motor neurones. In the same year Landmesser and Honig (1986) investigated the projection of sensory axons following early motoneurone ablation. They found that following these experiments, muscle nerves either did not form or were

dramatically reduced in diameter. Those that did form failed to enter muscle tissue and ran along its surface for great distances. They concluded that "motorneurones have a profound affect on sensory neurone outgrowth". Interestingly cutaneous nerves either appeared normal or were increased in diameter. This suggests that some of those axons which had not innervated muscle had instead projected down cutaneous pathways. Scott (1988) has shown that cutaneous innervation is relatively unaffected following motoneurone ablation. Swanson and Lewis (1986) in their similar study arrived at the same conclusion. In a further experiment, presumptive myoblasts were destroyed early in development, resulting in the production of a muscle-less wing (Lewis et al., 1981). Muscle nerves failed to form while cutaneous nerves remainedunaffected. Recently, Phelan and Hollyday (1990) surgically created muscleless wings, and showed that under these conditions muscle nerves were formed. It was not noted whether these contained both motor and sensory axons, however one would predict this from earlier work.

These experiments suggest that DRG axons are able to follow specific guidance cues within the limb, at least in proximal regions, so allowing the formation of mixed nerve trunks in the absence of motoneurones. However, in the absence of motoneurones, DRG axons are unable to form motor nerves. As muscle nerves are able to form in the absence of muscle, the axons must be navigating by other non-muscle cues.

Martin *et al.* (1989) carried out an experiment in which limbs were partially denuded of ectoderm (the target tissue of cutaneous axons). Under these conditions, mixed and muscle nerve trunks formed, but cutaneous nerve branches were absent even from the earliest stages examined. Again DRG neurones fail to form specific branches in the absence of target tissue, except that in this case control cannot be attributed to another neural population as in the previous experiment.

These studies suggest that DRG axons may not be pre-specified for their peripheral targets. This is demonstrated by the condition following motoneurone ablation, when many of the sensory axons which would normally have projected to muscle are re-routed into cutaneous nerve branches. A cell-surface glycolipid has been identified using a monoclonal antibody (SN1) which is only expressed by DRG neurones subsequent to target field innervation (Marusich *et al.*, 1986a and b; Marusich and Weston, 1988). They speculate that this is a marker for cutaneous sensory neurones, as it is most widely found in the thoracic ganglia, which contain less proprioceptive neurones than limb innervating ganglia. As this marker is also expressed on motoneurones in the lateral motor column, it is proposed that interaction with the periphery specifies the central connections of the neurones.

To summarize, DRG axons are able to form mixed nerve trunks in

response to environmental cues. They will only form muscle nerves when motoneurones are present to act as "pioneers" and cutaneous branches will only form in the presence of target tissue. It appears that even the nature of the targets to which DRG neurones project can be modulated by the limb. Finally, and most importantly, the limb appears to be the major controlling influence for innervation patterning. It is worth mentioning at this point that a normal limb, in terms of muscle, bone and cartilage, will develop in the absence of its normal source of innervation, motor and sensory (Lewis, 1980). Thus the limb develops autonomously.

1.4 FACTORS CONTROLLING PATTERNING

What factors present in the limb might be capable of controlling the pattern of innervation? Al-Ghaith and Lewis (1982) used electron microscopy to study neural growth cones as they traversed the limb. They found that the growth cones were associated with either other axons or mesenchyme cells over their entire surface. They propose that these intimate interactions allow for the laying down of "highways" (Lewis, 1983) within the limb for axon growth. In contrast to this Tosney et al. (1988) have demonstrated cell death and phagocytes present along prospective axon growth tracts in advance and independent of the axon growth cones. They do not state what cell types are being phagocytosed, but the non-muscle mesenchyme would seem a good candidate. They suggest that

phagocytes may either open up pathways for growth cone advancement, or release products from degenerating cells which have trophic properties. These two observations appear incompatible.

1.4.1 EXTRACELLULAR MATRIX MOLECULES

Al-Ghaith and Lewis (1982) in their study find no evidence for an association between pioneering growth cones and fibrils of the extracellular matrix. However immunohistochemical studies of the distribution of extracellular matrix molecules has revealed their presence in the vicinity of axon tracts. Rogers *et al*. (1986) have observed laminin whilst Tomasek *et al*. (1982), Yip and Yip (1990) have shown fibronectin to be present around nerve roots and along axon pathways within the limb. Though their distribution does not precisely match that of nerve trunks and their appearance prior to growth cones has not been convincingly demonstrated. Riggott and Moody (1987) working in a different system have shown a punctate array of laminin extending from the trigeminal ganglion into its target field, the mandibular process. Further, its presence has been demonstrated prior to the outgrowth of axons from the ganglion. Davies (1987b) suggests that such arrays may provide permissive pathways over which more specific guidance cues, such as trophic factors could act. Both fibronectin and laminin have been demonstrated to be suitable substrates for neurite extension from DRG neurones in culture. (Rogers *et al*., 1983). In

addition the Neural Cell Adhesion Molecule (NCAM) is present in the presumptive plexus region prior to the ingrowth of axons (Tosney et al., 1986). It has also been visualised in preformed tracts for optic axons (Silver and Rutishauser 1984). Therefore some role for adhesive molecules during limb innervation cannot be ruled out.

Recently Tanaka et al. (1989) using monoclonal antibodies, have identified a molecule of approximately 70kDa located on mesenchymal cells along major nerve trunk pathways in the developing limb. It is present prior to axon invasion and appears to mark out the paths of the early dorsal and ventral nerve trunks. However its expression in posterior sclerotome, which is non-permissive for axon outgrowth is confusing. It appears that this molecule may be permissive for axon outgrowth, and may be important in the establishment of non-specific pathways within the limb. The nature of the molecule is still unclear.

1.4.2 CHEMOTROPHIC FACTORS

The effects of trophic molecules on the establishment of innervation, have so far not be discussed. Two major trophic molecules are implicated in the regulation of sensory neurone populations: Nerve Growth Factor (NGF: Thoenen and Barde, 1980) which is synthesised in peripheral fields and Brain Derived Neurotrophic Factor (BDNF) which is synthesised in central

fields (Barde *et al.*, 1982).

Much work has been carried out on the properties of NGF, both *in vivo* and *in vitro*, and has led to the hypothesis that NGF attracts sensory and sympathetic neurones to peripheral targets by chemotropism (Levi-Montalchini, 1982) and promotes the survival of responsive neurones *in vivo* (Hamburger and Yip, 1984). Injections of NGF into the cranium of neo-natal rats *in vivo* results in the aberrant growth of sympathetic neurones into the spinal cord and along the dorsal columns toward the injection site (Menesini-Chen *et al.*, 1978). DRG explants in culture show the greatest degree of outgrowth from the side facing a source of NGF (Charlwood *et al.*, 1972; Ebendal and Jacobson, 1977). Further sensory neurites show an orientation response to higher concentrations of NGF (Letourneau, 1978) and in the almost classic experiment of Gunderson and Barret (1979, 1980) will turn toward a focal source of NGF, *in vitro*.

In many of these experiments, however, extremely high concentrations of NGF have been used. Gunderson and Barret (1979, 1980) for example, used NGF concentrations >25 000 times the maximum found in the most densely innervated cutaneous field in the mouse (Davies, 1987b). It has been demonstrated that at such massive concentrations, NGF is adsorbed onto culture surfaces and so increases adhesivity (Schubert and Whitlock, 1977). As neurites are known to grow preferentially on more adhesive surfaces (Letourneau, 1975b) many of the experiments *in*

vitro may simply be explained in terms of growth onto more adhesive substrate (Davies, 1987b). Springer and Loy (1985) have shown that sympathetic nerve fibres sprout in response to elevated NGF concentrations *in vitro*. Further, Campenot (1982,a,b) using an ingenious compartmentalised culture chamber has demonstrated that NGF can selectively maintain the branches of sympathetic neurones in its locale. Davies (1987b) again believes that these results can explain some of the earlier work *in vivo* and *in vitro*. If neurites or axons grow at random from a ganglion or explant, and those that by chance grow toward an NGF source were selectively maintained, while those growing in other directions degenerated. In the final outcome the many branches seen on the side of the NGF source might be viewed as evidence for attraction, which is not necessarily the case.

Further, it has been demonstrated that the growth and survival of sensory neurones is independent of NGF and other target derived factors prior to target field innervation (Davies *et al.*, 1987; Davies and Lumsden, 1984; Ernsberger and Rohrer, 1988; Ernsberger *et al.* 1989). Davies *et al.* (1987), working on the innervation of the maxillary process by the trigeminal ganglion, have elegantly demonstrated that in this case, NGF synthesis in the target field and NGF receptor expression by neurones does not occur until target innervation is seen. This would argue against NGF acting as a chemotropic (attractant) factor, but rather as a chemotrophic (survival) factor. Incidentally, this precise timing and correlation between NGF

expression and reception, stresses the importance of studying innervation/target interactions at the appropriate time. Many earlier studies have used denervated targets and axotomised neurones, which may provide little information as to the initial interactions during innervation.

BDNF is a protein present in extremely small quantities in the CNS which has been isolated from pig brain (Barde *et al.*, 1982). It has been shown to promote the growth and survival of neurones derived from the neural crest and ectodermal placodes, both *in vivo* (Hofer and Barde, 1988; Kalchiem *et al.* 1987) and *in vitro* (Lindsay *et al.*, 1985). Earlier, Yip and Johnson (1985) had demonstrated that following rhizotomy (severing of the dorsal roots) many DRG neurones died, which suggested the importance of a centrally derived factor for neural survival, though they incorrectly identified this as NGF. The work of Hofer and Barde, (1988) showed that following the same procedure neurones could be "saved" by implanting a foreign membrane coated with laminin and BDNF. The laminin appears to be permissive for BDNF activity though no mechanism has been proposed. Interestingly, DRG neurones show different times for the onset of receptivity to BDNF and NGF. These begin at E4 and peak at E10 for BDNF, and E5 with an E8 - 11 peak for NGF (Davies *et al.*, 1986; Davies and Lindsay, 1985). This timetable does not correlate with the formation of connections which occurs earlier in the periphery E5.5 (st28) than centrally E7 (st31; Honig, 1982). However, the first processes do enter the spinal cord as early as E3 (Cajal,

1929 in Guth, 1960) therefore growth cones could take up growth factors from the CNS even though they have not developed contacts with it. There appears to be some degree of overlap between the activities of NGF and BDNF on neurones. 60% of E6 DRG neurones survive in the presence of NGF or BDNF in saturating concentrations, but this increases when the two are present together. (Lindsay *et al.*, 1985; Davies and Lindsay 1985). In a detailed molecular study, Rodriguez-Tebar *et al.*, 1990) have demonstrated that while high affinity receptors for the two molecules are distinct, they appear to share a common low affinity receptor with a slightly higher affinity for BDNF. It is speculated that as the two molecules show a 60% homology in amino-acid sequence , that the low affinity receptor might be common to a whole family of as yet undiscovered trophic molecules. One such factor has been identified by Lumsden and Davies (1983, 1986) in the peripheral field of the mouse. This is specific for neurones of the trigeminal ganglion and its activity is distinct from either laminin or BDNF. This epithelial derived target factor has been tentatively named TNF, trigeminal neurotrophic factor.

It appears then, that target derived neurotrophic factors play a key role in the development and maintenance of specific neural populations. But some debate remains as to whether they can act as chemoattractants and actively guide neurone to their targets over what can be quite large distances.

1.5 CONTROL OF INNERVATION RATE

For many of the studies cited, it is clear that limbs can cause foreign nerves, not only to form a normal pattern of innervation, but in so doing elaborate processes over considerably greater distances than they would normally grow. Compare, for example, the distance which cranial or thoracic sensory neurones grow to their normal peripheral targets, with the most distant targets in the limbs which they can be caused to innervate. The latter will be approximately 2 - 3 times greater than the former. Most of these studies have been concerned with patterning, but it would appear from their results that the limb is also capable of controlling the rate of innervation. Only one piece of work has directly addressed this question, that of Swanson and Lewis (1982). They transplanted limbs between embryos at two different, early stages of development (sts 18 and 22, both prior to axon invasion) and compared innervation rates with control limbs at sts 29 and 31. They found that where an older limb-bud was grafted onto a younger embryo, axon growth was accelerated, resulting in an innervation pattern appropriate for the grafted limb. Conversely, when a younger limb was grafted onto an older embryo, axon growth was retarded, and the progression of innervation was again appropriate for the age of the grafted limb. They concluded from this that it is the limb which governs the rate of its innervation and not some intrinsic properties of the neurones. It has previously been stated that the "waiting

period" observed during limb plexus formation between sts 21 and 23 may reflect the non-permissive nature of early limb-bud mesenchyme for axon growth (Tosney and Landmesser, 1985a; Landmesser, 1987). This could then be a mechanism for controlling the rate of axon ingrowth and would be directly related to the stage of development of the limb. Do nerve fibres have a similar potential for growth which is modulated by the opening up of pathways within the limb? Are these similar to those in the neck and thorax, where axon growth is observed to be slower *in vivo*, rather than simply limited by the extent of their target fields? In short are there any intrinsic differences in the growth potential of neurones.

1.5.1 INTRINSIC CONTROL OF INNERVATION RATES

Recently, Davies (1989) examined the growth rates of cranial sensory ganglia both *in vivo* and *in vitro*. Prior to this very little work had been carried out on neural growth rates (Speidel, 1941; Bray, 1973; Jacobson, 1985; Davies, 1987), and although differences had been noted, no comparisons between these and target distances were made. The four cranial ganglia studied here; geniculate, vestibular, petrosal and nodose are derived from ectodermal placodes and show a similar ontogeny. Their targets are located at varying distances from the ganglia. Vestibular neurones have the closest targets, through geniculate and petrosal to nodose neurones which have the most distant

targets. Using wholemount, silver staining techniques he demonstrated that the first processes leave all the ganglia at the same stage of development. Following this they all show characteristically different rates of growth toward their targets. More importantly, these rates are directly related to target distance. Vestibular neurones have the slowest growth rates, then geniculate, petrosal, and finally nodose neurones which have the fastest growth rates. Davies then isolated ganglia at early stages, prior to axon outgrowth and prepared dissociated cell cultures. When growth rates were measured for the bipolar neurites, comparable rates to those seen *in vivo* were recorded. The neurones still showed differential growth rates, which were proportional to their *in vivo* target distances, even though they were now isolated from their normal environment. Davies hypothesised from these studies that the growth rate of these neurones is intrinsically programmed and related to the target distances found *in vivo*.

It would be interesting to know how these neurones would respond to a grafted limb in their immediate environment. Would their intrinsic programme be overridden by the "attractive" properties of the limb, and lead to them elaborating much longer processes, and possibly at greater rates than would normally occur. Such a possibility is suggested by the work of Swanson and Lewis (1982), where trigeminal and facial nerves were shown to innervate an ectopic limb placed at the level of the jaw. The pattern of innervation received by the limb is seen to be

appropriate for its stage of development.

1.6 CONCLUSION

From the historical data presented, it is clear that there is much conflicting evidence concerning the control of peripheral sensory innervation, particularly in the limbs. The majority of the work on DRG cited has been carried out *in vivo*, where a great many complicating factors surround each experiment. There seems to be good reason to conduct a detailed study of the developmental potential, particularly in terms of neurite growth of DRG neurones at different stages of development and from different segmental levels under controlled, cell culture conditions.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

Domestic fowl (Gallus domesticus) were obtained from either the Animal Physiology and Genetics Research Station, Roslin, Edinburgh (strain J-line), or Ross Breeders, Newbridge, Edinburgh (strain MPZ). Data from the two strains showed no systematic differences. Throughout, eggs were maintained in a humidified, force draft incubator at 37°C.

2.2 DISSECTION AND CULTURE

On the day of experiments, eggs were removed from the incubator and a small window cut in the shell, above the air sac. The chorio-allantoic membrane was removed revealing the embryo, which was staged according to Hamburger and Hamilton (1956). Embryos which had reached the appropriate stage of development were removed from the egg and placed into ice-cold Phosphate Buffered Saline (PBS), decapitated, cleaned of attached extra-embryonic membranes and eviscerated.

The embryos were then transferred to a clean Sylgard (BDH) bottomed dish in PBS and pinned dorsal surface up with the limb-buds splayed out. At these early stages of development the neural tube was clearly visible, so enabling the tissue which

overlies it to be removed from the embryo without damage to the underlying structures. The neural tube and paired Dorsal Root Ganglia (DRG) were exposed from brachial to lumbo-sacral segmental levels. In younger embryos the neural tube and DRG were freed from surrounding tissues and taking care to keep apart the different segmental levels to be studied, neural tube and DRG were removed together.

The DRG were then separated from the neural tube with fine forceps using a stripping action. Frequently the ganglia from one side of the neural tube remained attached and resembled a string of pearls on removal. In older embryos following exposure of the neural tube, ganglia were usually singly plucked from the appropriate segmental levels. Is some instances only lower thoracic and lumbo-sacral levels were exposed. A 1% solution of Phenol Red in PBS was dropped onto this region to aid in the specific identification of the ganglia which contribute to the lumbo-sacral plexus, and ganglia which contribute to the crural and sciatic plexi were removed separately (LS 1 - 3 and 4 - 7 respectively Scott, 1987). In all cases DRG explants were placed directly into a 0.05% Trypsin solution in Ca/Mg free Trizma (Sigma) buffered saline (T.D) at 37° C for 45 min. Following this the explants were spun down by a brief pulsatile action (<5 sec at 60,000 rpm, Eppendorf microfuge) and resuspended in T.D. containing 0.025% Soybean Trypsin Inhibiter (Sigma) and 0.02% DNase (Sigma) this denatured DNA released from lysed cells which would otherwise lead to

cellular re-aggregation. The explants were then triturated by not more than 10 passages through a fire-polished pasture pipette   to give a single cell suspension. This was again spun down (5 - 10 sec at 60,000 rpm) and resuspended in Ham's F12 media supplemented with: antibiotics (Gentamycin sulphate; 50ug/ml, Kanamycin sulphate; 100ug/ml), Conalbumin; 40ug/ml, L-Glutamine; 2mM (All from Sigma) and Fetal Calf Serum (FCS:Flow) at a final concentration of 10%.   50 ul of the resultant cell suspension was added to each of 24, pre-coated 9mm glass coverslips in individual 16 mm wells (Linbro space saver : Flow laboratories).   The culture dish was then placed into the humidified incubator at 37°C for 1 hr to allow cells to adhere, after which time a further 450ul of pre-incubated, complete media was added to each well to make 500ul in total.   In some cases Nerve Growth Factor (NGF: Sigma) was added to selected wells at a final concentration of 20 ng/ml. Culture wells were then replaced in the incubator for a further 23 hr.

After the 24 hr culture period, media was removed from each well and replaced with PBS, which was in turn replaced with 4% Paraformaldehyde in PBS for 10 min.   Finally the culture was rinsed in PBS , coverslips were removed from the culture wells, drained, mounted on glass slides in Aquapolymount (Polysciences) and allowed to dry at room temperature in preparation for microscopy.

2.3 COATING OF COVERSLIPS

2.3.1 POLY-DL-ORNITHINE

Coverslips were coated with the poly-cationic amino-acid Poly-DL-ornithine (PORN:Sigma) which ensures the attachment of all viable cells in culture and increases subsequent laminin binding (Edgar *et al.*, 1984). 9mm glass coverslips were flame-sterilised in absolute alcohol, cooled and placed into each 16mm well of a 24 well culture dish. 75ul of 250ul/ml PORN in Borate buffer (pH8.4) was added to each coverslip and incubated at 20°C for 30 min. The PORN was removed, each coverslip was washed three times in sterile, double-distilled water and allowed to dry and sterilise under a U.V. lamp for 60 min at 20°C.

2.3.2 LAMININ

In the majority of cultures, Laminin coating was carried out subsequent to PORN coating. Laminin (Bethesda Research Laboratories) at 100ug/ml in sterile T.D. was added at 50 ul/coverslip, these were then placed into the humidified incubator for 30 min at 37°C. Following this the Laminin solution was drawn off and the coverslips washed once in sterile, double-distilled water. Laminin coating was always carried out on the day of experimentation.

2.3.3 FIBRONECTIN

In some instances Fibronectin rather than Laminin was used to coat the coverslips. Fibronectin at 100ug/ml in T.D. was applied in an identical manner to that indicated above for Laminin.

2.4 STAINING PROTOCOLS

2.4.1 STAINING FOR THE 68Kd NEUROFILAMENT SUB-UNIT PROTEIN

Staining was carried out using a monoclonal antibody raised against the neuron-specific, 68Kd neurofilament protein in 24 hr cultures according to the following schedule:

1. wash three times in PBS

2. incubate for 30 min with FCS at 20°C

3. wash three times in PBS

4. fix and permeabilise for 4 min in acetone at -20°C

5. wash three times in PBS

6. incubate for 60 min in 68Kd neurofilament antibody at
    1:50 dilution in PBS at 37°C

7. wash three times in PBS

8. incubate for 60 min in Fluoroscein Iso Thio Cyanate
    (FITC) conjugated, goat-anti-mouse IgG secondary at 1:5
    dilution in PBS at 37°C

9. wash three times in PBS

10. mount in Aquapolymount on glass slides.

2.4.2 STAINING WITH PROTARGOL (SILVER-PROTEINATE) IN WHOLEMOUNTS

A modified silver/Protargol method (Lewis, 1978) was used to investigate the extent of innervation in wholemount embryos. Whole, decapitated, eviscerated embryos at stage 28 of development were fixed in a mixture of; alcohol, water, formalin and glacial acetic acid in the ratio; 75:15:5:5 for 3 day. After which time they were stained according to the following schedule;

1. rinse for three day in 70% alcohol (four changes)

2. incubate for 19 hr in the dark at 37°C with agitation in a 1% aqueous Protargol solution (Strong silver protein: Roques was sprinkled onto the surface of dH$_2$O at 37°C and allowed to dissolve without agitation.) with a piece of bright copper (0.9mm copper; BDH wire (BDH) was cleaned in detergent, rinsed and added to the Protargol solution with the tissue at 1.25g/100ml)

3. rinse in dH$_2$O for 10 min

4. reduce for 60 min at 4°C with agitation in a 75% aqueous solution of Sodium sulphite

5. rinse for 2 hr at 4°C with agitation in dH$_2$O (three changes)

6. tone for 60 min at 4°C with acidified 1% yellow gold chloride (acidification was by the addition of three drops/100ml Acetic acid)

7. rinse for 60 min in dH$_2$O (three changes)

8. bleach for approximately three hr in a fresh 5% aqueous solution of Potassium fericyanide

9. rinse for 60 min in dH_2O (two changes)

10. soak for 2 hr in a fresh 5% alkalinised aqueous solution of Sodium thiosulphate (alkalination was by the addition of 0.5ml/100ml NaOH)

11. rinse for 60 min in dH_2O (two changes)

12. dehydrate in alcohol

13. clear in Methyl salicylate

2.5 MICROSCOPY AND PHOTOGRAPHY

A Nikon Diaphot inverted microscope fitted with phase-contrast optics was used throughout to observe both living and fixed cultures. Still photographs were taken on a 35mm Nikon F-301 camera using Kodak : TMax 400ASA, Ektachrome 200ASA and Ektachrome Professional 800/1600 ASA films. Video images were collected on a JVC TK-10 monochrome camera and displayed on a JVC TM-9060 monochrome monitor. Fluorescence microscopy was carried out using a high pressure mercury light source and a blue (450-490nm) excitation block (Nikon :B2-H) to view FITC conjugated antibody labelled cultures (Peak emission 535nm : Green).

A Nikon Labophot microscope equipped with bright-field illumination and a camera lucida was used to view the wholemount, silver/Protargol stained embryos.

2.6 MEASUREMENT AND ANALYSIS OF NEURITE LENGTHS

Fixed neural cultures were systematically assayed and all cells which fulfilled the following criteria were recorded :

i) Cell bodies; rounded, discreet structures, not clustered or associated with glia.

ii) Neurites; fine, not flattened and terminating freely not on other neurites, cell bodies or glia. An often substantial minority of cell bodies which fulfilled the first criterion but grew no neurites after 24 hr in culture were recorded as "failures". For each neurone a small sketch of its neuritic arbor was made and the length of each neurite branch was recorded.

Measurement of neurite length, and in some cases cell body diameter were made from digitized video images (Watford Electronics) displayed on an Acorn Archimedes computer. The monitor display was configured to maintain the correct aspect ratio and calibrated against a microscope slide graticule. Distance between two points, located by their x and y co-ordinates on the screen were made automatically using Pythagoras' theorem. Thus, by clicking the computer mouse along a neurite and thereby tracing its course, the total length was calculated from the sum of distances between successive points. Such measurements were accurate to within 1um which is approximately 1/100th of the mean neurite length measured, thus

any minor inaccuracies are negligible.

Results were stored on a commercial spread-sheet package (Minerva:Sigma-sheet), in such a way as to enable the later sorting of data for single neurite length, mean neurite length and inter-branch lengths. Using our own software again, data was taken from the spreadsheet, placed into 25um 'bins' and displayed as histograms, which also showed the number of failures present.

2.7 CURVE FITTING

This procedure is described in greater detail in the results section, but briefly; histograms of neurite length were tested against a Poisson model of neurite growth. Two important assumptions are made when applying the Poisson statistic: first, underlying events which give rise to neurite outgrowth occur with a low probability. This would predict the occurence of some neurones with no neurites. Second, each "event" occurs independently of another in the same and different neurones, therefore the amount of growth occurring from one neurone is unaffected by those around it.

2.7.1 THE POISSON STATISTIC

The Poisson statistic calculates the probability; (P) of an amount of growth; (x) occurring, given knowledge of the mean

amount of growth; (m). This equation is however only suitable for describing discontinuous variables, eg if x = were to equal the number of neurites expressed by a neuron. Neurite length is obviously a continuous variable and in order to modify the equation the distribution of values about each "unit length" must be taken into account. The error function, which describes a Gaussian distribution was used to calculate values for each quanta, and the areas under the individual curves were summed to give the final curve. This must then be appropriately scaled to fit the displayed data, (see Results). Curve fits were calculated using our own software for the Acorn Archimedes computer.

2.8 STATISTICS

2.8.1 DESCRIPTIVE STATISTICS

Descriptive statistics were used to quantify the forms of the illustrated distributions of neurite lengths. Mean and standard deviation were not used as the data is not normally distributed. Instead mode, median and inter-quartile ranges were calculated. The mode is that class which contains the most observations, and generally located the mean of the unitary neurite growth event. The median is the central observation when all values are ranked, not including failures, the difference between mode and median is a measure of the skewness of the distribution. Finally inter-quartile range is the range

of values -25% of the population from the median and is an
indication of the spread of values present. All three figures
are quoted as the upper limit of the histogram bins in which
they occur.

2.8.2 STATISTICAL TESTS

A Mann-Whitney non-parametric test was used to compare the
medians recorded under different conditions. Tests were carried
out using the "First" statistical package for the Archimedes
computer.

3 RESULTS

3.1 SEGMENTAL SENSORY INNERVATION

In the chick, as in all vertebrates innervation of peripheral tissues (mainly skin and muscle) develops in a segmental pattern. Dorsal root ganglia form in pairs along the length of the spinal cord in a rostro-caudal sequence. Axons grow out from these sensory ganglia into the periphery from as early as 50 - 60 hr of development (st 17/18). It has previously been shown that the initial growth rate of axons innervating trunk skin and musculature is considerably slower than axonal growth rates from neurones in ganglia which innervate the limbs.

Experiments were carried out in order to ascertain whether these differences in the growth rates observed *in vivo* are intrinsic to the neurones; that is, are their differences a consequence of the segmental location of the ganglionic neurones? Subsequent growth would then occur at a rate independent of the local environment of the axonal growth cone.

To investigate this issue, ganglia from different segmental levels, at different stages of development were isolated, enzymically dissociated and cultured under identical conditions. If the differences in outgrowth rates seen in vivo were intrinsically regulated by the segmental position of the ganglia, then these differences would be preserved in culture.

However, if the differences seen in the rates of outgrowth were determined by the environment of the growing axon, one would not expect to see any differences in the rates of neurite outgrowth. The data presented below suggests that the latter of these two possibilities is more likely. Following this, some caveats in the technique will be addressed and the effects of some common environmental growth promoters and modifiers (extracellular matrix and trophic molecules) will be examined.

3.1.1 PERIPHERAL INNERVATION *IN-VIVO*

Chick embryos were stained using a Silver/Protargol method, modified for use on wholemounts by Lewis (1978 see Methods). The results, which are summarised in Fig.3.1 show a composite drawing taken from three embryos stained at st28 of development (approximately 5.5 days of incubation at 37°C). No distinction between sensory and motor axons is made in the drawing, but as sensory and motor axon growth cones have been shown to leave the brachial and lumbo-sacral plexi at the same time (Tosney & Landmesser, 1985 a,b,c; Honig, 1982) and to innervate distal areas of the limbs at similar times, the illustration may be taken as an accurate reflection of the extent of sensory innervation. These observations show that axons in fore and hindlimb regions are approximately three times longer than those in the trunk, 18mm compared with 6mm respectively. Axons emerge from condensing ganglia at slightly different times, with

FIGURE 3.1

Innervation of the limbs and trunk of the stage 28 chick embryo, compiled from camera lucida drawings of three separate embryos fixed and stained with a silver/protargol method (Materials and Methods) to show nerve branches.

Scale bar = 1mm
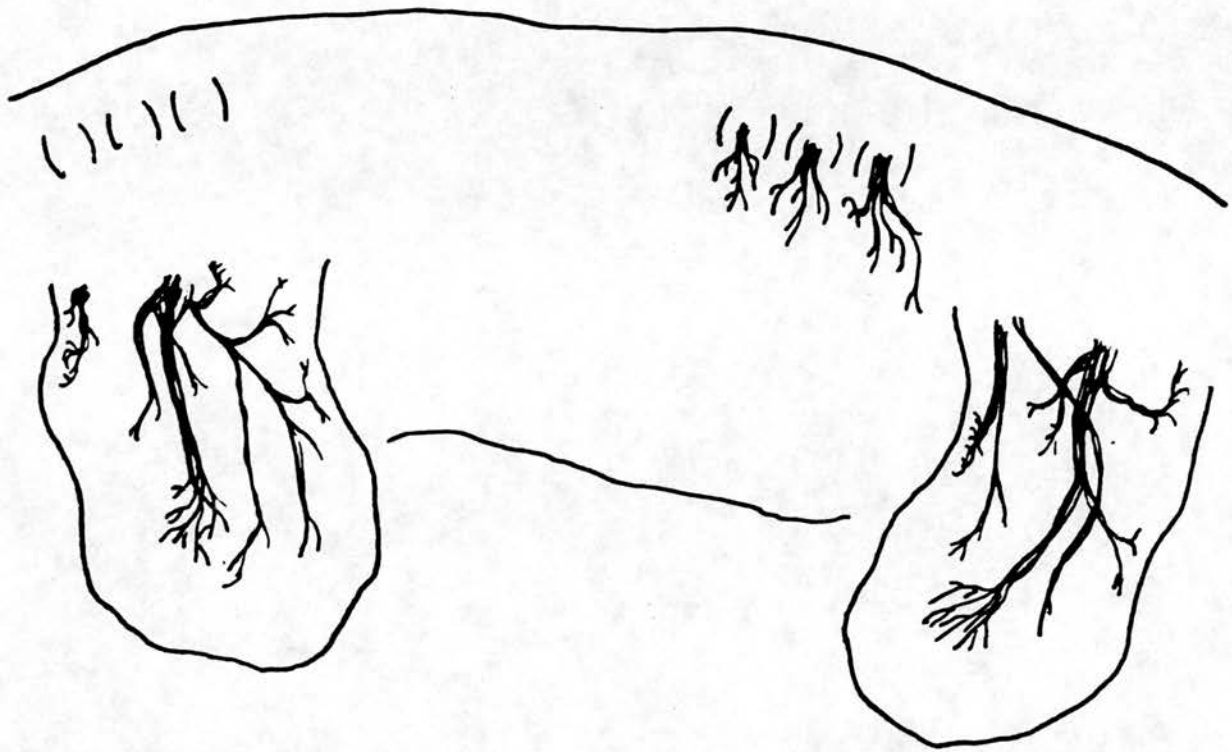
1mm

those which lie most caudally in the embryo showing a developmental lag over those located more rostrally therefore, the extent of innervation in the hind limb compared with that in the trunk is even more striking, representing a greater than three fold increase in the rate of axonal growth into the limb.

3.2 APPEARANCE OF CULTURES

Enzymically dissociated neurones were cultured on Laminin coated glass coverslips, as detailed in Methods. Fig.3.2 shows a series of micrographs taken of fixed preparations after 24hr in culture. These cultures contained both neural and non-neural cell types. Neurones were identified according to a standard criterium; those cells with a discreet rounded cell body, from which one or more fine processes (neurites) extended. Flattened growth cones were occasionally visible at the neurite tips. Two quite different sub-classes of neurones were visible within this classification; first, cells with a large, phase-bright cell body from which single or multiple, branched neurites extended, these were categorised as Large, Bright pseudo-Multipolar (LBM) neurones (Fig.3.2a). Second, cells with small, phase-dark cell bodies and bipolar, unbranched neurites, these were categorised as Small, Dark, Bipolar (SDB) neurones (Fig.3.2b). A third category of cells which had large, phase bright cell bodies, but no neurites after 24hr in culture were present (Fig.3.2c) and were recorded as "failures". Fig.3.3 shows preparations stained

FIGURE 3.2

Phase-contrast and fluorescence micrographs of neurones which had been fixed after 24hr in culture.

a) A large, phase-bright,pseudo-multipolar (LBM) neurone, phase contrast

b) A small, phase-dark bi-polar (SDB) neurone, phase contrast

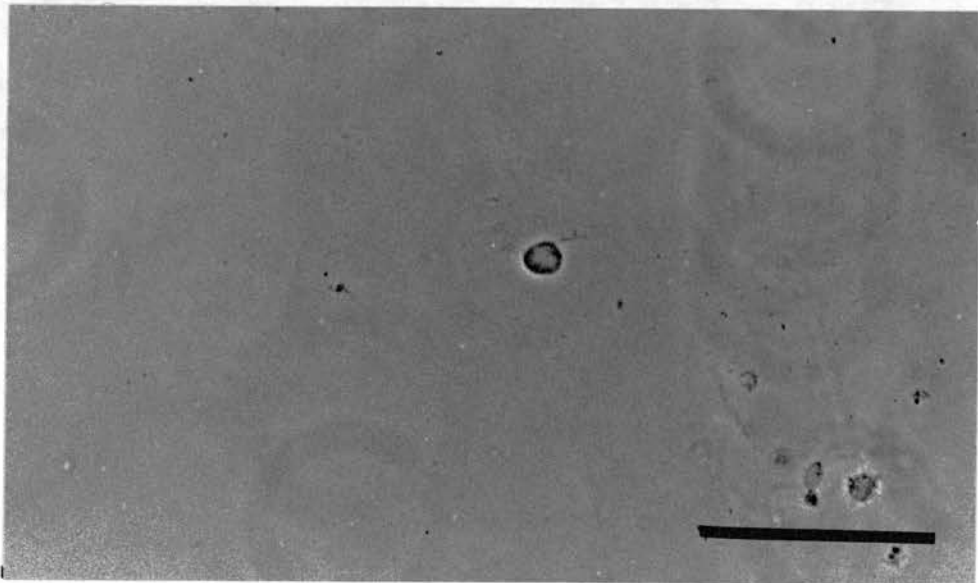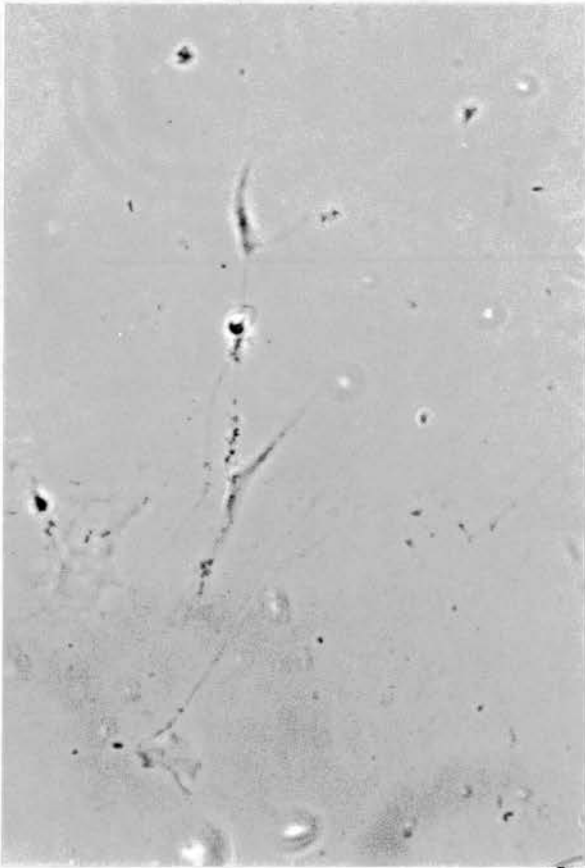c) A failure neurone which had produced no neurites after 24hr in culture. Scale bar = 100um
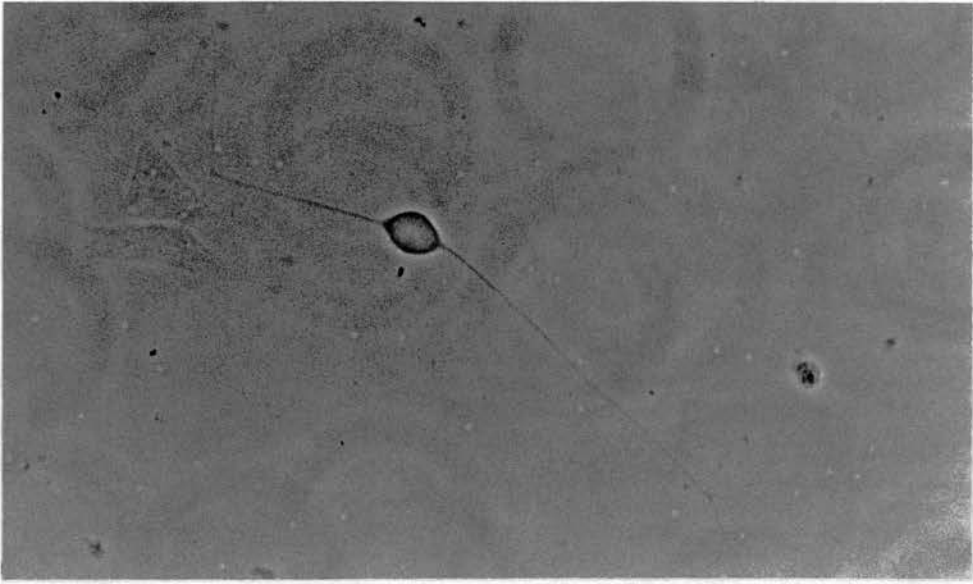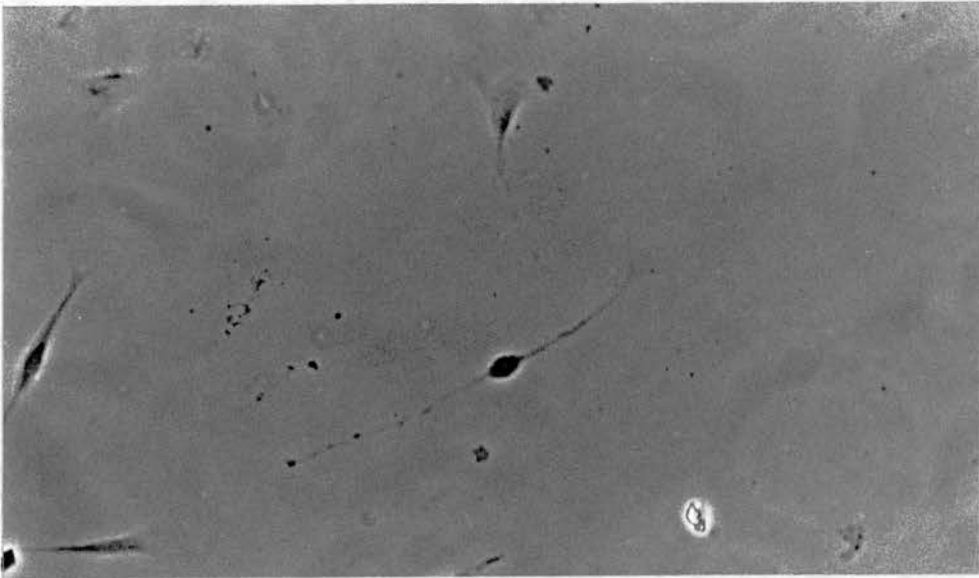
FIGURE 3.3

Neurones stained with the 68kD anti-neurofilament subunit protein.

a and c) Phase contrast of an E5.5 (st28) culture

b and d) Fluorescence of the same fields as (a and c)

Scale bar = 100um
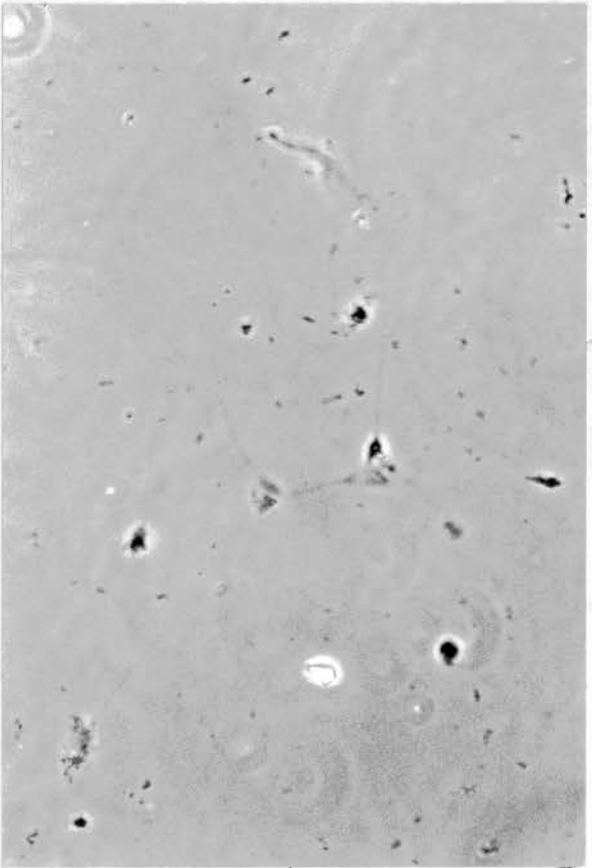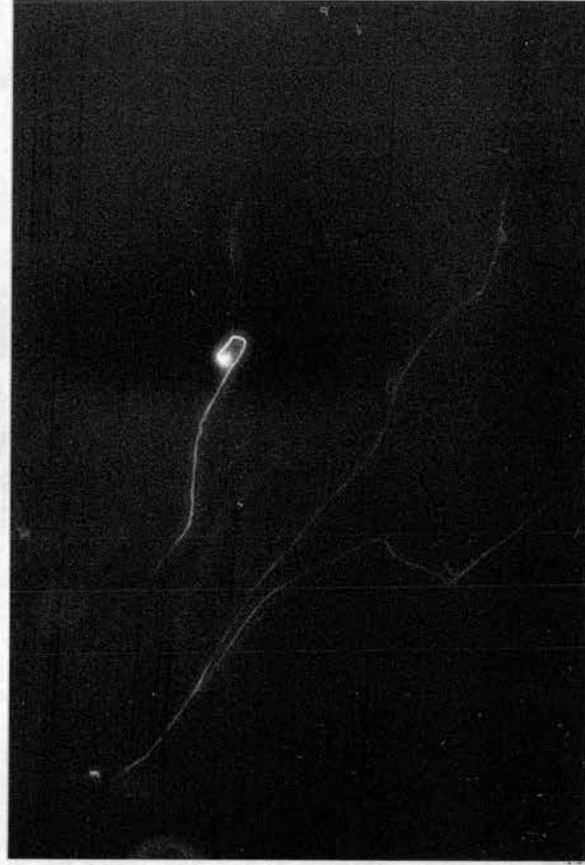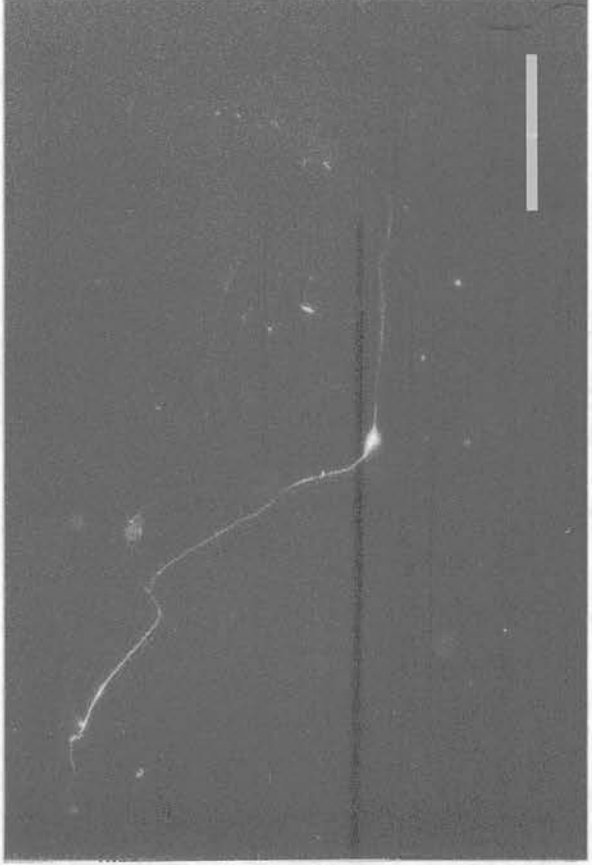
for the neurone specific 68kD neurofilament subunit, it is seen to label the fine processes of neurones but not flattened cells. Due to the poor morphology of cell bodies following the staining protocol, it was not possible to differentiate between SDB and LBM neurone types. Further, failure neurones were impossible to identify with phase-contrast optics in these preparations, therefore their identity as neurones could not be confirmed by this staining technique. However, their morphology suggested that they were neurones and not non-neuronal cell types. This final cell type constituted a significant minority of the population in all cultures. Finally, non-neuronal cell types were present in cultures. These either had a flattened, granular cell body with no neurites : fibroblasts or were small dark and spindle-shaped : schwann cells. Both cell types were easily identified and discounted from the analysis.

3.3 SEGMENTAL NEURITE GROWTH

Three different stages during the development of the chick embryo were chosen for this study. These represent stages over which innervation of the trunk and limbs by both motor and sensory nerves is occurring. At stage 25 axons are beginning to enter the proximal portion of fore and hind limbs following the establishment of brachial and lumbo-sacral plexi. By stage 28 the most distal growth cones have reached the level of the elbow and knee in fore and hind limbs, also at this stage the first

central responses to peripheral stimuli in the limbs have been recorded. Finally, by stage 30 the basic pattern of adult innervation has been established in the hindlimb and distal axon tips have reached the wrist of the developing forelimb. These three stages of development represent 4.5, 5.5 and 6.5 days of incubation at 37°C.

Cultures were prepared from three different segmental levels; brachial, thoracic and lumbo-sacral. The total length of the neuritic arbor of each neurone was measured after 24hr in culture under identical conditions. Histograms of total neurite length per neuron, including a category of failure neurones were constructed to enable the comparison of neurite lengths from ganglia which normally innervate limb and nonlimb tissues to be made.

3.3.1 RESULTS

Histograms of total neurite length per neuron for three different segmental levels from each of three developmental stages are shown in Figs 3.4, 3.5 and 3.6. Descriptive statistics for these histograms are shown in Table 3.1 to aid direct comparison between them.

Three important features common to all the histograms are immediately apparent: i) the data do not have a normal distribution, but show a strong positive skew.

FIGURE 3.4

Histograms of total neurite length per neurone, measured from digitised video images after 24hr in culture. Embryos were at st25 of development, the substrate was laminin and media was supplemented with NGF. Results are taken from six repeat cultures from separate animals. The furthest left histogram bar represents the number of failure neurones recorded. a) Brachial, b) Thoracic and c) Lumbo-sacral segmental levels.
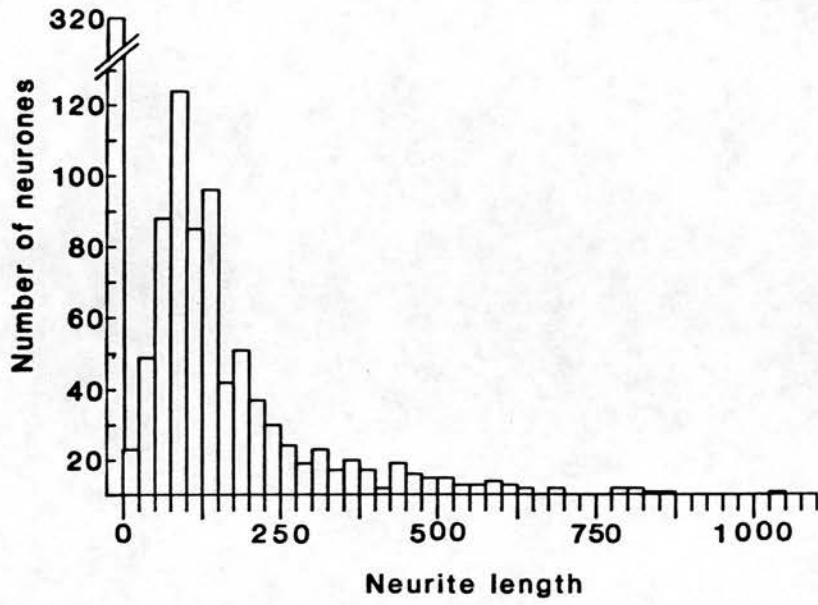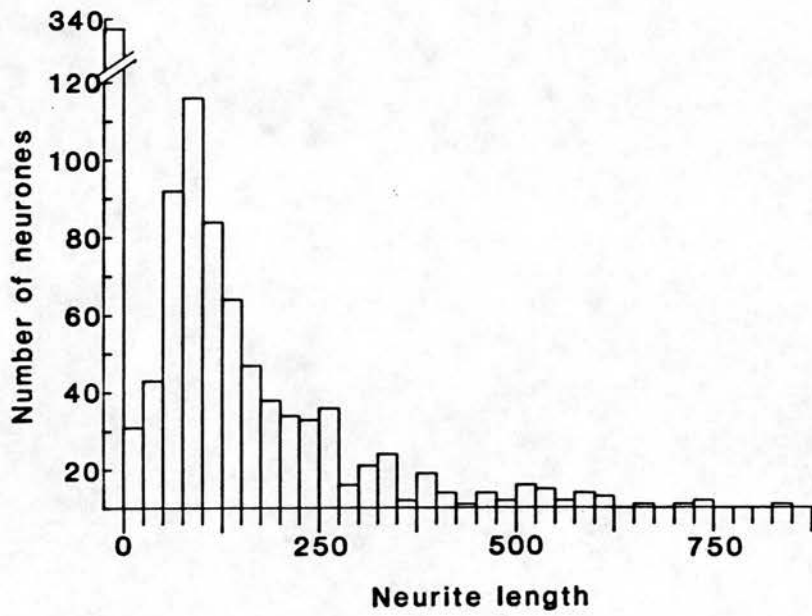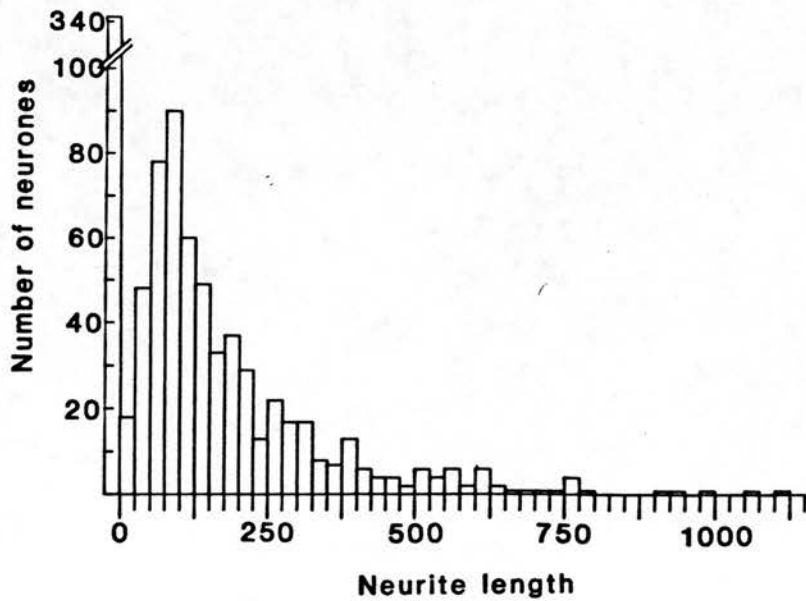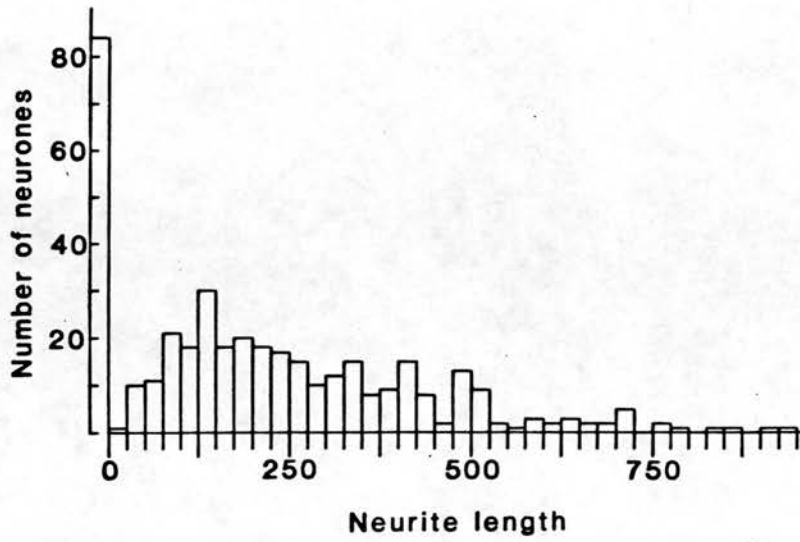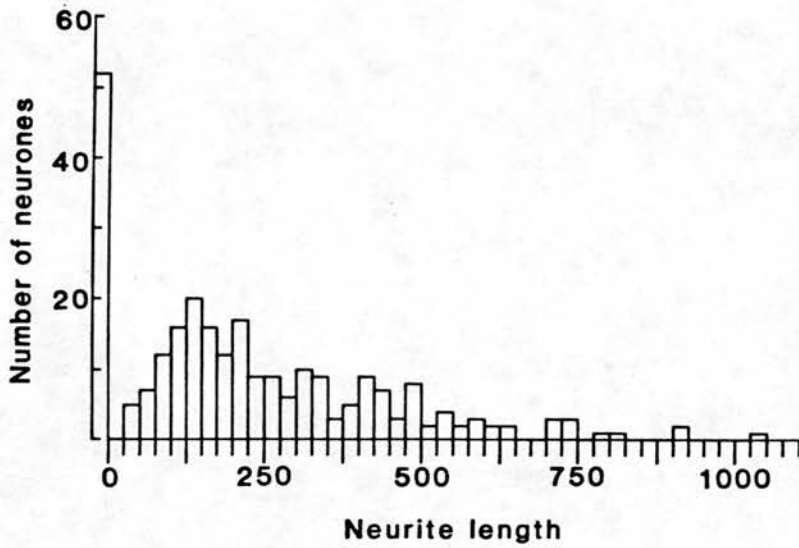
a

Number of neurones

Neurite length

b

Number of neurones

Neurite length

c

Number of neurones

Neurite length

FIGURE 3.5

Histograms of total neurite length per neuron, measured from digitised video images after 24hr in culture. Embryos were at st28 of development, the substrate was laminin and media was supplemented with NGF. Results are taken from four repeat cultures from separate animals. The furthest left histogram bar represents the number of failure neurones recorded. a) Brachial, b) Thoracic and c)Lumbo-sacral segmental levels.
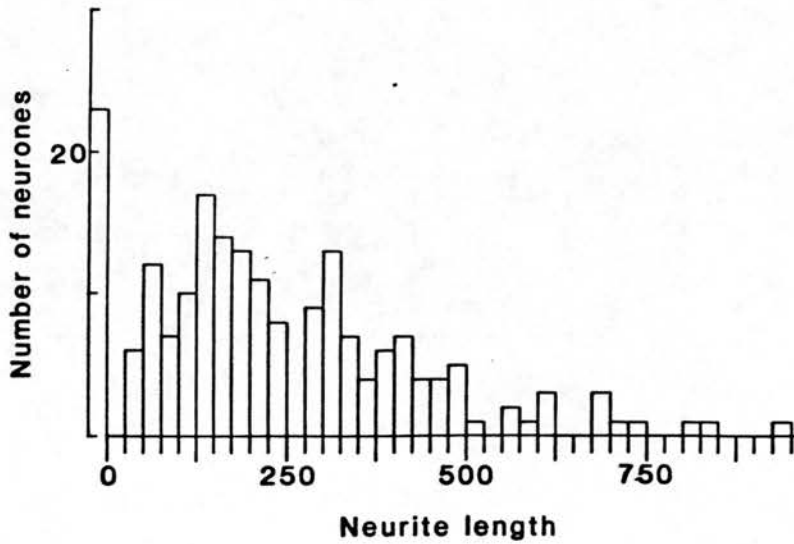
a

b

c

FIGURE 3.6

Histograms of total neurite length per neurone, measured from digitised video images after 24hr in culture. Embryos were at st30 of development, the substrate was laminin and the media was supplemented with NGF. Results are taken from four repeat cultures from separate animals. The furthest left histogram bar represents the number of failure neurones recorded. a) Brachial, b) Thoracic and c) Lumbo-sacral segmental levels.
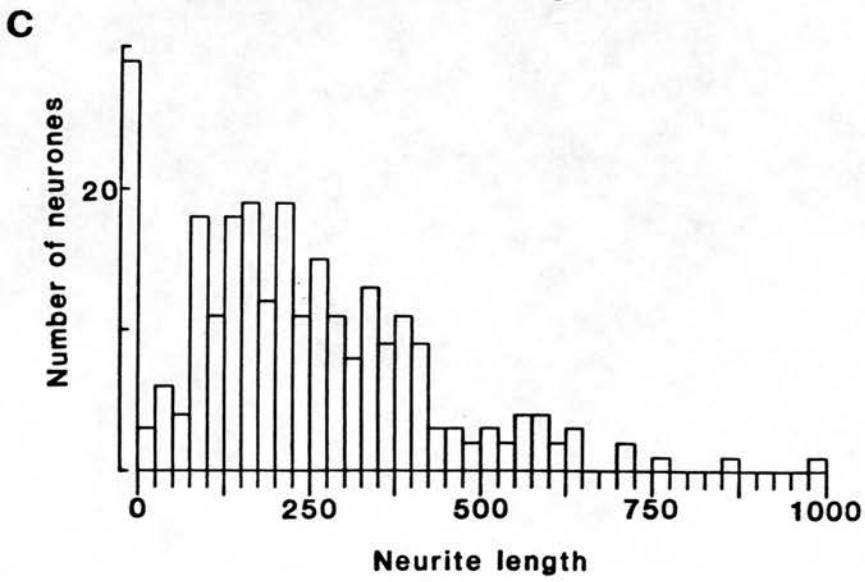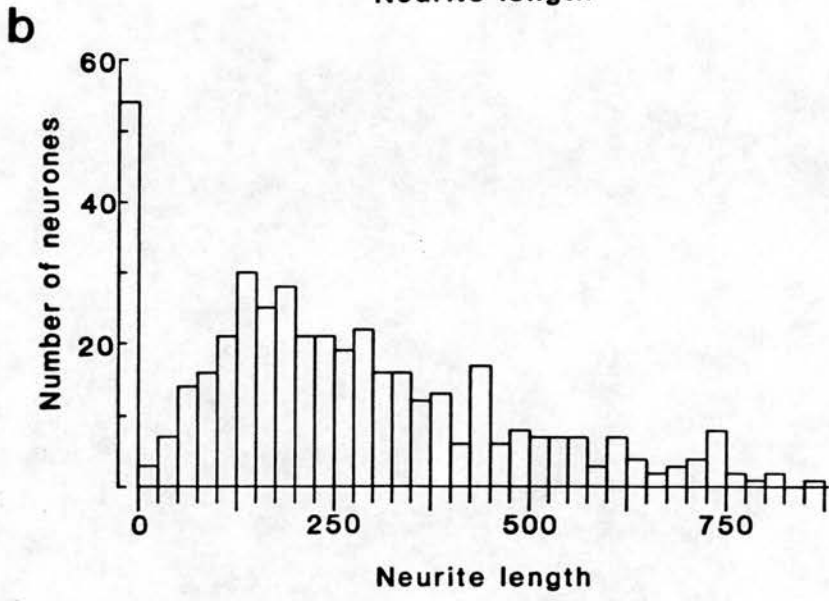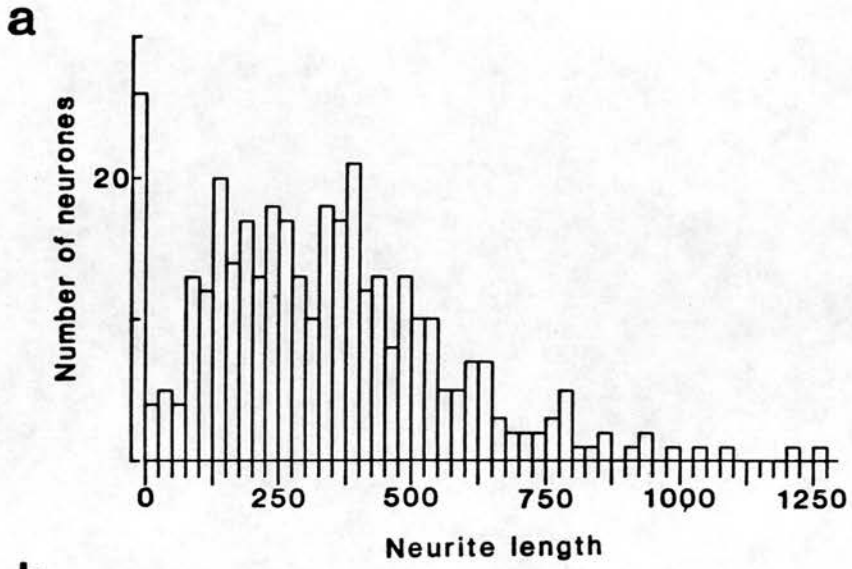
Table 3.1

Descriptive statistics for the histograms shown in Figs. 3.4,

3.5 and 3.6.

61

Stage	Segmental level	Number of neurones	% failures	Mode	Median	Lower quartile point	Upper quartile point	Inter-quartile range
25	B	875	35	100	125	100	175	75
	T	823	40	100	100	75	150	75
	L	865	40	100	125	75	200	125
28	B	393	22	150	250	150	375	225
	T	262	20	150	225	150	425	275
	L	195	12	150	225	150	400	250
30	B	358	7	150	350	200	500	300
	T	458	13	150	275	175	475	300
	L	302	11	175	250	150	350	200

Table 3.2

Values of probability from the Mann-Whitney test for the difference between distributions of neurite lengths taken from Figs. 3.4, 3.5 and 3.6. * denotes a significant difference at the 5% level.

	Stage		
Levels tested	25	28	30
B v T	0.0009*	0.4872	0.4147
T v L	0.0601	0.1797	< 0.0001*
B v L	0.0554	0.2207	< 0.0001*

ii) the "tail" of the distribution does not show a smooth fall, rather appearing multi-peaked, and further each subsequent peak occurs at approximate integer multiples of the first (modal) peak.

iii)a significant minority of failure neurones which had not produced a neurite after 24hr in culture are present.

Taking each of the three stages of development in turn, there is no evidence of differential growth between segmental levels. Statistically values for mode, median and inter-quartile range show little variation between different segmental levels. Where differences are present they are inconsistent and there is certainly no evidence for either the predominance of short neurites from the thoracic region or long neurites from the limb and particularly the hind limb regions as might have been expected (Table 3.1). Similarly, the numbers of failures recorded show no consistent variation between segmental levels. The Distributions were tested against one another by the Mann-Whitney U-test of significance and the results of this are shown in Table 3.2. Where a significant difference is seen (st25 brachial longer than thoracic neurites, st30 lumbo sacral shorter than brachial and thoracic) the difference in medians are only 25 and 100um respectively. These are inconsistent, one being in favour of limb the other non-limb ganglia growing the longest neurites. Further the differences would need to be in the region of 400-500um to account for the differences seen in-

vivo. Mann-Whitney U-tests were carried out on the geometric means of the distributions, following log transformations of the data. These showed no important or significant differences.

In conclusion, there is no evidence in culture for a three fold difference in axonal growth rates between limb and non-limb innervating neurones, as is seen in-vivo.

It is apparent however that differences do exist between the stages of development. The value of the first peak in the distribution (the mode) increases with development, as does the median. The inter-quartile ranges recorded are relatively small throughout. As this is a measure of the variability about the median, a small value gives greater confidence in the differences quoted between medians. Most striking is the fall in the percentage of failure neurones recorded with increasing age. Mean values for the three segmental levels fall from 39% at st25, through 19% at st28 to 17% at st30. Differences in the morphology of the neurones with the stage of development are also seen. The numbers of tri and quadrapolar neurones on the cultures rise from 0.02 through 2.1 to 3.2%, between st's 25, 28 and 30 respectively. Similarly the percentage of branched to unbranched neurites increases from 9.8 through 7.6 to 18.4%, over the same set of stages.

These data suggest an increasing competence and complexity of neurite production within the population as development

proceeds. This results in fewer failures, longer and more highly branched neurites being recorded at the later developmental stages.

No evidence for an intrinsic mechanism, conferred by the segmental position of the ganglia, in controlling neurite growth has been found, when neurones were isolated and cultured under identical conditions. There does however appear to be a developmentally controlled, intrinsic regulation of neurite growth from all ganglia when isolated at different stages of development. This seems to control both the production of neurites per se and the length to which these neurites grow in culture.

3.4 SPLIT LUMBAR PREPARATION

The ganglia in the previous series of experiments were taken from the different segmental levels of single embryos, at a particular stage of development. One potential problem with this approach is the rostro-caudal sequence of neural development. This results in the later development of DRG located in the lumbo-sacral region compared with those in the brachial and thoracic regions. These minor differences in ontogeny could possibly be masking subtle variations in neurite outgrowth between segmental levels.

To address this potential problem, advantage was taken of the organisation of innervation to the hindlimb. Here, lumbo-sacral

(LS) ganglia 1-8 project to the limb through two distinct plexi,
sciatic and crural. The crural plexus receives its input from
LS1-3, which then project to targets in the thigh and knee.
While the sciatic plexus receives axons from LS4-8, which
project to targets in the knee and shank (Scott, 1987). In this
system then, differences in target innervation distance exist.
Innervation of the dermatomes of the hindlimb have been shown to
occur simultaneously across the hindlimb (Scott, 1982). It must
then be assumed that axons innervating distal targets (through
the sciatic plexus), have grown at greater rates than those
innervating more proximal targets (through the crural plexus).
In this system, the close proximity of the ganglia, effectively
controls against any differences in their respective stages of
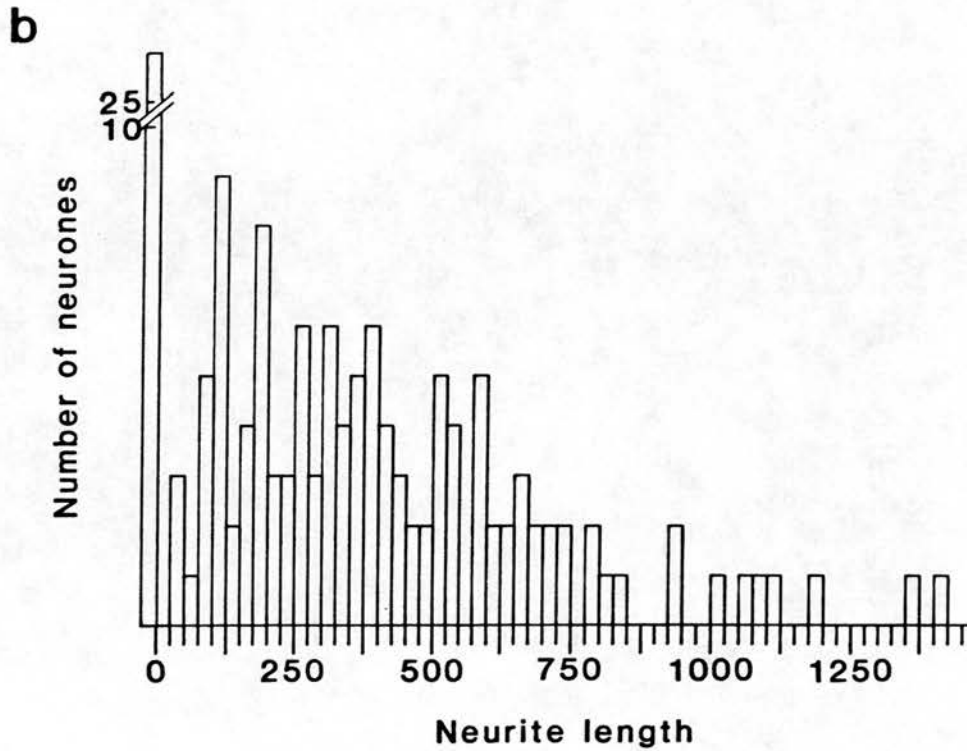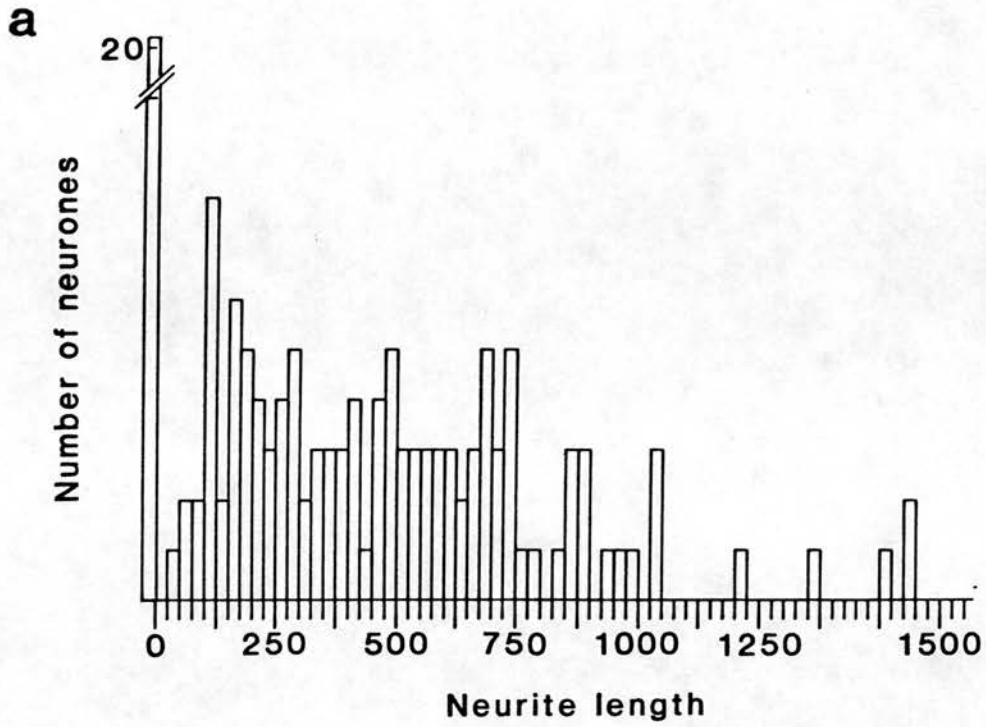development.

3.4.1 RESULTS

Ganglia were isolated from DRG LS1-3 and LS4-8, of st28 embryos.
St28 was chosen as the earliest stage at which the ganglia could
be reliably separated. Dissociated neurones from the two sources
were cultured separately under identical conditions, and neurite
lengths measured after 24hr.

Histograms of total neurite length for the two different
segmental levels are shown in Fig.3.7. First, both show the same
positively skewed, non-normal form which has been described in

FIGURE 3.7

Histograms of total neurite length per neurone, measured from
digitised video-images after 24hr in culture. Embryoś were at
st28 of development, the substrate was laminin and the media was
supplemented with NGF.   Results were taken from two repeat
cultures from separate animals. The furthest left histogram bar
represents the number of failure neurones recorded. a) Lumbo-
sacral ganglia 1- 3,   b) Lumbo-sacral ganglia 4 - 7.

a

Number of neurones

20

0   250   500   750   1000   1250   1500

Neurite length

b

Number of neurones

25
10

0   250   500   750   1000   1250

Neurite length

the preceding section. Second, the multi-modal shape of the distribution is apparent, even in this relatively small sample. Third, a significant minority of failures are present in both cases.

There are no differences between the lengths of neurites produced by neurones isolated from the different segmental levels. There is no evidence that the neurones from DRG LS4-8 are growing longer neurites than those from LS1-3. Modal values are similar, 125 and 100um, with medians being slightly more disparate, 450 and 375um, for LS1-3 and LS4-8 respectively. Inter-quartile ranges are large (500 and 400um), which is to be expected from relatively small samples. When the two populations were statistically tested, no significant differences were found (Mann-Whitney U-test; P>0.05). These results add further strength to the conclusion made in the previous section, that the differential rates of growth seen in-vivo are not determined by the nature of the neurones.

3.5 SUBSTRATE MANIPULATIONS

What factors in the environment of the growing axons might be capable of causing the differential rates of neurite growth observed in-vivo? It has previously been stated that the limb appears to have attractive properties (Hamburger, 1939). Molecules of the extracellular matrix are known to be permissive substrates for the extension of neurites from sensory neurones

(Rogers et al, 1983). The expression of two molecules, laminin and fibronectin has been demonstrated in chick limbs during innervation (Tomasek *et al.*, 1982; Rogers *et al.*, 1986; Yip and Yip, 1990). In another system the importance of pre-formed pathways of laminin in peripheral innervation has been demonstrated (Riggott and Moody, 1986). Could the presence of specific extacellular matrix molecules in the developing limb be responsible for the increased rates of growth observed from DRG neurones?

If the neurite lengths recorded on the two substrates were significantly different, this would suggest a possible role for the differential expression of these molecules in controlling innervation. It should be noted that if this is the case a similar response would be expected at all segmental levels, rather than one which was specific to particular ones.

In these experiments a possible role for fibronectin in the regulation of forelimb innervation will be demonstrated, though in itself this would appear to be insufficient to explain the situation in-vivo.

3.5.1 RESULTS

Cultures were prepared in which the substrate for neurite growth was either laminin or fibronectin at the same concentrations. Parallel sets of cultures were set up, in which neurones from

different segmental levels of st25 embryos were grown on both substrates. Total neurite lengths from each culture were measured, and the histograms of these measurements are displayed in Figs 3.8, 3.9 and 3.10. Statistics taken from these are displayed in Table 3.2. All the histograms show the now typical positively skewed, multimodal form of distribution. Taking each segmental level in turn, median values are higher in each case, though only by 25um (one histogram bin). Failures only show a marked variation between the two substrates at the brachial level, being lower on fibronectin. Inter-quartile ranges are small, and only in the brachial region again are the positions of both lower and upper quartile points shifted upwards on fibronectin compared with laminin. This data suggests that on the whole, outgrowth is similar on the two substrates. However there is a suggestion of greater outgrowth on a fibronectin substrate at the brachial level (significantly different, Mann-Whitney U-test, P<0.001). Comparing results from different segmental levels, the only apparent difference is the greater neurite growth from brachial cultures on both substrates. This is reflected in a decrease in the percentages of failures recorded, and an upward shift in the limits of the inter-quartile ranges. These differences are obviously insufficient alone to explain the growth characteristics observed in-vivo. There is a suggestion of a role for fibronectin in forelimb innervation, which correlates well with the demonstration of this molecule in this position in-vivo (Tomasek, 1982).

FIGURE 3.8

Histograms of total neurite length per neuron, measured from digitised video-images after 24 hr in culture. Embryos were at st25 of development, substrate was varied and media was supplemented with NGF. Results were taken from six repeat cultures from separate animals. The furthest left histogram bar represents the number of failure neurones recorded. a) Brachial segmental level, laminin substrate, b) Brachial segmental level. Fibronectin substrate.
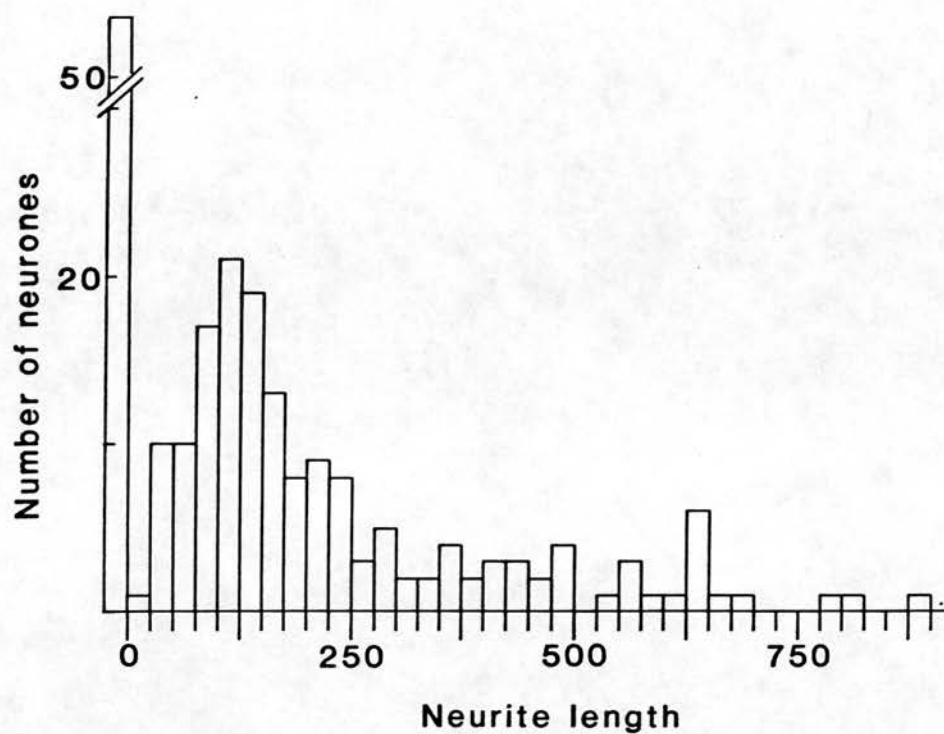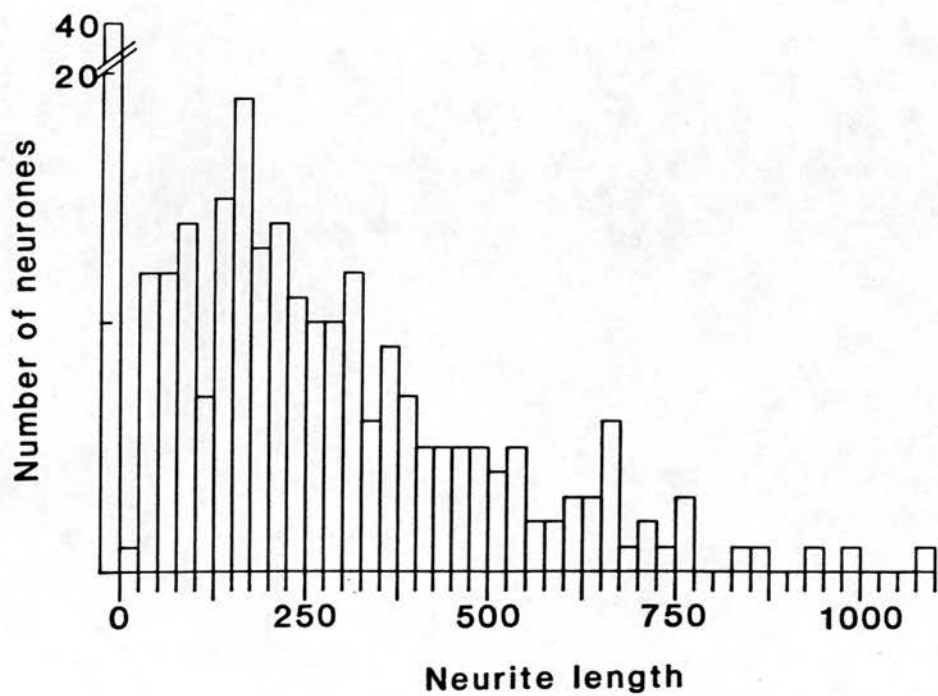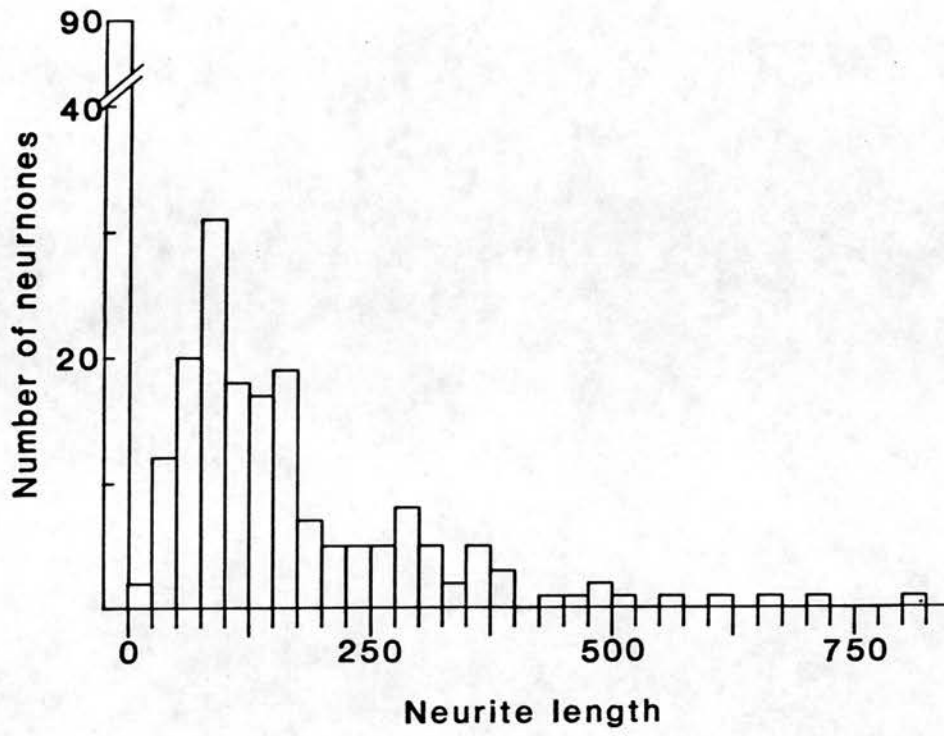
FIGURE 3.9

Histograms of total neurite length per neurone, measured from digitised video images after 24hr in culture. Embryos were at st25 of development, substrate was varied and media was supplemented with NGF. Results were taken from six repeat cultures from separate animals. The furthest left histogram bar represents the number of failure neurones recorded.

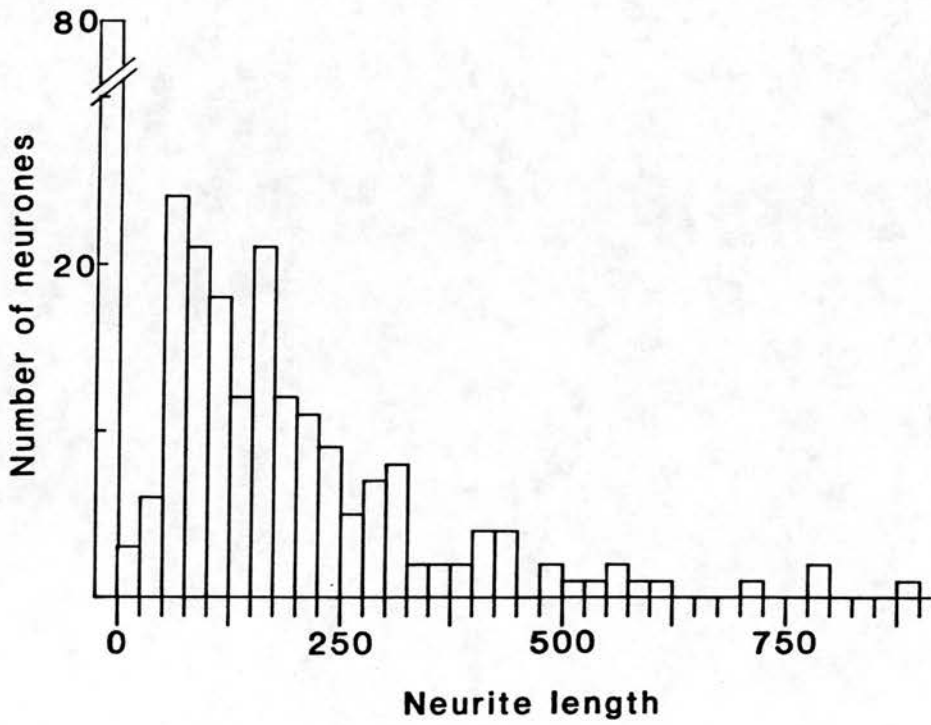a) Thoracic segmental level, laminin substrate, b) Thoracic segmental level. Fibronectin substrate.

a

Number of neurnones (y-axis)
Neurite length (x-axis)

b

Number of neurones (y-axis)
Neurite length (x-axis)

FIGURE 3.10

Histograms of total neurite length per neurone, measured from digitised video images after 24hr in culture. Embryos were at st25 of development, substrate was varied and media was supplemented with NGF. Results were taken from six repeat cultures from separate animals. The furthest left histogram bar represents the number of failure neurones recorded. a) Lumbo-sacral segmental level, Laminin substrate, b) Lumbo-sacral segmental level. Fibronectin substrate.
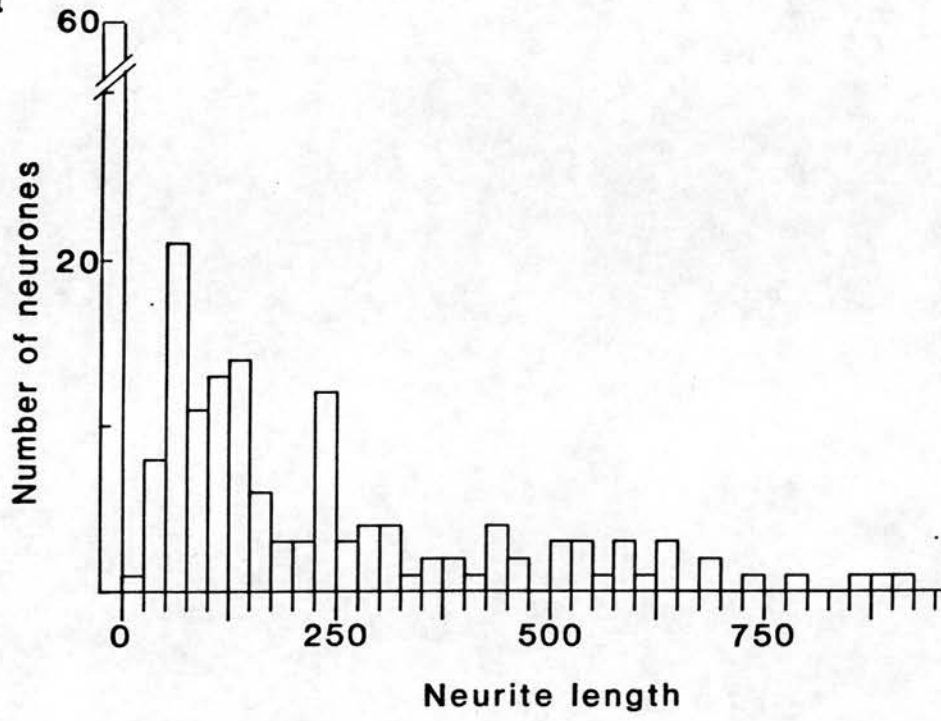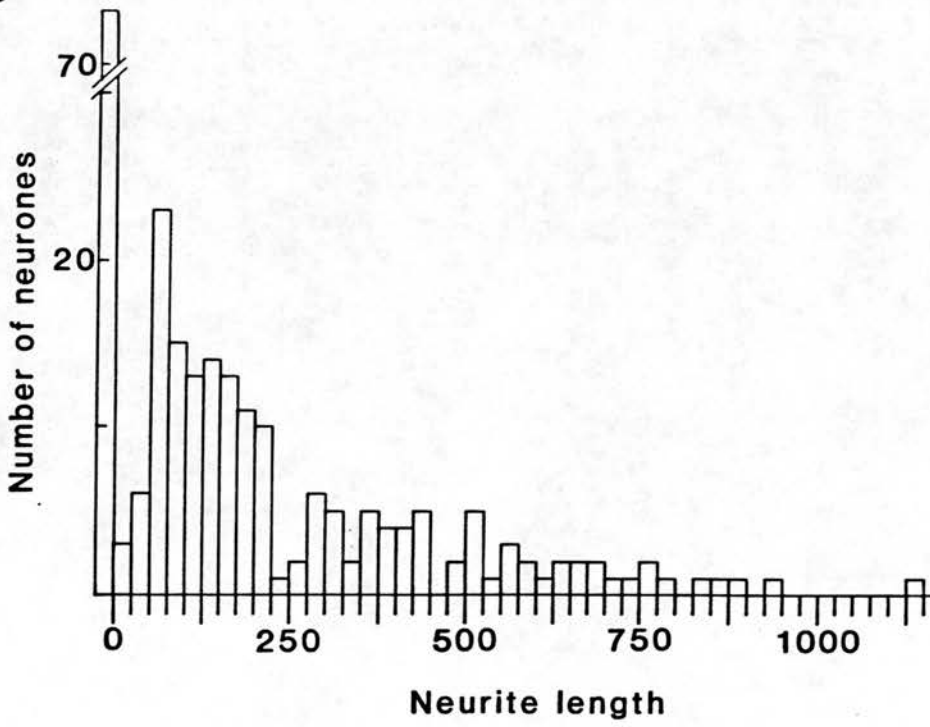
Table 3.3

Descriptive statistics for histograms shown in Figs. 3.8, 3.9 and 3.10.

Segmental level	Substrate coating	Number of neurones	% failures	Mode	Median	Lower quartile point	Upper quartile point	Inter-quartile range
B	Lam	215	24	125	175	125	300	175
B	Fn	269	15	175	200	150	275	125
T	Lam	264	34	100	150	100	275	175
T	Fn	263	30	75	175	100	250	150
L	Lam	197	30	75	150	100	325	225
L	Fn	245	30	75	175	100	375	275

3.6 TROPHIC MOLECULES

NGF is a potent trophic molecule for many neural crest derived neurones, which include those of the DRG (Levi-Montalchini, 1982). NGF is present in the target fields of many peripherally projecting neurones, where it appears to regulate innervation density (Davies et al, 1987). Further it has been demonstrated to be an attractant stimuli to responsive neurones in culture (Campenot, 1987a,b; Gunderson and Barret, 1979, 1980). Might the presence of NGF in certain areas in the chick embryo be capable of orchestrating innervation rates? Davies (1987), has suggested possible interactions between trophic molecules and permissive substrates such as laminin. Experiments were carried out in which substrate coating was varied in the presence or absence of NGF. NGF will be shown to be ineffective in promoting neurite growth, but a possible role in stimulating neurite production per se is discussed.

3.6.1 RESULTS

Parallel cultures were set up from st25 embryos, on a laminin or fibronectin substrate in the presence or absence of NGF. Histograms of total neurite lengths recorded are shown in Figs 3.11, 3.12, 3.13, 3.14, 3.15 and 3.16. Descriptive statistics are shown in Table 3.4. NGF deprivation has no visible effects on the total length of neurites produced by a neurone. At all segmental levels studied and on both substrates, no reduction in

neurite growth in the absence of NGF is seen. Median values never show a larger variation than 25um in neurite lengths between the presence and absence of NGF. It does however seem to affect the production of neurites within the population per se. Fewer failures are recorded in the presence of NGF under all conditions, except for lumbo-sacral neurones on a fibronectin substrate. The differences are often small, and a more comprehensive study would need to be carried out to confirm these observations. As survival analysis was not carried out, it is uncertain whether the decrease in the number of failures represents the production of neurites by cells which would have been quiescent in the absence of NGF. Alternately, it could be due to the survival of cells which would otherwise have died in the absence of NGF, but have survived and produced neurites. In summary, NGF is ineffective in increasing neurite growth from competent neurones, but appears to affect the numbers of neurones within the population which are able to produce a neurite.

The previous data suggested that neurite outgrowth from brachial neurones was greater on fibronectin than laminin substrates. Data from this series of experiments shows the same result. Median values are greater, inter-quartile limits are raised and the percentage of failures is decreased on fibronectin.
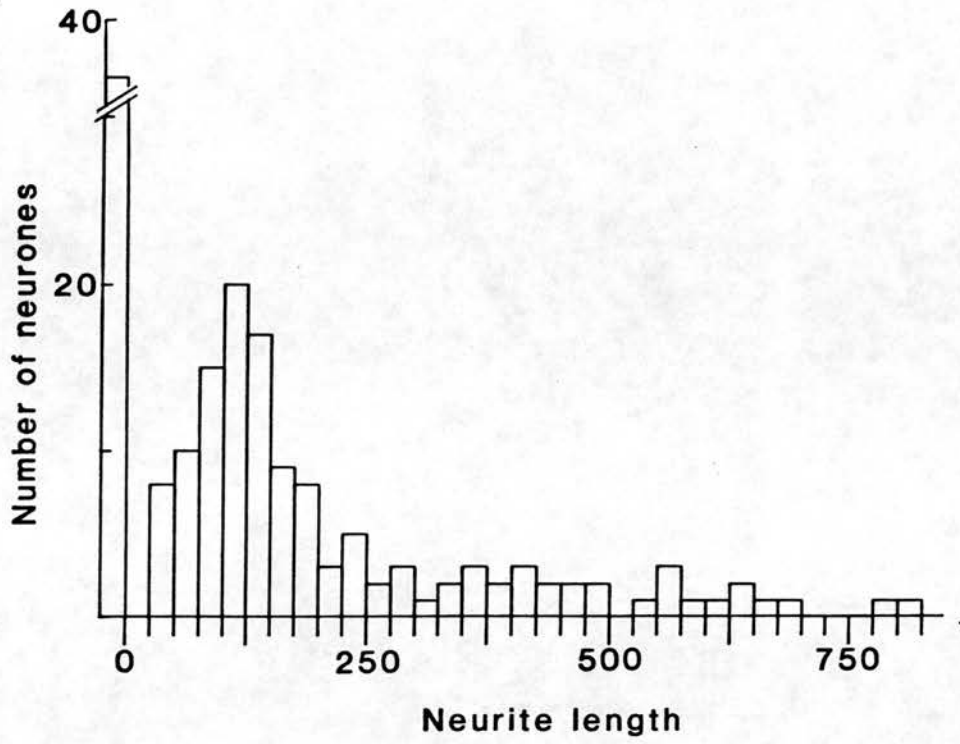
FIGURE 3.11

Histograms of total neurite length per neurone, measured from digitised video images after 24hr in culture. Embryos were at st25 of development, the substrate was varied and NGF was either present or absent as a supplement. Results were taken from five repeat cultures from separate animals. The furthest left column represents the number of failure neurones recorded.

a) Brachial segmental level, laminin substrate, NGF supplemented

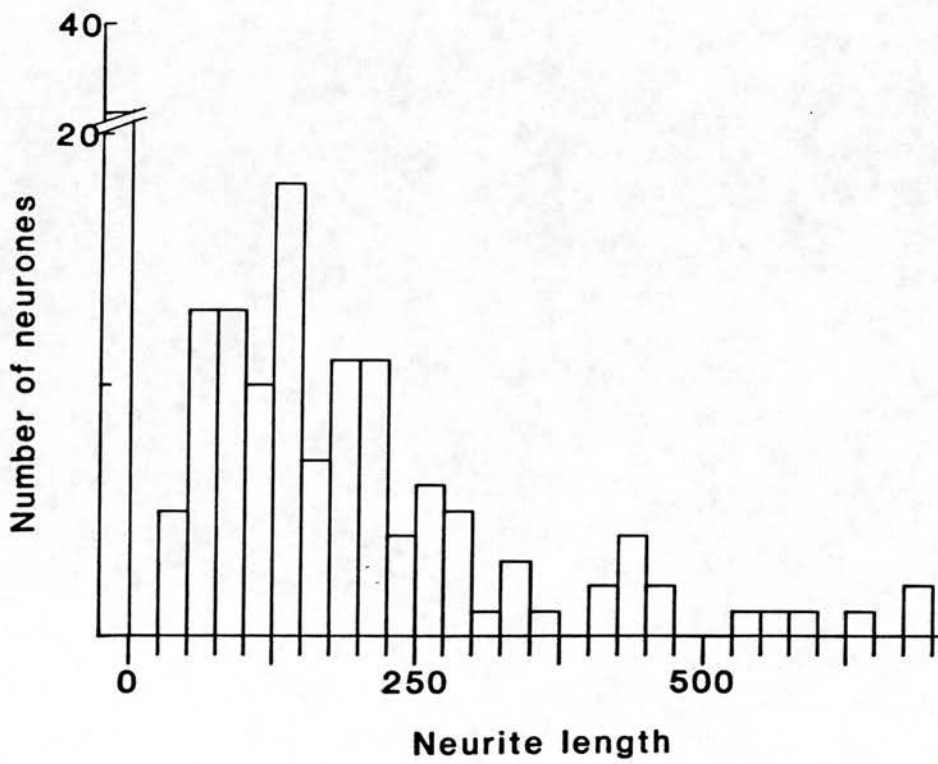b) Brachial segmental level, laminin substrate NGF absent

FIGURE 3.12

Legend as for Fig 3.11.

a) Brachial segmental level, fibronectin substrate NGF supplemented b) Brachial segmental level, fibronectin substrate, NGF absent.
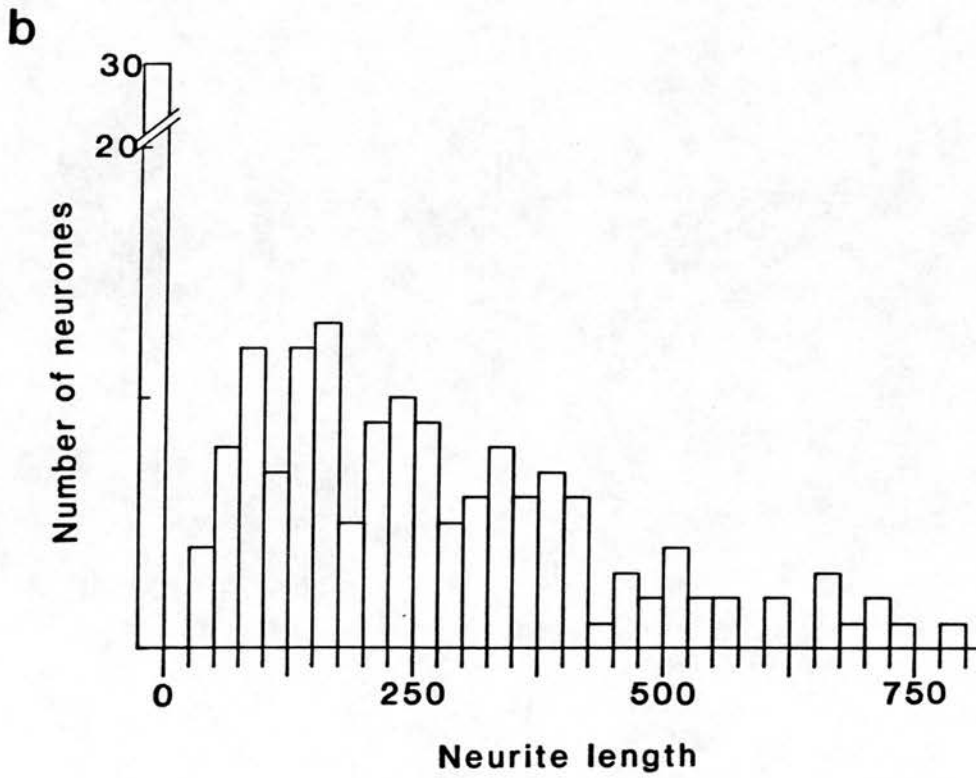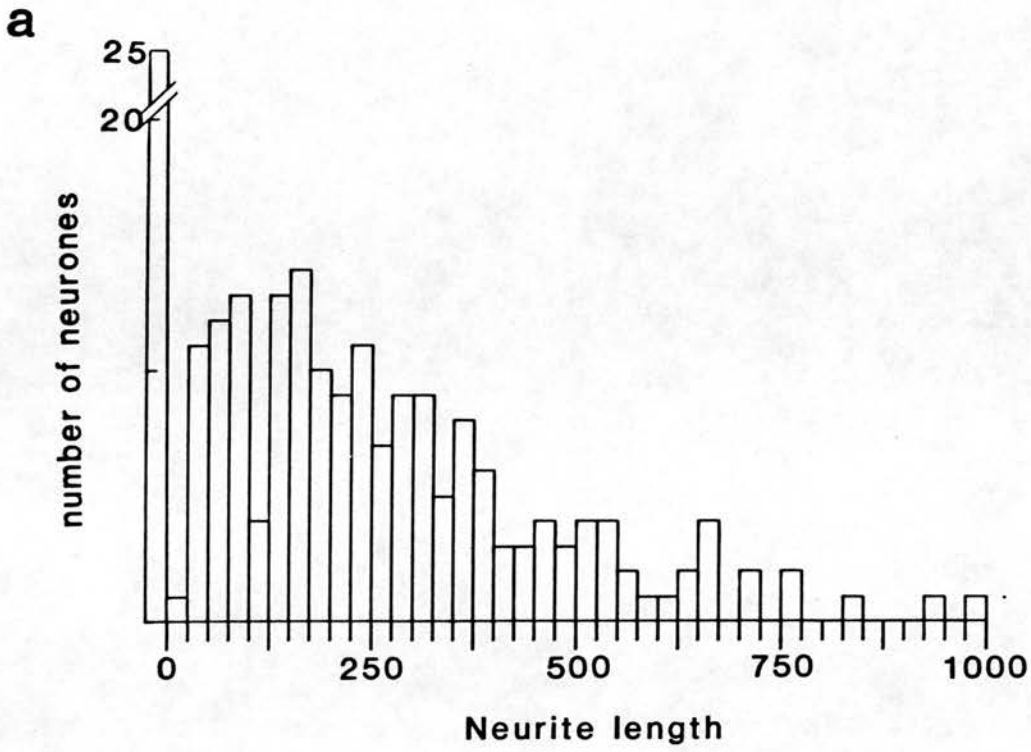
FIG 3.13

Histograms of total neurite length per neurone, measured from digitised video images after 24hr in culture. Embryos were at st25 of development, the substrate was varied and NGF was either present or absent as a supplement. Results were taken from five repeat cultures from separate animals. The furthest left column represents the number of failure neurones recorded.

a) Thoracic segmental level, laminin substrate, NGF supplemented

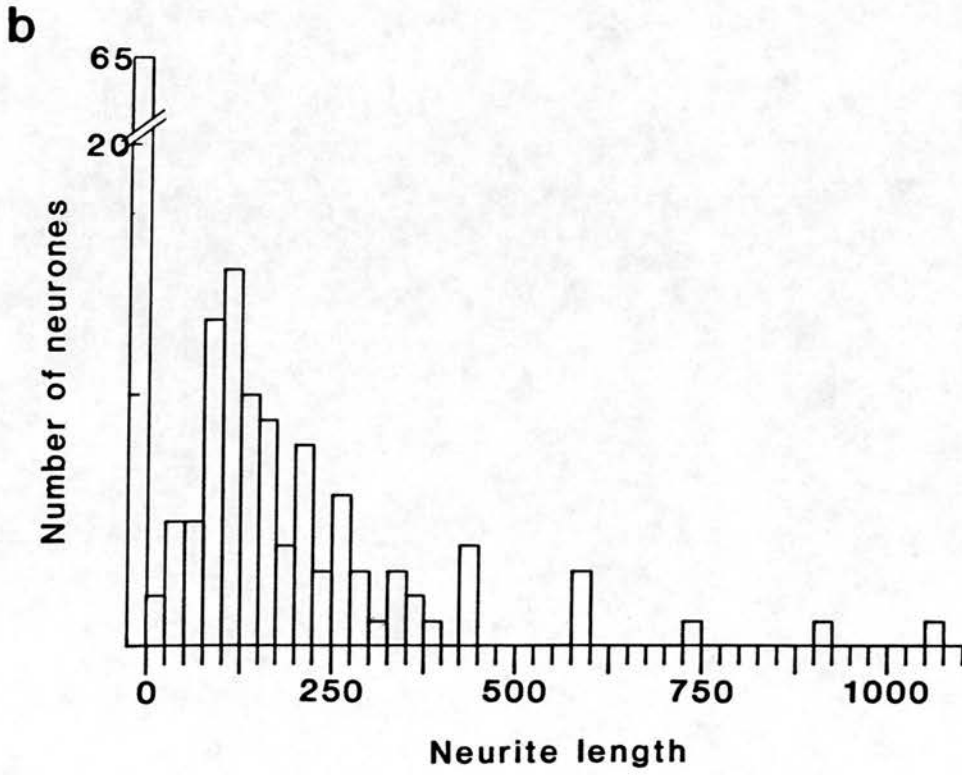b) Thoracic segmental level, laminin substrate NGF absent

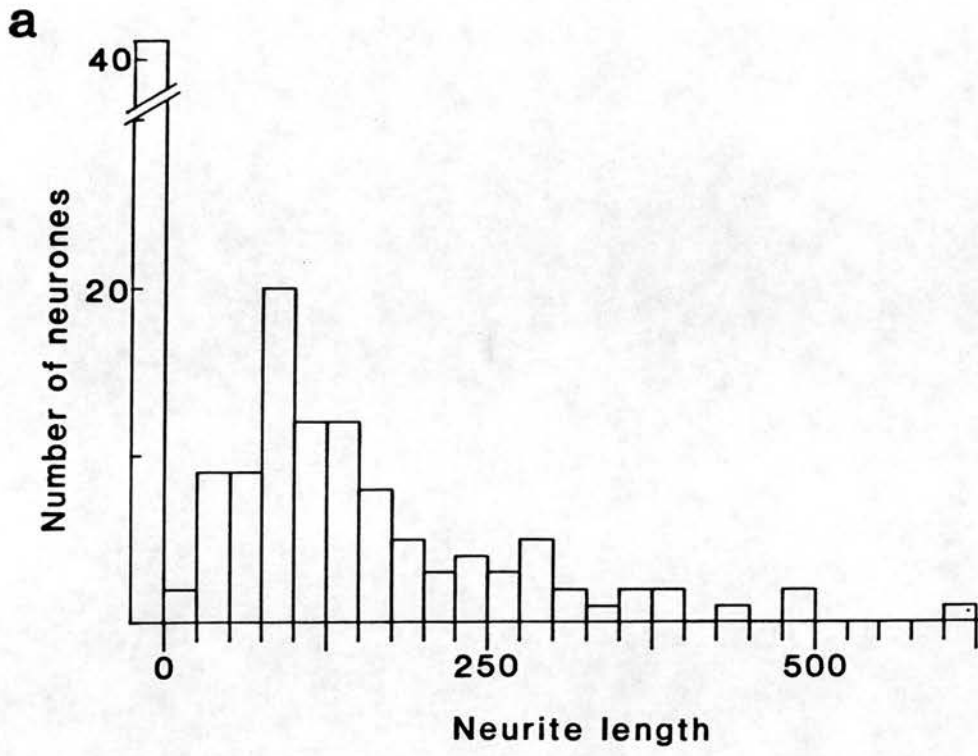FIGURE 3.14

Legend as for Fig 3.13

a) Thoracic segmental level, fibronectin substrate NGF
   supplemented

b) Thoracic segmental level, fibronectin substrate, NGF absent

a

Number of neurones

Neurite length
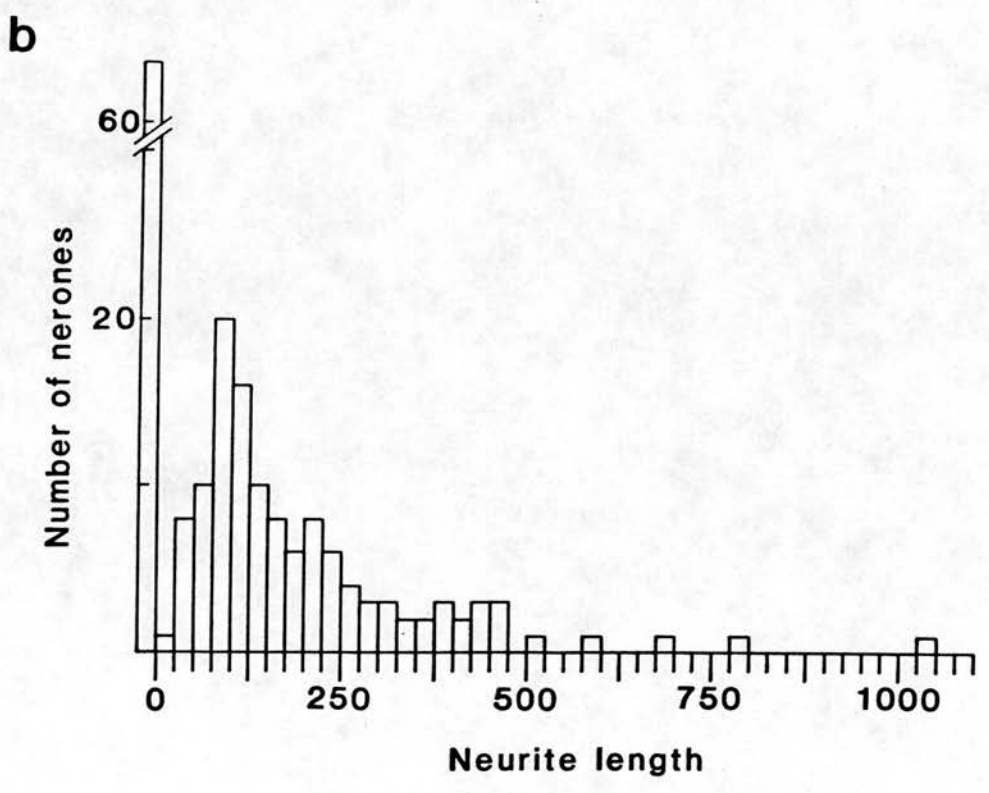
b

Number of nerones
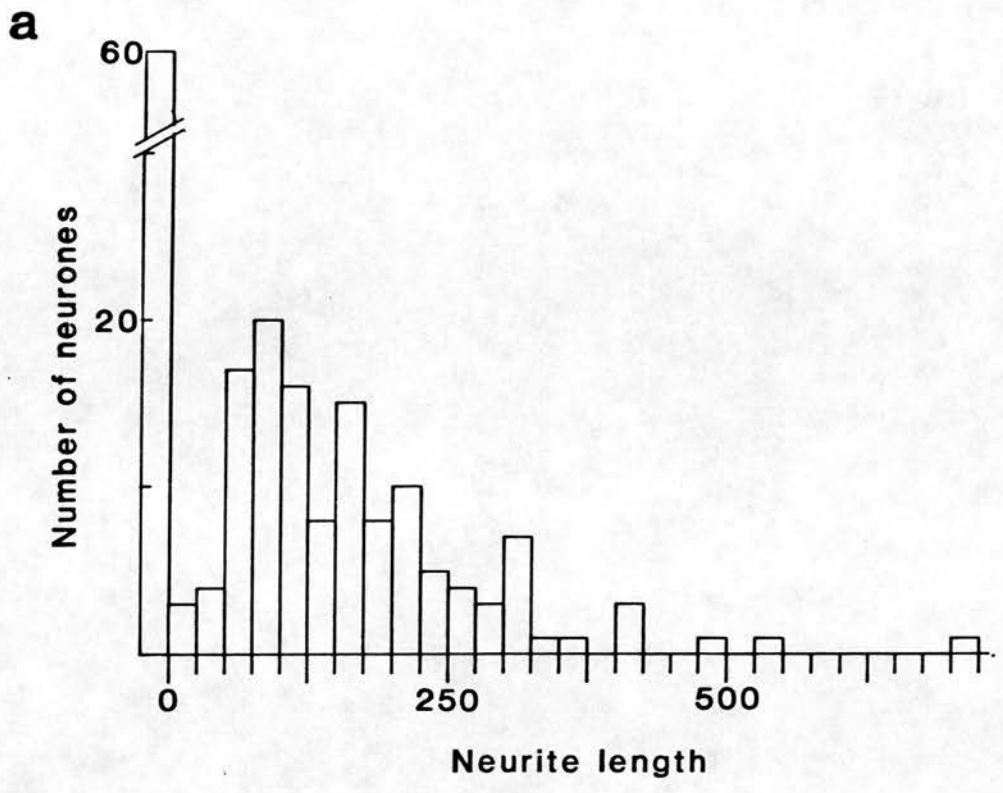
Neurite length

FIGURE 3.15

Histograms of total neurite length per neurone, measured from digitised video images after 24hr in culture. Embryos were at st25 of development, the substrate was varied and NGF was either present or absent as a supplement. Results were taken from five repeat cultures from separate animals. The furthest left column represents the number of failure neurones recorded.

a) Lumbo-sacral segmental level, laminin substrate, NGF supplemented

b) Lumbo-sacral segmental level, laminin substrate NGF absent

FIGURE 3.16

Legend as for Fig 3.15

a) Lumbo-sacral segmental level, fibronectin substrate NGF
   supplemented

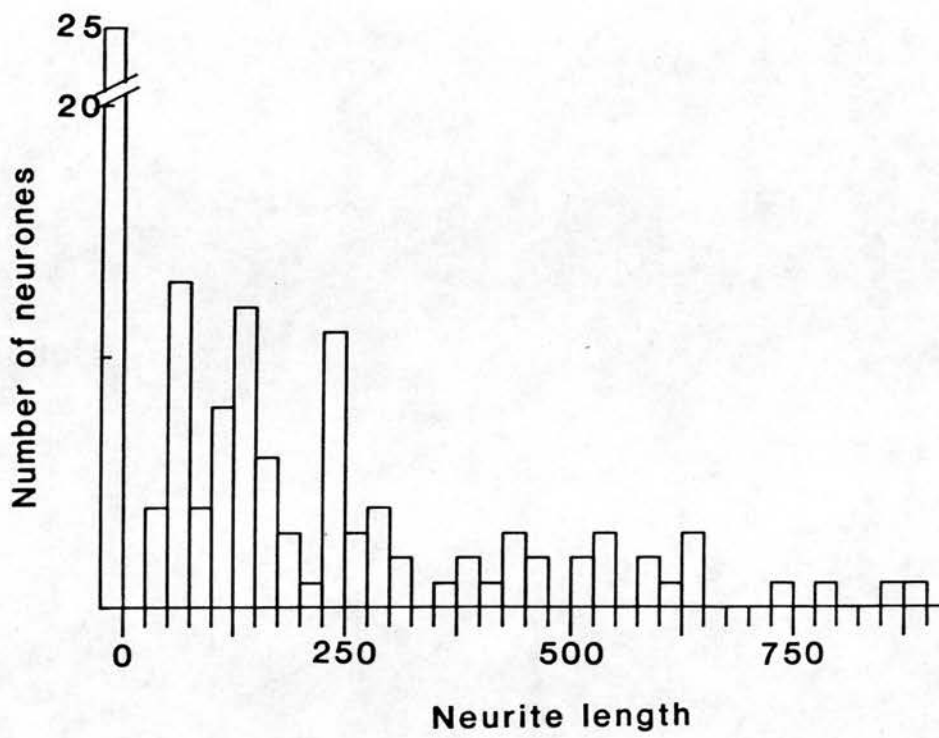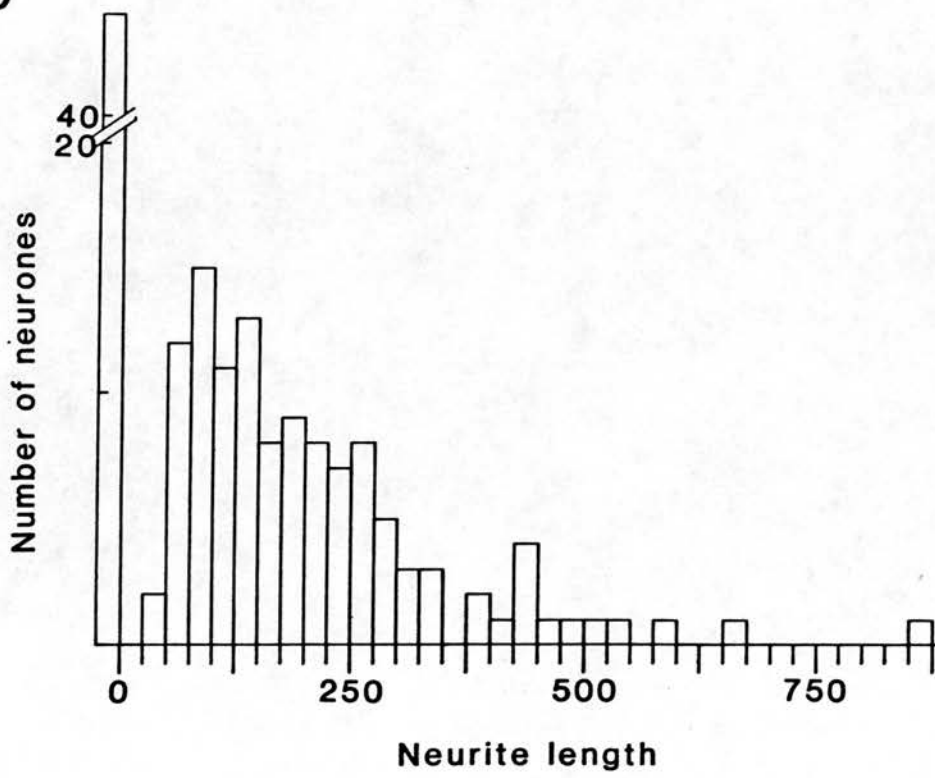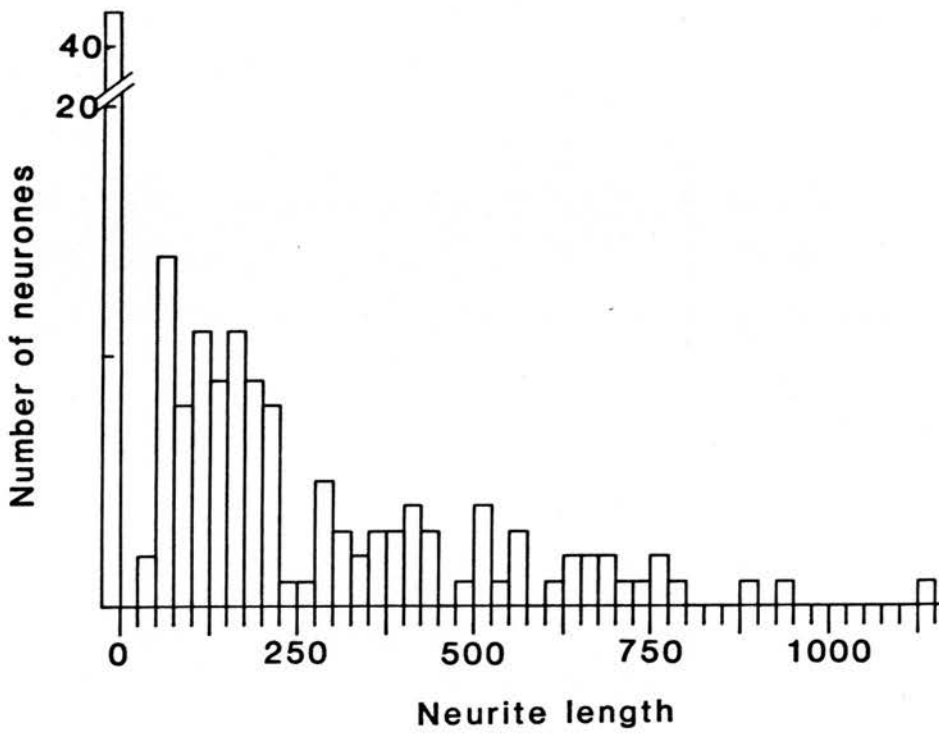b) Brachial segmental level, fibronectin substrate, NGF absent.

a

Number of neurones

Neurite length

b

Number of neurones

Neurite length
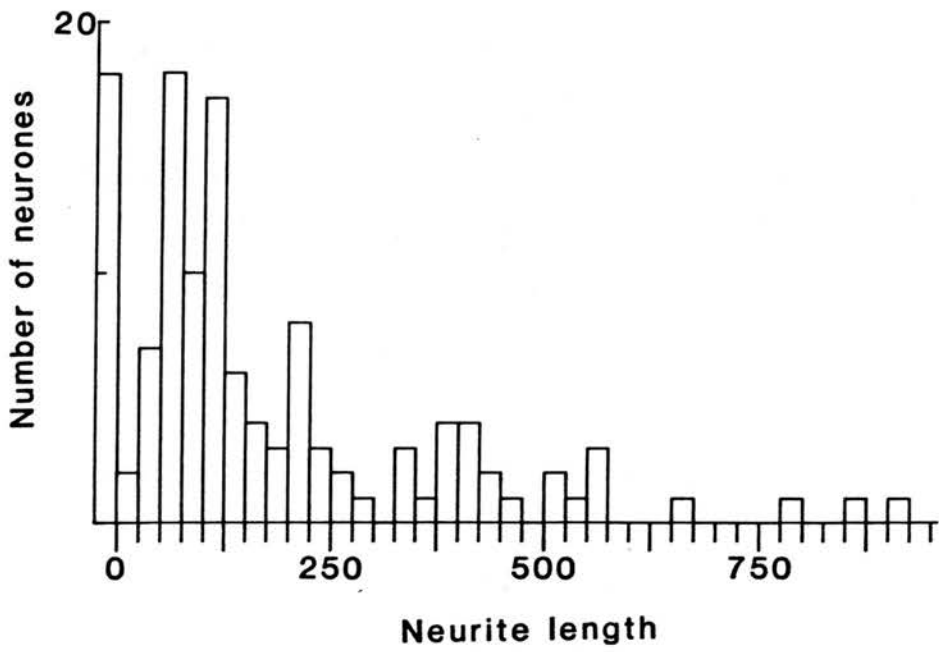
Table 3.4

Descriptive statistics for histograms shown in Figs. 3.11, 3.12, 3.13, 3.14, 3.15 and 3.16.

Segmental level	Substrate coating	NGF	Number of neurones	% failures	Mode	Median	Lower quartile point	Upper quartile point	Inter-quartile range
B	Lam	+	167	23	125	150	100	225	125
B	Lam	-	159	23	150	150	100	375	275
B	Fn	+	205	12	175	250	150	375	225
B	Fn	-	181	17	175	250	150	375	225
T	Lam	+	144	29	100	125	100	200	100
T	Lam	-	165	39	125	150	100	275	175
T	Fn	+	188	32	100	150	100	250	150
T	Fn	-	185	34	100	175	100	325	225
L	Lam	+	120	20	75	175	125	325	200
L	Lam	-	161	27	100	175	100	275	175
L	Fn	+	162	25	75	200	125	425	300
L	Fn	-	124	15	75	125	100	300	200

4 ANALYSIS OF NEURITE GROWTH IN CULTURE

4.1 ANALYSIS OF NEURITE LENGTHS

The histograms of total neurite lengths have several characteristic features. They are all positively skewed, multimodal and show a significant minority of failures. What characteristics of the dissociated neurones might generate this form of distribution? It has already been shown that the neurones are heterogeneous in terms of age and morphology. One can ask whether the skewed multimodal form of the distribution is a reflection of the different behaviour of subsets within the total population? In the following section, some of the more accessible heterogeneities of the neurones will be investigated. It will be shown that none of these are responsible for the form of the distributions of total neurite length. In light of this a novel hypothesis for the control of neurite growth will be put forward and modelled.

4.1.1 NEURONE POLARITY

The number of neurites per neurone varied between zero and four in the cultures, although uni and bipolar cells are by far the most common. Neurites are known to grow at a constant rate once initiated (Bray, 1973). If we assume that all the neurites of a neurone are initiated simultaneously, as suggested by Ernsberger and Rohrer (1988), the multimodal form of the distributions of

neurite length might simply represent the amount which a neurite grows during the culture period. If this were the case, the first, modal peak would be made up of unipolar cells, the second would be bipolar cells and so on. It was noted during the experiments that the frequency of multipolar cells increased with the stage of development. This might then explain the increase in median values seen with development, as an increase in the number of neurites per neurone, not of neurite length itself.

If this hypothesis was true, one would expect each neurite to be approximately the same length.

4.1.2 RESULTS

The data presented in Figs 3.4, 3.5 and 3.6 were re-analyzed and expressed as the total length of each neurite plus branches. Histograms of these measurements are shown in Figs 4.1, 4.2 and 4.3. Failures are not included in these observations, as the number of observations is no longer equal to the number of neurones measured. Descriptive statistics for the histograms are shown in Table 4.1.

First, and most importantly all the histograms still show a strong positive skew. This observation immediately rejects the hypothesis that the multimodal form of the distributions of neurite length is caused by the differential polarity of

FIGURE 4.1

Histograms of total individual neurite lengths for data first shown in Fig.3.4 from st25 embryos. a) Brachial, b) Thoracic and c) Lumbo-sacral segmental levels

FIGURE 4.2

Histograms of total individual neurite lengths for data first shown in Fig.3.5 from st28 embryos. a) Brachial, b) Thoracic and c) Lumbo-sacral segmental levels
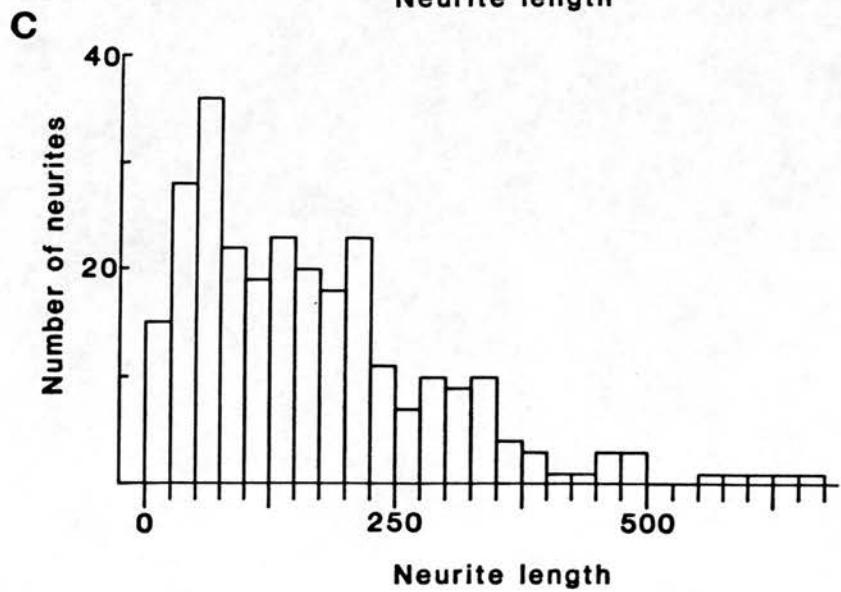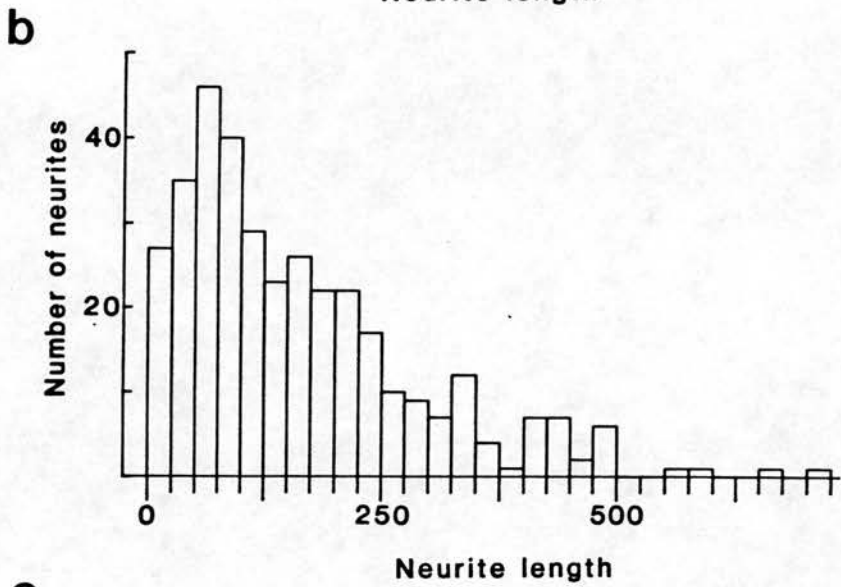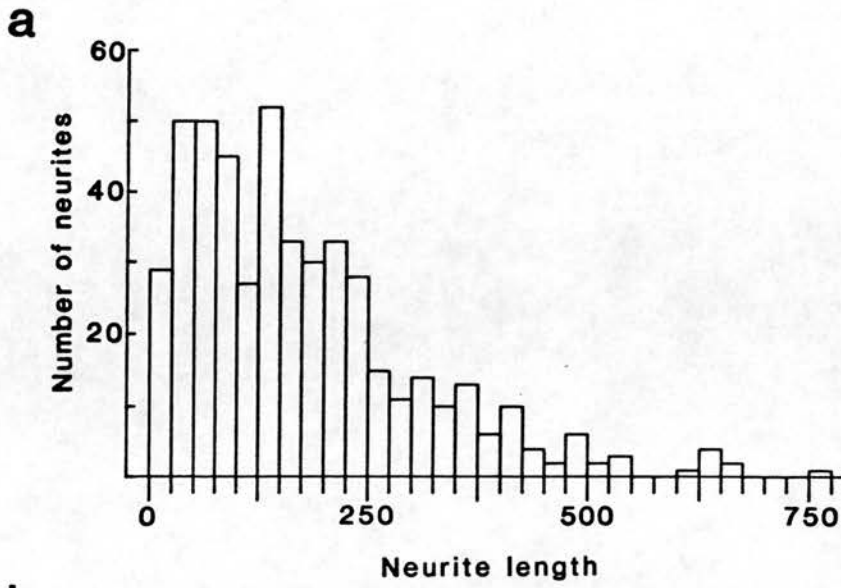
FIGURE 4.3

Histograms of total individual neurite lengths for data first shown in Fig.3.6 from st30 embryos. a) Brachial, b) Thoracic and c) Lumbo-sacral segmental levels
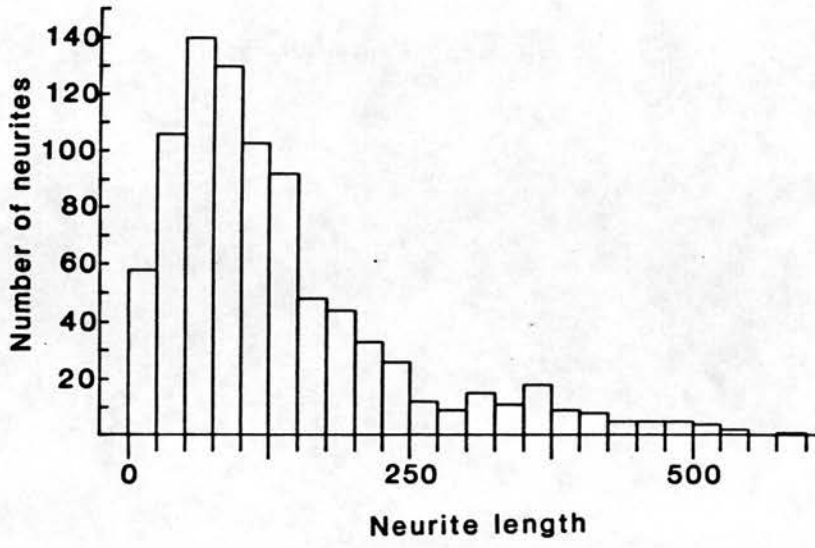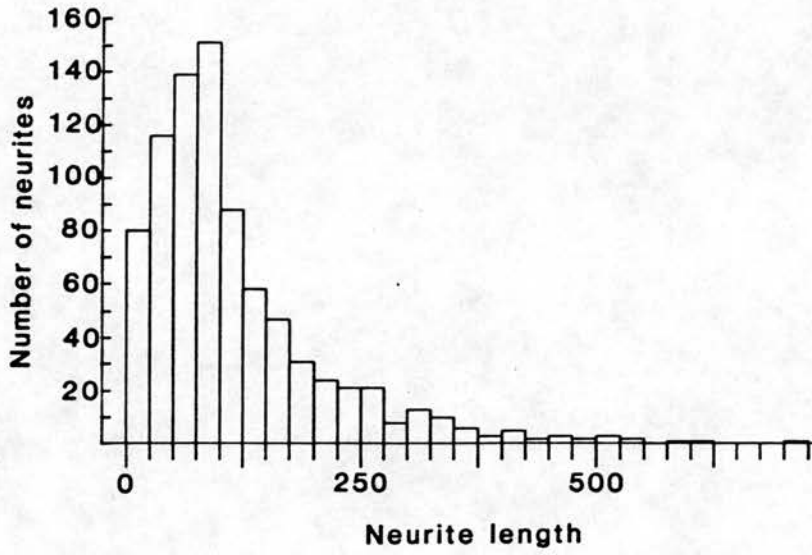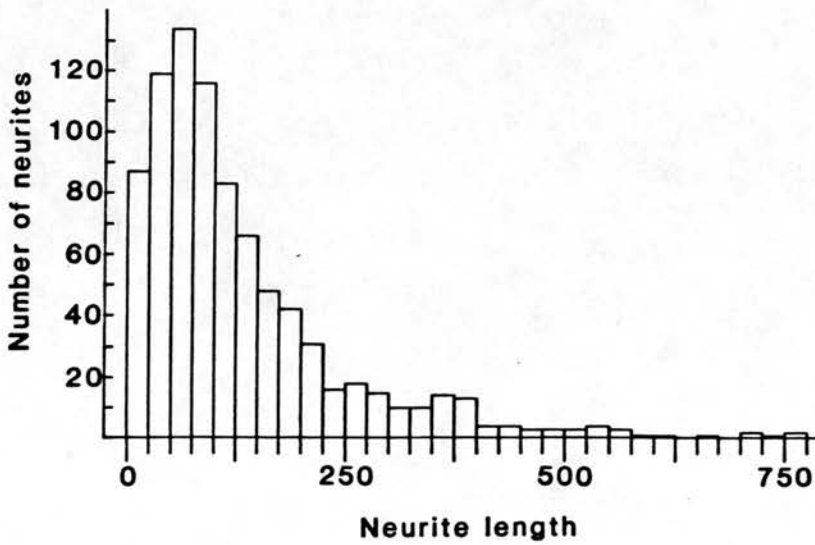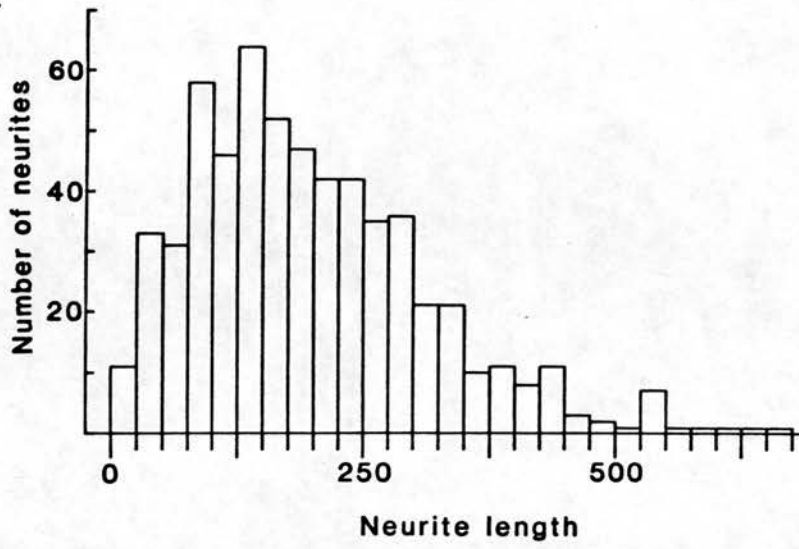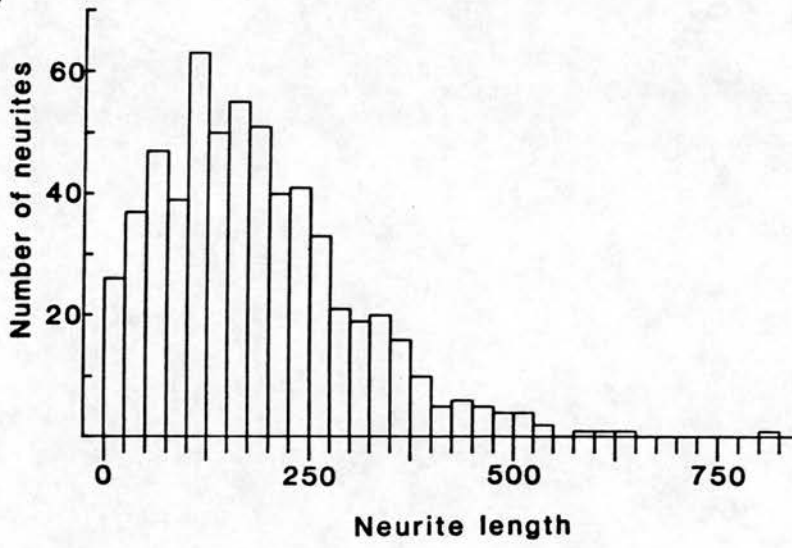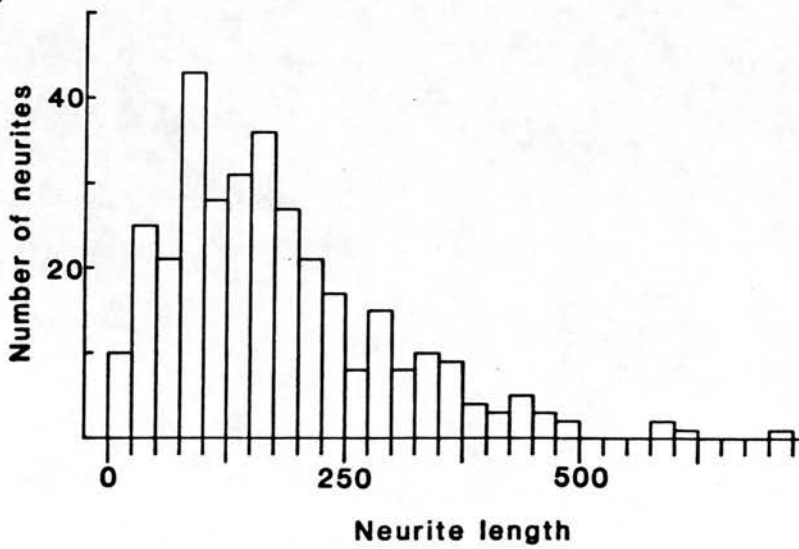
Table 4.1

Descriptive statistics for the histograms shown in Fig.4.1, 4.2
and 4.3.

Stage	Segmental level	Number of neurites	Mode	Median	Lower quartile point	Upper quartile point	Inter- quartile range
25	B	885	75	125	75	175	106
25	T	838	100	100	75	175	100
25	L	854	75	100	75	175	100
28	B	484	150	150	75	275	200
28	T	356	75	150	75	225	150
28	L	274	75	150	75	225	150
30	B	598	150	175	100	275	175
30	T	598	125	175	100	250	150
30	L	332	100	175	100	250	150

neurones within the cultures. If this had been true one would have expected to see a normal distribution of neurite lengths about a mean of approximately 100um, the typical value for the modal peak in the original data sets. Second, the histograms still show a multimodal form. This suggests that the event responsible for the form of the distribution of total neurite lengths is fundamentally expressed in the lengths of single neurites.

No differences in single neurite lengths between segmental levels are seen. If anything, the histograms are more alike than originally. Modal and median values are decreased relative to the original data set. This is obviously due to the division of neurite lengths from multipolar cells and is most apparent at the later stages of development where multipolarity is greatest. Inter-quartile ranges are also smaller which indicates that there is less variability in the lengths of single than total neurite lengths.

Median neurite lengths increase with development. This indicates that neurones from older ganglia grow longer neurites as well as more of them. Modal values are generally in the order of 75-100um. If the histograms in which larger values are recorded are examined carefully, it is seen that generally there is a slightly smaller peak at 75-100um (see Figs 4.2a and 4.3a). It seems then that all these histograms are based on a unitary event, resulting in 75-100um of neurite growth during the culture period.

4.2.1 NEURITE BRANCHING

Many of the neurites measured in this study were branched. Further the degree of branching varies with the stage of development of the embryo. The percentages of branched to unbranched neurites are; 9.8, 7.6 and 18.4% for st's 25, 28 and 30 respectively. Single neurite lengths were shown above to demonstrate the same characteristics as total neurite lengths. If the branching of neurites were regulated, and the interval between subsequent branch points were equal, then the multimodal form would be a reflection of the number of branches per neuron. Variation in the degree of branching would then explain the increase in neurite growth with stage of development seen. To investigate this measurements were made from a population of highly branched neurites. From these it appears that the distance between branch points is random.

4.2.2 RESULTS

Measurements of the inter-branch distances of neurites in a series of highly branched neurones were made from st30 cultures. A histogram of these measurements is shown in Fig.4.4a. Inter-branch neurite lengths are randomly distributed, there is no evidence of a regular pattern.

FIGURE 4.4

Histograms of interbranch neurite lengths taken from pseudo-multipolar cells from the brachial region of one st30 embryo.

a) inter-branch neurite lengths, b) interbranch neurite lengths, not including proximal segments.

a

Number of segments

40

20

0   250

Neurite length

b

Number of segments

20

0   250

Neurite length
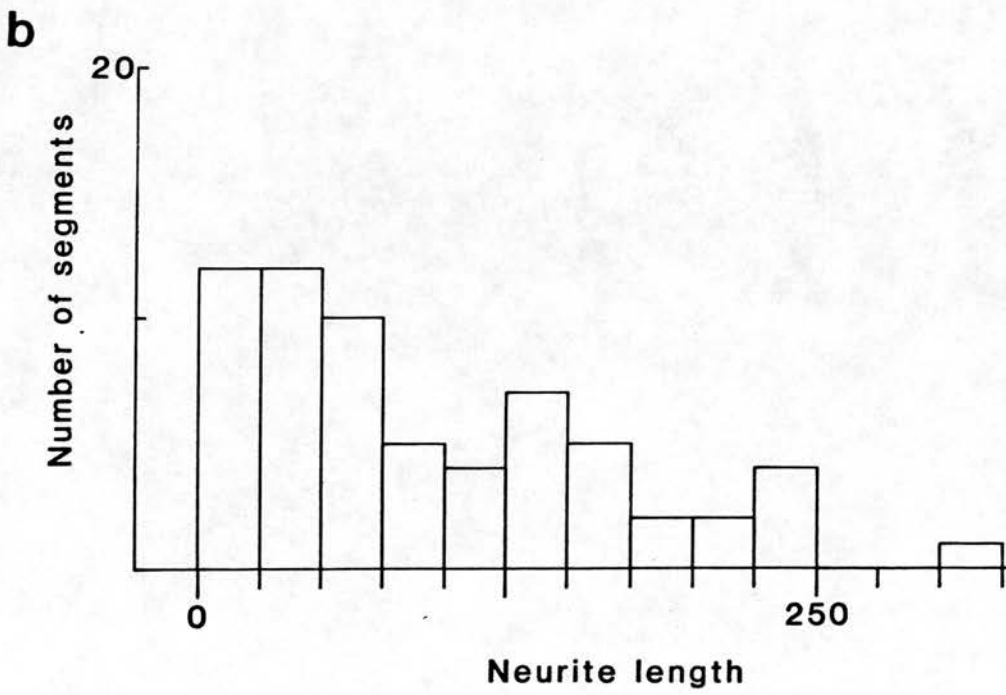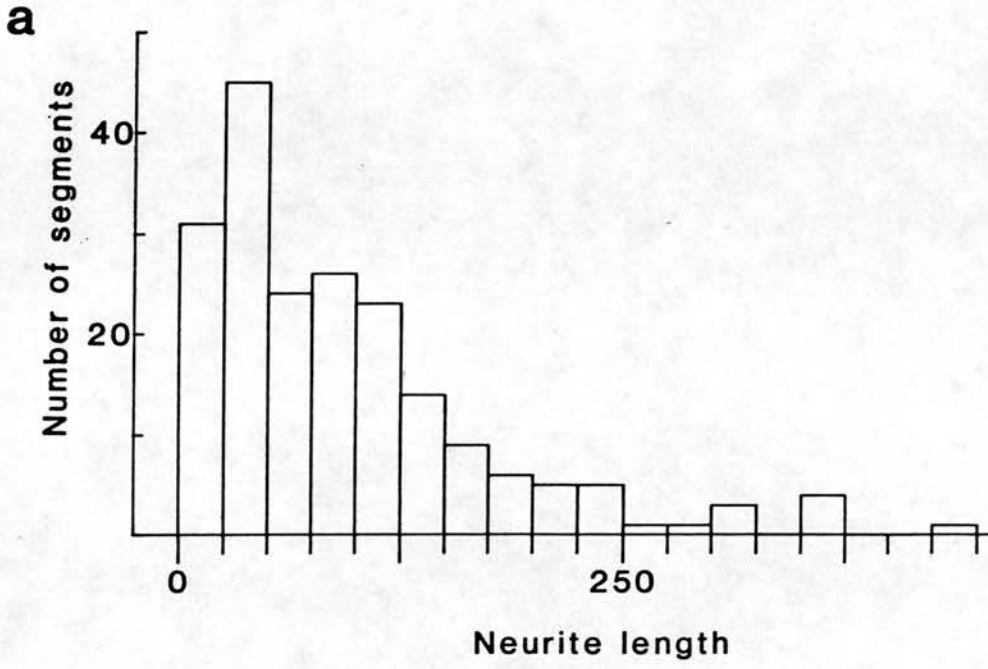
There is one potential problem with this analysis. The terminal segments of each neurite were still actively growing at the time the cultures were fixed. They may not then have been able to express their full growth potential. To exclude this possibility, Fig.4.4b was constructed to include only those segments which were complete, ignoring all the distal segments. The distribution is again random, and there is no suggestion of a unit, segment length.

4.3.1 NEURONAL AGE

DRG contain a temporally heterogeneous population of neurones. Development from progenitors and maturation of neurones occurs over a protracted period, which includes the three stages studied here (Carr & Simpson, 1978; Hamburger, 1981). At any of these stages of development individual ganglia will contain neuronal progenitors (indifferent cells), primitive neuroblasts and intermediate neuroblasts (Pannese, 1974). Mature pseudo-unipolar cells do not appear until E8 (st34). Some of the neurones will have grown axons *in vivo* and been axotomised during the isolation procedure. Others may have become post-mitotic prior to isolation and differentiated in culture (Rohrer *et al.*, 1985). Might these cells behave differently in culture, resulting in the production of skewed histograms, one peak representing single neurite lengths normally distributed about a mean of approximately 75-100um, and the other at 200um, but with a greater variance. Such a model would produce at least a

bimodal, positively skewed distribution of neurite lengths.

Fortunately, the two classes of neurones described above can be distinguished *in vitro* on purely morphological grounds (Rohrer *et al.*, 1985; Davies, personal communication). Those neurones which have differentiated in-vitro, and are therefore "inexperienced", have small, phase-dark cell bodies and bipolar neurites (SDB, Fig.3.2b). Those which were axotomised during the isolation procedure, and are "experienced", have large, phase-bright cell bodies and pseudo-multipolar neurites (LBM, Fig.3.2a). This criterion was used to investigate the behaviour of these two classes of neurones in culture.

4.3.2 RESULTS

Measurements of neurite lengths were made from the brachial region of st25 cultures and are displayed in Fig.4.5a. Data for LBM neurones is shown overlain by that for SDB neurones, failures represent the total for the whole culture. First, there are no striking differences in the two histograms, both show a skewed multimodal distribution of neurite lengths. Most strikingly the multiple peaks for SDB data are discontinuous. Second, there are no significant differences between mode (125 and 175um), or median (225 and 250um), inter-quartile ranges are also similar (300 and 250um, data for LBM and SDB respectively). Thus the two distinct populations, behave identically when placed into culture. Fig.4.5b shows the mean cell body diameters

FIGURE 4.5

a) Histograms of total neurite length per neurone for different classifications of neurone taken from part of the data set shown in Fig.3.4 from four repeat cultures from separate animals. Open bars are Large, Phase-Bright, pseudo-Multipolar neurones (LMB). Filled bars are Small, Phase-Dark, Bipolar neurones. (SDB). Failures were estimated as a percentage of those recorded in the original data set. b) The means of two measures of cell body diameters for the two populations. The first measure was taken at the longest cord and the second at right angles to the first.
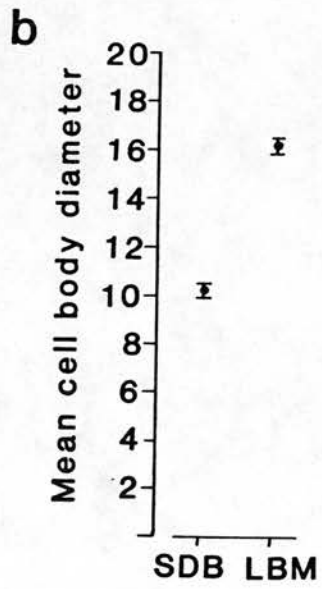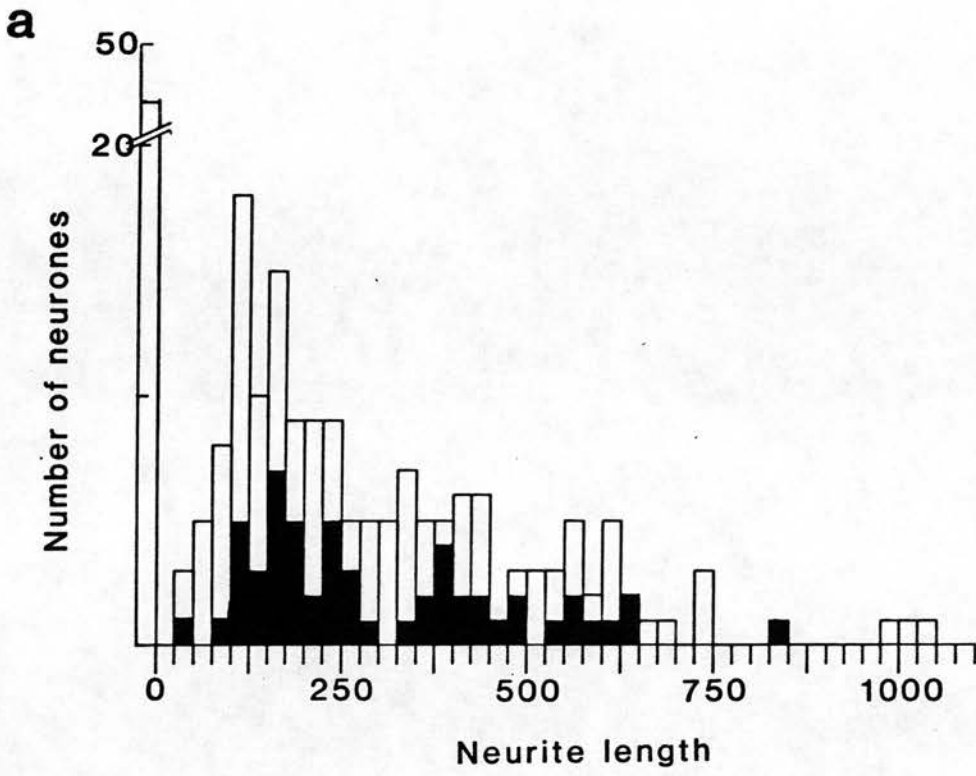
for the two classifications of neurone. The separation of the two indicates that differentiation, at least in terms of this parameter was successful.

Earlier observations have suggested that the event leading to the multimodality of the distributions is fundamentally expressed at the single neurite level. Data from Fig.4.5a was re-analyzed and displayed as single neurite lengths in Fig.4.6. Both retain their skewed, multimodality, only now they share the same modal value (75um) which has been suggested as the size of the event on which the neurite lengths are based. The differences In polarity of the neurones within the two populations, presumably cause the variation in total neurite lengths seen. LBM containing many unipolar, branched and multipolar cells, while SDB contains only bipolar cells.

It appears that the two, ontogenetically distinct populations of cells studies here show the same behaviour when isolated and placed into culture.

4.4 MODELLING THE DISTRIBUTION

The heterogeneities observed within the neuronal culture, are insufficient to account for the characteristic distribution of total neurite lengths. The data suggests that an event leading to the multimodal form of the distributions is fundamentally expressed in the lengths of single neurites. How can this be

FIGURE 4.6

Histograms of total individual neurite lengths for SDB and LBM populations, open bars represent data for LBM neurones and filled bars for SDB neurones.
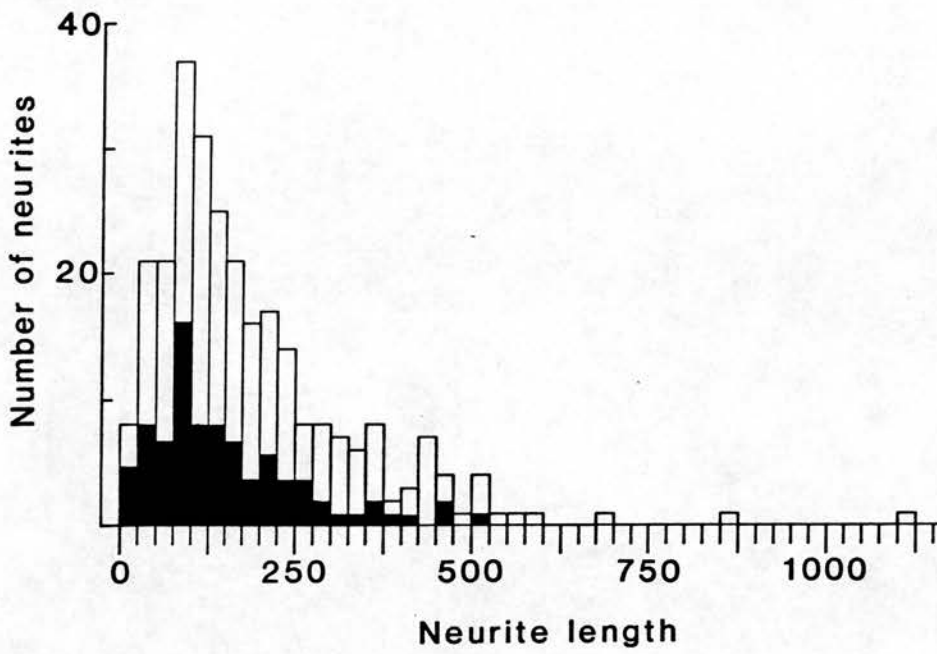
explained?

The features of the distribution I have described; skewness, multimodality and a significant minority of failures, are then characteristic of a Poisson distribution of quantal components. Thus we may propose that the growing neurone expresses some component affecting neurite growth which occurs in unitary quantities. If the unit event is distributed with a low probability, we would expect a percentage of the population to fail to produce a neurite. The event itself is unitary, as its expression occurs in integer multiples of a fixed value. The occurrence of an event leads to the growth of a set length of neurite, this is termed a quantum. Quantal mechanisms such as this, have previously been described by the Poisson theory, for example, single channel noise analysis (Colquhoun & Hawks, 1983), miniature end plate potential, interval analysis (Fatt & Katz, 1952) and vesicular release of neurotransmitter at the neuromuscular junction (delCastillo & Katz, 1954).

In the following section, this form of analysis based on the Poisson theory will be applied to the histograms of neurite lengths. The method of construction of Poisson, predicted curve fits will be described, and these will be shown to agree well with the data.

4.4.1 POISSON CURVE FITTING

The poisson statistic was used as a theoretical basis, from which curve fits for the histogram data were constructed.

$$P_x = \frac{m_x \cdot e^{-m}}{x!}$$

        x = number of quanta

        m = mean quantal content

        P = probability of "x" quanta occurring

The mean quantal content is calculated from ln(trials/failures), which in this case is equal to ln(total neurones/neurones without processes), after 24 hr in culture. Thus, as the probability of expression of a quantum of neurite growth increases, the mean quantal content increases and the number of failures recorded will decrease.

The Poisson equation in this form is only suitable for describing a discontinuously variable population. For instance the occurrence of neurones with one, two or three neurites in a culture might be described in this way. To apply the Poisson equation to a continuously variable population, such as the neurite lengths measured in the present study, some modifications must be made. The variability of events about any

quantum of neurite growth must be taken into account. Unit growth was assumed to be normally distributed about a mean value which represents one or more quanta of neurite growth. This Normal (Gaussian) distribution of events is described by the error function:

$$f(x) = \frac{1}{2\pi \sigma^2} \cdot \exp\left[\frac{-(x-\bar{x})^2}{2\sigma^2}\right]$$

    x = neurite length

    $\bar{x}$ = mean quantal neurite length

    σ^2 = variance

This must be calculated for each of the multiple quanta, one, two, three etc. The areas under these individual curves for the error function must be summed, to give the final curve for neurite lengths according to a Poisson model of neurite growth. In the curves shown here, calculations were carried out between the limits of 0-10 quanta, as the probability of expression is by definition assumed to be low. Finally the derived curve must be appropriately scaled to fit the histogram data. To do this the total size of the population, and the class width of the histogram "bins" must be known. The final equation, containing all of these factors, which has been used to calculate the curve fits presented is:

$$P_{(x)} = N_{Cw} \sum_{m_x 0}^{m_x 10} \frac{m_x . e^{-m}}{x!} \left[\frac{1}{2\pi m_x 6^2} . exp \left(\frac{-(x - m_x \bar{x})^2}{2 m_x 6^2} \right) \right]$$

N = total size of the population

Cw = class width of the histogram bins

$\sum_{m_x 0}^{m_x 10}$ = sum of the values for quanta between the limits 0 and 10

Px = probability of any neurite length (x) occurring

Our own software for the Archimedes computer was used to calculate the curve fits. Following data entry the program requires only three variables to be entered; i) the mean neurite length of the unitary quantal event (the mode in the majority of cases). ii) an estimate of the variance of the population about the mean. iii) the mean quantal content (calculated from; ln trials/failures).

4.4.2 RESULTS

Figs 4.7, 4.8 and 4.9 show Poisson curve fits (solid lines) overlying histogram data for total segmental neurite outgrowth initially shown in Figs 3.4, 3.5 and 3.6 respectively. The curve fits were constructed as described above, using the values for mean unitary quantal event, variance and mean quantal content

shown in Table 4.2.

The histogram data and Poisson curve fits show a remarkable degree of agreement at all the stages examined. It is interesting to note that for any particular stage of development, the values which have been used to construct the curves are remarkably similar. This further strengthens the conclusion made in the previous section, that there are no differences between neurite growth from different segmental levels. Equally, the differences between the stages of development studied are reflected in the different values used to construct the curve fits for them. Values for the mean quantal content increase with the stage of development, from 1.12 through 1.54 to 2.63 in brachial cultures, and similarly for the other segmental levels. This is a direct result of the decreasing numbers of failures recorded as development proceeds. The rise in mean quantal content is an indicator of an increase in the probability of expression of a quantum of neurite growth by a neurone. In the histograms the numbers of neurones with neurites in the bi and tri modal peaks of the distribution increase. This is well modelled by the curve fits as mean quantal content increases. This is most prominent at st30 in the brachial region. Here mean quantal content is 2.63, a value sufficiently large to increase the probability of a neurone expressing two quanta to the same level as that for a single quantum. The curve produced fits the broad distribution of neurite lengths obtained. It can be seen the that the

FIGURE 4.7

Histogram data first presented in Fig.3.4 for st25 of development, the solid black line represents the sum of Gaussian curves whose mean and variance are distributed according to the Poisson statistic $Px = e^{-m}.m^x/x!$ Mean quantal content; m was calculated from the number of failure.

a) Brachial, b) Thoracic and c) Lumbo-sacral segmental levels.
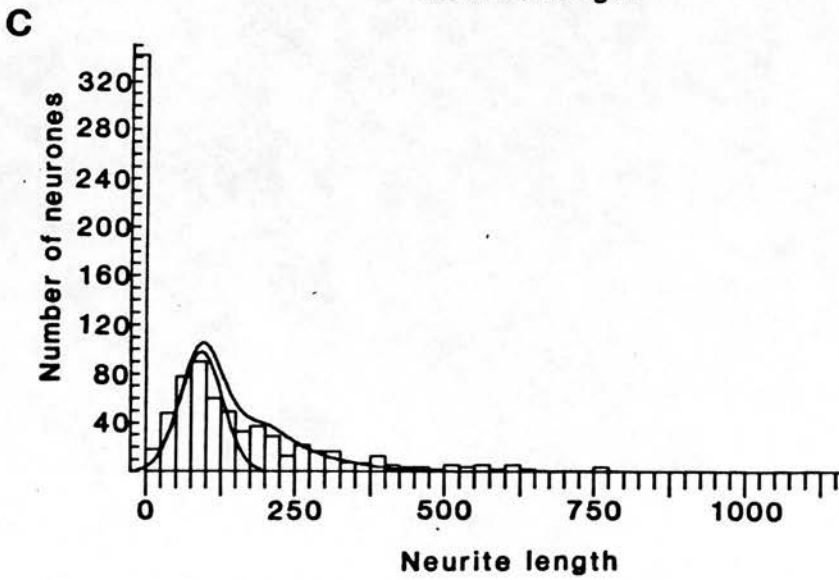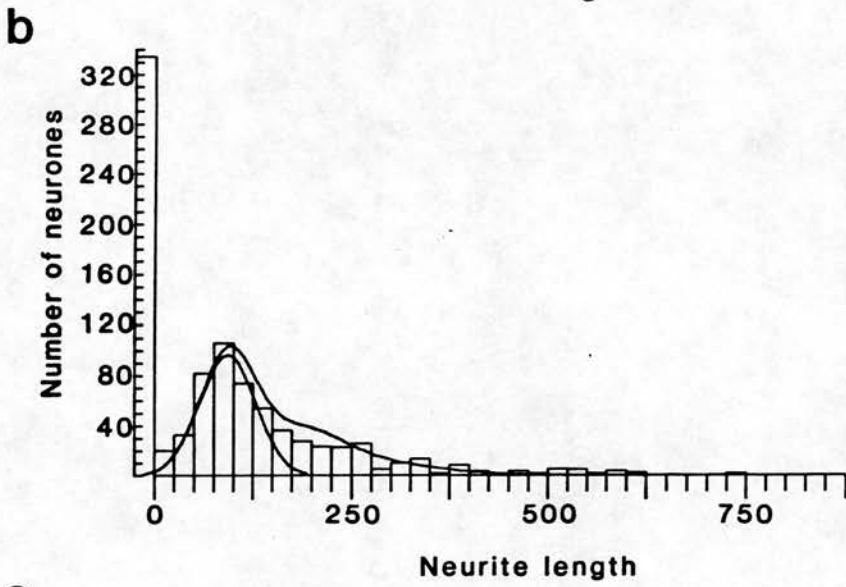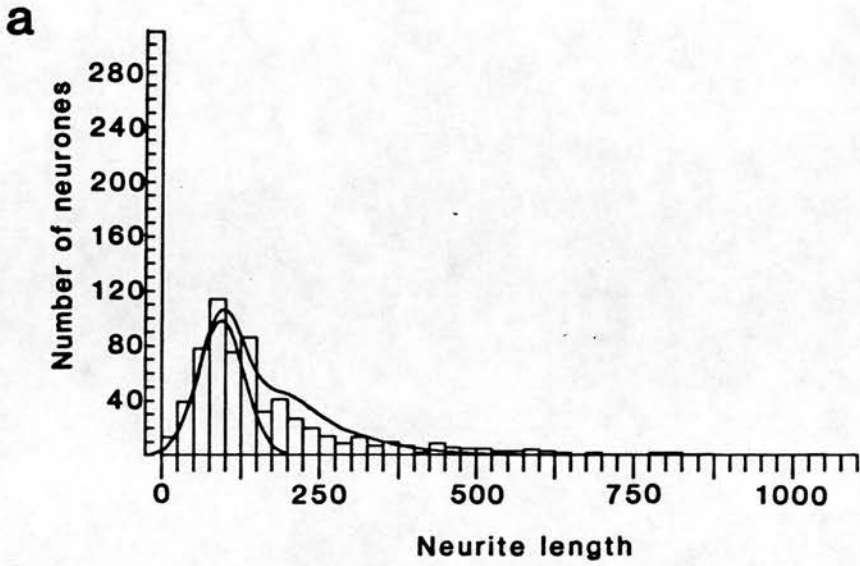
FIGURE 4.8

Histogram data first presented in Fig.3.5 for st28 of development, the solid black line represents the sum of Gaussian curves whose mean and variance are distributed according to the Poisson statistic $Px = e^{-m}.m^x/x!$ Mean quantal content; m was calculated from the number of failure.

a) Brachial, b) Thoracic and c) Lumbo-sacral segmental levels.
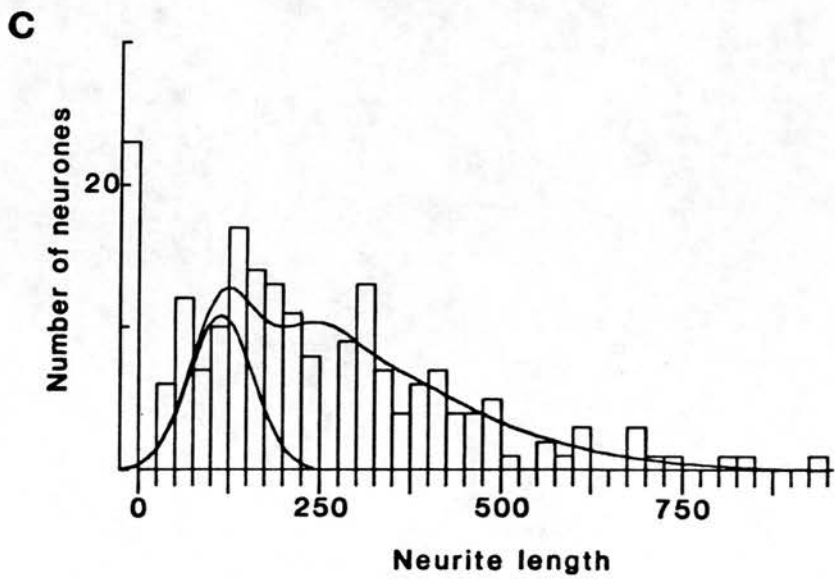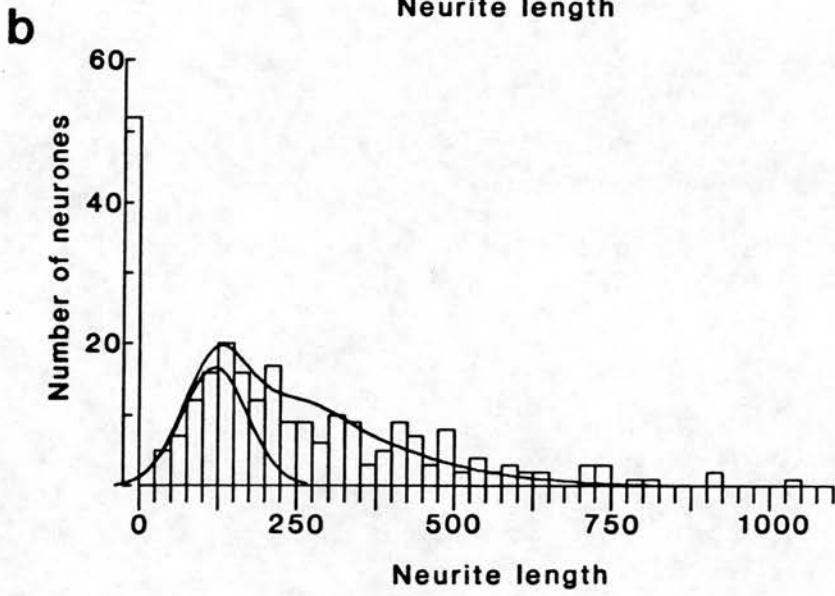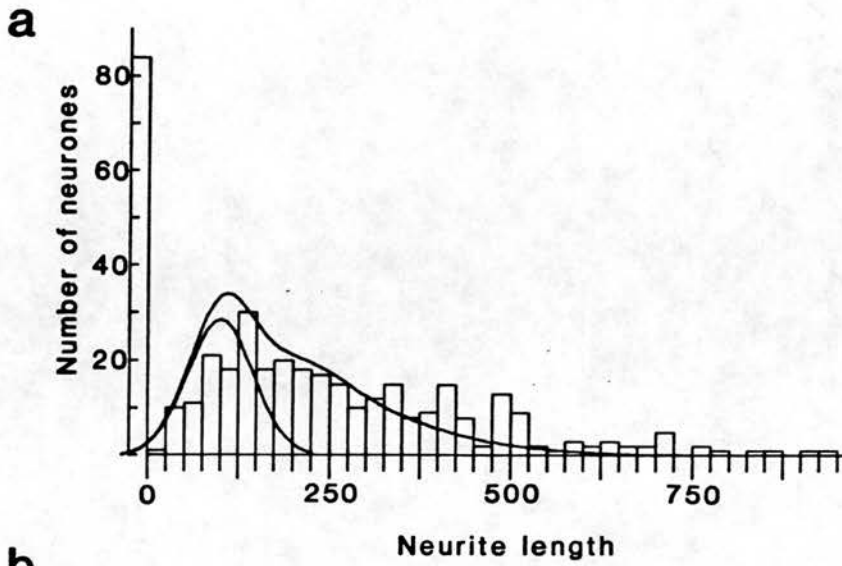
FIGURE 4.9

Histogram data first presented in Fig.3.6 for st30 of development, the solid black line represents the sum of Gaussian curves whose mean and variance are distributed according to the Poisson statistic $Px = e^{-m}.m^x/x!$ Mean quantal content; m was calculated from the number of failure.

a) Brachial, b) Thoracic and c) Lumbo-sacral segmental levels.
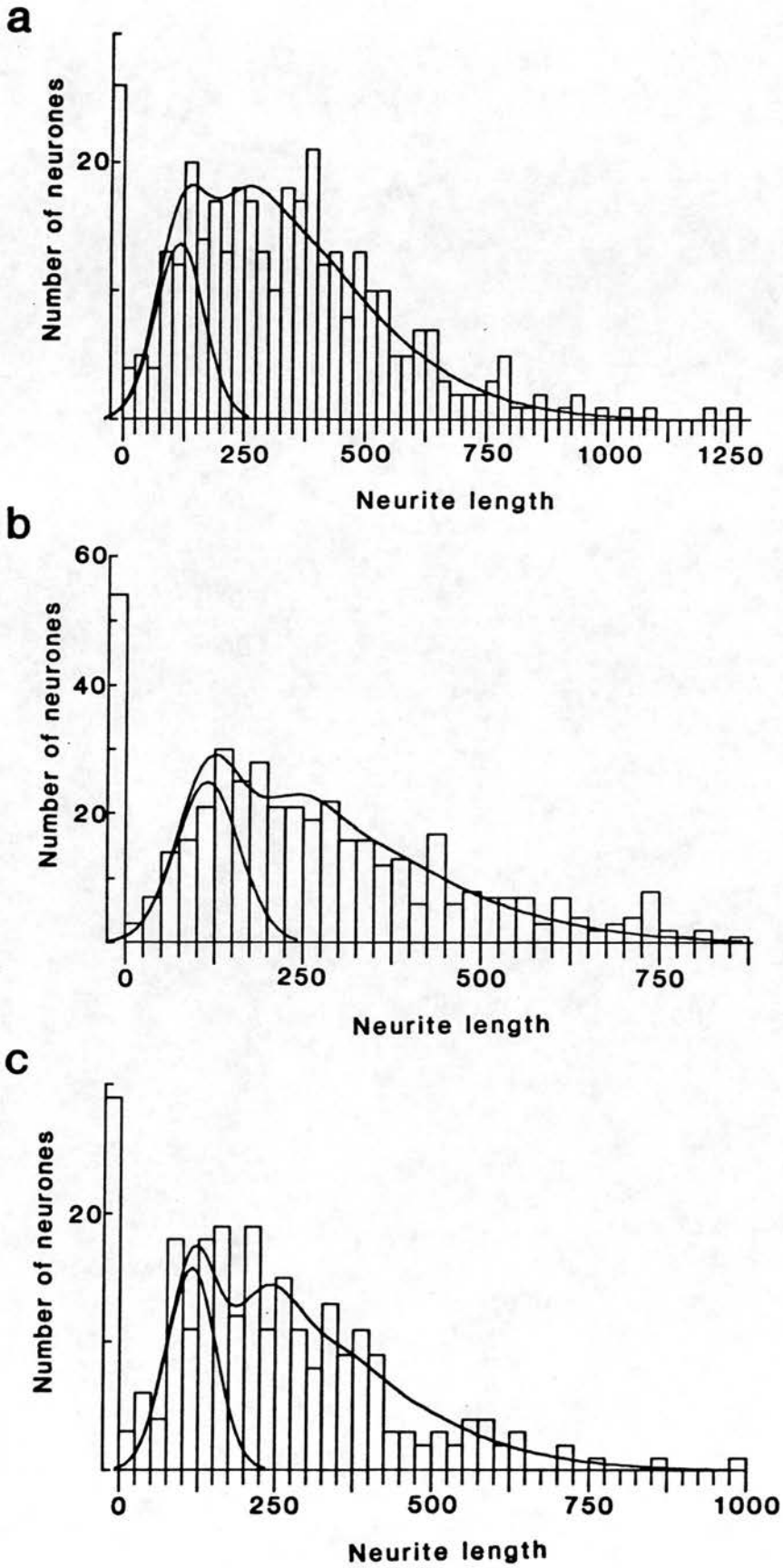
Table 4.2

Values used to construct the Poisson curves shown in Figs.4.7, 4.8 and 4.9.

Stage	Segmental level	Mean unitary event	Variance	Mean quantal content
25	B	90	35	1.12
25	T	90	35	1.01
25	L	90	35	1.01
28	B	100	45	1.54
28	T	115	50	1.61
28	L	115	45	2.14
30	B	115	50	2.63
30	T	115	45	2.08
30	L	115	40	2.18

percentages of failures recorded is directly linked to neurite growth within the population. Modal values fluctuate between 90 and 115um (one histogram bin). In a perfect quantal theory, these values would all be the same, indicating that the same event was underlying neurite growth at all the stages of development studied. Variance also increases with development, this reflects the broadening of the distributions as more neurones grow longer neurites.

It should be stressed that the curves illustrated are not "best fits" calculated by altering the three variables at random, to the point of minimum deviation. They are predicted curves calculated for values obtained from the data. The mean of the unitary quantal event was deduced from the modal peak. Mean quantal content was calculated from the number of failures. Variance is the only factor which is estimated. The agreement between these curves then, indicates that a Poisson model of neurite growth is consistent with the data recorded. If best fits had been constructed, the agreement with the data may have been better, but nothing would have been added to the overall conclusion.

It has been shown that the multimodality of total neurite lengths are due to that of single neurite lengths. Pursuing this idea, Poisson curve fits were produced for some of the single neurite length histograms. Fig.4.10 shows Poisson fits for the brachial segmental level at the three different stages of

development. Values for mean quantal content were taken from Table 4.2 as no independent estimate of the numbers of failures in this study was available. The number of observations in these histograms is much larger than the number of neurones recorded, therefore a direct count of failures would be inaccurate. The same value of 75um was used for all three curve fits, while variance ranged from 25 to 35um. Once again the agreement between data and theoretical curves is remarkable. More importantly, the use of the same value for the size of the unitary quantal event throughout, demonstrates that the same event is regulating neurite growth at all the stages of development examined. Note again that when mean quantal content rises sufficiently, the probability of a neurite growing two units of neurite length is the same as that for a single unit length (see fig. 4.10). This explains the increase in mode to 150um, as the expression of two quanta.

Thus we may formally propose the hypothesis that the growth of single neurites is quantal in nature, and that the probability of expression of a quantum of growth increases with development. At older stages of development, fewer neurones have no neurites and single neurites are greater in length.

Poisson fits were also calculated for the two populations of SDB and LBM neurones separately, and are shown in Figs 4.11a and b. Failures were estimated as a percentage of the total, any independent estimate for the two being impossible. The two show

FIGURE 4.10

Histogram data for total individual neurite lengths shown in Figs.4.1, 4.2 and 4.3. The solid black line represents the sum of Gaussian curves whose mean and variance are distributed according to the Poisson statistic $Px = e^{-m}.m^x/x!$ Values for mean quantal content were taken for table 4.1.

a) Brachial segmental level st25 of development, b) Brachial segmental level st28 of development, c) Brachial segmental level st30 of development.

a

b

c
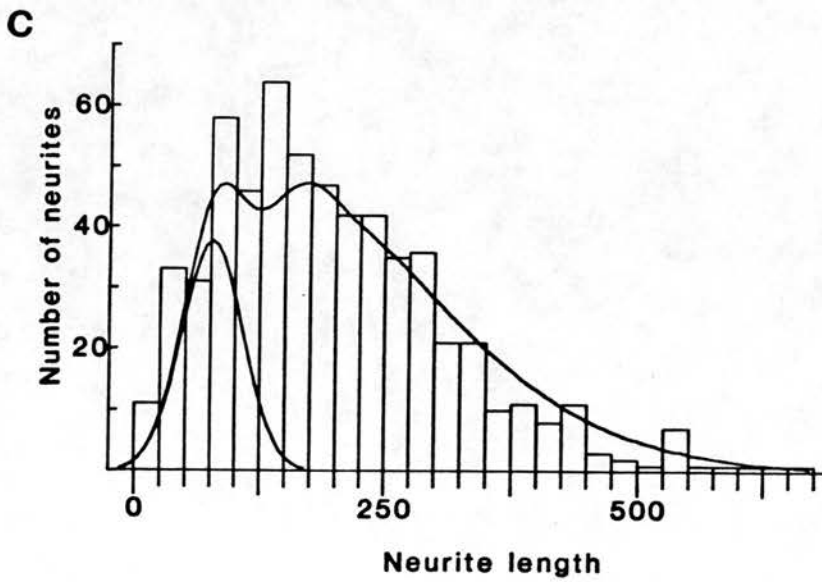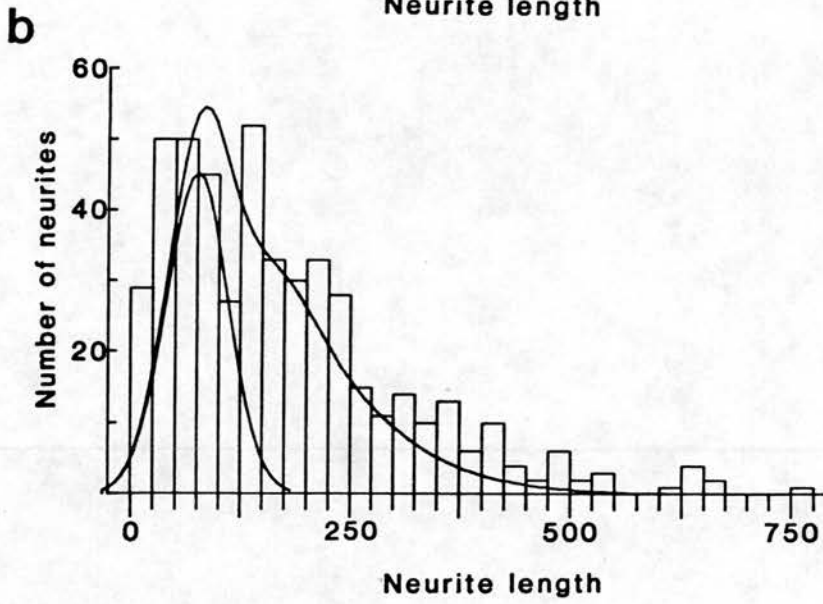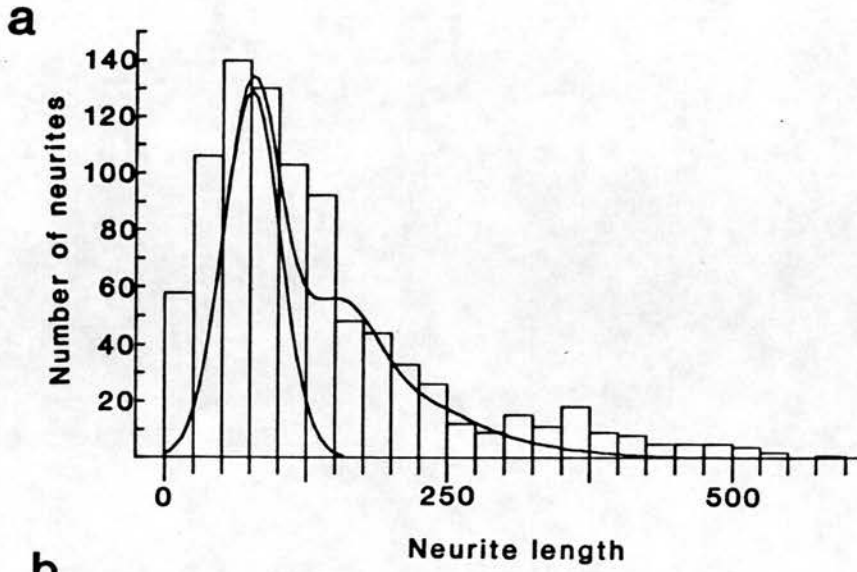
FIGURE 4.11

Histograms for LBM and SDB populations, first shown in Fig.4.5a, the solid line represents the sum of Gaussian curves whose mean and variance are distributed according to the Poisson statistic $Px = e^{-m}.m^x/x!$

a) LBM neurones, b) SDB neurones. Failures were estimated as a percentage of those recorded in the original data set.
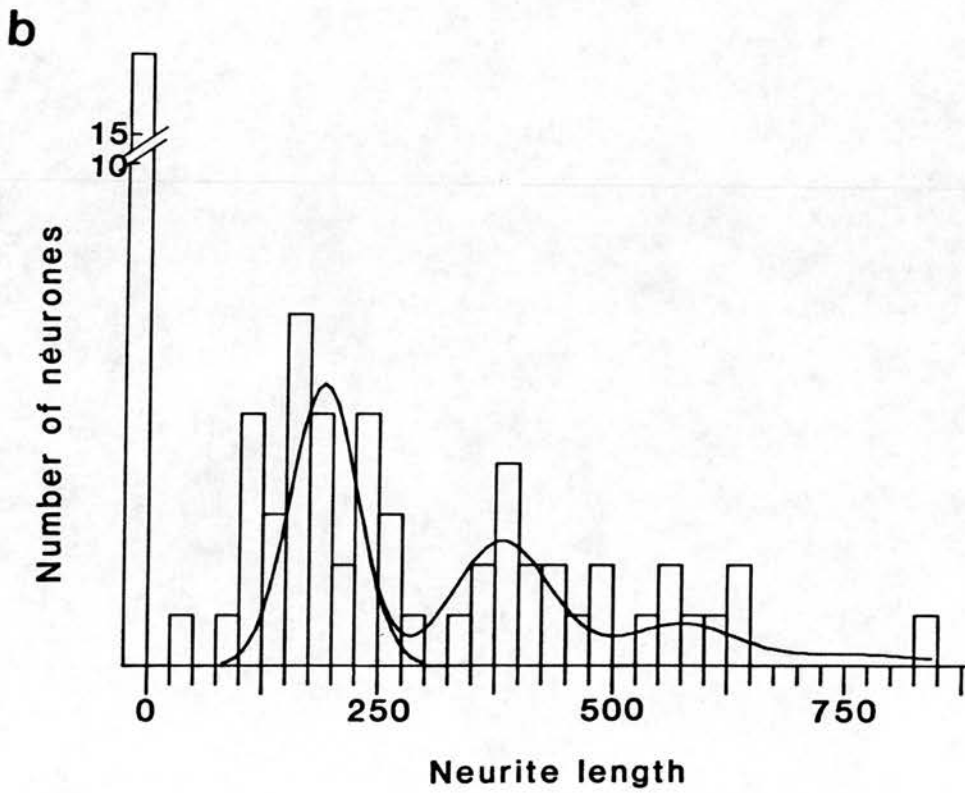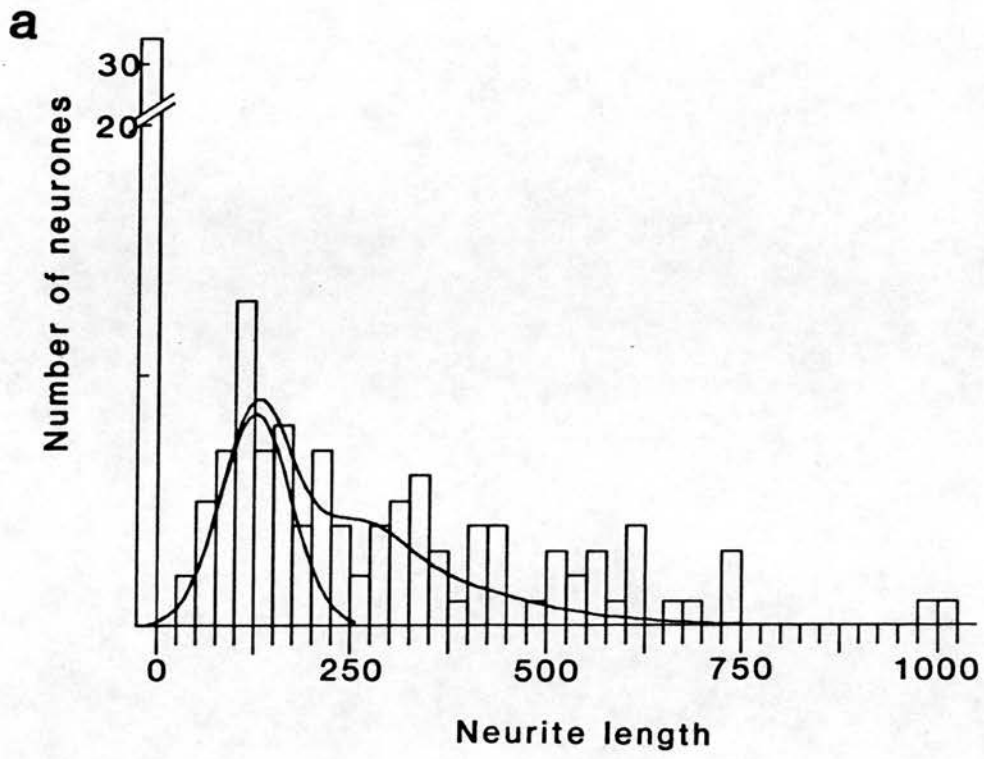
FIGURE 4.12

Poisson fits as previously described, for SDB and LBM neurones separately, drawn as a solid line over the data taken from Fig.4.6.   a) LBM neurones, b) SDB neurones.
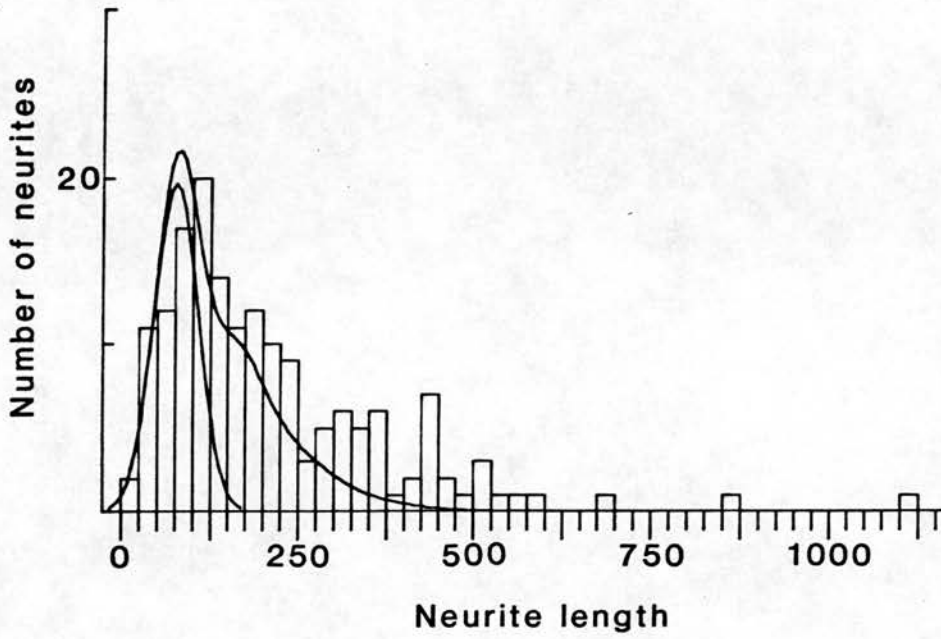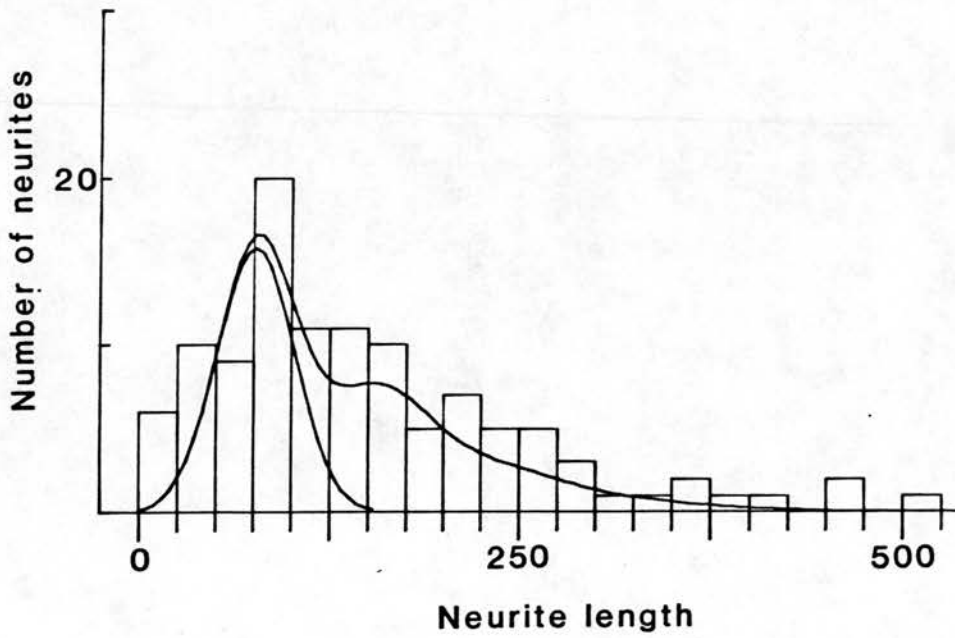
a good agreement, especially so for the SDB neurones, where three discontinuous peaks are seen. This may suggest that quantisation is most clearly expressed in these neurones which are growing their first process. The mean of the unitary quantal event for the SDB population is surprisingly high at 185um, but it must be remembered that all the neurones in this category are bipolar.

Poisson curves were fitted to the data for single neurite lengths and are shown in Figs 4.12a and b. The same values for mean quantal content (1.25; taken from Figs 4.11a and b) and mean unitary event (75um) were used in both cases. Both populations of neurones grow single neurites which can be described by an almost identical set of parameters for the Poisson equation. This indicates that the two behave the same when isolated and placed into a foreign culture environment. This is perhaps surprising considering the different developmental histories prior to culturing which they have.

4.5 CONCLUSION

The results of these experiments suggests that neurite growth may be somehow quantised. It has been shown that all the neurones studied grow processes which appear to be based on a unit multiple of a 75-100um length. This was true for all segmental levels and all stages of development. The multimodal form of the distribution was also unaffected by branching,

polarity, or the developmental history of the neurones. Culturing the neurones on different substrates in the presence or absence of trophic factors did not affect the expression of the neurite length quanta. A Poisson model for a low probability, independently occurring, quantised event provided an acceptable fit to the data.

5 DISCUSSION

The aim of these experiments was to determine whether the differential, axonal growth rates seen *in vivo* were intrinsically controlled as has been suggested in another system by Davies (1989). In addition to this, a detailed study of neurite growth from isolated DRG neurones in culture has been carried out. This part of the study has revealed several interesting phenomena, which are previously undescribed.

In answer to the initial aim of the study. Neurite growth was measured from DRG neurones isolated from different segmental levels in the embryo, but maintained under identical conditions in culture. When observed after 24hr, neurones demonstrated a great heterogeneity in polarity, branch pattern and neurite length. Total neurite lengths per neurone were measured , and the distribution of these measurements compared. No differences were found between cultures obtained from brachial, thoracic and lumbo-sacral segmental levels. This result then, is consistent with the conclusions of earlier authors following experiments *in vivo* . Namely that it is the nature of the limb which controls both the pattern and rate of its own innervation (Landmesser, 1984. Lewis, 1983. Davies, 1987. Scott, 1987. Davies and Lumsden, 1990 for reviews). When limbs were grafted to ectopic sites at early stages of development it was shown that they came to receive a relatively normal pattern of innervation from a foreign nerve supply. Further, this appeared to occur over a

time scale appropriate to the stage of development of the grafted limb. Swanson and Lewis (1982) confirmed this in experiments where they swapped limb-buds between embryos at different stages of development (prior to innervation). They concluded, that the stage of development of the limb was the controlling influence on the rate at which it became innervated. Tosney (1988) carried out deletions of the dermomyotome (presumptive muscle and dermis), and subsequently studied the innervation of the limb. She found that axons reached the base of the limb much earlier than normal, because of the reduced innervation distances following the experimental procedure. However they failed to enter the limb prematurely. This was speculated to be caused by the non-permissive nature of early, limb-bud mesenchyme, in agreement with the studies *in vitro* of Landmesser (1987). This phenomenon may be responsible for the "waiting period" observed following the early colonisation of the limb plexus at st21, but the failure to enter the limb until st24/25 (Hollyday, 1983. Tosney and Landmesser, 1985a). Hamburger and Brunso-Bechtold (1981) suggested that the limb mesenchyme might be a source of trophic factors for the developing DRG neurones. If this were the case, then the early non-permissive nature of the mesenchyme might be prior to the synthesis of such a molecule. The alterative hypothesis is that the early mesenchyme is inhibitory to axon advancement. Such a system of inhibitory interactions has recently been described in the posterior half of the sclerotome (Keynes and Stern, 1984; Davies *et al.*, 1990). Here, both motor and sensory axons are

channelled through the anterior half of the sclerotome. This is due to the inhibitory properties of the posterior, rather than the positive affect of anterior sclerotome on axon growth. So, it seems that the stage at which innervating growth cones invade the limb, is regulated by the limb itself, and not by the intrinsic properties of the neurones.

Once the growth cones invade the limb, the axons advance at much greater rates than those which innervate non-limb tissues. This is perhaps most striking in those ganglia which only partially supply a limb plexus. The differences in axon length between those growing to limb and non-limb sites from the same ganglion is remarkable (eg. T7; see Swanson and Lewis, 1982; Fig.3a for example). This clearly shows that only those neurones which specifically enter the limb, show an increased rate of growth, and not all the axons from a particular ganglion. This information however, did not preclude an intrinsic component to innervation control. Work has shown that the allocation of neural crest to DRG is arbitrary, and a pre-formed segmental gradient would not necessarily be constrained by the limits of the ganglia.

Davies' (1989) work on cranial sensory ganglia showed that their differential, target-distance related, growth rates, *in vivo* were maintained following isolation and culture. He concluded, that in this system, growth rates are intrinsically controlled. DRG neurones are derived from the neural crest, which is a

multi-potent, plastic population of cells (Le-Dourain, 1982), which form many different structures in the adult. It is perhaps not surprising that they do not show a strict, intrinsic growth program as is found in the ectodermal placode derived, cranial sensory ganglia which have a much more restricted set of developmental fates.

Two molecules of the extracellular matrix, laminin and fibronectin are known to be suitable substrates for the growth and differentiation of DRG neurones (Ernsberger and Rohrer, 1988), and to show differential patterns of expression in association with nerve trunks during development (Tomasek, 1982; Rogers *et al.*, 1986; Yip and Yip, 1990). DRG neurones grow longer, more highly branched neurites on laminin than on fibronectin (Rogers et al, 1983). In the present study neurones from st25 embryos were cultured under identical conditions on either laminin or fibronectin substrates. No differences in the total length of neurites grown on the two substrates were recorded for thoracic and lumbo-sacral levels. But, in cultures obtained from the brachial level, neurite outgrowth was greater on fibronectin than laminin substrates, by up to 60%. Following the demonstration of fibronectin in the developing wing by Tomasek (1982), Yip and Yip (1990), it appears that it may have a role in controlling innervation, in the fore-limb at least. Yip and Yip (1990) indicate an inverse relationship between the presence of fibronectin and axons in the developing limb. They have suggested that the fibronectin which is present prior to

axon ingrowth, may either be destroyed or modified by the invading axons, possibly by the release of proteolytic enzymes. If fibronectin was causing increased neurite growth, it is interesting that only brachial neurones appear able to respond to it with supra-basal growth. It is difficult to envisage how this could occur and additional work would be needed to confirm such a theory.

The results obtained are contrary to those obtained by Rogers *et al.* (1983) and Ernsberger and Rohrer (1988), who demonstrated that laminin was the preferred substrate for DRG neurite advancement. In Rogers' study, mean neurite lengths with standard deviations are presented. The present study has shown that neurite lengths in cultures of DRG neurones are not normally distributed. Therefore, presenting data in this way could be misleading. I suggest the use of median and inter-quartile range as a more accurate representation of the distribution of neurite lengths within a population of neurones. Further, Rogers *et al.*, (1983) in their study argue that the large standard deviations of mean neurite lengths recorded, are due to the asynchronous development of neurones in culture, although they present no evidence for this. In fact, these are probably caused by an unobserved, large, positive skew in the distribution of the neurite lengths.

On the whole it appears unlikely that extracellular matrix molecules alone, are capable of orchestrating the enhanced rates

of axon growth into limbs. However, a possible role for them in axon guidance has been suggested by Riggott and Moody (1987), who have demonstrated a punctate array of laminin extending between the trigeminal ganglion and its target field, the mandibular process. This is present prior to axon outgrowth, and has been suggested to act as a permissive substrate over which more complex, trophic interactions can take place (Davies, 1987b). In such a system, laminin would serve to channel axons (stereotypic guidance) and target derived trophic molecules would act to direct the growth (chemotropic guidance). Such a mechanism is similar to the "public highway" theory of limb innervation put forward by Swanson and Lewis (1982) and Lewis et al. (1983). In this model two factors are required, one to channel axons and another to attract them.

In the light of these postulates, experiments to investigate the activity of the commonest, peripheral, trophic molecule; NGF, was carried out. Much work, both *in vivo* and *in vitro* has lead to the hypothesis that NGF is capable of guiding receptive neurones (such as those from DRG), to their target fields (Levi-Montalchini, 1982). However, Davies (1987) and Davies and Lumsden (1990), present evidence from other systems to refute this idea (Introduction). In the present experiments, NGF was found to have no activity in promoting neurite outgrowth from E4.5 (st25) neurones, from any segmental level when cultured on laminin or fibronectin. Ernsberger and Rohrer (1988) have demonstrated the dependence of older neurones, isolated at E6

(st29), on NGF for the growth of "long" neurites (> four cell body diameters), but not for the growth of "short" neurites (< four cell body diameters). The presence of either laminin or fibronectin as a substrate in both cases was essential for the production of neurites. They further showed the complete dependence of E9 (st35) DRG neurones on NGF for neurite production. In one system, it has been demonstrated that NGF is not synthesised in target fields until the arrival of the first innervating axons, which, in turn do not become receptive to NGF until after its expression in the target field (Davies *et al.*, 1987). At E4.5 (st25) DRG growth cones have just begun to invade the limbs (Lance-Jones and Landmesser, 1981a and b; Honig, 1982; Tosney and Landmesser, 1985a,b) and target innervation will be in its very earliest stages. It is therefore possible that the neurones isolated at these early stages in the present experiments will be, as yet, unresponsive to NGF. In these early cultures then, the neurones appear to be refractive to NGF in terms of neurite growth, although it did appear to reduce the percentage of failure neurones recorded. However, this is not to say that it may not be important at later stages of development.

The limb clearly contains many different types of signal; i) one which prevents the early invasion of the limb by growth cones and leads to plexus formation, ii) one which attracts axons into the limb, iii) another which channels growing axons into nerve trunks, iv) and further ones which determine the branch points of intramuscular and cutaneous nerve branches. Most of these

still remain relatively obscure, and where an interaction has been identified, no specific molecular mechanism has been proposed. There is scope for a great deal of work to elucidate the specific mechanism through which the limb controls the pattern and timing of its own innervation.

Returning to the behaviour of the isolated neurones. No intrinsic differences were found in the growth potential of neurones from different segmental levels, at any of the developmental stages studied. But differences between the stages of development were apparent. An increase in median neurite lengths with the stage of development was seen. How then might this relate to the behaviour of the neurones *in vivo*? When estimates of axon growth rates are made from stained preparations, it is only possible to accurately measure the rate of advancement of the axon bundle tip. These measurements are then effectively a measure of the rate of growth of "pioneering" axons (those which are the first to invade the tissues). DRG neurones, show a protracted period of proliferation and differentiation (Carr and Simpson, 1978a and b), and therefore project along axon pathways over a similarly protracted period. Many of the later differentiating neurones are thus able to follow pre-formed pathways to their target tissues. One might therefore predict that these late differentiating neurones will grow more rapidly along the forged highways. In comparison, the trial and error,"pioneering" of the early differentiating neurones will be slower, through the relatively unstructured

mass of mesenchyme cells which confront them (Al-Ghaith and Lewis, 1982). This would of course be true for limb and non-limb innervating neurones, and in this study median neurite lengths increase in cultures from all three segmental levels studied. One would suspect that any such differences were the product of the environment, these results may suggest otherwise.

The occurrence of multipolar cells (tri and quadrapolar) also increased with the stage of development as did the numbers of highly branched neurones. Bunge et al. (1987) and Bray et al. (1987) demonstrated a similar rise in branching and polarity of DRG neurones in vitro between E8 - E15. They suggest that the control of this is intrinsic, but offer no explanation. Further they show that more adhesive substates increase the onset of axon initiation and neurite growth rates. Rogers et al. (1983) showed a similar increase in polarity and branching of DRG neurones with increasing substrate concentrations of laminin and fibronectin. It has been suggested that this is caused by increased adhesive interactions as substrate concentration rises. In the present experiments however, substrate concentrations were not varied. Thus, any increase in adhesivity would have to occur at the growth cone, presumably through an increase in receptor molecules as development proceeds. It has also been speculated that when adhesion is too great neurite growth may not occur.

As cell survival was not studied in these experiments, it remains a possibility that somehow, different sub-sets of neurones were being selected for at each stage of development. These neurones could then show different growth potentials in culture.

One important characteristic of every experiment carried out during this study, was the presence of apparently healthy neurones which had not grown a process during the culture period. These cells which were christened failures, were present in variable but significant numbers, and are displayed in all histograms of neurite lengths. Their numbers showed no consistent variation between segmental levels, but decreased with increasing stage of development. In fact, in all experiments the numbers of failures were inversely proportional to the median total neurite length recorded. If one regards all the neurones within the cultures as having the potential to grow neurites, but the probability of expression of this potential varies. The "growth potential" of the neurones will be reflected, both in the lengths of neurites and the numbers of failures recorded. Thus when growth potential is low, many failures and shorter neurite lengths will be recorded, the opposite will also be true. From the present experiments it would appear that growth potential increases with the stage of development (at least over the range of stages studied here). It should be noted that no attempt has been made to measure neurite growth rates in this study. The data presented, simply

represents the neurite lengths grown after an arbitrary 24 hr culture period. Before continuing, it is worth considering whether this value of total neurite length is a reasonable measure of the behaviour of the neurones during the culture period.

Do all the neurones which are going to initiate processes do so simultaneously, or with some degree of heterogeneity? Collins (1978a,b) has studied axon initiation in cranial sensory ganglia. In this system 75% of neurones cultured on a heart conditioned media treated, PORN coated substrate, initiated processes within 1hr, and 100% within 3hr. He further demonstrated that multipolar neurones tended to initiate all their processes simultaneously. Rohrer et al. (1985) has demonstrated that DRG neurones which have recently differentiated from precursors, show a relative synchronicity of axon initiation. Therefore axon initiation in culture may be both rapid and synchronous.

Is the rate of growth of neurites constant throughout the culture period, or do they speed up and slow down, stop and start? Bray (1973) in a detailed study of sympathetic neurones in culture, has shown that once initiated all the neurites of a neurone grow at similar and constant rates. He also states that following the bifurcation of a neurite, both branches will continue to extend at the same rate as prior to the bifurcation. Katz (1984) has measured the rate of axonal elongation at a zone

immediately proximal to the growth cone in cultures of chick DRG and frog neural tube. He reports that although short term rates (<1min) show a great variety, long term rates (>20min) were far more constant and consistent betweendifferent neurones.

Do neurones exhibit their normal characteristics in culture, or does the isolation procedure result in their loss? Rohrer *et al.* (1985) has shown that DRG precursors differentiate in-vitro to adult neurones, using biochemical and electrophysiological techniques. These are cells which underwent their terminal mitosis 8-16 hr prior to isolation and have continued their normal developmental program in culture.

This evidence together suggests that in culture, neurones innitiate processes synchronously, grow neurites at similar rates and develop normally. It seems reasonable then, to compare neurite lengths between different cultures, and to draw conclusions concerning the factors which might be regulating them.

It may be interesting at this point to compare the amounts of neurite growth recorded in the present series of experiments *in vivo* and *in vitro*. From the silver stained preparations, an estimate of axon growth may be gained. If axons are assumed to leave the ganglia as early as E3 (st18/19), The axon lengths at E5.5 (st28) represent 60hr of growth. Taking the measurements quoted in the Results gives values of 240um/D and 720um/D for limb and non-limb innervating axons respectively. These compare

well with measurements taken from the similar preparations of Swanson and Lewis (1982) of 150um/D and 675um/D for thoracic and brachial axons respectively. At the same stages of development, median neurite lengths are between 100 and 250um after 24hr in culture, though some neurites of greater than four times this length are also recorded at these stages. The amount of neurite growth seen in culture, is closest to that from thoracic ganglia *in vivo*. This appears to be the basal level of neurite growth, and that growth into limb regions is accelerated, by some as yet undetermined factors. These values are generally lower than those recorded in other systems; 480um/D in the mouse (Davies, 1987), 1000um/D in the tadpole (Speidel, 1941), 600-1800um/D in frog neurones (Jacobson and Huang, 1985), 600um/D in frog explants (Harrison, 1907) and 500um/D in cultured chick sympathetic neurones (Bray, 1973).

It may be concluded then that DRG neurones show an intrinsic growth program, which is regulated by the stage of development of the embryo, but not by segmental level. This is manifested as an increase in the length and complexity of neurites with stage of development. The significance of this to the situation *in vivo* is unclear. Mature DRG neurones have a pseudo-unipolar morphology and the complexity of terminal elaboration varies considerably between neuron types. It is therefore unlikely that the form of the neurones in culture is an accurate reflection of that *in vivo*. It is more likely that it simply reflects a change in the properties of the neurones with development. This could

be either in terms of cytoskeletal organisation or cell surface expression of receptors.

It should be noted that the production of neurites per se is an intrinsic capacity which varies between different types of neurone. For example, the pattern of outgrowth from DRG neurones is very different from that of the NT cells studied in the next section, these cells grow much shorter and less complex neurites over a similar culture period. Further, the neurites produced by sympathetic neurones are more complex and highly branched than those from the DRG neurones in the present study (Bray, 1973).

In the experiments presented here, one important fact has emerged. The neurite lengths within a population are not normally distributed. They show a strong positive skew, the tail of which is multipeaked. These multiple peaks occur at approximate integer multiples of the first, major, modal peak. In addition to this, each population contained a small, but significant minority of neurones which failed to grow a process during the culture period. The characteristics of neurite growth from the neurones was investigated to discern the nature of these unusual distributions. Single neurite lengths displayed the same multimodal distribution as total neurite lengths. This demonstrated that it was a process fundamental to the growth of single neurites which was leading to the multimodal form of the histograms for total neurite length. Branching of neurites was found to be random, thus it is the total length of neurite produced which is regulated. Finally, cultures contain neurones

at differing stages of development, particularly in that some
had been axotomised during the isolation procedure, while others
had only become post-mitotic several hours prior to isolation.
These two major sub-sets of neurones were distinguished on
purely morphological grounds. Surprisingly the two were found
to behave almost identically in culture in terms of the growth
of neurites. There was no evidence that differential behaviour
of the two sub-sets could be responsible for the form of the
distribution of neurite lengths recorded. At st25, when these
experiments were carried out, only those early differentiating,
VL neurones will have extended axons into the periphery. These
then will make up the majority, if not the whole of the LBM
population of axotomised neurones. Whereas, the SDB population
will contain both early, VL and late DM differentiating
neurones. Therefore, it seems that the two different categories
of neurones which have been identified *in vitro*, also behave
similarly when isolated and placed into culture.

The same multimodal distribution was seen under all conditions
examined, including different substrate, stage of development
and segmental level. This suggested that there was some basic
phenomena underlying neurite growth, which was expressed as
unitary quantities of neurite growth, which is contrary to the
view of neurite growth as infinitely variable. Returning to the
data histograms, the mean neurite length of the first, modal
peak determines the size of the unit, and subsequent peaks
represent multiples of it. The presence of failures in the

cultures indicates that the expression of the unit of growth occurs with a relatively low probability. This occurence of events in multiples of a unitary size has been termed "quantisation".

A modification of the Poisson theory was used to predict the occurence of independent, low probability, unitary events. The agreement between these predicted distributions and the data was remarkable. This fact however does not prove that a quantised process underlies neurite growth. It simply demonstrates that such a theorem can adequetly describe the events recorded. The original predicted Poisson curves were fitted to histograms for total neurite length per neurone and indicated some degree of variability in the size of the unit of growth. When distributions for the lengths of single neurites were plotted, they were found to be multimodal. More importantly; the size of the unitary event was consistent, both between segmental levels and stages of development. This indicated that the growth of single neurites was regulated through the expression of multiples of a 75-100um unit of growth. That is, single neurite length is quantised.

A working hypothesis for such a system is presented. It is proposed that neurite growth in this system, is regulated in a quantised manner. This quantisation is seen as the production of between 75-100um of neurite growth. The expression of the underlying event which leads to this occurs with a low

probability. This probability varies directly with the stage of development of the embryo. The major drawback with this hypothesis is the inability to make an independent estimation of the size of the unitary, quantal event. When a similar analysis was applied to transmitter release at the neuromuscular junction (del-Castillo and Katz, 1954), spontaneously occuring miniature end-plate potentials provided an independent estimate for the size of transmitter vesicles. In the present system there is no way to measure "spontaneous" neurite growth. If the stimulus for growth is removed, namely the permissive substrate, no neurite growth will occur. Further, at the neuromuscular junction, the probability of transmitter release can be raised and lowered pharmacologically. Under these conditions, it is possible to modulate mean quantal content, something which is not possible in the present system. In the future,the advent of specific neurite growth promoters, either genetic or metabolic might enable this type of study to be carried out.

Many studies have been carried out into the mechanics of neurite growth. The results obtained and the conclusions made are often contradictory. It is difficult to reconcile these observations with the results obtained in the present study, however some of the relevant work will be discussed. It should be remembered however, that an investigation into the mechanism of neurite outgrowth and an application of this to the proposed quantal hypothesis was not the aim of this study.

Bray *et al.* (1987) has proposed that the control of polarity, branching and neurite length are likely to depend upon the rate of macromolecular synthesis and detailed internal organisation of a particular nerve cell. He demonstrates that neurite production from DRG neurones increases with the stage of development of the embryo (between E8 - E15). Over the same time period cell body sizes also increase. He argues that the increased neurite growth is a consequence of an increased capacity to synthesise essential macromolecules as cell body size increases. In the present study cell body diameters were not correlated with neurite length. However the LBM cells studied do have larger cell bodies and are older than the SDB neurones, they produce more complex but not longer neurites in culture. In Bray's study almost all the neurones will have been axotomised during isolation, which may be a contributary factor in their behaviour in culture. Bray (1979) demonstrated that growth cones put active tension on the neurite, and that this tension is proportional to the degree of adhesivity present. When the tension in the growth cone is sufficient to overcome that in the neurite, advancement will occur. Katz (1984) has calculated parameters for the probability of neurite advancement or retraction based on Bray's mechanical model. He proposes that neurite outgrowth occurs in small steps, and for each one of these there is a probability of advancement or retraction. This probability will vary with the type of neurone and substrate. Interestingly he has used Binomial statistics to describe this "stochastic walk model of neurite growth". The Poisson statistic

used in the present study is a special derivative of the Binomial distribution. Whether the process Katz describes of short term neurite growth rates is related to that described here is unclear. Bray (1984) further showed that neurite extension occured in response to tension from the growth cone, which suggests that it is the advancement of the growth cone and its specific interactions with the environment which govern neurite extension. Processes which lead to neurite growth may then simply be a passive consequence of growth cone advancement. In an aside to his towing experiment Bray (1979) noted the changes in neurite diameter following bifurcations. He determined that neurite diameter decreased at each branch point, but the sum of the two daughter neurites was always greater than that of the parent. In another study, Bray (1973) has indicated that neurite growth rates are similar before and after a bifurcation. This suggests that the limiting factor on neurite growth is not the production of membrane. The production of microtubules which extend along the length of neurites could still be important, and it is interesting that the average length of these is 108um (Bray and Bunge, 1981). This value is very close to the quantal neurite length proposed here.

Whether the event which underlies the quantisation of neurite growth described here is active through metabolism, gene expression or macromolecular synthesis is unclear. Its importance in the control of neurite growth is clear and demands further study.

NEURONAL TOXICITY OF CYTOSINE-B-D-ARABINOFURANOSIDE

6 INTRODUCTION

In this section I shall describe experiments which were carried out to investigate the effects on neurite outgrowth and neuronal survival of a pharmacological agent, whose action on post-mitotic cells such as neurones was previously unknown.

Cytosine B-D arabinofuranoside (AraC) is an anti-mitotic agent which is widely used to suppress the proliferation of fibroblasts and other rapidly-dividing cell types in cultures of neural or muscle cells (Furth and Cohen, 1968; Cohen, 1966; Burry, 1983; Fischbach, 1972). Following addition of AraC to a culture, the progession of dividing cells from the G1 phase to the S phase of the cell cycle is retarded, and cell division is prevented in the S phase (Graham and Whitmore, 1970). At the molecular level, AraC acts as a competitive inhibitor of DNA polymerase (Furth and Cohen, 1968; Furlong and Gresham , 1971). Kufe and Major (1982) showed that after entry into a cell, AraC is phosphorylated to an active intermediary which can substitute for dCTP in DNA strand elongation. Subsequent deoxynucleosides are then unable to add efficiently to the AraC and DNA synthesis stalls.

Although post-mitotic cells are normally thought to be unaffected, evidence for toxicity of high concentration of AraC

on neurones has been found, for instance in cerebellar explant (Messer, 1977; Seil *et al.*, 1980; Patel *et al.*, 1988), or following its clinical application in the treatment of myelogenous leukaemia (Breuer *et al.*, 1977). However, recent studies have suggested that low concentrations of AraC have a neurite growth-promoting effect on axotomised neurones (Oorshot and Jones, 1987); and on axonal growth into a spinal lesion *in vivo* (Guth *et al.*, 1988).

The present study was carried out to evaluate the effects of AraC on the growth of neurites and on the survival of neurones over a range of concentrations of the drug. Neurones were obtained from dissociated chick neural tube (Henderson *et al.*, 1984). In this system I was unable to confirm the reported nerve growth promoting effects of AraC and further, I found AraC to be mildly toxic to neural cells even at relatively low concentrations. Finally, AraC appeared to have independent effects on neuronal survival and neurite growth.

7 MATERIALS AND METHODS

Domestic fowl (Gallus domesticus) were obtained fertile from the Animal Physiology and Genetics Research Station, Roslin, Edinburgh (strain: J-line) and maintained in a humidified, force-draft incubator at 37°C until the day of experimentation.

7.1 DISSECTION AND CULTURE

Procedures were identical to those detailed in section 1, only differing in the following areas: whole neural tube explants from brachial to lumbo-sacral levels were taken, cleaned of associated meninges and dorsal root ganglion rudiments prior to trypsinisation; media was serum free or supplemented with 10% FCS, NGF was not added; 750ul of cell suspension (20-40 x 10^6 cells/ml) were added per 16mm well of a 24 well culture plate (Linbro-flow).

In one experiment the four limb-buds of an E6 (st.26) embryo were removed, dissociated and cultured in an identical manner to the neural tubes. These cultures were used to ascertain the specificity of the anti 68Kd neurofilament sub-unit.

All cultures were maintained in a force draft incubator at 37°C throughout.

7.2 ADDITION OF CYTOSINE-B-D-ARABINOFURANOSIDE, 2'DEOXYCYTIDINE AND CYTOSINE

Cytosine-B-D-Arabinofuranoside (AraC:Sigma) was added to the cultures prior to plating out, to give final concentrations ranging from 100nM to 1mM (10^{-7} - 10^{-3}M) and 0 in controls. In selected cultures either 2' Deoxycytidine (2'DC:Sigma) or Cytosine (Cyt:Sigma) were added at final concentrations of 100um (10^{-4}M) in addition to AraC.

7.3 COATING OF CULTURE WELLS

Cell culture treated, 16mm wells (Linbo-Flow) were prepared according to the following procedures.

7.3.1 POLYORNITHINE

PORN coating was carried out essentially as described in section 2.3.

7.3.2 LAMININ

Subsequent to PORN coating, half of the wells were coated with laminin. 1.2ml of 1mg/ml laminin (Bethesda Research Laboratories) was added to each well, spread across the surface and allowed to air dry at room temperature. Laminin coating was always carried out just prior to plating the neurones. A few experiments were carried out to examine the specificity of

laminin in stimulating neurite outgrowth, using laminin antiserum. In these experiments wells were coated with a laminin solution diluted to concentrations of 1-100 ug/ml following the protocol in section 1 in PBS, as preliminary assessments indicated that even neat antiserum did not prevent neurite growth in wells where laminin was allowed to dry onto the culture surface. Coverslips were incubated in laminin antiserum (Bethesda Research Laboratories) for 60 min followed by three washes in PBS. The wells were air dried at room temperature prior to the addition of cell suspensions.

7.4 ASSAYS

7.4.1 QUANTITATIVE ASSAY OF NEURITE OUTGROWTH AND CELL NUMBERS

In order to evaluate neurite growth rapidly and reliably in large numbers of culture wells, we adopted the following procedure, based on reference (but essentially arbitrary) neurite length. After 22-24 hours of culture, five central fields from each culture well were examined. Each field normally contained 10-50 viable cells. These were defined as cells with small, phase dark, unflattened somata. Cells possessing one or more thin processes (neurites) terminating in growth cones were then scored. Cells with one or more neurites longer than a 20um projection on an eyepiece graticule (corresponding to approximately two cell-body diameters) were classified as "long process" cells. Cells with neurites shorter

than this arbitrary length were defined as "short process" cells. Flattened cells were not included in the assay, but they never constituted more than 5% of the total. Viable cells thus fell into three categories: cells without a process; and cells with short or long processes.

For each culture well, the total numbers of cells with short or long processes were assigned as A or B, such that the number of cells in B was always greater than that in A. Then the expression No. = 1-(A/B) was calculated, to give a value between 0 and 1. This was defined as positive when B was equal to the number of cells with long processes, and negative when B equalled the number of cells with short processes. The value of No thus gave a linear measure of neurite outgrowth ranging from -1 (all cells with short processes) to +1 (all cells with long processes). A value of zero would represent a culture with equal numbers of cells with short and long processes. This method of analysis was based on one used for other purposes, described by Ridge and Betz (1984).

7.4.2 ASSAY OF CELL SURVIVAL

After 24hr in culture five central wells from each culture cell were examined. Each field generally contained between 10 - 50 neural cells. Viable cells were defined as having round, unflattened, phase bright cell bodies, whereas dead neurones were crenated and phase-dark. survival was expressed as the

mean percentage of surviving neurones per well.

8.RESULTS

8.1 APPEARANCE OF CULTURES

Cultures on PORN and PORN/laminin substrates differed characteristically in appearance. In both cases, the cells had rounded, phase-dark nuclei from which processes extended. These took the form of a halo of short, stubby neurites on PORN (Fig.5.1a), or fewer, long and thin neurites on PORN/laminin (Fig.5.1b). At high concentrations of AraC many neurones appeared dark, crenated and had no processes (Fig.5.1c).

One-day cultures were stained with antibody against neurofilament 68Kd subunit, which is present in most post-mitotic neurones (Henderson et al., 1984). All cell bodies were lightly stained, but the long processes were intensely stained in their entirety (Fig.5.1d, 5.2b). Often a bright calyx was visible at the axon hillock, near the point of emergence of a neurite. No cells which appeared flattened under phase-contrast illumination were ever brightly stained (Fig.5.2a,b arrowed). As a further control, cultures of E6 dissociated limb-buds were made and incubated with the neurofilament 68Kd antibody. In these cultures only light staining of the cell body was seen. Some of the cells bore short, spiky processes but these were also unstained (Fig.5.2c,d).

Fig 5.1

Phase contrast micrographs of typical cultures after 24hr (a) PORN substrate 10uM AraC serum supplemented. (b) PORN/Laminin substrate 10um AraC serum supplemented. (c) 1mM AraC PORN/Laminin substrate serum supplemented. Micrograph of preparation stained for the 68kD neurofilament subunit with fluorochrome labelled anti-sera (d) Single neurone from the same culture as Fig 5.2a, fluorescence at high power.

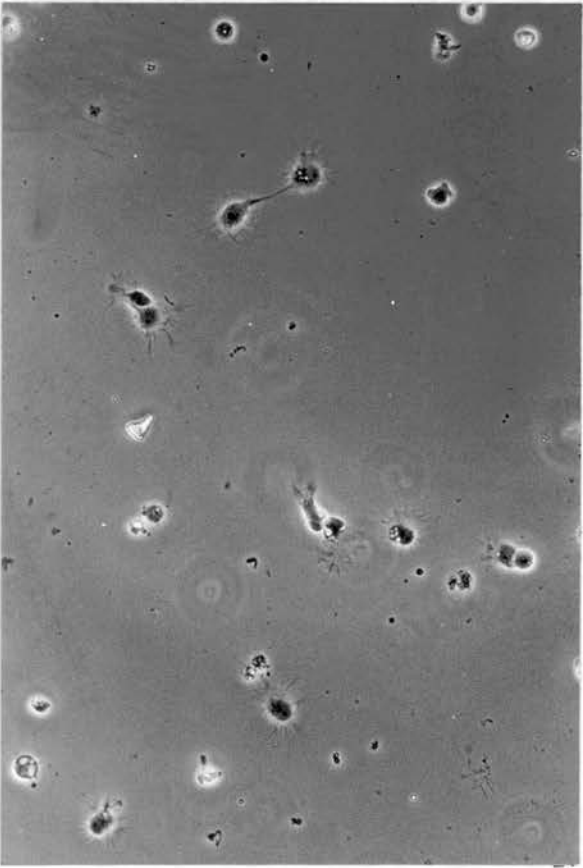Scale bar = 50um.
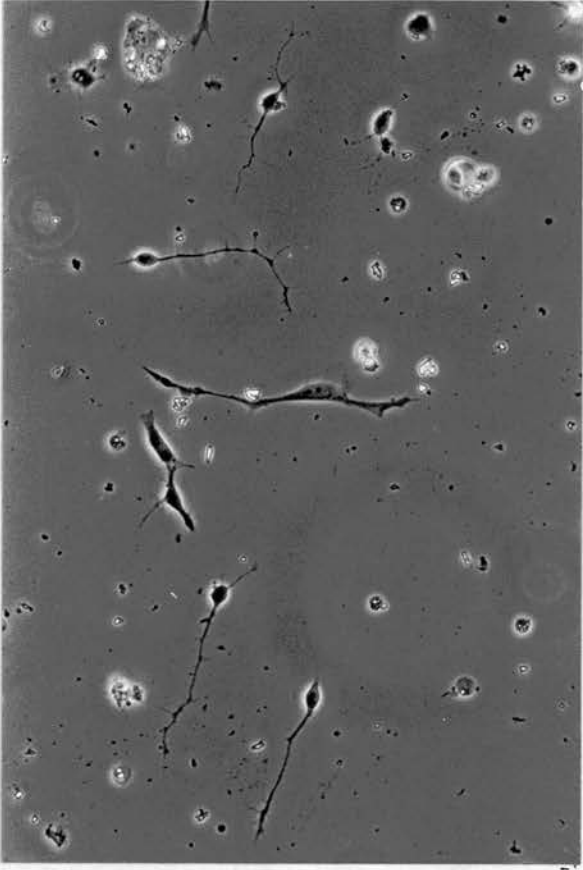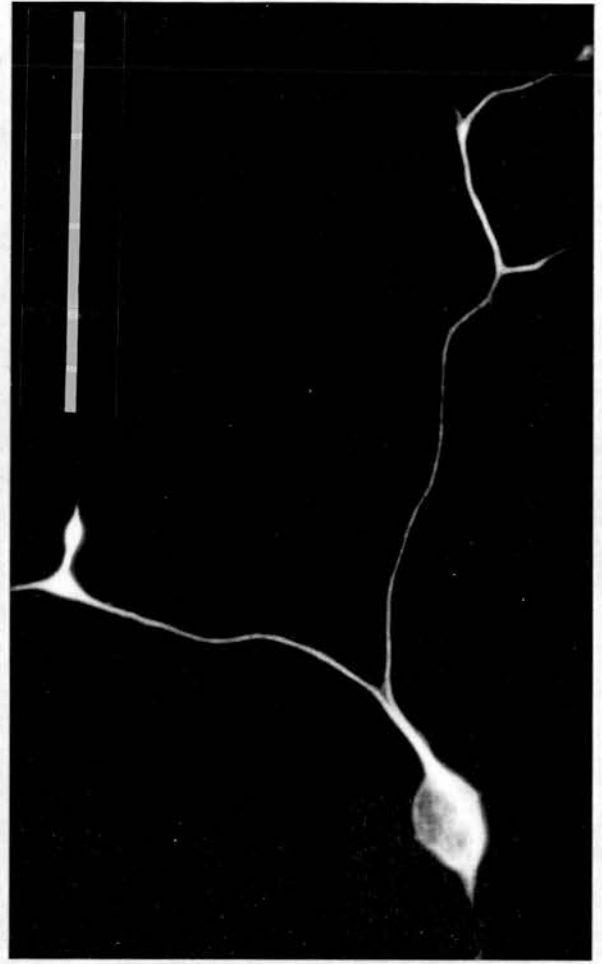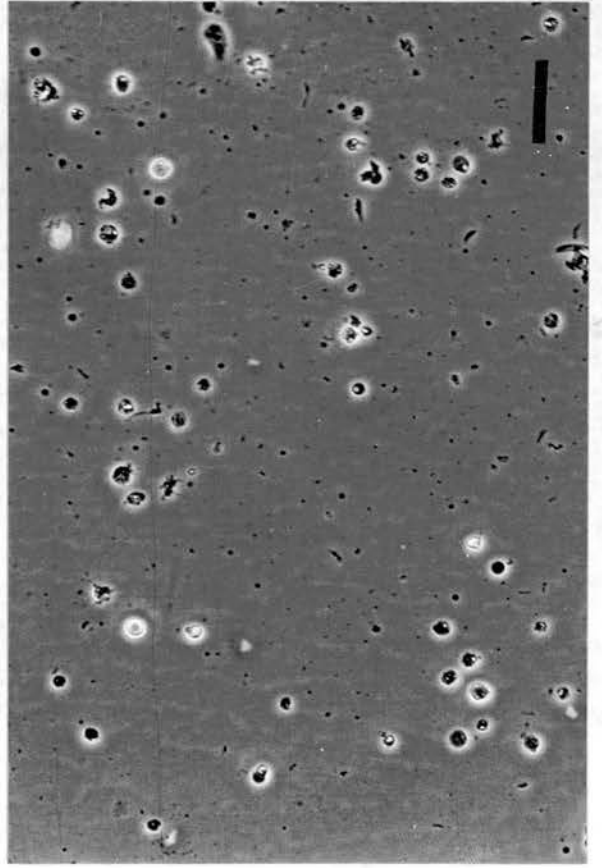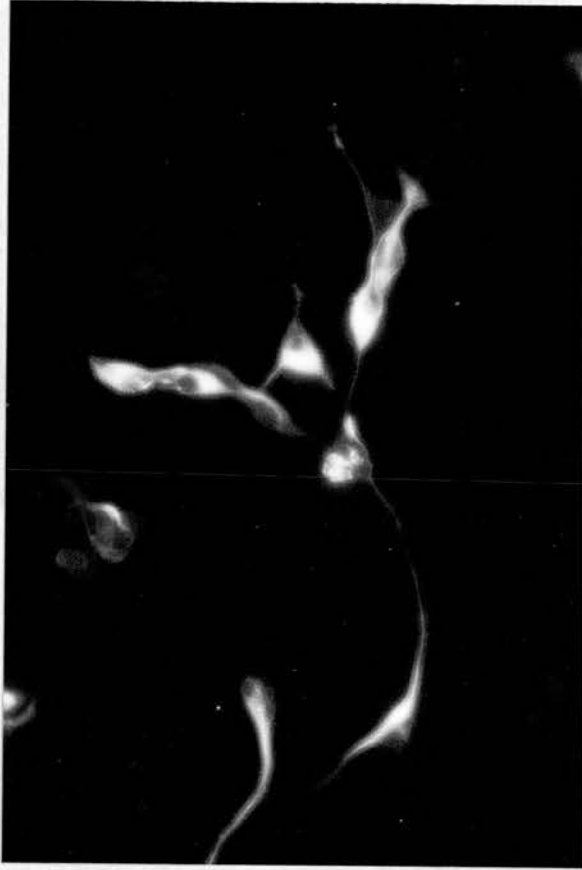
Fig 5.2

Preparations stained for the 68kD anti-neurofilament subunit

protein with fluorochrome labelled anti-sera (a) Phase contrast

PORN/Laminin substrate, serum supplemented, note the arrowed

flattened cell which is not stained (b) Fluorescence of

identical field (c) Phase contrast E6 limb bud culture (d)
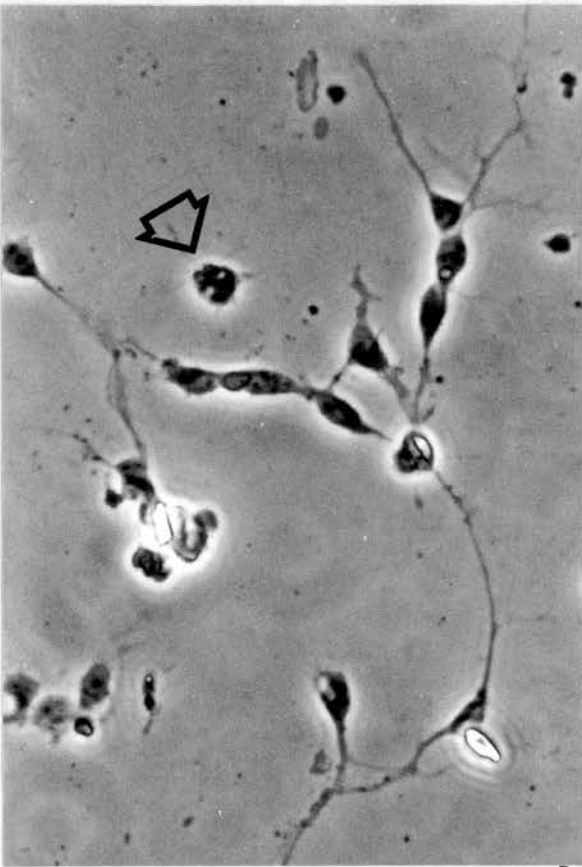
Identical field under fluorescence illumination.

Scale bar = 50um
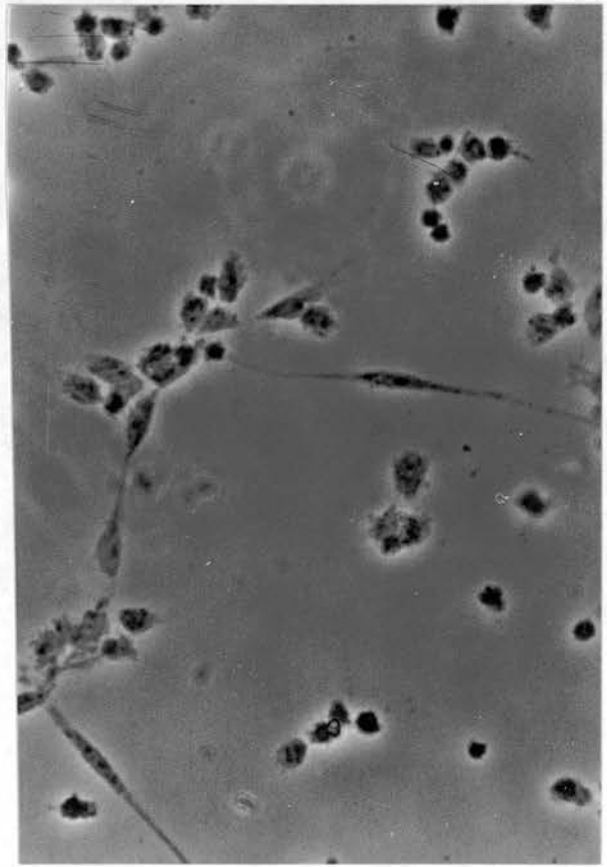
In five preparations cell counts were made on neurofilament stained preparations under both phase-contrast and fluorescence illumination (Fig5.2). Values for the percentages of cells with short or long processes were not significantly different (P>0.001; t-test) for assays under these two conditions, confirming that the criteria used to exclude non-neuronal cells in our protocol were effective.

8.2 CELL VIABILITY

Pre-treatment of dishes with PORN optimised the adhesion of initially viable cells to the culture surface (Letourneau, 1975a) and increases laminin binding (Edgar *et al.*, 1984). Equal plating densities were used in each experiment, so we were able to compare cell viability in different wells.

Figures 5.4 and 5.5 show a dose-dependent effect of AraC on neuronal survival under all culture conditions for the different embryonic ages. The effect is shown most clearly in cultures from E4 embryos, where survival in 1mM AraC is less than 5% of that in control (AraC free) media.

At high concentrations of AraC cell bodies were smaller, less rounded and phase-bright. Neurites were sparse or absent from these cells and when present, they had a beaded appearance. Such cell types were classed as non-viable and were excluded from the assay.

Fig 5.3

Neurite outgrowth after fluorescence labelling. E4 neural tube
preparations were stained and mounted after 24 hr in culture for
the 68Kd neurofilament subunit. Then assays were carried as
previously under Phase-contrast <Ph>, and fluorescent <Fl>
illumination for identical cultures. Values are shown of means
+/-SEM for the percentage of (A), "short neurite" and (B), "long
neurite" cells of the total count from 5 central fields of 5
repeat cultures.

Fig 5.4

Survival of dissociated neurones at varying concentrations of AraC after 22-24 hr in culture. Data from each experimental day were normalised with respect to the maximum single well cell count recorded, before mean and SEM values were calculated for four repeats in each case. Mean percentages for cell survival were then plotted for age, substrate and media variant experimental conditions. (A), PORN 250ug/ml, serum free. (B), PORN 250ug/ml, 10% FCS.

AraC concentrations;

(a), 0

(b), 100nM   (10^{-7}M)

(c), 1uM   (10^{-6}M)

(d), 10uM   (10^{-5}M)

(e), 100uM   (10^{-4}M)

(f), 1mM   (10^{-3}M)
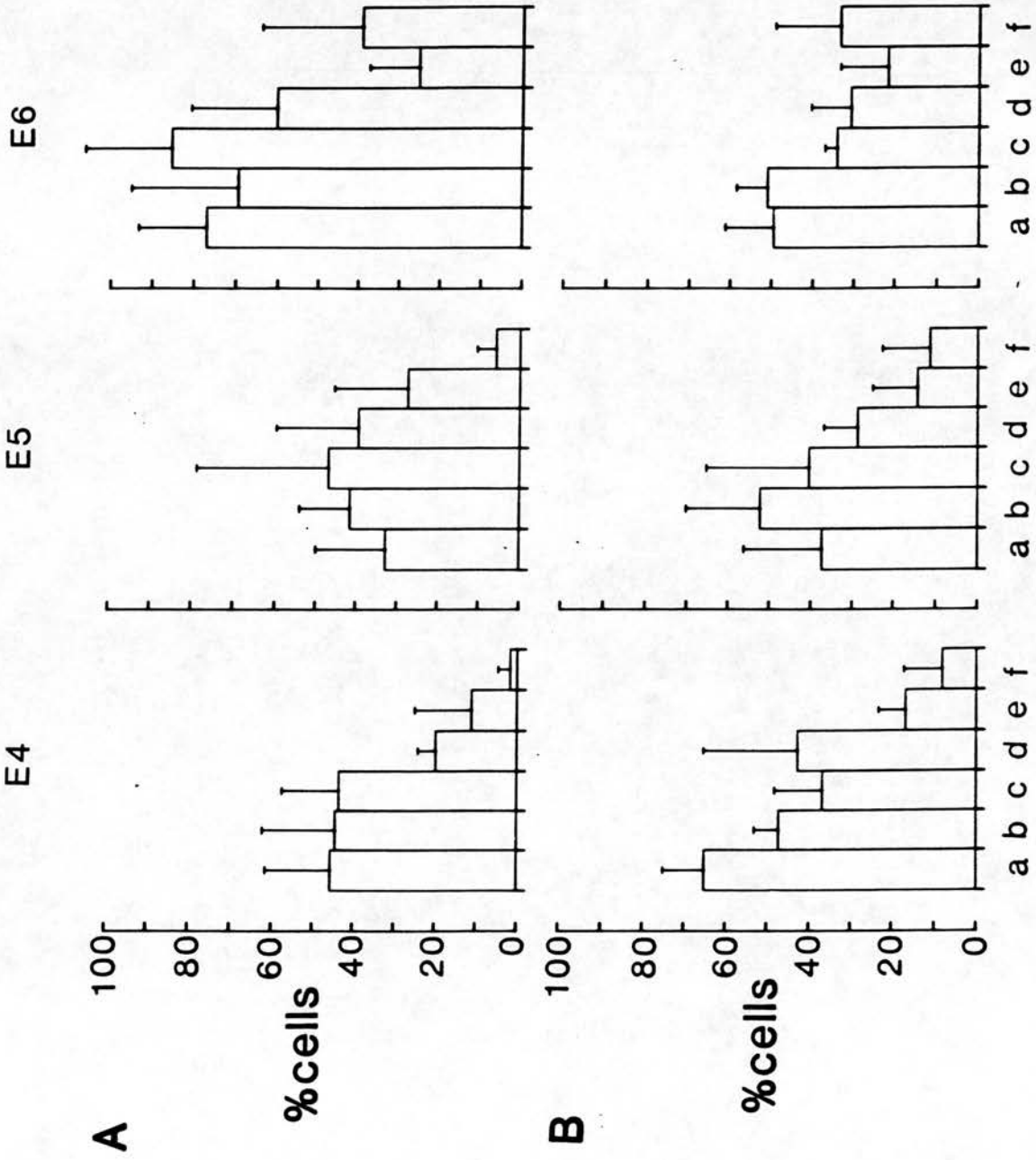
Fig 5.5

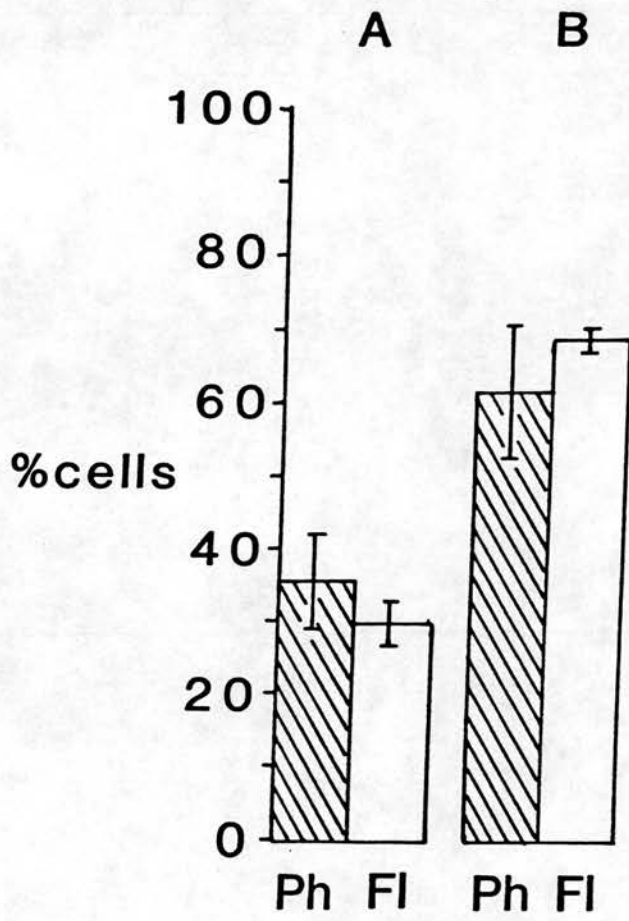Survival of dissociated neurones at varying concentrations of AraC after 24hr in culture. Data from each experimental day were normalised with respect to the maximum single well cell count recorded, before mean and SEM values were calculated for four repeats in each case. (A), PORN 250ug/ml, Laminin 1mg/ml, serum free. (B), PORN 250ug/ml, Laminin 1mg/ml, 10% FCS. AraC concentrations as for Fig 5.4
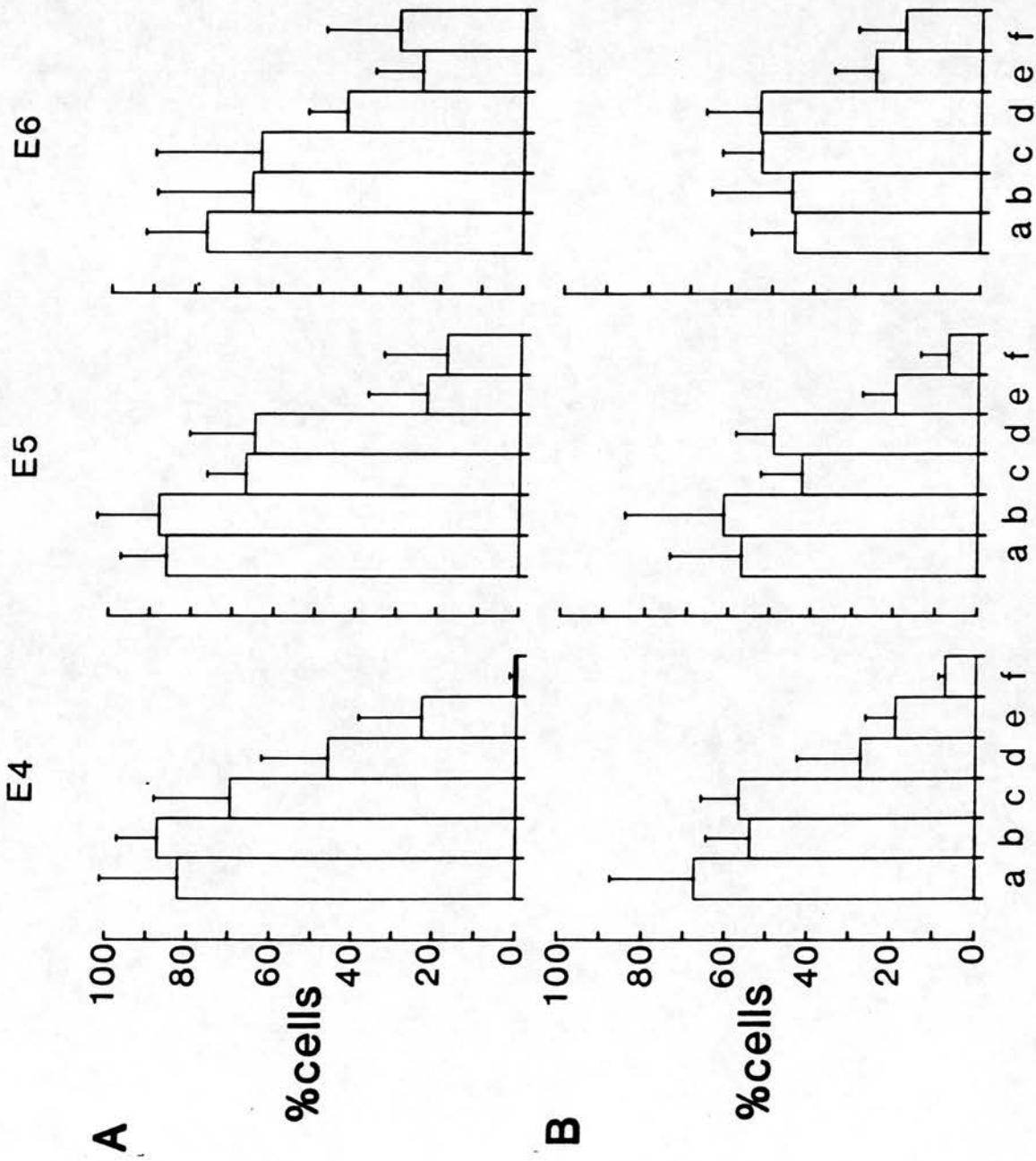
Cell survival was greatest in serum-free medium when the cells from E4-E5 day embryos were plated on a PORN/laminin substrate. Here cell viability ranged between 80-90% compared with 30-55% on PORN substrate alone. In E6 cultures, although the same high level of cell survival was seen on PORN/laminin, an increased level of survival to 80% was also seen on PORN substrate alone.

Serum appeared to cause a 10-15% reduction in the potentiation of cell survival on laminin substrate, but there was no effect on PORN substrate (Fig.5.5A,B).

Cultures from E6 day embryos also showed a marked resistance to high concentration AraC under all conditions. Viabilities greater than 20% were recorded at 1mM AraC against less than 10% in comparable E4/5 cultures (Figs.5.4, 5.5).

AraC appeared to be non-specific in its toxicity, as both neural and non-neural cell types were evidently killed in equal numbers. At concentrations of 100 uM AraC, values for cell survival were significantly smaller than those in control cultures (P<0.05; t-test).

8.3 NEURITE OUTGROWTH

The effect of AraC on neurite outgrowth was seen most clearly on a PORN/laminin substrate. Sprouting was progressively inhibited by increasing concentrations of AraC (Fig.5.6A,B). Outgrowth was reduced from an index above +0.8 in the control PORN/laminin cultures to -1.0 at a concentration of 1mM. This apparent profound inhibition of neurite growth by the highest concentrations of AraC should perhaps be treated with caution, because there were so few cells in these cultures (Figs.5.3, 5.4).

The effects on neurite outgrowth were far less apparent on PORN substrate where outgrowth was minimal (Fig.5.6A,B). There was no evidence for a potentiation of neurite growth in cultures containing low concentrations of AraC, even when the neurones were cultured on PORN substrate alone, when a stimulation of growth would have been easiest to detect (Fig.5.6, 5.7).

Neurite outgrowth was greatest in cultures from E4 neural tubes and progressively less in E5 and E6 cultures. This was unexpected but it may correlate with the stage of formation of synaptic connections in the embryo (see Discussion).

Neurite outgrowth in serum free and serum supplemented media was similar, in contrast to the inhibitory effect of serum on cell viability.

Fig 5.6

Neurite outgrowth from dissociated fetal spinal cord neurones, assayed after 22-24 hr as detailed in the materials and methods section to give a score value for N_0 between +1 and -1. Results from four separate experiments were used to calculate mean +/- SEM values shown for dose dependency. (A), PORN 250ug/ml, serum free. (B), PORN 250ug/ml 10% FCS.

AraC concentrations as for Fig 5.4

Fig 5.7

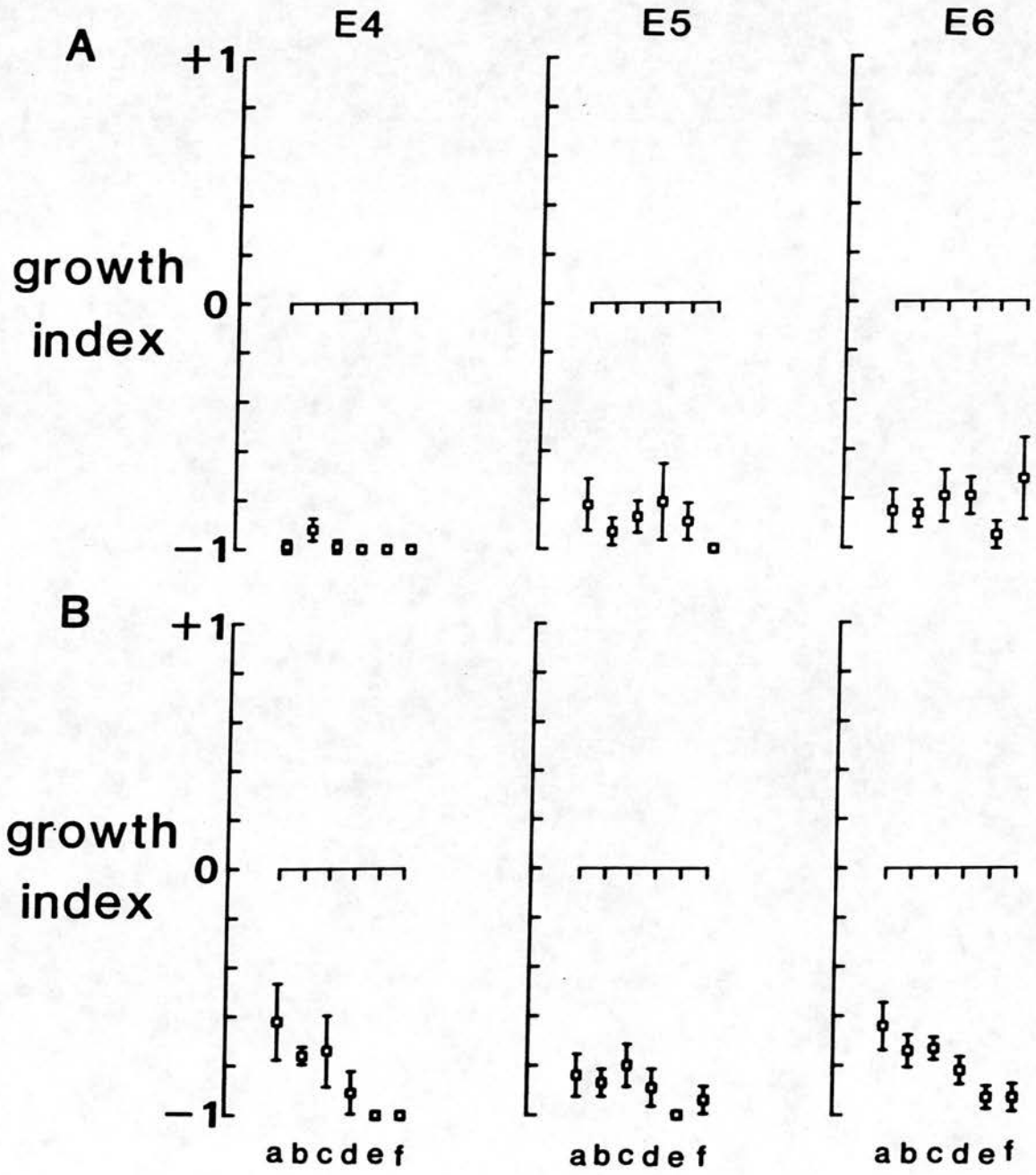Neurite outgrowth from dissociated fetal spinal cord neurones, assayed after 22-24 hr as detailed in the materials and methods section to give a score value for N_o between +1 and -1. Results from four separate experiments were used to calculate mean and SEM values shown for dose dependency. (A), PORN 250ug/ml, laminin 1mg/ml, serum free. (B) PORN 250ug/ml, laminin 1mg/ml, 10% FCS.

AraC concentrations as for Fig 5.4
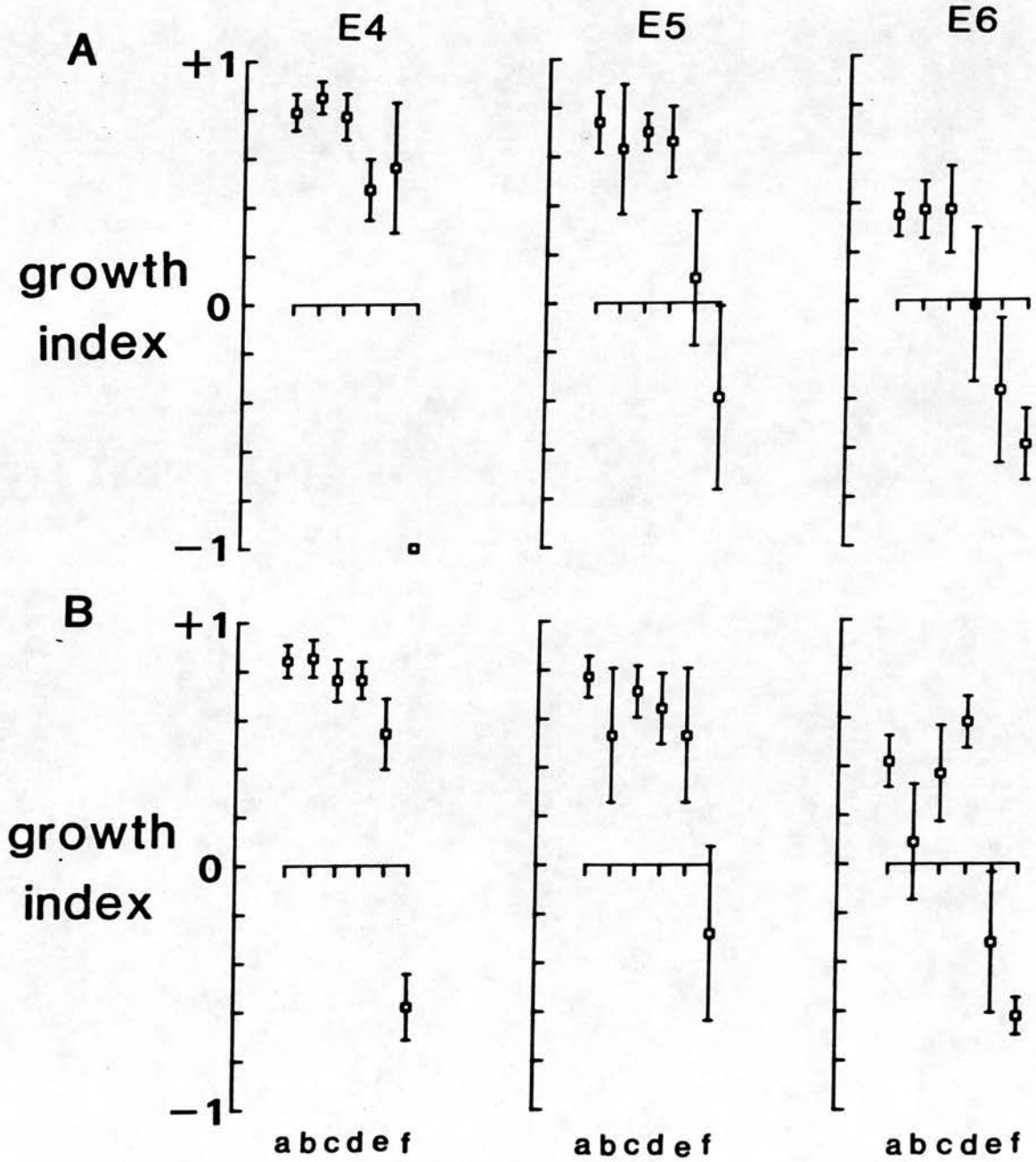
8.4 EFFECTS OF LAMININ ANTISERUM

In other culture systems, laminin-induced neurite outgrowth is blocked by laminin antisera (Edgar et al., 1984). It was therefore of interest to determine whether a similar effect could be shown using the present assay. Plates were coated with various amounts of laminin and then treated with varying concentrations of laminin antiserum prior to setting up the cultures.

Neurite outgrowth was correlated with laminin substrate concentration, decreasing from an index of +0.4 at 1 mg/ml to -0.8 at 1ug/ml (Fig.5.8). The numbers of neurones with neurites were no different, comparing different concentrations of laminin and laminin antiserum (data not shown). This suggests that the capacity of a neurone to produce neurites was independent of the presence of laminin, and only the degree of outgrowth was sensitive to the amount of substrate bound laminin.

Laminin antiserum effectively blocked the neurite promoting activity of laminin. This effect was seen most clearly at 100 and 10ug/ml where 1:1000 and 1:500 dilutions of antiserum respectively caused a half maximum reduction in neurite outgrowth. At 1ug/ml a response was unclear, perhaps reflecting the low levels of substrate bound laminin and neurite outgrowth produced at this concentration.

Fig 5.8

Neurite outgrowth after treatment with Anti-Laminin. Substrate coatings were carried out as previously but Laminin concentrations were reduced and subsequently, coated coverslips were treated with an Anti-body specifically raised against the Laminin used. All cultures were embryonic day 4 and PORN was added at 250ug/ml. Coverslips were incubated in laminin for 1 hr at 37°C, then in Anti-laminin for a further hour prior to the addition of dissociated neurones. Results are shown for composite plots of neurite outgrowth and cell survival as detailed in Figs.5.3, 5.5, except that means alone are shown for the two experimental runs carried out. (A), laminin 1mg/ml. (B), laminin 100ug/ml. (C), laminin 10ug/ml. (D) laminin 1ug/ml. (E) PORN, control.

Anti-laminin dilutions; (a) 1:10. (b) 1:50. (c) 1:100. (d) 1:500. (e) 1:1000. (f) Control.
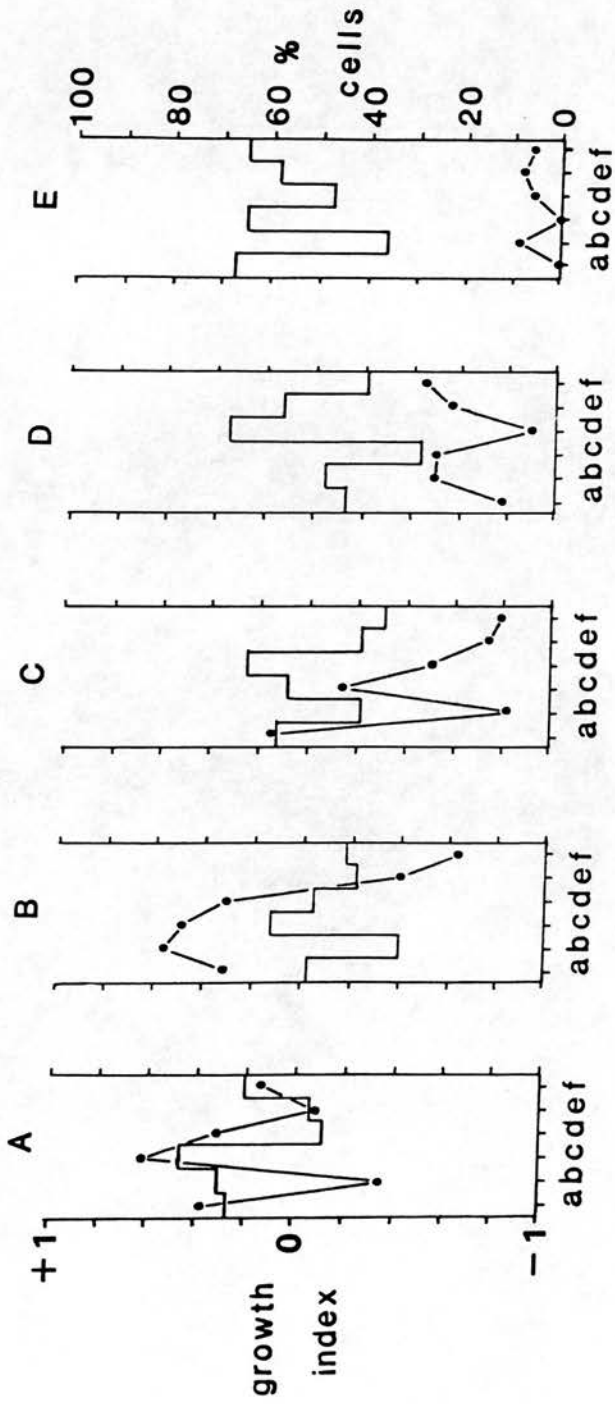
Cell viability was unaffected by decreasing substrate laminin concentration or by increasing concentrations of antiserum compared with the effects seen on neurite outgrowth (Figs. 5.6, 5.7). This providesfurther evidence that neuronal survival and neurite promotion may be independently affected by the laminin molecule.

8.5 THE MODIFICATION OF AraC TOXICITY BY 2'DC and CYTOSINE

Wallace and Johnson (1989) demonstrated that the cytotoxic affects of AraC were blocked by low concentrations of 2'DC. Experiments were carried out to examine the effects of 2'DC and Cytosine in this system. Cell survival was assayed (Materials and Methods) after 24hr in culture on PORN/laminin substrate supplemented with 10% FCS. Data for the three experiments are presented in Fig.5.9.

AraC caused a potent, concentration dependent, decrease in neuronal survival. 50% inhibition of cell survival compared with controls (IC_{50}) was seen at 7.5×10^{-5}M. A similar result to that obtained previously (Fig.5.5; E4,b).

In the additional presence of 2'DC at 10^{-4}M, the effects of AraC were eliminated at all concentrations examined, up to 10^{-3}M AraC.

Fig 5.9

The percentage survival of neural tube cells after 24 hr in culture. Cells were cultured in the presence of AraC (Δ) at varying concentrations, either alone or in the presence of 10^{-4}M 2'DC ($\square$) or 10^{-4}M Cytosine ($\blacksquare$). Values represent means +/- SEM from seven replicate experiments from different embryos.

Cytosine arabinoside (M)
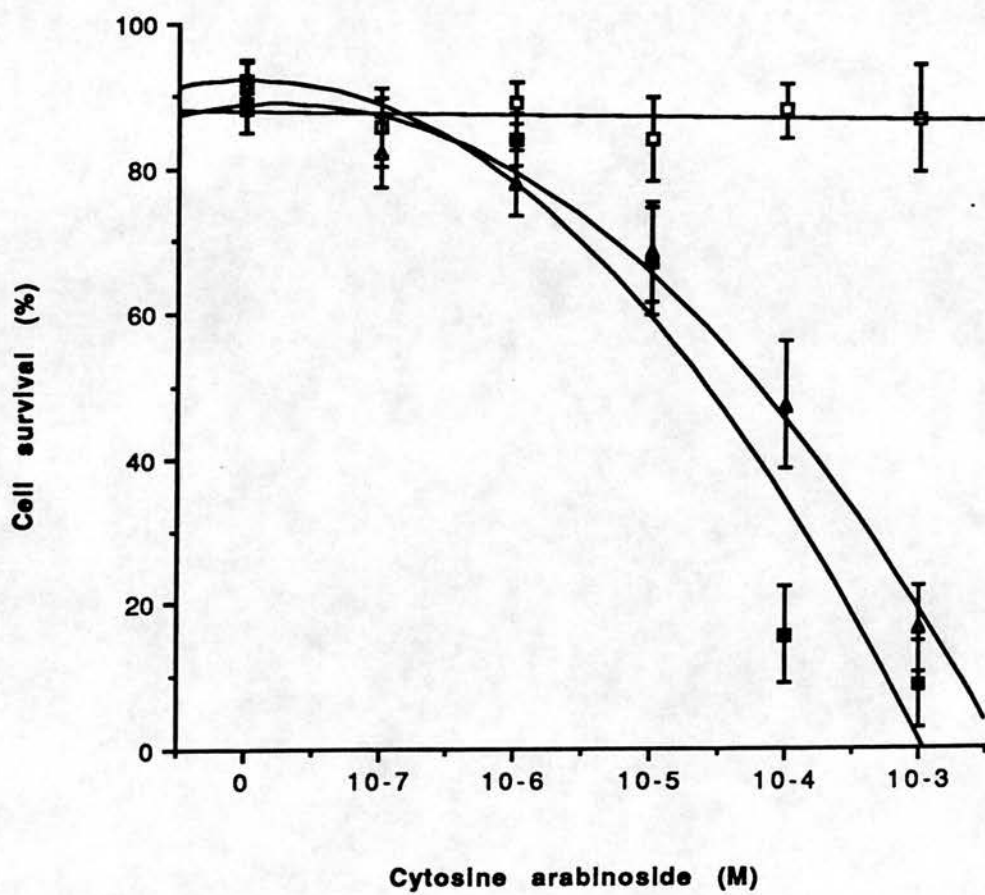
Cytosine, which is a pre-cursor of 2'DC (modification simply requiring the addition of a ribose ring, (Lehninger, 1982) was added in the place of 2'DC at 10^{-4}M. In this case, no inhibitory activity of the effects of AraC were apparent, even at the lowest concentrations.

9 DISCUSSION

Our data confirm the neurotoxicity of high concentrations of AraC, but the results also suggest that relatively low concentrations, in the range normally used to control non-neural cell proliferation, inhibit neurite outgrowth and reduce neuronal survival. We are therefore unable to confirm previous reports of neurite promotion by low concentrations of AraC (Oorschot and Jones, 1987; Siskin et al., 1988; Guth et al., 1988). In those previous studies, either explant cultures were used, or the experiments were carried out by topical application of the drug in vivo . In both situations, the site of action of the drug is unknown. The present experiment were carried out using a relatively pure culture system. As non-neuronal (flattened) cell types were always at a low density in our cultures, the effects on neurite outgrowth and cell survival may be assumed to be direct and not secondary to trophic interactions mediated by the control of non-neural cell types as previously suggested.

Our data also show the effects of the substratum and of the medium on both neurite growth and cell survival. A promotion of neuronal survival was seen on a PORN/laminin substrate in the absence of serum (Fig.5.5A), but this effect was reduced in the presence of serum (Fig.5.5B). The simplest explanation for this is that serum proteins cause a non-specific block of an important membrane protein (Davis et al., 1984), perhaps of a

laminin receptor or of the heparin binding site of the laminin molecule which has been implicated in the potentiation of cells survival (Edgar *et al.*, 1984; Dillner *et al.*, 1988). This site also appears to be important in the neurite outgrowth promoting activity of the laminin molecule, though in the present study no FCS block of neurite promotion was seen (Fig.5.5A,B). This, coupled with the observation that cell viability was not reduced in the presence of laminin antiserum, whereas neurite outgrowth was inhibited (Fig.5.8), supports the proposed separation of cell attachment and neurite promoting sites on the laminin molecule (Dillner *et al.*, 1988).

2'DC has been shown to block the cytotoxic effects *of* AraC on neural tube cells in this study. This observation extends that of Wallace & Johnson (1989) who showed such an interaction in ciliary ganglion and DRG neurones. Cytosine (a pre-cursor of 2'DC) was however ineffective, this suggests that some specific 2'DC dependent process is responsible for mediating the activity of AraC.

More recently Martin *et al.* (1990) have shown that cell death mediated by AraC resembles that due to NGF deprivation in sympathetic ganglion neurones. This was specific to AraC and not to other commonly used anti-mitotic agents. Agents which prevent death through NGF deprivation such as inhibitors of DNA and protein synthesis and a cAMP analog, were also inactive in inhibiting the toxic effects of AraC. They speculate that 2'DC

may be an important intermediary in signal transduction in response to trophic factors. In the presence of AraC this system fails, which leads to the cell following a "death program". This then resembles NGF deprivation which may also lead to the expression of a similar sequence of events. In such a system, cell death results from the active production of "killer proteins" within the neurone. In the present study, the neural tube cells are at very early stages in development, at st24.5 motorneurones are first entering the limbs. (see Landmesser, 1984 for review). It is unlikely that these cells, though making up only a small proportion of neural tube cells, are responsive to trophic factors. They would not then be expected to express the mechanisms necessary for trophic factor reception and signal transduction Davies *et al.* (1987). Further, in the present study, not only was cell survival reduced, but also neurite growth in the presence of AraC. It is difficult to envisage how the scenario presented by Martin *et al.* (1990) could account for these observations.

The mechanism of AraC toxicity in post-mitotic neurones remains unclear. The cellular mechanism of AraC toxicity has been studied in some detail in mitotic cell lines (Woodcock, 1987). Here an improper chromosome segregation following a DNA replication error has been implicated. This cannot explain AraC toxicity in post-mitotic cells however, One possibility is that a gene or genes whose expression is determined by metabolism or by the metabolic demands of cell may be selectively inhibited.

In chick retinal neurones, for instance, AraC reduces the synthesis of RNA and many proteins contrary to Martin *et al.* (1990) who predict increased protein synthesis, but the transcription and translation of one enzyme, glutamine synthetase is increased. This is not due to a direct effect of AraC on transcription or translation of the structural genes for this enzyme, however, it is instead due to an inhibition of the formation or activity of a repressor gene product which regulates the enzymes synthesis (Jones, 1974). Perhaps cells on which high metabolic demands are placed are severely affected by AraC. Substrate stimulation of neurite outgrowth from neurones should put a high metabolic demand upon a cell. Accordingly, we might expect to find that cells cultured on a laminin substrate, which are most actively producing neurites, would be the most sensitive to AraC, compared with essentially quiescent cells on a PORN substrate. There is a suggestion of this in our cultures (see Figs. 5.4, 5.5). Further, cells with long "beaded" neurites characteristic of degenerating neurones (Markesbery and Lapham, 1974) were often seen at 1mM AraC on a laminin substrate.

Several other groups have observed membrane disorders in cells treated with high concentrations of AraC. "Atypical" membrane inclusions have been reported in non-neural cells in explant cultures (Oorschot and Jones, 1985); also, post-mortem microscopical examinations of patients treated intrathecally with high dosesAraC for myelogenous leukaemia have shown micro-

vacuolisation, axonal swelling, demyelination and fat laden macrophages (Breuer *et al.*, 1977). Both these observations may be linked to the "gene regulation" hypothesis, assuming that metabolic demand is related to membrane production.

Another hypothesis which is consistent with these observations is one which directly involves membrane synthesis. Wallace and Johnson (1989) speculate that glycoprotein and glycolipid synthesis in addition to gene expression may be altered in the presence of AraC. More recently, it has been found that tritiated 2'DC incorporates into the phospholipid precursors dCDP-choline and dCDP-ethanolamine in human tonsillar lymphocytes (Spasokukotskaja *et al.*, 1988). These deoxyliponucleotides are converted into phosphatidyl choline and phosphatidyl ethonalamine (Fig.5.10). Further, Lauzon *et al.* (1978) have demonstrated the production of the analogous AraC lipids by cells. Thus, AraC could interfere with the incorporation of 2'DC into membrane precursors, so limiting the capacity for neurite production, or the AraC analogous lipids could directly interfere with the membranes thermodynamic properties, leading to instability.

Finally, the present study shows the need for caution in the use of AraC as a mitotic inhibitor in short-term neural preparations, as demonstrated by its toxicity even at those concentrations normally used to control non-neural cell proliferation in culture.

Fig 5.10

Part of the metabolic pathway for the biosynthesis of phosphatidylcholine, which is the major phospholipid in animal cells. dCTP*; indicates the site at which phosphorylated 2'DC is involved. DG ; Diacylglycerol. From Vance and Choy, 1979.

Choline

ATP

ADP

Phosphocholine

(dCTP)* CTP

PP₁

Cytidyltransferase

CDP-choline

DG

CMP

Phosphocholinetransferase

Phosphatidylcholine

Cell membrane
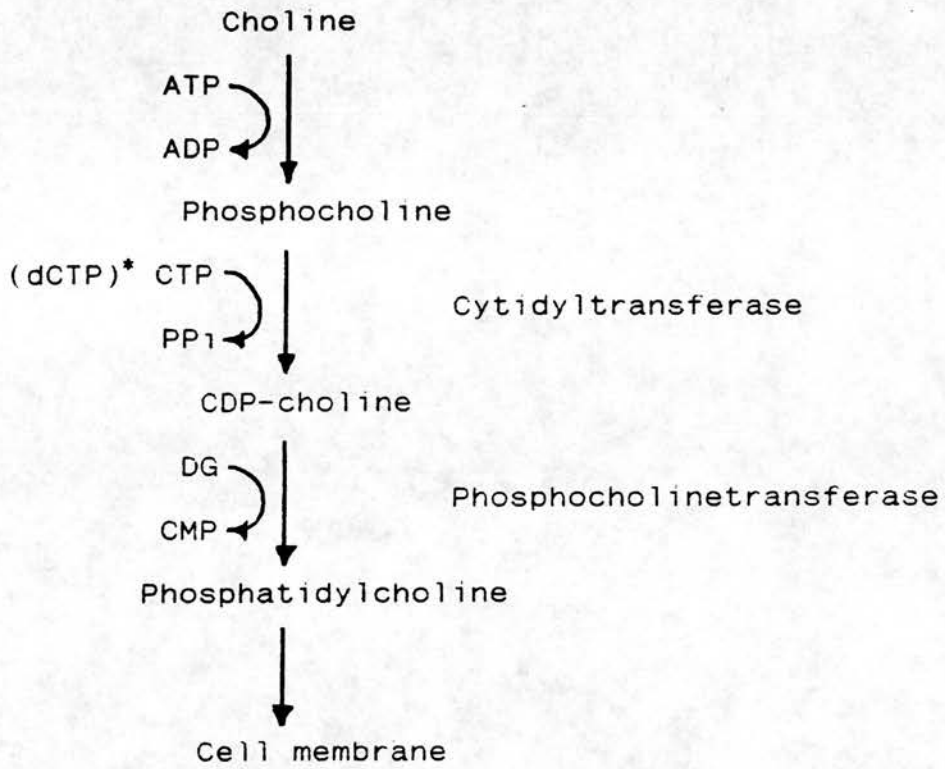
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