Observations on the Innervation of the Integument of Amphioxus, Branchiostoma lanceolatum

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With two plates (figs. 1 and 2)

SUMMARY

In this paper the pattern of innervation of one of the simplest forms of skin, the integument of amphioxus (covering the dorsal fin in the middle third of the animal) is compared and contrasted with that of the cornea and of the skin in man.

An account is given of the results of stimulation experiments in which the integument in this region was irradiated with infra-red rays.

The observations which have been made can be summarized briefly as follows:

1. No neuro-epithelial cells were encountered in the epidermis in the region chosen for study.
2. Evidence is given which suggests that the nuclei along the course of the nerve-trunks which combine to form the ‘dorsal roots’ of the nerve-cord are not those of the first sensory neurone. They appear to be analogous to the Schwann-cell nuclei of vertebrates.
3. The ratio of epidermal cells to parent sensory axons in randomly selected metameres of integument in the selected region is as low as 7:1. From this it has been calculated that the density of innervation is comparable with that in the cornea and in the skin of vertebrates.
4. Every epidermal cell has two or more terminal filaments ending in relationship to it.
5. Pre-terminal axons from parent axons of different diameters are widely and apparently randomly scattered beneath the epidermis.
6. Each and every epidermal cell is related to terminals which approach from different directions and serve more than one parent axon.
7. The patterned arrangement of the nerves serving the integument of amphioxus is comparable with that observed in the skin of teleost fish, in the cornea of a number of vertebrate species, and in the skin of man.
8. The nerves in the integument in the selected region can transduce non-injurious infra-red stimuli, although the stimuli were of a kind not normally encountered by the animal in its natural habitat.
9. In the light of these observations and of experimental observations in man (Weddell, 1955; Lele and Weddell, 1956; Weddell, 1957), it is difficult to subscribe to the notion that information concerning the environment is transmitted to the central nervous system from the skin by a restricted series of nerves having terminals which only transduce stimuli having quite specific physical attributes. Rather, it seems likely that information reaches the central nervous system in the form of a space-time pattern of action potentials from endings which are either more or less available to a range of stimuli having different physical characteristics. In other words, different stimuli must evoke different patterns of activity, which are analysed by the central nervous system acting in a role of an analogue as opposed to a digital computer.

INTRODUCTION

In 1908 Parker demonstrated that four characteristic reactions can be elicited from amphioxus by the application to its integument of stimuli which in man would be expected to evoke reports of touch, warmth, cold, and pain. This led him to postulate that the receptors for mechanical, thermal, and chemical stimuli must be at least physiologically distinct from one another.

In 1938, however, Ten Cate (1938a, b) observed that, in his experience, studies confined to noting the reaction of amphioxus to stimuli which obviously link man with the changes in his environment may be a very misleading basis upon which to formulate theories as to the mechanisms underlying its behaviour.

From the neurohistological standpoint, no author has claimed that there are morphologically specialized nerve-endings in the integument of amphioxus; on the contrary, it is agreed that the nerves which do not originate in neuro-epithelial cells arise in free nerve-terminals. On the other hand, the number, size, and patterned arrangement of the sensory nerves serving the integument does not appear to have been investigated. In this paper an account is given of an attempt to fill this gap, together with the results of some stimulation experiments with infra-red rays and other stimuli which supplement the observations made by Parker and others.

Weddell and his colleagues have recently suggested that 'free' nerve-terminals in the cornea, and possibly also in human skin, are capable of transducing stimuli having different physical characteristics. Further, they submit that the patterned arrangement of non-specific nerve-terminals is the mechanism whereby environmental changes are transduced in man (Weddell, 1957). Thus, the chief purpose of this investigation was to establish that, from the histological point of view, there are no specific nerve-terminals in the integument of amphioxus and then to examine its behaviour in the light of the above hypothesis.

LITERATURE

The integumental innervation of amphioxus has been described in terms suggesting its unimpressive nature, especially compared with that of its atrial nervous system (Holmes, 1953). It is, however, agreed that in each metamere there is a plexus of fine sub-epidermal nerves which give rise to terminals which end freely at the base of the epidermal cells. The nerves of the sub-epidermal plexus are gathered into small nerve-bundles in the cutis, which, in turn, lead into the nerve-cord through the dorsal nerve-root (Retzius, 1898; Franz, 1927).

On the functional side, Parker (1908) has shown that the movements induced by visible light only occur when the 'eye-cups' in the wall of the nerve-cord are illuminated. A narrow beam of light which illuminates the integument only is without effect. He also concluded, as the result of a
Integument of Amphioxus

number of experiments, that the integument must contain at least physiologically distinct mechanical, thermal, and chemical receptors.

Franz (1924) confirmed Parker's (1908) observations but noted that the most effective mechanical stimulus was a natural brushing contact against the integument anywhere over the body-surface by the undulatory movements of another amphioxus in the immediate neighbourhood.

Finally, Ten Cate (1938a), in a series of experimental observations on animals in their natural habitat, has countered Parker's conclusions by pointing out that many of the experimental procedures employed by Parker in his studies on the animal's behaviour were too artificial to throw any light on its functional organization.

Material and Methods

Material

This was obtained from the Marine Biological Station at Plymouth during the months of September and October 1954, February 1955, and December 1956. The specimens were adults of different sizes and at different stages of sexual development. No larvae were examined. Immediately upon arrival the animals were placed in a large tank containing continuously aerated sea-water in a semi-darkened, cool room at a nearly constant temperature (17°C ± 1°C). The bottom of the tank was covered with a layer of sea-sand to a depth of 3 cm and sea-water surmounted the sand to a depth of 15 cm. Experimental observations were carried out within a week of the arrival of each batch of animals, although surplus animals survived for 3 months without any apparent alteration in size and behaviour.

Histological methods

Twelve animals were immersed for 20 min in an 0.02% solution of methylene blue in sea-water containing 50 units of hyaluronidase per ml. They were then transferred to fresh sea-water and examined under a binocular microscope.

Fifteen animals were anaesthetized with urethane immersed in sea-water containing 50 units of hyaluronidase per ml and then fixed in 10% formalin made neutral to litmus paper with calcium carbonate. After fixation they were impregnated with silver by a modified Bielschowsky-Gros technique and sections of integument were cut in various planes.

Ten animals were anaesthetized with urethane, left for 20 min in sea-water containing 50 units of hyaluronidase per ml, fixed in Bouin's solution or Susa, embedded in wax, and cut in serial section, some in the longitudinal, others in the transverse plane. The sections were stained with haematoxylin and eosin.

Ten animals were anaesthetized with urethane and tangential slices of body wall removed with a safety razor blade and placed on glass slides either in a drop of plain sea-water or sea-water containing 0.05% methylene blue.
Experimental methods with intact animals

Controls. These were carried out on 5 specimens in a quiet room illuminated by a darkroom safe-light fitted with a red filter. Each animal in turn was placed in a dish 16 cm in diameter, containing enough fresh sea-water to cover it completely when it lay still. As soon as it had come to rest, the time was noted and the animal was observed continuously for 30 min, during which time it received no extraneous stimuli of which we were aware. Any movements seen (other than the rhythmic ones of respiration) were recorded. A note was made of the time of onset of the movement, its duration, and whether or not it resulted in a change in the position of the animal. The animal was returned to the tank as soon as the experiment was over.

Stimulation. Each animal in turn was again placed in the dish and after it had come to rest, a total of not less than 10 stimuli of each of those listed below was delivered in random order to the integument covering the dorso-lateral surface of the middle third of the body; the time of onset, duration, and nature of any movement evoked being recorded.

(ii) A bevelled-edged disk of hardwood having a circular area of 19.6 sq. mm.
(iii) A fine sharp-pointed needle.
(iv) A circular beam of white light, 1 cm in diameter, containing less than 5% of red and infra-red rays.
(v) A circular beam of infra-red rays 1 cm in diameter, wavelength 1 to 3 μ, energy value 0.1 to 2 cal/cm²/sec (Lele and Weddell, 1956).

Experimental methods with operated animals

Controls. Integument covering the middle third of the dorsal aspect of each of another series of 6 animals was irradiated with infra-red rays (1.8 cal/cm²/sec), first on one side of the mid-line, then on the other. The time between the onset of stimulation and any movement evoked was recorded.

Operation. The animals in turn were laid on cotton-gauze soaked in sea-water and the integument covering the dorso-lateral aspect of the middle and caudal thirds on one side was removed. To do this two incisions were made, one extending from the junction of the middle and cranial thirds of the animal down the whole extent of the mid-dorsal line and the other from the same level down the mid-ventral line. The anterior ends of the incisions were united and the integument lying between the longitudinal incisions peeled off. This caused the animal to wriggle around in the dish at intervals for 10 to 15 min.

Stimulation. Thirty minutes after operation and again 2 h later, each animal was stimulated with infra-red rays as in the control experiments. The lapse of time between the onset of stimulation and a movement causing displacement was recorded.
FIG. 1

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Integument of Amphioxus

At the end of the experiment, each animal was fixed in Bouin’s fluid and subsequently cut into serial transverse sections, which were stained with haematoxylin and eosin.

HISTOLOGICAL OBSERVATIONS

No cells giving rise to nerve-fibres passing along bundles towards the dorsal nerve-cord were seen in the epidermis of the dorsal fin. Indeed, no cells having distinctive morphological or staining features were seen in this region in sections from active uninjured animals which had been carefully handled during their preparation for microscopical examination.

In the sections stained with haematoxylin and eosin both dorsal and ventral nerve-roots are seen as nucleated tracts comparable to those in vertebrates, but the nuclei are rounder and less evenly spaced than the Schwann-cell nuclei of vertebrates (fig. 1, A). The ratio of the length to the breadth of the nuclei under consideration is $2 \pm 0.25$, whereas that of Schwann-cell nuclei in the rabbit ear is $3 \pm 0.5$. In silver preparations, the nuclei were never deeply impregnated and no processes appeared to extend from their neighbourhood in either direction along the nerve-trunk. However, both impregnated axons and nuclei lay within a common, faintly outlined, and apparently structureless sheath.

Further to test the hypothesis that these nuclei are those of the cells of the first sensory neurone, counts were made (in relation to three different dorsal nerve-roots) of the numbers lying between the origin of the ramus dorsalis from the dorsal nerve-root and the termination in the integument of the nerves serving it. The counts were compared with the number of impregnated sensory axons entering the dorsal nerve-roots from the ramus dorsalis of Amphioxus.
corresponding metameres in a different animal of about the same length (fig. 1, c). The counts are given in table 1. The size of the nuclei varies little from region to region; they were $3.0 \pm 0.5 \mu$ in length and $1.5 \pm 0.5 \mu$ in breadth and they contained no visible nucleoli, although these were always seen in the nuclei of cells in the nerve-cord; moreover, the nuclei in the nerve-cord are larger than those in the nerve-roots. In a random sample of 20 cells the size ranged from 5 to 9 $\mu$ in length and from 3 to 8 $\mu$ in breadth.

**Table 1**

_Specimens nos. 1 and 12, 6 cm and 6.1 cm long respectively_

<table>
<thead>
<tr>
<th>Roots</th>
<th>No. of axons</th>
<th>No. of nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>70</td>
<td>108</td>
</tr>
<tr>
<td>B</td>
<td>63</td>
<td>102</td>
</tr>
<tr>
<td>C</td>
<td>60</td>
<td>102</td>
</tr>
</tbody>
</table>

Table 1 shows the number of axons in sensory ramus dorsalis serving dorsal nerve-roots in 3 closely adjacent metameres in the middle third of specimen no. 1 and the number of nuclei in sensory nerve-trunks serving dorsal nerve-roots in 3 adjacent metameres in the middle third of no. 12.

Taken at their face value, then, these figures suggest that the nuclei are not those of the first sensory neurones.

The general features of the layout of the nervous system was studied in the whole specimens in which the nerves were stained with the methylene blue which had been added to the sea-water in which they were living. Fig. 1, B is a low-power photomicrograph showing bundles of nerve-fibres passing towards the integument covering the dorsal fin in the middle third of such an animal. It was noted in specimens stained in this way that the size of the nerve-bundles seemed to bear a fairly close relationship to the areas of the segmental integument which they subserved, being distinctly smaller in metameres in the posterior third of the animal. This suggested that some measure of the density of innervation of the integument covering the dorsum of amphioxus might be obtained by counting the number of axons contained in the supero-dorsal cutaneous nerve (one of the parent divisions of the sensory ramus dorsalis of the mixed dorsal nerve-root, see fig. 1, E) and comparing this figure with the number of epidermal cells covering one-half of the dorsal fin in a single metamere, the territory of integument which is seen to be supplied exclusively by this nerve.

In only one animal and only in relation to three different bundles was the plane of section approximately transverse and silver impregnation deep and selective enough for counts to be reliable. The numbers recorded are likely to be somewhat on the low side, for only axons about whose identity there was no doubt were included in the count. There is, however, no reason to suppose that many axons normally impregnated by silver escaped enumeration.

Further details of the innervation of the integument will be described in
relation to photomicrographs obtained from different specimens which had been prepared for examination in different ways.

Table 2

<table>
<thead>
<tr>
<th>Specimen no. 14, length 6 cm</th>
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</thead>
<tbody>
<tr>
<td>Metamere</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
</tbody>
</table>

Table 2 shows relationship between the number and surface area occupied by epidermal cells covering one-half of the integument of the dorsal fin of a single metamere and the number of parent dorsal root sensory axons which serve the area.

Axons passing towards the integument in the ramus dorsalis of the dorsal nerve-root vary in thickness; most of them are too fine to measure accurately so that a histogram could not be constructed. The majority of axons appear to be between 1 and 2 μ in diameter; the largest reach 4 μ and the smallest are less than 1 μ in diameter (fig. 1, c). The ramus dorsalis leaves the myoseptum at a point just above the V-bend (fig. 1, e). It soon divides without collateralization of axons into two: a small branch (approximately one-quarter of the axons), the latero-dorsal cutaneous nerve; and a larger branch (approximately three-quarters of the axons), the supero-dorsal cutaneous nerve.

Each dorsal root parent axon on entering the subcutis (which consists of a network of collagen fibres permeated by blood-vessels) gives rise to numerous collaterals which pass obliquely towards the epidermis. They proceed singly, in bundles of three or four, rarely in bundles of up to eight axons. The juxtaposition of axons of different diameters is apparently random, for some bundles contain relatively few thick axons, others about equal numbers of axons of all diameters, whilst solitary axons in the subcutis are sometimes thick and sometimes thin (fig. 1, f).

The collaterals then traverse the cutis; this consists of a fibrillary layer of connective tissue containing no nuclei situated immediately below the basement membrane and difficult to separate from it; the basement membrane is also non-cellular and made up of a lattice work of closely knit fibres running at right angles to one another. The axons pass vertically through the basement membrane (figs. 1, g; 2, a, b). The 'apertures' in the basement membrane through which they pass are conspicuous in fresh specimens of integument under phase-contrast microscopy and in selected silver preparations (fig. 1, d). The axons, still of widely different diameters, in a random mixture, emerge from the basement membrane void of visible sheaths and come to lie immediately subjacent to the epidermal cells. Here they again collateralize extensively. The branches change direction through a right angle on leaving the parent axons and travel for various distances, interweaving with and crossing one another to form a complicated sub-epidermal plexus (fig. 1, g).
The collaterals from a single parent axon vary in number and size. They diverge from one another at their origin and so become disseminated in different directions. The particular direction depends partly upon the number of collaterals which arise, for when numerous they radiate from their point of origin in a stellate fashion (fig. 2 D). Some of the collaterals (most commonly the thicker ones) give off branches while proceeding for a comparatively long distance (in one section equivalent to the sum of the breadth of 15 epidermal cells) beneath the epidermis before terminating (fig. 2, c). Others proceed for shorter distances and still others apparently terminate immediately after piercing the basement membrane. In this way, not only do terminals derived from different parent axons end in close proximity to one another but parent axons serve numerous terminals which are widely and apparently randomly scattered throughout the epidermis.

In agreement with Retzius, we found it difficult to determine, in either fixed methylene blue or silver impregnated sections, exactly how the finest collaterals terminate. However, in sections which showed the least evidence of shrinkage and/or irregularity, fine filaments appeared to end by attenuation near the plane of junction between the epidermal cells.

By phase-contrast microscopy, terminal axoplasmic filaments cannot be seen in freshly cut sections immediately after mounting either in sea-water or methylene blue in sea-water. After 10 to 20 min, parent axons, some of which are surmounted by fine thread-like extensions, can be seen (fig. 2, e). At the same time, small gaps become visible between the epidermal cells into which these filaments appear to pass, often seeming to proceed as far as the cuticle covering the surface of the integument. After 30 to 60 min, particularly in the case of specimens which have been mounted in sea-water containing methylene blue, the terminal filaments form a series of interconnected beads lying in gaps between epidermal cells (fig. 2, f). Such appearances are also

![Fig. 2 (plate).](image-url)

A, oblique section through the integument. Axons can be seen in the subcutis on the right of the picture. Some are passing through the apertures in the basement membrane, a, b. Photograph taken by phase-contrast microscopy 10 min after mounting the specimen from a living animal. Compare with fig. 1, c.

B, vertical section through the integument. Shows an axon (a) passing through the subcutis towards the basement membrane which it pierces at b and gives rise to sub-epidermal filaments, one of which is seen at c. Modified Bielschowsky-Gros preparation.

C, oblique section through the integument. Shows the long sub-epidermal course pursued by the thicker of the two collateral axoplasmic filaments. a, aperture in basement membrane; b, thick collateral; c, finer collateral. Modified Bielschowsky-Gros preparation.

D, tangential section through the integument. Shows parent axons giving rise to axoplasmic filaments in the sub-epidermal zone. They diverge from one another and run in a plane at right angles to that taken by their parent axons. Modified Bielschowsky-Gros preparation.

E, oblique section through the integument. Shows threadlike extensions from a parent axon; they form the sub-epidermal plexus. Specimen from a living animal examined by phase-contrast microscopy; picture taken 20 min after mounting.

F, oblique section through the integument. A swollen parent axon can be seen giving rise to a beaded filament lying in a space between two epidermal cells. Picture taken by phase-contrast microscopy 20 min after mounting the specimen (which was taken from a living animal) in 0.02% methylene blue in sea-water.
FIG. 2
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Integument of Amphioxus

seen in 'fixed' methylene blue preparations when high concentrations of dye are used.

Details of our observations on the innervation of the integument covering the dorsal fin in the middle third of amphioxus are summarized in fig. 3.

**FIG. 3.** This summarizes in diagrammatic form the patterned arrangement of the nerves serving the integument covering the dorsal fin in the middle third of amphioxus.

Briefly, it seeks to illustrate that in relation to the integument covering the dorsal fin in a given metamere, every epidermal cell has two or more terminal filaments ending in relation to it; moreover, pre-terminal fibres from parent axons of different diameters are widely and apparently randomly scattered beneath the epidermis. Thus each and every epidermal cell is in contact with terminals approaching from different directions and serving more than one parent dorsal root axon.

**Experimental Observations**

*Unoperated animals*

*Control experiments.* Within less than a minute of being placed in the dish, each animal came to rest on its side. For the next 30 min the position of the animals in the dish did not change appreciably, although some of them exhibited a few feeble wriggling movements at random intervals after observation had begun. These movements, which lasted for less than 5 sec, apparently arose spontaneously but they were not vigorous enough to displace the animal. The maximum number of such movements was 4 in the 30 min during which any of the animals was under observation. Although every effort was made to secure silence, the observer inadvertently introduced noises from time to time.
time but they did not appear to be connected with the movements observed. The deliberate introduction of loud noises evoked no movements.

In view of this it was decided that any movement deemed to have been evoked by stimulation of the integument would have to satisfy two criteria: they would have to be vigorous enough to displace the animal, and they would have to follow stimulation so that the correlation was significant ($P < 0.01$).

**Mechanical stimulation.** A no. 1 nylon suture brought briskly into momentary contact with the dorsal fin in the middle third of the animal evoked no movements.

Light contact of the hardwood probe against the middle third of the dorsal fin and the adjacent dorso-lateral integument on one side invariably caused the animal to wriggle away from the stimulus and immediately to come to rest in a new position in the dish. A rapid brush-like motion of the probe against the integument invariably caused the animal not only to wriggle away from the stimulus but to continue to wriggle around in the dish for up to 10 sec before taking up a new position.

The brisk insertion of a fine sharp needle into the integument covering the lateral aspect of the middle third of the dorsal fin invariably caused the animal to wriggle away from the stimulus and to continue to exhibit wriggling movements for between 5 and 10 sec after the stimulus had been withdrawn. To evoke a movement the point of the needle had to pierce the integument. A blunt needle evoked no movements, even if it was applied vigorously enough to displace the animal in the dish.

**Stimulation with visible light.** These observations were the outcome of an attempt to employ the easiest method of restricting a beam of infra-red rays accurately to specified areas of integument. After the heat-absorbing filter had been exchanged for an infra-red filter, the lamp was switched on for a brief period. This allowed a beam of visible light normally excluded by the infra-red filter to be brought quickly on to the selected area. Illumination of the fin in the middle third of the animal evoked no movement, but as soon as the beam struck the nerve-cord, it invariably and immediately caused the animal to execute vigorous wriggling movements and to thrash around for from 15 sec to 2 min in the dish; the time depended upon the intensity of the illumination.

**Stimulation with infra-red rays.** In view of the effect of visible light, the infra-red rays were focused by sighting and measurement. A single freely suspended thermocouple (40 S.W.G. wire) was gently lowered on to the integument in the centre of the area selected for stimulation (a manœuvre which evoked no movements) and the infra-red generator positioned accordingly. The temperature of the integument was then recorded and the lamp switched on. Both the rise in surface temperature and the time which elapsed between the onset of stimulation and the onset of movement were noted.

Over 20 stimuli, ranging from transfers of 1.2 to 1.8 cal/cm²/sec, were delivered in random order to each of the 5 animals. The total number of stimuli delivered was 170.
Part of a protocol from one of the experimental runs is given in table 3.

**Table 3**

<table>
<thead>
<tr>
<th>Strength of stimulus (cal/cm²/sec)</th>
<th>Interval from previous stimulus (min)</th>
<th>Temperature (°C) of integument</th>
<th>Rise in temp (°C)</th>
<th>Movements exhibited</th>
<th>Duration of stimulus (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>First stimulus</td>
<td>Initial 13.5</td>
<td>Final 18.0</td>
<td>4.5</td>
<td>Sprang out of water</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>13.5</td>
<td>16.0</td>
<td>2.5</td>
<td>&quot;&quot;</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>14.0</td>
<td>17.0</td>
<td>3.0</td>
<td>Wriggled away from stimulus</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>13.5</td>
<td>18.5</td>
<td>5.0</td>
<td>Sprang out of water</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>13.5</td>
<td>17.0</td>
<td>3.5</td>
<td>Wriggled away from stimulus</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>14.0</td>
<td>19.0</td>
<td>5.0</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Table 3 shows the effect of irradiation on specimen no. 18. Length, 5·1 cm. Temperature of water, 13·5°C. Site of irradiation, right postero-lateral middle third of animal.

It was clear from these experiments that the surface temperature of the integument in the area chosen rose by an amount which was dependent upon the intensity of the radiation. It was also found that the relationship between stimulus and movement was significant to a level $P > 0.01$ provided that the rate of transfer exceeded 1.2 cal/cm²/sec. The actual threshold varied, however, from animal to animal and from time to time in the same animal.

In two of the animals in which the thresholds were consistently low, the effect of exposure to a low rate of heat transfer over a fairly long period was investigated. The rate used was below threshold and chosen arbitrarily to cause a rise in surface temperature of approximately 1°C per min. In the case of the first animal, the surface temperature at the centre of the area irradiated had risen by 10·5°C after 10 min but the animal had not moved. In the case of the second animal, the surface temperature at the centre of the area irradiated had risen by 11°C after 10 min, but it likewise had not moved. The first animal was similarly irradiated on three subsequent occasions and the second animal on five subsequent occasions at intervals of not less than 10 min. On no occasion were any movements evoked. After an interval of 2 h the first animal was again exposed to a low rate of heat transfer for 5 min and the temperature at the centre of the area irradiated rose by 5°C; the stimulus was then abruptly increased in strength to 1·2 cal/cm²/sec. Five seconds later, when the surface temperature had risen by a further 1°C, the animal suddenly started to wriggle vigorously around the dish. The second animal reacted in a comparable manner 3 sec after the stimulus had been abruptly increased in strength and the surface temperature at the centre of the area irradiated had risen by a further 0·55 to 0·75°C.
At the end of these experiments, both animals appeared to be just as responsive and appeared to move just as vigorously when stimulated by white light as they had before stimulation with infra-red rays. Twenty-five hours later, both animals were fixed in Bouin's fluid and cut into serial transverse sections, which were stained with haematoxylin and eosin and examined under the microscope. The appearance of the integument, in the areas which had been irradiated, was comparable in every way with that on the opposite side and with that in animals which had not been irradiated.

Operated animals (in which the integument covering the dorso-lateral aspect of the middle and caudal thirds on one side had been removed).

Control experiments. In each of 6 further animals which were similarly irradiated, first on one side and then on the other, with a stimulus of 1.8 cal/cm²/sec, the figures obtained fell within the range of those determined previously.

Stimulation of operated animals. Half an hour after operation, during which time it had not changed its position, each animal was again irradiated on both sides (in random order) with a stimulus of 1.8 cal/cm²/sec. Five animals were similarly irradiated for a second time after an interval of 2 h and two of them for a third time an hour later. The results of the experiments in each of the 6 animals are set out in Table 4.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Length in cm</th>
<th>Time of onset of movement in sec</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal side</td>
</tr>
<tr>
<td>K</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>L</td>
<td>3.7</td>
<td>5</td>
</tr>
<tr>
<td>M</td>
<td>5.3</td>
<td>13</td>
</tr>
<tr>
<td>N</td>
<td>5.2</td>
<td>4.5</td>
</tr>
<tr>
<td>O</td>
<td>3.5</td>
<td>10</td>
</tr>
<tr>
<td>P</td>
<td>5.7</td>
<td>31</td>
</tr>
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</table>

Table 4 shows the response evoked by transferring heat at the rate of 1.8 cal/cm²/sec to the dorsal surface of the middle third of amphioxus on the normal and operated sides.

The examination of serial transverse sections showed that the nerve-cord and the integument on the unoperated side had apparently suffered no damage and that no integument remained on the operated side.

These experiments favour the view that non-injurious infra-red stimuli applied to normal animals are transduced by nerve-endings in the integument as opposed to either the nerve-cord or the 'eye-cups' associated with it.
Integument of Amphioxus

Discussion

In this series of animals, neighboring epidermal cells covering the dorsal fin were indistinguishable in respect of their shape and staining properties. A number of authors, however, have reported seeing a few isolated (commonly darkly staining) cells with distinctive outlines in the epidermis (Joseph, 1908; Krause, 1921). They labelled them neuro-epithelial cells although they had no means of knowing whether or not axons arose from them. In this connexion, however, Franz (1923) voiced the view that in one sense every epithelial cell was a sensory cell, for nerves terminated at the base of each of them. By contrast, Dogiel (1903) explicitly states that he reserved the term neuro-epithelial for cells which not only stained darkly with methylene blue but appeared to fuse with nerve-fibres. He only came across such cells on very rare occasions. Thus, there is general agreement, which is supported by our observations, that specialized cells (presumably specialized receptors) are rare in the epidermis of amphioxus. In seeking to explain why the descriptions of neuro-epithelial cells which have been given lack precision, it occurred to us that the cells in question might have been ordinary epithelial cells but either injured or in a dying condition; dying cells do change in shape and in staining properties. For instance, they shrink and become deep blue before normal cells in tissues stained with methylene blue. Under such conditions they might well appear to fuse with blue-stained nerve-terminals which end in relation to every epidermal cell. There can be no proof of this suggestion, but because such cells are only seen sporadically it is a possibility which cannot be excluded in any discussion on the presence of neuro-epithelial cells in the epidermis of amphioxus.

The exact nature of the nuclei along the course of the nerve-trunks which combine to form the 'dorsal roots' of the nerve-cord has been a matter for dispute in the literature. Some authors (e.g. Hatschek, 1892) have considered that they are the cell-bodies of the first sensory neurone. They argue that, in this respect, amphioxus is primitive and is displaying a characteristic which might have been expected. Most of its sensory nervous system has evolved beyond that of the neuro-epithelial stage towards, but has not yet reached, that acquired in vertebrates, i.e. a definitive dorsal root ganglion system (Young, 1954). Others are emphatic that there are no nerve-cells outside the nerve-cord in amphioxus (Heymans and Van der Stricht, 1898; also Retzius). It was suggested by Fusari (1889) that they were the nuclei of cells similar to those in the dura mater. Franz (1927), on the other hand, suggested that they were the nuclei of cells akin to glial cells. Those who rejected the hypothesis that they were the nuclei of the first sensory neurone (e.g. Heymans and Van der Stricht) pointed out that they can neither be seen clearly when techniques selective for nerve-cells and fibres are employed nor can processes be traced passing from the neighbourhood of the nuclei towards or away from the nerve-cord.
The evidence against the hypothesis that they are the nuclei of the first sensory neurones is growing and now outweighs anything in its favour. In the first place, we have shown that comparable nuclei can be seen along the course of the nerve-trunks which spring from the 'anterior roots', which are wholly motor in function in amphioxus. Secondly, no discrete cell-bodies envelop the nuclei and no processes can be seen passing from their immediate neighbourhood towards or away from the nerve-cord. Moreover, the nuclei are not selectively stained by techniques which can be successfully used to demonstrate nerve-fibres and nerve-cells elsewhere. Thirdly, the nuclei, which appear to contain no sharply defined nucleoli, are smaller than those nerve-cells in the cord; their size is remarkably constant and their number along the course of the ramus dorsalis of the 'dorsal root' and the nerves which subserve it far exceeds the number of parent sensory nerve-fibres which can be impregnated with silver in this purely sensory trunk. In the light of Gasser's (1956) observations, it is possible to argue that the technique which we employed did not impregnate all the axons in the nerve-trunk. On the other hand, the work of Ranson, Droegemueller, Davenport, and Fisher (1935) concerning the relationship between the number of dorsal-root ganglion cells and the number of dorsal-root axons which can be impregnated with silver in mammals suggests that any discrepancy encountered is unlikely to be of the order revealed by our counts, particularly in view of the high ratio we found between the number of parent axons and the area of integument which they collectively served. Thus, the nuclei in question are probably associated with the sheaths which accompany sensory nerves from the basement membrane beneath the epidermis to the nerve-cord, and are therefore likely to be of the nature of the nuclei of the Schwann cells of vertebrates, rather than the nuclei of nerve-cells.

Observations reported in this paper on the patterned arrangement of the nerves serving the integument elaborate upon previous descriptions in two important respects. In the first place, the ratio of epidermal cells to parent axons serving them in randomly selected metameres of integument covering the lateral aspect of the dorsal fin in the middle third of the trunk has been estimated to be as low as 7:1. Moreover, the surface area in square mm divided by the number of dorsal roots serving it is 0.034. In man the smallest figure obtained in this way, which has been reported, is 0.65 (Ranson and co-workers); in the ear of a 3-week-old rabbit it is about 0.175 (figure calculated from Weddell, Pallie, and Palmer, 1955; Weddell and Pallie, 1955; Weddell, Taylor, and Williams, 1955). These figures are not, of course, strictly comparable, and their full significance is unknown, but they do suggest that the density of innervation of the integument of amphioxus in the region of the dorsal fin can hardly be described as meagre. At first glance it certainly appears to be meagre in comparison with that of, say, the cornea; but this is due to the fact that in amphioxus both the collaterals derived from the parent axons and their terminal arborizations are extremely fine. In addition, the terminal arborizations are confined within a narrow zone which is
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difficult to prepare for examination in the form of a continuous sheet of undamaged tissue, for the epidermis consists of a single layer of cells only, and what is more, the thickness of the integument over the dorsal fin is nowhere greater than 30 μ. Thus the only nerves to catch the eye are the bundles lying in the subcutis.

In the second place, a description has been given of the patterned arrangement of the collaterals and pre-terminal branches of parent sensory nerves serving the integument, together with an account of the relationships one to another of the terminals derived from parent axons of different diameters. The examination of successive metameres suggests that these relationships are entirely random although the large number of, and complex pathways pursued by, the fine axons forming the sub-epithelial plexus, make it impossible to be dogmatic on this point. Thus, despite the simple nature of the integument in amphioxus, the patterned arrangement of the nerves serving it is comparable with that in the skin of teleost fish (Whitear, 1952) and the cornea of a number of vertebrate species (Zander and Weddell, 1951a). (Incidentally, the latter comparison was foreshadowed by Zelinka as long ago as 1882.)

Numerous factors determine the characteristic patterned arrangement of the axons and terminals in the integument of lower vertebrates. Among other things, Weiss (1950) cites experimental evidence demonstrating the influence of (1) differences and changes in metabolic equilibrium on the specific growth potential possessed by primary sensory neuroblasts; (2) the specific properties of the tissues through which the tips of their growing processes come to pass; (3) the influence of the growing processes of other neurones which terminate within the same area of integument; and (4) the particular nature of the goal towards which the processes are advancing.

Additional evidence that the final pattern displayed by nerve-fibres in integument is not a matter of pure chance is suggested by the events which take place in the cornea after keratotomy. Zander and Weddell (1951b) have shown that the denervated zone is rapidly re-innervated by sprouts which grow in from surrounding undamaged axons. Remodelling (by successive waves of invasion and degeneration of axons) then takes place until all the severed nerves that are not permanently obstructed have given rise to new processes which re-enter and terminate within the cornea to form a pattern comparable with that seen on the unoperated side.

The fact that a series of morphological readjustments of this kind consistently take place in the cornea after keratotomy might be used to support the generalization of Sperry (1950) that peripheral sensory neurones are each endowed with specificities of a highly refined nature. Further, the close similarity between the arrangement of the nerves in the integument of amphioxus and of those in the cornea make it not unreasonable to suppose that each of the sensory neurones serving the integument in each of the metameres in amphioxus is endowed with a high degree of refined specificity, but there is, of course, no proof either that this suggestion is correct or that,
if correct, it is of any significance in the reaction the animal displays to stimuli received in the course of daily life.

Our observations did not establish the precise mode and place of termination of the nerves serving the integument. In well-fixed preparations the nerves appear to end by attenuation at the base of the epidermal cells as described by Retzius, Franz, and others. By contrast, in fresh (but dying) preparations, filaments can be seen passing between epithelial cells towards the cuticle, beneath which they appear to end blindly, observations more in accord with those of Dogiel. Clearly, the solution of this problem lies in the successful application of methods of microscopy capable of yielding higher magnifications without loss of resolution. Preliminary observations made by Whitear (1957) with the electron microscope indicate that some of the nerve-fibres in the corneal epithelium are situated in deep grooves on the base of epithelial cells. Indeed, casual observation suggests that they are lying within the cytoplasm of the cell-bodies, whereas in fact they invaginate the walls of the cells and remain at all times extracellular.

Parker's (1908) experimental observations have been amplified by the demonstration that nerves in the integument of amphioxus can transduce non-injurious stimuli resulting from heat transfer by irradiation (at a rate of 1-2 millical/cm²/sec or more) into nervous activity sufficient to cause displacement of the animal by undulating movements involving the whole trunk.

Some of the stimuli chosen by Parker for application to the integument had physical attributes equivalent to those which (by common consent), when delivered to the skin in man, evoke reports of touch, warmth, cold, and pain. Most of the other stimuli he chose (solutions of chemical substances) would evoke reports of pain if applied to exposed mucous membranes in man. In addition, he observed the behaviour of animals in the tanks in which they were living and noted in particular that amphioxus is very sensitive to sound. Even the noise Parker made on entering the darkened tank room as quietly as he could caused the animals to wriggle more deeply into the sand. He advanced evidence in favour of the view that noise was effective because it gave rise to vibration and hence mechanical stimuli which were transduced by nerve-terminals in the integument. We have confirmed Parker's observations in relation to animals living in a tank room but we failed to evoke any significant reactions to sound from animals which were lying in a glass-bottomed dish.

Franz (1924) also reported an interesting observation in relation to the stimulation of relatively large areas of integument. He noted that the mechanical stimulus which invariably invoked the most vigorous and prolonged outbursts of movement was a brushing contact of the integument anywhere over the body-surface delivered by another amphioxus swimming in the immediate neighbourhood.

Ten Cate (1938b) was the first to draw critical attention to the artificial nature of the experimental procedures employed by many workers in this
field. For example, he demonstrated that a standard mechanical stimulus, such as a needle prick, was far more effective when applied to animals which had been permitted to bury their bodies in the sand than it was when similarly applied to animals isolated in glass-bottomed dishes. He also demonstrated, in relation to needle pricks, that the nervous system in amphioxus is so organized that the only response which can be evoked is displacement forwards or backwards (away from the stimulus) resulting from co-ordinated undulatory movements involving the musculature throughout the body. This reaction, as he pointed out, is closely related to the particular needs of amphioxus in the environment in which it lives. In other words, in the case of amphioxus (as opposed to man), in its normal habitat, the effectiveness of a needle as a stimulus object is related both to the fact that it pierces the integument locally and the fact that it gives rise concomitantly to a series of punctate stimuli over a wide area of integument by grains of sand tending to resist the displacement of the animal by the needle.

In this connexion, Rohon (1882) and Kohl (1890) deduced that light was not an effective stimulus; but their experiments were carried out on amphioxus almost completely buried in the sand, as they are normally in nature. In both Krause's (1897) and our own experiments there was no doubt about the effectiveness of light stimuli, but in both cases the animal was completely uncovered in a flat-bottomed dish so that all the eye-cups in the cord were exposed, a condition which occurs but rarely in nature (Ten Cate, 19386).

From the evidence available, then, it appears that the gross motor reactions of amphioxus to stimulation are most easily explicable on the basis that they are dependent upon the site of origin as well as the number and patterned arrangement of the nerve impulses which reach the cord; they are not necessarily dependent upon the immediate physical attributes of the stimulus which evokes them. As Ten Cate has already pointed out, to make a really critical assessment of the organization of the mechanisms underlying the functional activity of the nervous system, it is necessary to study further the pattern of the reactions of amphioxus to the changing environmental conditions in its natural habitat. A study confined to noting its reactions to stimuli which obviously link man with the changes in his own environment may be very misleading.

When considered from this point of view, it is clear that in its normal habitat amphioxus is unlikely to receive stimuli having physical attributes similar to some of those employed by Parker. The choice of radiant heat as a stimulus to be delivered to a small area of integument in the region of the dorsal fin of an animal isolated in a glass dish was clearly in the nature of an academic exercise and far more unnatural than any of the experiments carried out by Parker. However, the results of these experiments demonstrate quite unequivocally that free nerve-endings in the integument of amphioxus can transduce non-injurious stimuli having physical attributes which are of a kind not encountered in the natural habitat of the animal.
It is of some interest that unencapsulated nerve-endings in the integument (though apparently not in the case of amphioxus) can, under certain conditions, transduce light stimuli. For instance, Parker (1903) advanced experimental evidence for the view that light can be transduced by nerve-endings of spinal origin in the skin of the frog. In the case of the lamprey there is experimental evidence that unencapsulated nerve-terminals in the skin of the tail transduce light stimuli (Young, 1935). However, the nerves serving these particular terminals are of cranial origin and the terminals themselves are related to epidermal cells which appear to be specialized to assist in the transducing of light stimuli (Steven, 1951). Such observations serve to add to the number of stimuli with different physical attributes which unencapsulated nerve-terminals in the integument of different animals are capable of transducing. They also make it clear that certain unencapsulated nerve-terminals (morphologically indistinguishable from their neighbours) in the integument are in some way differentiated to transduce stimuli having specific attributes. Thus it is possible to argue that some terminals in the integument of amphioxus may have a low threshold to either positive or negative heat transfer, as Parker originally claimed. However, our experimental observations in relation to the effectiveness of unnatural stimuli suggest that it is most necessary to remember that functional specificity may only apply in relation to one or more of the range of stimuli which normally impinge on a specific zone of integument covering a particular species of animal living in surroundings that are natural to it.

Precise information as to the specificity of nerve-terminals in the integument of amphioxus in relation to stimuli having physical attributes likely to be encountered in its normal habitat can only be obtained directly by recording from single sensory nerve-fibres—technically a difficult task. Moreover, it will not be sufficient to record from single parent axons serving a given area of integument at random. The activity in each and every axon serving the area will have to be recorded in relation to each stimulus object, for Lele (1954) has shown that in human skin free nerve-endings are not related to thermal sensibility in a direct and simple way. Indeed, his work forces one to the conclusion that the temporo-spatial pattern of activity reaching the central nervous system must be a factor of some importance in relation to modality discrimination. This in turn indicates that the patterned arrangement of nerve-fibres and their terminals in the skin is also of importance in this respect. There is thus every reason to suppose that the complex patterned arrangement of nerve-fibres and endings in the integument of amphioxus, which in many respects resembles that in the skin of man very closely, may, in addition to the possible existence of 'modality specific' nerve-terminals, play a part in determining the response evoked by a given stimulus object.

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