A STUDY OF SPINAL CORD DEVELOPMENT IN SILVER-STAINED SHEEP EMBRYOS CORRELATED WITH EARLY SOMATIC MOVEMENTS

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A PRECEDING paper (Barcroft *et al.* 1936) dealt with the course of development of early somatic movement in sheep embryos. The present article takes as its scope a histological study of growth changes in the rostral part of the spinal cord of these same embryos and attempts to interpret some of the early behaviour in the light of these changes.

Twenty-one sheep embryos ranging from 32 to 38 days of age were tested physiologically and their behaviour was observed at the physiological laboratory in Cambridge. Twelve were prepared for histological study after this had been done and forwarded to our laboratory in Chicago. Fifteen additional untested embryos ranging from 4 mm. to and including 20 mm. crown-rump length were obtained. All specimens were stained by the pyridine-silver method of Ranson, modified by reducing the time in some of the reagents (Davenport *et al.* 1934). Those studied in detail are listed in Table I.

Table I. *Material studied*

Embryo No.	Size mm., C.R. length	Age days	Embryo No.	Size mm., C.R. length	Age days
121	4	19 ± 1	764	20*	?
113	7	?	136	23	32 ± 1
117 A	8	22 ± 1	144	23·5	33 ± 1
760	12*	?	134	23·5	34 ± 1
759	13*	?	146	24	34 ± 1
761	14*	?	141	24·5	33 ± 1
762	16·5*	?	147	25	34 ± 1
763	16·5*	?			

* Approximate size determined after fixation. The other specimens were accurately measured before fixation.

OBSERVATIONS

Motor neurons. The precocity of the motor apparatus is even more clearly demonstrated in the sheep than it was in the cat (Windle, 1932 *a*, *b*). In the 4 mm. embryo neurofibrils are appearing in neuroblasts at the rostral end of the spinal and bulbar motor column. The cells are unipolar and bipolar and not until the 8 mm. stage do they start to become multipolar. In the 7 mm. embryo the cell bodies of the visceral motor nerves such as the vagus are found more medial and ventral than those of the somatic nerves such as the

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hypoglossal and the spinal ventral roots. By the time the sheep has reached a length of 16·5 mm. the visceral nuclei have shifted to their lateral stations. This process of migration has been described in cat embryos (Windle, 1933). There is no distinct subdivision of the spinal motor column in the smaller sheep embryos but at the 16·5 mm. stage the column has begun to divide into medial and lateral nuclei for axial and appendicular innervation respectively. Frontal and saggital sections demonstrate that motor nerve cells extend their processes in a plane at right angles to the long axis of the spinal cord. In the 16·5 mm. embryos the intermedio-lateral cell column of the thoracic region is just beginning to differentiate and in the 20 mm. embryo this cell group has definitely separated from the ventral horn. At the same time the cell bodies of the spinal accessory nerve have come to lie dorsolateral to most of the ventral horn cells.

Internuncial neurons. Unipolar secondary neurons have differentiated in the lateral wall of the spinal cord of the 7 mm. embryo. Most are found in the alar plates but a small number are located more ventrally. The axons of all secondary neurons of the 7 mm. embryo course ventrad and give rise to a very small ventral spinal commissure and, on the opposite side, a longitudinal tract, the anlage of the ventral funiculus. This funiculus lies along the lateral side of the spinal cord superficial to the motor cell column. In the 8 mm. embryo the commissural fibres have increased in number and part of the ventral funiculus now lies ventral to the emerging motor root fibres (Pl. I, fig. 4). At 13 mm. the commissure and funiculus are well developed. In sheep embryos of 8 mm. and larger the commissure is found in all spinal cord segments. A study of frontal sections of the spinal cord indicates that the course of most of the commissural fibres after crossing the floor plate is rostrad.

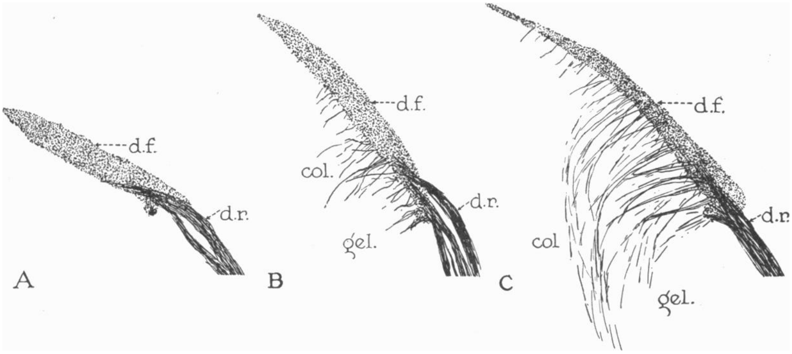
Non-crossing associational neurons could not be observed in specimens smaller than 14 mm. long. The associational fibres arise from cell bodies in the alar plates and course laterad to enter into the formation of a lateral funiculus. In the 16·5 mm. embryos this direct system is well established. The axons of associational neurons course through the dorsal part of the brachial motor nucleus where they come in contact with dendrons of the motor cells. Prior to the 20 mm. stage local motor and internuncial elements form the predominant neuronal pattern of the spinal cord. As yet there is no indication of a significant downward growth of pathways from the brain.

Sensory neurons. The development of the sensory lags behind that of the motor mechanism. Dorsal ganglion cells, roots and funiculus are lacking in the 4 mm. embryo. By 7 mm. a few bipolar neuroblasts are present in the ganglia (Pl. I, fig. 2). In the second cervical segment a few fibres from these bipolar cells enter the dorsal lateral part of the cord and course toward the central canal without building a longitudinal tract. In the 8 mm. embryo dorsal root fibres form the anlage of the dorsal funiculus (Pl. I, fig. 4). This funiculus is a prominent compact bundle of afferent fibres at 13 mm.

Dorsal root ganglion cells retain their bipolar character and the dorsal funiculus appears as a sickle-shaped tract upon the dorso-lateral surface of the spinal cord in all the larger sheep embryos. In those of 20 mm. the substantia gelatinosa makes its first appearance as a cellular mass lacking fibrils and located just ventral to the dorsal funiculus where it displaces the associational fibres medially and ventrally.

Prior to the 20 mm. stage the tractus solitarius is not a separate bundle but appears to be located in the lateral and ventral part of the dorsal funiculus. It lies just medial and ventral to the dorsal funiculus at 20 mm. and is quite large in the 23 mm. embryo. The tractus solitarius receives fibres from the dorsal funiculus and the spinal accessory nerve in addition to those from the cranial visceral afferent nerves. The tractus solitarius also receives some associational fibres from the anlage of the recticular formation. It descends into the third cervical spinal cord segment.

It is clear that all intrinsic elements which go to make up simple spinal reflex arcs have been formed before the sheep reaches the 32nd day of gestation (23 mm.). Associational and motor neurons are closely related in position but sensory neurons, late in formation, are isolated from the other spinal structures in a compact tract upon the dorso-lateral surface of the cord.



Text-fig. 1. Detail camera lucida sketches of the dorsal funiculus and dorsal roots at the seventh cervical spinal cord segment of sheep embryos of (A) 20 mm., (B) 23 mm., and (C) 24 mm. C.R. length. Somatic movements were elicited in C but not in B; the sensory collaterals reach the association centre in C but not in B. Pyridine-silver stain; magnification × 72.

Table II. *Number of sensory collaterals in representative spinal cord segments*

Embryo No.	Age days	Size mm.	C 3	C 5	C 7	T 1	Total
136	32	23·0	3914	3567	4607	2761	14,849
144	33	23·5	6100	5982	6111	3569	21,762
134	34	23·5	3808	5544	6741	3859	19,952
146	34	24·0	7033	8710	9408	5009	30,160
141	33	24·5	2410	3604	5660	2428	14,102
147	34	25·0	7913	9289	9686	6182	33,070

In the 23 mm. embryo the first sensory collaterals have appeared along the medial border of the dorsal funiculus. At first they are short and most of them are located near the lateral end of the tract (Text-fig. 1 B and Pl. II, fig. 6). The collaterals increase in number between 23 and 25 mm. Many swing mesad and ventrad around the substantia gelatinosa in specimens greater than 23 mm. long (Text-fig. 1 C and Pl. II, fig. 7), converge upon the associational nucleus and deploy among the dendrites and cell bodies of these internuncial neurons.

In order to obtain an index for comparing the degree of sensory development the collateral fibres of the dorsal funiculus were counted unilaterally in spinal cord segments C 3, C 5, C 7 and T 1 of a group of physiologically tested embryos. Results are shown in Table II. The greatest number of collaterals were encountered at C 7 in all embryos; the least, at T 1. It should be emphasized that all the embryos were darkly stained with the silver reduction and all are coloured to about the same degree with the exception of embryo No. 141 in which the stain was somewhat uneven. A binocular compound microscope with 2 mm. oil immersion objective was used. Both authors checked the results.

Peripheral nerves. The spinal nerves are well formed in the 13 mm. embryo and can be followed far toward the periphery. The growth tips of the brachial plexus fibres can be seen in the forelimb at 16·5 mm. Some terminate in groups among mesodermal cells of the premuscle masses and others ramify beneath the epithelium; no specialized endings of adult type exist. In the 20 mm. embryo some of the shoulder muscle are differentiating. Both sensory and motor nerves can be recognized. In the 23 mm. embryo the fibres which are running beneath the epithelium end in free endings and little bulbs upon the bases of the epithelial cells. Other nerve filaments enter muscles, run parallel to the myoblasts and terminate in little swellings and in clusters of bulbs resembling grape-like endings upon the myoblasts (Pl. I, fig. 5). In the 24 and 25 mm. embryos skeletal muscles are well differentiated and numerous motor nerve fibres appear in them; superficial cutaneous nerves ramify beneath the integumental epithelium in many places. Thus both motor and sensory nerve endings of a primitive kind are present at the time the embryos become motile.

DISCUSSION

The fundamental plan of spinal cord development is alike in all mammalian embryos studied (Windle, 1933, 1934; Windle & Baxter, 1936; Windle & Fitzgerald, 1937). Species differences are for the most part minor and seem to be of little significance. An exception is found in the substantia gelatinosa. This nucleus is similar in the sheep, cat and man but is more highly developed in the rat, occupying so much space in the dorso-lateral part of the spinal cord that the sensory collaterals must course a considerably greater distance to reach the associational centre than in the other animals. Correlated with this, local reflexes do not manifest themselves quite so early in the rat as in other mammals (Windle & Baxter, 1936).

Studies in silver-stained embryos of the several species of mammals clearly demonstrate that motor neurons appear first, to be followed by internuncial and then by sensory neurons. That such a sequence holds in parts of the nervous system above the spinal cord was indicated in a previous article (Windle, 1935). It seems to be a general rule that neurofibrillar differentiation proceeds from efferent to afferent side within any specific reflex system. From the standpoint of functional studies it is clear that myogenic responses precede neurogenic.

The appearance of neurofibrils in a cell marks it a nerve cell. Neurofibrils form concomitant with the outgrowth of processes, the nerve fibres. No proof exists that the cell is better able to conduct immediately after fibrils develop than it was before but it is natural to assume that as the cell grows to resemble the neuron, as we know it in the adult organism, its property of conduction improves. Previous to the appearance of effector organs it will be difficult to demonstrate nervous function even in the cells with long processes filled with fibrils. For a short time after myoblasts and primitive motor nerve endings are developed it is still impossible to demonstrate nervous function as expressed by muscular activity even though muscular contraction can be induced by stimulating the muscles directly. Since many long motor, internuncial and sensory neurons can be demonstrated at this time by the silver technique, it is evident that something more than the development of neurofibrils in nerve cells and fibres must occur before reflex activity can take place. The present and other studies (Windle, 1934; Windle & Baxter, 1936) offer an explanation of the first embryonic movements in terms of anatomic structures.

The only fundamental structural change occurring in the spinal cord of sheep embryos between 20 and 25 mm. long involves the number and especially the length of the sensory collateral fibres. The 20 mm. embryo has no collaterals. The 23 mm. embryo has a large number but they are short and fail to reach the associational nucleus. In all embryos larger than 23 mm. long, collaterals increase in number and some reach the associational nucleus. The associational and brachial motor neurons bear intimate relationship throughout their development and are in structural contact. All the embryos larger than 23 mm. long which were studied histologically had responded to stimuli (Barcroft *et al.*, 1936). Faradic shocks applied to the back over the spinal cord were followed by forelimb movements. In those 24–25 mm. long mechanical stimulation (pressure upon the amnion) produced similar results. We believe that it was possible to obtain these responses because the specimens in question had reached a stage in development at which nerve impulses could flow from afferent to efferent neurons over newly formed collaterals in the spinal cord. That this correlation between structure and function is not simply coincidence is indicated by the fact that other species of animals (cat, rat and chick) have shown the same (Windle, 1934; Windle & Baxter, 1936; Windle & Orr, 1934).

The assumption of Barcroft *et al.* (1936) that two neurons and a synapse were involved in the embryonic forelimb reactions is conservative. The histological observations allow us to suggest that three neurons and two synapses may have been involved. The impulse could have travelled over an afferent fibre of the dorsal funiculus and its collateral branches to a synapse with an associational neuron in the spinal cord, then to the brachial motor nucleus where a second synapse would have led to discharge of a motor neuron. The peripheral terminations of motor neurons on myoblasts are clearly formed.

Recent studies in cat embryos (Windle, 1937) have shown that localized stimulation of afferent endings in the fore paw produce responses like those seen in the sheep. These involve all the neural elements of simple unilateral, unisegmental, spinal reflex arcs from receptor ending to effector ending.

SUMMARY

A series of silver-stained sheep embryos demonstrate the precocity of development of the motor apparatus. As early as the 4 mm. stage neurofibrils are present in motor cells at the rostral end of the spinal cord and in the medulla oblongata. Internuncial neurons are first seen in the 7 mm. embryo. These form the commissural fibres and ventral funiculus of the opposite side of the spinal cord. The direct associational neurons were first observed in specimens 14 mm. long. Their axons enter into the formation of the lateral funiculus of the same side.

A few bipolar neuroblasts are present in the dorsal root ganglia of the 7 mm. specimen. At 8 mm. dorsal root fibres form the anlage of the dorsal funiculus which becomes a prominent tract at 13 mm. The first collaterals are given off to the grey matter by this tract in the 23 mm. sheep embryo. Earlier than the 23 mm. stage the associational and motor neurons are closely related in position and structural contact between them does exist, but the sensory nerve fibres are isolated from other intrinsic spinal cord structures in a compact tract upon the dorsal surface of the cord.

The peripheral nerves, both motor and sensory, and the myoblasts in certain muscle masses are well developed at the 23 mm. stage. Motor nerve fibres terminate in free endings and little bulbs upon the myoblasts. Free sensory endings are in contact with the epithelium in some places, notably the forelimb.

Prior to the 23·5 mm. stage physiological responses could be elicited only by stimulating muscle directly. Physiological activity which seemingly involved nerve conduction was elicited in the 23·5 mm. and all larger embryos. At this time some of the sensory collateral fibres have grown out from the dorsal funiculus and have reached the region of the internuncial neurons thus completing the first structural reflex arcs.

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KEY TO LETTERING

as.	associational neurons.	*gel.*	substantia gelatinosa.
as.n.	nucleus of associational neurons.	*l.f.*	lateral funiculus.
c.	ventral commissure.	*mot.*	motor cell column.
col.	collaterals of dorsal funiculus.	*mot.l.*	appendicular motor nucleus.
d.f.	dorsal funiculus.	*mot.m.*	axial motor nucleus.
d.r.	dorsal root.	*v.f.*	ventral funiculus.
d.r.g.	dorsal root ganglia.	*v.r.*	ventral root.
end.	endings of motor nerve fibres.	XI.	spinal accessory nerve.

EXPLANATION OF PLATES I AND II

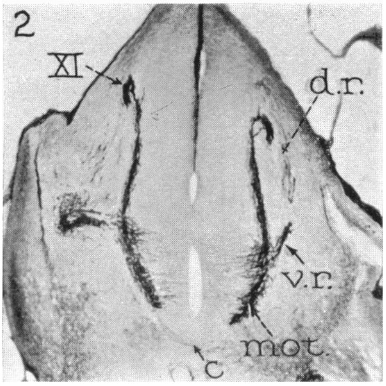
Plate I

Unretouched photomicrographs of pyridine-silver stained sections of sheep embryos.

Fig. 2. The first cervical segment of embryo No. 113; 7 mm. long. The prominent darkly stained motor cell column and ventral motor root appears in the lateral part of the spinal cord. The spinal part of the accessory nerve is prominent in a dorsal position. The anterior commissure is faintly visible. The sensory dorsal roots and ganglion cells are beginning to form. Magnification: ×100.

Fig. 3. The lumbar region of embryo No. 113; 7 mm. long. The motor columns and ventral roots are present but no sensory elements have neurofibrils. The anterior commissure is represented by two or three fibres which cannot be seen in the photograph. Magnification: ×100.

Fig. 4. The eighth cervical segment of embryo No. 117 A; 8 mm. long. Many sensory elements have appeared. The ventral commissure is larger and the ventral funiculus is seen on either side of emerging ventral roots. Magnification: ×100.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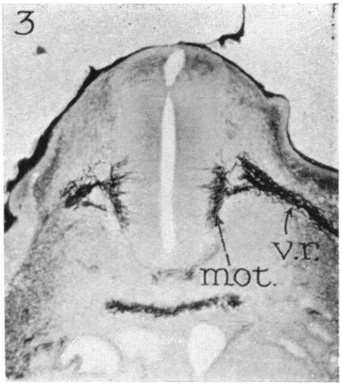
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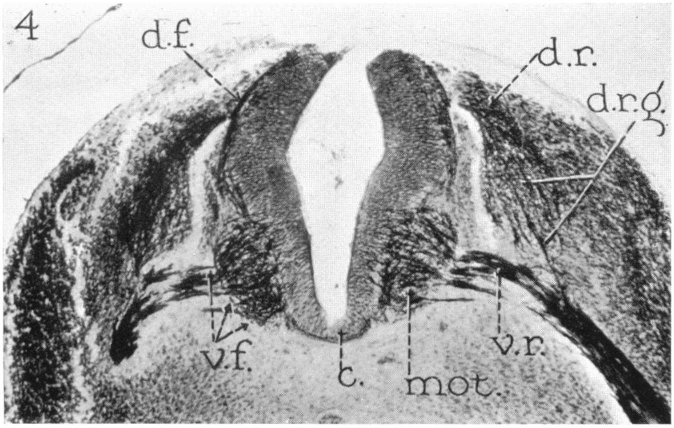
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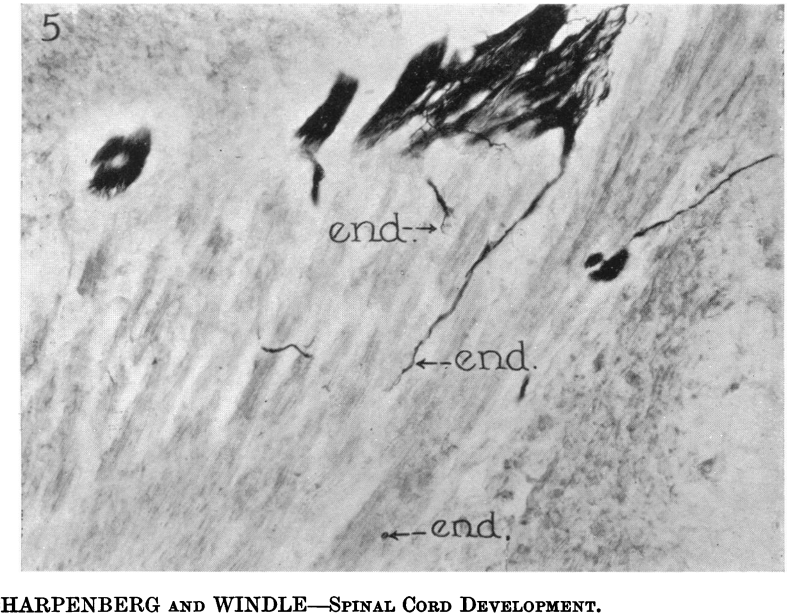
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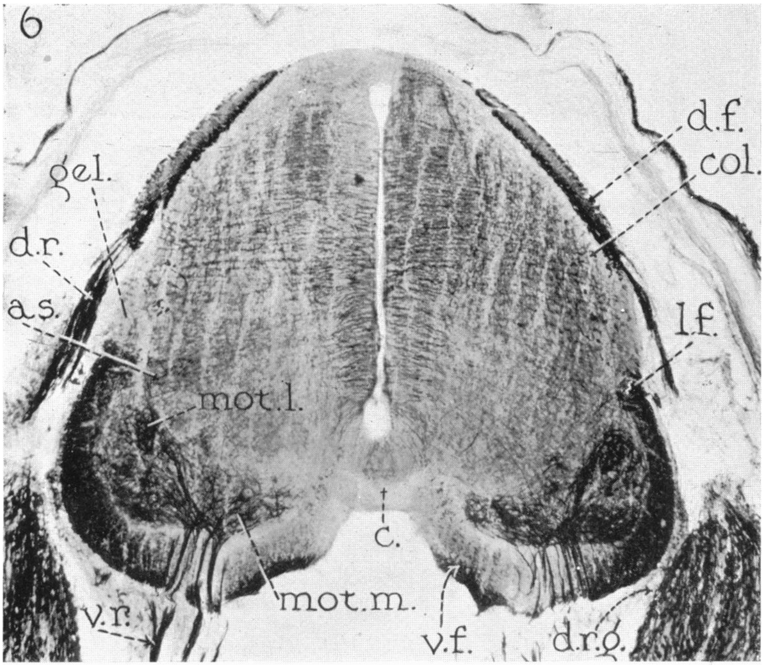

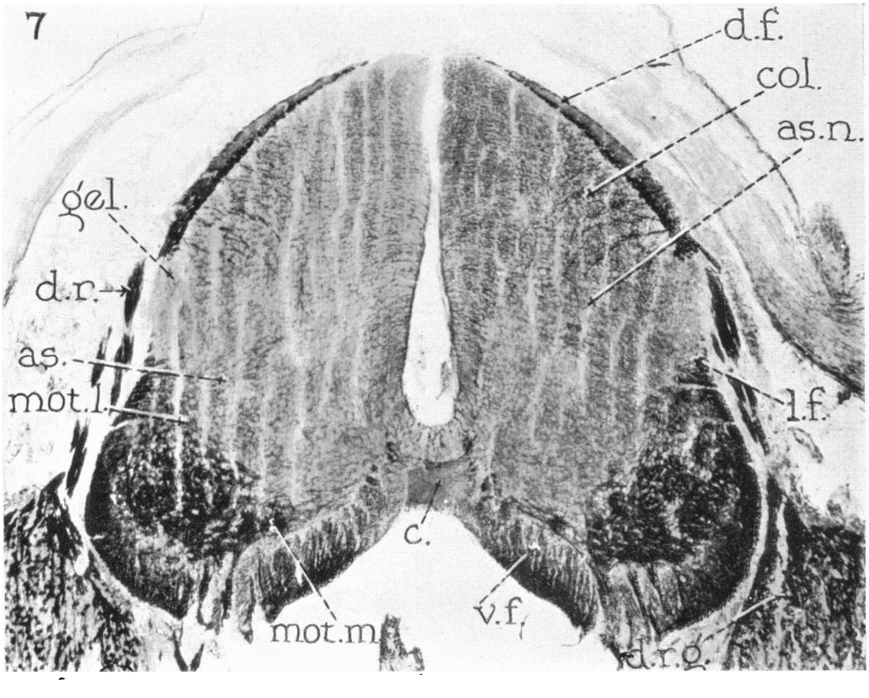

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Fig. 5. From a section through shoulder musculature of embryo No. 146; 24 mm. long. Myoblasts form a background against which motor nerve fibres and endings are seen. Magnification: × 320.

PLATE II

Unretouched photomicrographs of pyridine-silver stained sections of sheep embryos.

Fig. 6. The sixth cervical segment of embryo No. 136; 23 mm. long. The motor column has divided into medial and lateral nuclei. The associational fibres, not clearly illustrated, course into the prominent lateral funiculus. The dorsal funiculus is large and along its ventral edge a few short collaterals may be seen. The substantia gelatinosa is indistinct. Magnification: × 100.

Fig. 7. The eighth cervical segment of embryo No. 146; 24 mm. long; compare with Pl. I, fig. 4. Prominent in this specimen are the collateral branches of dorsal root fibres of the dorsal funiculus which course into the nucleus of the associational neurons. Fibres from this nucleus form part of the lateral funiculus adjacent to the appendicular (brachial) motor nucleus. Magnification: × 100.