A quantitative study of the growth and development of the ventral root in normal and experimental conditions

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\textbf{SUMMARY}

1. The development of the ventral root (VR) in \textit{Xenopus} has been studied by electron microscopy. Total fibre counts, and counts of classes of fibres were made from large photomontages of the whole of VR 9 at $\times15000$.

2. The total number of fibres in the root shows the same pattern of initial rise, peak, and subsequent decline that previous ventral horn (VH) cell counts had shown. The two curves overlay each other initially, but after the decline, there were apparently more cells than fibres.

3. Promyelin and myelin formation was seen at the time of the decline. There was no evidence that dying axons had started to myelinate.

4. In some animals the limb-bud was removed at the time of its first penetration by nerve fibres. The ventral roots developed normally for a week, but thereafter fibre loss was accentuated, advanced and more profound, so that after another week, no fibres were left. In these roots, no promyelin or myelin was formed.

5. In other animals, it was shown that there is no evidence for collateral sprouting in the ventral roots during normal development.

6. It is argued that the axons which die in normal development have already reached the limb-bud.

7. The correspondence between axon and cell number is discussed.

\textbf{INTRODUCTION}

Several authors have recently made accurate counts of the number of nerve fibres in developing nerves. Wilson (1971) compared the number of retinal ganglion cells and optic nerve axons in developing \textit{Xenopus}; she found that both increased together so that there was an approximate 1:1 relationship at all times. Prestige \& Wilson (1972) did the same for lumbar lateral ventral horn cells and ventral root axons in segment 9: they found that initially numbers rose, later falling steeply, but the 1:1 correspondence was similarly maintained. At the same time, Hughes \& Egar (1972), using another Anuran \textit{Eleutherodactylus}, were counting the numbers of fibres in the sciatic nerve.

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throughout development. Since this is a mixed nerve, the pattern they found was correspondingly more complex: they tentatively identified within it a decrease due to loss of motor fibres and a subsequent peak and slow increase thereafter due to sensory fibres. Aguayo, Terry & Bray (1973) counted fibres in perinatal rat cervical sympathetic trunks (which are over 99% unmyelinated when adult). They also found a striking reduction in the axonal population. And most recently, Fraher (1974, personal communication) has found a similar post-natal decline in cervical ventral root fibre numbers in the rat.

There are a number of topics of interest which have been raised by these observations, apart from the general issue of why there should be axonal loss, presumably related to cell death (recently reviewed by Cowan, 1973). The first is a timing question: have the axons which are lost reached their intended destination? The second is also on timing: have the axons which die started to myelinate? The third is the question already referred to, that of the correspondence between axon and cell number. The fourth is the branching problem, and the fifth the question of how many fibres are still lengthening. The evidence already obtained shows that axon loss is not an exclusive property of myelinated nerves; nor of motor, sympathetic or sensory nerves. It is possible that axon loss was also taking place in the growing optic nerve, for if it had, it would have been obscured by the further addition of new fibres. To understand some of these problems we have confirmed and extended the data of Prestige & Wilson (1972), and examined the effects on ventral root axonal number of removing the tissue which these fibres are destined to innervate. We also raise the question of collateral sprouting.

**MATERIALS AND METHODS**

The technical problems to be overcome arose from the absolute requirement to be certain of identifying segment 9. This required a block of tissue to be fixed that was larger than is usually considered optimal for electron microscopy. As a result, fibres were pressed against each other with reduction of extra-cellular space, some tearing of connective tissue occurred due to shrinkage, and in a small proportion of fibres (<1%) smooth endoplasmic reticulum was dilated. Ultimately, none of these artifacts interfered with the accuracy or ease of counting.

Tadpoles were reared and staged according to Nieuwkoop & Faber (1956). In this table, at stage 50 the hind limb-bud is round, ventral horn cells are first detectable (Prestige, 1970) and axons of ventral horn cells first reach the limb-bud (Lamb, 1974). At stage 54, all the toes of the hind-leg are first delineated, and movement of the limb starts (Hughes & Prestige, 1967). At stage 58, metamorphic climax starts and at stage 66, metamorphosis is complete. Each stage lasts two to four days.

In some experiments, tadpoles of stage 50 were anaesthetized in MS 222 and
Development of ventral root 821

the left hind limb-bud (length 0-4 mm) removed with forceps. On recovery from the anaesthetic, they swam away without stress. After five to seven days, they were examined for signs of limb regeneration, and if necessary, the operation was repeated. The maximum regeneration that occurred was equivalent in length and form to a stage 50 limb-bud.

In other experiments, tadpoles of stage 50 were reared in 0-1% KClO₄. This acts as a goitrogen (Barrington, 1963), and prevents metamorphosis. Under these conditions, tadpoles are produced, which are abnormally large for the stage reached; in our hands tadpoles reached early stage 54, but no further. Some of these were anaesthetized in MS 222 and the left leg removed above the hip with forceps. These animals also showed no stress on recovery.

For electron microscopy, tadpoles were anaesthetized in MS 222, and staged. They were then immersed in cold Karnovsky's fixative (1965), and a block containing the lumbar spinal cord with the adjacent tissues was dissected, and skinned. The time between immersion in fixative and the end of the dissection was about 2 min. Fixation continued for at least 2 h. After washing overnight in 0-1 M cacodylate buffer, pH 7-3, the blocks were post-fixed for 1–2 h in 1% OsO₄ in 0-2 M cacodylate buffer, dehydrated and embedded in Araldite. 1 μm sections were cut in the longitudinal, horizontal plane, starting dorsally, and segment 9 identified. A slight tilt was then necessary to ensure that the plane of thin sections was normal to the ventral roots.

The identification of segment 9 is complicated by the fact that there are two different systems of enumerating segments in Anura. Following Gaupp (1899) and Hughes & Tschumi (1958), we have taken segment 1 to be that trunk myotome lost in early larval life, segment 2 to be that supplying the hypoglossal nerve, segments 3 and 4 those to the fore limbs, segments 5, 6 and 7 those to the trunk, segments 8, 9 and 10 those to the hind limbs, and segments 11 onwards those to the tail: segment 7 or segment 11, but rarely both, may also contribute to the hind legs. This represents the embryological view, and has been used in all the Xenopus papers of this series. The anatomical view is represented by Ecker (1889) who starts by allocating spinal nerve 1 to the hypoglossal nerve; thus the segments to the hind limbs are numbered 7, 8 and 9. Only a careful search of each paper can ensure knowledge of which system the author is using. Nieuwkoop & Faber (1956) use the anatomical enumeration.

Segment 9 was identified by the relative sizes of the dorsal root ganglia. Hughes & Tschumi (1958) showed that 11 ≤ 10 > 9 > 8 > 7 > 6 > 5 ≤ 4, 3 and 2 is absent. These sizes are reflected in the cross-sectional area of the dorsal roots. In addition, in Xenopus, they found a fortunate and conspicuous criterion in that ganglia 8, 9 and 10 lack the large early-differentiating ventrolateral neurones and are initially composed only of small cells. As additional confirmation, the column of ventral horn cells runs from anterior to root 8 to posterior to root 10. By repeatedly examining 1 μm sections at about 5 μm intervals, all the bundles of ventral root fibres were identified (Fig. 1). Thin
sections were then taken at just above the level of the base of the dorsal root ganglion. The section size was large enough to include the whole ventral root, but not large enough to cover the adjacent or opposite ones. When it was required to take sections from both sides of the same animal at the same level, one side was tilted slightly up and the block face trimmed to include only the ganglion and ventral roots. The depth of the pyramid was very shallow so that once the thin sections had been obtained, it was only necessary to cut a few thick sections before the whole face of the block was again being cut and then the pyramid was trimmed on the opposite side. Sections were mounted on 0.5% formvar films, double stained with uranyl acetate and lead citrate, and photographed at ×5000 on an AEI EM6B. Montages were constructed from prints off overlapping plates at ×15000 and from these counts were made.

The fibres were scored separately as myelinated, singly wrapped or naked. Unfortunately, there are as many terminologies as authors for the classification of growing axons. Since it is desirable that quantitative studies should be related to each other, we have endeavoured to bring together the various classifications in Table 1. In our definitions, singly wrapped axons possess a complete layer of non-neuronal cytoplasm around them, but are excluded if they have compact myelin and hence classed as myelinated. Naked fibres are those remaining and are almost invariably in bundles.

Cell counts of lateral ventral horn (lateral motor column) cells were obtained from previous experiments, or by similar means to previous experiments (Prestige, 1967).

The errors involved in fibre counting have been recently reviewed (Prestige & Wilson, 1974). They are (a) in montage assembly; (b) in inconsistent criteria of identification; (c) in incorrect criteria of identification; (d) failure to count. In experiments done for another purpose, the estimated error due to (a) or (b) is such that individual counts will lie within 5.2% (P > 0.95) of the mean obtained by many repetitions of the montage assembly and count (using the
Table 1. Comparison of the classifications used by authors for developing nerve fibres

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Friede &amp; Samorajski (1968)</td>
<td>Myelinated</td>
<td>Myelinated</td>
<td>Singly wrapped, including myelinated</td>
<td>-</td>
<td>-</td>
<td>Compact myelin</td>
<td>1:1 axons with mesaxon or myelinated</td>
</tr>
<tr>
<td>Prestige &amp; Wilson (1972)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Non-compact myelin, with mesaxon Promyelin</td>
<td></td>
</tr>
<tr>
<td>Wilson (1971)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Transitional</td>
<td>Segregated axons</td>
</tr>
<tr>
<td>Hughes &amp; Egar (1972)</td>
<td>Myelinated</td>
<td>Singly wrapped</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>Axons in bundles</td>
</tr>
<tr>
<td>Aguayo et al. (1973)</td>
<td>Unmyelinated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Foetal</td>
<td>Axons in bundles</td>
</tr>
<tr>
<td>Fraher (1972, 1974)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Compacted myelin</td>
<td></td>
</tr>
<tr>
<td>Webster, Martin &amp; O’Connell (1973)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:1 axons with mesaxon or myelinated</td>
<td></td>
</tr>
</tbody>
</table>
method suggested by Prestige & Wilson, 1974). Error (d) is minimized by using an electromechanical counter triggered by the crayon marker. It is assumed that each axon passes only once through the plane of the section. Since the sections are all taken at corresponding levels, there will be no error due to differences between levels.

The errors involved in cell counting have been reviewed by Konigsmark (1970). Since the nucleolus is sometimes not bisected by the microtome knife, but is displaced whole into one or other section, no correction for double scoring has been used. The cell counts are therefore overestimates, perhaps even by as much as 15–20%. These arguments are more necessary in older animals, when the nucleolus is much larger.

RESULTS

Normal development

We have confirmed and extended the data of Prestige & Wilson (1972). Fig. 2 shows the number of axons in VR 9 in 24 animals from stage 49 onwards, plotted against a background representing the range of cell counts of the corresponding region of lateral ventral horn. The approximate 1:1 relationship between cell and axon holds at all times except at stages 55 and 58, when there is an excess of cells in the ventral horn. In other words, the loss of cells is apparently less steep than the loss of axons.

Fig. 3A shows that singly wrapped fibres are not seen until after stage 52, but thereafter increase to a peak in number at stage 55, after which they become less frequent, though some are still present in the juvenile. Fig. 3B shows that the steep rise in the number of myelinated fibres comes at stage 56, and it continues rising until the end of metamorphosis. Fig. 3A is an approximate time derivative of Fig. 3B, but displaced by one stage. The duration of the
Development of ventral root

Fig. 3. A, Number of singly wrapped fibres in VR 9 at stages throughout development. B, Number of myelinated fibres in VR 9 at stages throughout development. Abscissa: stage.

singly wrapped period for each fibre is thus about one stage, or two to four days.

Some myelinated fibres are already present at stage 49 before the lateral ventral horn starts to form, and there is a steady increase of these up to stage 55. Some of these may be large, up to 10 μm in diameter. They can be followed in silver preparations, or in serial 1 μm araldite sections, from the large primary motoneurons in the medial ventral horn either into the dorsal ramus or via the ventral ramus to the myotomes (Hughes, 1959). It is not yet known when the earliest nerve fibre to the limb is myelinated. Certainly, none are present at stage 51 (unpublished observations).

Effect of limb bud removal at stage 50

It was intended to remove the limb-bud before it was penetrated by nerve fibres. However, Lamb (1974) using retrograde uptake of peroxidase, has since shown that a few ventral horn cells have axons in the limb-bud as early as stage 50. So the initial operation will have injured some nerve fibres. The majority of nerve cells divide finally after this, and their axons will be undamaged. Changes in ventral root constitution will thus largely (but maybe not exclusively) be due to the inability of nerve fibres to find suitable tissue for termination sites, rather than to injury.

The results in which both sides of the same animals are available for comparison are presented in Table 2, the unoperated side serving as a control. The two sides of the same animal might be expected to be more similar than
those of two different ones, but they will not always be identical, for the precise detail of whether a small rootlet becomes part of spinal nerve 9 or goes on caudally to become attached to 10 is a bit arbitrary. Differences of up to 200 fibres (10%) have been recorded at early stages.

Up to stage 52, limb-bud removal is apparently without effect; any difference between the sides is within the experimental error. After this stage, a progressive loss of axons ensues so that the normal decline seen on the control side is, on the amputated side, advanced, accentuated and more profound. This is shown graphically in Fig. 4B together with data from animals in which it was only possible to get sections from one side. By stage 56 ventral root 9 has only a tiny proportion of fibres left, probably innervating the myotomes.

These results on ventral root fibre number can be compared with the corresponding cell counts in Fig. 4A. This expresses in quantitative form what Hughes & Tschumi (1958) reported as occurring after early limb-bud removal — that the initial differentiation of ventral horn cells was normal, but that there was a failure of maintenance after stage 52. The two sets of data on ventral horn cell and ventral root fibre number correspond as well as do the data on normal animals in Fig. 2.

Axons which are prevented from reaching the limb fail to show any signs of myelination or single wrapping. This is shown in Table 3. The expected rise in single wrapping in stages 54 and 55 does not occur: neither does the number of myelinated fibres increase above the figure present at stage 51 when they all go to myotome muscle. It may be that contact with the periphery is the signal and thus a necessary condition for the initiation of myelin wrapping:

Table 2. The growth of the ventral root after removal of one limb-bud: counts of the number and type of the axons in VR 9

<table>
<thead>
<tr>
<th>Stage</th>
<th>Hind leg length mm</th>
<th>Control</th>
<th>Amputated</th>
<th>Number of axons</th>
<th>% remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Myelinated</td>
<td>Singly wrapped</td>
<td>Naked</td>
<td>Total</td>
</tr>
<tr>
<td>51</td>
<td>0.8</td>
<td>31</td>
<td>0</td>
<td>1875</td>
<td>1906</td>
</tr>
<tr>
<td>52</td>
<td>1.0</td>
<td>31</td>
<td>1</td>
<td>1673</td>
<td>1705</td>
</tr>
<tr>
<td>52/53</td>
<td>1.2</td>
<td>16</td>
<td>3</td>
<td>1803</td>
<td>1822</td>
</tr>
<tr>
<td>54</td>
<td>2.2</td>
<td>26</td>
<td>1</td>
<td>1675</td>
<td>1702</td>
</tr>
<tr>
<td>54</td>
<td>2.8</td>
<td>22</td>
<td>3</td>
<td>1726</td>
<td>1751</td>
</tr>
<tr>
<td>55</td>
<td>4.7</td>
<td>23</td>
<td>1</td>
<td>1128</td>
<td>1152</td>
</tr>
</tbody>
</table>

those of two different ones, but they will not always be identical, for the precise detail of whether a small rootlet becomes part of spinal nerve 9 or goes on caudally to become attached to 10 is a bit arbitrary. Differences of up to 200 fibres (10%) have been recorded at early stages.

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Development of ventral root

Fig. 4. The effect of removing one limb bud at stage 50 on the subsequent development of (A) ventral horn cells and (B) ventral root axons. ●, ▲, Control side; ○, △, amputated side. Where data from both sides of the same animal are used, the points are linked. In B, other data (crosses) from control animals are included.

Table 3. Data on the failure of ventral root axons to myelinate after removal of the limb-bud into which they are growing

<table>
<thead>
<tr>
<th>Stage</th>
<th>51</th>
<th>52</th>
<th>53</th>
<th>54</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singly wrapped axons</td>
<td>0</td>
<td>3, 3, 3</td>
<td>4</td>
<td>9, 248</td>
<td>179, 133 control</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1, 1, 0</td>
<td>1</td>
<td>1, 1, 6, 6, 3</td>
<td>2, 1 amputated</td>
</tr>
<tr>
<td>Myelinated axons</td>
<td>31</td>
<td>16, 22, 14</td>
<td>19</td>
<td>34, 56</td>
<td>341, 267 control</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>26, 23, 31</td>
<td>24</td>
<td>20, 22, 32, 23, 29</td>
<td>30, 32 amputated</td>
</tr>
</tbody>
</table>

Each count is taken from a photomontage of VR 9, mostly from separate animals.

or it may be that cells and fibres do not survive long enough to begin wrapping: contact would then be a necessary condition for survival, and only indirectly for myelination.

Possibility of collateral sprouting

The evidence is that from stage 53 onwards, ventral horn cells and axons are sensitive to the absence of their peripheral musculature. It is not known
Table 4. The effect of removal of one leg upon the number and type of nerve fibres in VR 9 of chemically thyroidectomized tadpoles

<table>
<thead>
<tr>
<th>Stage</th>
<th>Body length (mm)</th>
<th>Limb length (mm)</th>
<th>Days after operation</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myelinated</td>
<td>Singly wrapped</td>
<td>Naked</td>
<td>Total</td>
<td>Myelinated</td>
<td>Singly wrapped</td>
<td>Naked</td>
<td>Total</td>
<td>% of control</td>
</tr>
<tr>
<td>54-</td>
<td>63</td>
<td>2-1</td>
<td>2</td>
<td>55</td>
<td>49</td>
<td>1526</td>
<td>1630</td>
<td>36</td>
<td>38</td>
</tr>
<tr>
<td>54-</td>
<td>55</td>
<td>2-0</td>
<td>3</td>
<td>26</td>
<td>20</td>
<td>1867</td>
<td>1913</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>54-</td>
<td>57</td>
<td>2-0</td>
<td>3</td>
<td>89</td>
<td>110</td>
<td>1735</td>
<td>1934</td>
<td>73</td>
<td>52</td>
</tr>
<tr>
<td>54-</td>
<td>53</td>
<td>2-1</td>
<td>7</td>
<td>24</td>
<td>18</td>
<td>2078</td>
<td>2120</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>54-</td>
<td>50</td>
<td>2-1</td>
<td>7</td>
<td>54</td>
<td>60</td>
<td>921</td>
<td>1035</td>
<td>69</td>
<td>28</td>
</tr>
<tr>
<td>54-</td>
<td>49</td>
<td>2-1</td>
<td>7</td>
<td>53</td>
<td>47</td>
<td>1934</td>
<td>2034</td>
<td>43</td>
<td>34</td>
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<tr>
<td>54-</td>
<td>52</td>
<td>1-7</td>
<td>7*</td>
<td>40</td>
<td>21</td>
<td>4215</td>
<td>4276</td>
<td>32</td>
<td>4</td>
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</tbody>
</table>

* VR 10 was chosen in this animal.
Development of ventral root

Table 5. The effect of amputating one leg on the number of ventral horn cells in segment 9 in chemically thyroidectomized tadpoles

<table>
<thead>
<tr>
<th>Body length (mm)</th>
<th>Limb length (mm)</th>
<th>Days after amputation</th>
<th>Stage at fixation</th>
<th>Unoperated side living cells</th>
<th>Operated side living cells</th>
<th>% of unoperated side</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>4</td>
<td>2</td>
<td>55</td>
<td>978</td>
<td>891</td>
<td>90</td>
</tr>
<tr>
<td>36</td>
<td>1.6</td>
<td>3</td>
<td>53</td>
<td>1578</td>
<td>1383</td>
<td>88</td>
</tr>
<tr>
<td>43</td>
<td>3.5</td>
<td>3</td>
<td>55</td>
<td>945</td>
<td>615</td>
<td>65</td>
</tr>
<tr>
<td>46</td>
<td>3.4</td>
<td>7</td>
<td>54</td>
<td>1665</td>
<td>1008</td>
<td>60</td>
</tr>
<tr>
<td>50</td>
<td>6</td>
<td>7</td>
<td>56</td>
<td>942</td>
<td>597</td>
<td>63</td>
</tr>
</tbody>
</table>

Amputations in normal animals at stage 53 or 54

<table>
<thead>
<tr>
<th>Amputations in KCIO₄ animals at stage 53–54</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length (mm)</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>53</td>
</tr>
<tr>
<td>52</td>
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<td>52</td>
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</table>

what is the stimulus in normal circumstances for their loss which starts in stage 54. One possibility which has been canvassed, though without supporting evidence, is that loss is connected with the process of establishing correct neuromuscular connections. The present evidence adds some support to this conjecture in so far that it demonstrates that dying cells do indeed have axons. If withdrawal of incorrect neuromuscular connexions were taking place in normal development, then a situation favourable for collateral sprouting would be produced. We therefore investigated whether such a process was detectable in the ventral roots.

For this purpose, tadpoles were used whose development had been halted by use of the anti-thyroid agent, KCIO₄. This does not prevent growth however, and the tadpoles were thus abnormally large for the stage reached. They were in stasis at early stage 54, that is, just before the decline in fibre number. Counts taken on the control side were normal for the stage (Tables 4 and 5) except for an increased number of myelinated fibres, probably corresponding with the increased myotome mass. These animals had their left leg removed, and were examined at two, three and seven days subsequently. It was argued that the axons present were thus deprived of their terminal connexions: since normal cell and axon death had been prevented by the virtual absence of thyroxin, they would have time to put out branches if they were capable of it.

The results show that the normal cell death which follows amputation does not take place in thyroidectomized animals (Table 5), and further, shows that with the exception of one animal at seven days, there is no great increase in the number of ventral root fibres (Table 4). The absence of cell death is an argument for expecting a similar absence of axonal death, and we therefore interpret Table 4 as indicating that collateral sprouting does not occur in the
ventral roots in these conditions. This does not preclude the possibility of sprouting at other more distal levels.

Since no sprouting occurred in these animals within seven days, it is unlikely that any could be significant in normal animals, for seven days is a greater length of time than the period of decline from stages 53–55. Late sprouting would thus be too late.

DISCUSSION

These observations have some bearing on three of the questions raised in the Introduction, as well as some other points:

*Have the axons which die reached their destination?*

The evidence that axonal loss on the operated side starts soon after stage 52 indicates that interaction between nerve fibres and limb-bud must be taking place, even before the expected decline at stage 54. In this experimental situation, there is no doubt that the extra axons which die do so because they have not been able to reach their destination. By stage 54, 1200 extra fibres will have died (out of the total 1900), all of which therefore must have innervated the limb. Since in the normal animal only 500 axons survive after stage 55, at least 700 of the total population present in normal ventral roots at stage 54 must innervate the limb and are about to die.

The same conclusion can be reached by a different argument (Lamb, 1974). Prestige & Wilson (1972) showed that there was no detectable delay between the first identification of ventral horn cells in the spinal cord and the appearance of their axons in the ventral roots. Lamb (1974) found that ventral horn axons are also to be detected in the limb-bud with a delay that was certainly less than three days, since they both first appear at stage 50. Since the ventral root fibre count closely follows the ventral horn cell count, and there is little extra delay between ventral root and limb-bud, he argued that the large majority of ventral horn cells also had axons into the limb-bud at all stages. Hence we argue that it is likely that most dying cells have had axons into the limb.

*Have the axons which die started to myelinate?*

The timing of these events appears to indicate that the increase in singly wrapped fibres occurs before the period of axonal decline. This conclusion is not justified, because of the local asynchrony of development of the ventral horn cells (Prestige, 1970). There may well be axons being lost and replaced during the plateau period of stage 52–54, that is, before the increase in singly wrapped fibres. The total number of singly wrapped and myelinated fibres taken together rises steadily and never exceeds the total number of fibres remaining after metamorphosis. There is thus no evidence here that myelinated or singly-wrapped fibres are among the population of dying axons, though
they may well be. In rat cervical ventral roots, Fraher (personal communication) has found that fibre loss is definitely occurring among promyelins fibres, as well as foetal fibres.

Is there 1:1 correspondence between ventral horn cells and ventral root axons?

The cell and fibre counts presented here suggest that there are more cells than fibres at stage 55. Before this observation is accepted, there are a number of factors to be considered:

(a) Criteria for identification of corresponding cell population. The method for deciding which fraction of ventral horn cells to use was described by Prestige & Wilson (1972). It depended on matching the juvenile horn and roots, and assessing which part of the juvenile horn matched VR 9. Since the horn elongates without distortion, the same relative part was taken for earlier stages. It is possible that this process introduces errors of the scale of the discrepancy at stage 55.

Another possible error is referred to in Methods. The cell counts are probably overestimates, especially in the older animals. If the cell counts from stage 55 onwards were reduced by 15–20\%, the discrepancy would be lost.

(b) There is no evidence as to whether axons are branched (though this would compound the discrepancy).

(c) There is no evidence as to when sympathetic pre-ganglionic fibres first appear in the ventral roots (though this too would aggravate the problem), nor whether there are any afferent fibres with them.

(d) The fibre counts at stage 55 would suggest that it is only the successful cells that have axons, and that the remainder of cells about to die have already lost theirs. This is difficult to give credence to because amputation of the leg at stage 57 delays the death of those cells about to die (Prestige, 1967). There must be an information pathway from limb to cells about to die which is still present at stage 57, and it is most likely that this is the cell’s own axon.

Identification of Phase I axons

Prestige (1967) defined a sub-population of developing ventral horn cells which he called Phase I. These cells were able to survive without the presence of the tissue they would normally innervate, and could be isolated by removing the limb. Only Phase I cells survived and thus they could be counted. The present experiments reveal a similar population of Phase I axons. They are all naked fibres. However, not all naked fibres are Phase I, because single wrapping does not start until stage 54, still with as many fibres, whereas the decline in Phase I axons starts after stage 52 (Fig. 4).

It might be tempting to identify Phase II axons (see Prestige, 1967) as those possessing single wrapping, for the counts of both show a peak in mid-larval stages. However, they certainly cannot be identical, for phase II cells show a peak at stage 54, while the singly wrapped fibres reach a peak at stage 55.
Why does sensitivity to limb removal begin earlier than the decline in normal axon number?

When the limb is absent, changes in ventral root fibre number become apparent soon after stage 52, during the plateau of normal fibre numbers. The decline on the normal side starts in stage 54. (This is also true of cell counts.) One possibility is to assume these are unrelated phenomena, but this is to argue against the evidence from hyperplastic changes following grafting of supernumerary limbs, which indicates that peripheral control of cell number is significant. Another possibility is to assume that fibres die because of a reduction in the number of sites available, and that this only becomes significant normally at stage 54. A third possibility is that fibre death is occurring normally from stage 53, but that it is initially masked by further fibre outgrowth. There is evidence that such a process of cell turnover exists in the ventral horn at these stages (Hughes, 1961). This last hypothesis requires a different mechanism for fibre death than exclusion from termination sites (redundancy), for the same number of fibres is maintained. Fibres could be rejected from sites on this basis.

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REFERENCES


Development of ventral root


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