

A Quantitative Study of the Organization and Trophic
Regulation of Cutaneous Mechanoreceptors in the Salamander

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ABSTRACT

Partial denervation of a shared target tissue leads to 'collateral' sprouting of the remaining nerve fibres supplying the same region. The mechanisms responsible for this widespread phenomenon are unknown, but it has generally been believed that products from the degenerating fibres constitute the sprouting stimulus. Recently it was shown that a similar sprouting of intact nerves occurred in the salamander hindlimb when the axoplasmic transport in one of the neighbouring nerves was blocked by colchicine. This block did not, however, appear to interfere with the functioning of the treated nerve. On this evidence it was proposed that nerves release factors which are concerned in the regulation of their peripheral fields. The experiments of this thesis test this hypothesis further. In particular this study quantitatively examines the organization of the cutaneous mechanoreceptors of the salamander hindlimb, and the effects of various nerve treatments on this organization.

A mechanical prodder of 10-50 μ tip diameter was used to determine the sensory threshold of the skin at selected spots. The mechanoreceptors were found to be all rapidly-adapting. An analysis of the distribution of their thresholds across the skin points to the existence of a single population of mechanosensory endings of similar threshold which are fairly uniformly distributed in the plane of the skin. A simple model, based on the results, indicates that these receptors have receptive field sizes of about 50 μ , and are spaced approximately 150-200 μ apart. Direct

inspection of data from systematically surveyed skin areas gave findings which were consistent with these estimates. These quantitative results made it possible to establish that the blocking of fast axoplasmic transport does not affect the sensitivity or distribution of the individual mechanosensory endings of the treated nerve. Nevertheless new sprouts appeared from the adjacent nerves, and it seemed that there was a tendency for these to grow preferentially to the site of the still-functioning endings of the treated nerve. Most significantly, after partial denervation of the skin the number of newly sprouted endings quantitatively matched the number of endings lost by nerve section.

It was concluded that there is a control mechanism which continuously regulate the density of the skin innervation. This control system probably involves the mutual interaction of substances continuously secreted from the target tissue, which cause nerves to sprout, and factors released from the nerve endings which offset the effects of the growth stimulus. The nerve field density reflects an equilibrium state between the neural and tissue influences.

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SUMMARY

1. The major objective of this thesis was to study the mechanisms responsible for collateral sprouting of nerves after partial denervation. The particular hypothesis investigated was that nerves are stimulated to sprout by growth substances released from the target tissue, and that this growth stimulation is offset by factors transported along the nerves and released from their terminals. Removal of these neural factors (by nerve section) results in a rise of target factors until the compensatory sprouting is sufficient to balance them out again.

2. The experimental preparation was the sensory innervation of the hindlimb skin in the salamander. The study aimed at a quantitative description of collateral sprouting, and this required the investigation of the organization of the mechanosensory axonal fields, and of the distribution of the individual mechanoreceptors in the skin. By selectively stimulating points on the skin with a 10 μ diameter prodder while simultaneously recording from the segmental nerve trunks it was possible to activate single sensory units. All units were rapidly adapting; there appears to be no slowly adapting mechanoreceptors in the salamander skin.

3. An occlusion technique involving two separate prodders was used to map the receptive fields of individual axons. This method did not require dissecting down the segmental nerves into single units, and

so facilitated the mapping of adjacent axonal fields. Each axon appeared to innervate its own territory of skin with only a little overlap of the neighbouring fields (i.e. there is a mosaic organization); these axonal fields ranged from 0.5 mm^2 to 4.0 mm^2 in area.

4. The distribution of mechanoreceptors in the skin was investigated by making a random survey of the sensitivity of the skin to point mechanical stimulation. Points in the highest sensitivity range for touch were located most often, and points needing the highest strength of stimuli were located least often. When the detailed distribution of sensitivity of the skin was analyzed, both by theoretical and by experimental means, the results strongly suggested that there is in fact only one population of mechanoreceptors in the skin. These receptors are uniformly distributed throughout the skin area and their thresholds are fairly constant, ranging from $0.5 \mu/\text{msec}$ to $1.0 \mu/\text{msec}$. The receptive fields of these receptors were estimated to be 50μ in radius, and they are spaced approximately $150\text{--}200\mu$ apart. These values agreed with ones based on inspection of the thresholds obtained in systematic surveys of all spots in selected areas of skin.

5. In previous experiments adjacent nerve sprouting was shown to occur after blocking fast axoplasmic transport in salamander nerves by treating the nerves with colchicine. This finding prompted the formulation of the hypothesis for collateral sprouting mentioned above. The possibility that the colchicine block caused a scattered degeneration

of some terminals of the treated nerves was excluded by showing that the distribution and sensitivity of the mechanosensory endings of treated nerves was no different from those of control nerves, on the opposite side of the animal.

6. Another possibility, that colchicine was causing sprouting by having a direct action on the target tissue on the treated side, was examined by using radioactive colchicine in similar doses to those needed for sprouting. The amount of labelled material reaching the skin was very low and was the same on both sides of the animal. It was concluded that adjacent nerve sprouting resulted from the interruption of factors carried in fast axoplasmic transport in the treated nerves.

7. A quantitative study was made on the effects of partial denervation on the sensory innervation of the skin. After partial denervation of one nerve to a shared skin region, the remaining intact nerves sprouted; the number of new sprouts quantitatively matched the number of endings which had degenerated. Furthermore, from analysis of the sprouting after colchicine treatment it appeared that the new sprouts grew preferentially to the sites of the endings of the treated nerves. These findings are also consistent with the proposed hypothesis.

8. A comparison of the response to partial denervation during the summer and winter months indicated that although collateral sprouting readily occurred during the summer it seldom happened in the winter. The process of nerve regeneration, however, resulting in normally functioning mechanosensory endings, did not appear to be affected by

seasonal changes. The significance of this finding in relationship to the proposed stimulus for collateral sprouting is discussed.

9. It is concluded from these experiments that there is a mechanism in the adult able to control the density of innervation of nerve fields continuously. This control system most probably involves the mutual interaction of substances released by the target tissue, which causes nerves to sprout, with factors released from nerves which offset the effects of the growth substance. Elimination of the neural factors, either by partial denervation or by blocking axoplasmic transport, leads to a build-up of the growth substances and therefore sprouting of intact nerves. Sprouting stops when the equilibrium between neural factors and the target substance is restored.

I. GENERAL INTRODUCTION

The search for changes in individual neurons which may underlie learning and memory in man is one of the most difficult in Neurobiology. In view of the complexity of this undertaking, many scientists have pursued relatively simple models in attempts to demonstrate basic mechanisms of neural behaviour that might be involved in such sophisticated activities of the brain. One approach has been particularly successful. It is the study of the nervous system at a time when a very large number of new connections are being made, that is, during early development. Among the many phenomena which such studies reveal is the ability of nerve cells to modify the pattern of their terminal arborizations by sprouting new branches and reabsorbing old ones. New connections made as a consequence of sprouting would lead to a change in neural circuitry, and this could well be a mechanism involved in, for example, learning.

The sprouting of nerve branches does not only occur during primary development, when the need is evident, but also during adult life. It is very well documented that when partial denervation of a target-tissue takes place, the undamaged nerves sprout new branches which can tend to restore the original innervation to the territory (Edds, 1953; Moore, 1974). A widely held explanation for this "denervation sprouting" is that the undamaged axons are stimulated to sprout by degeneration products of the sectioned nerves; the phenomenon could thus serve as a useful reparative mechanism. However, there are in-

stances where similar sprouting happens in the adult nervous system without denervation (Duchen and Strich, 1968; Olson and Malmfors, 1970; Aguilar, Bisby, Cooper and Diamond, 1973). Although in this case the stimulus which causes sprouting has not been identified, it certainly cannot be "products of degeneration". This raises the question as to whether such products of degeneration truly stimulate sprouting at all.

When nerves are sectioned, not only do they degenerate, but impulses in them are blocked, as is the axonal transport of material to and from the cell bodies. Conceivably, therefore, the interruption either of the transport system or of impulse conduction could be involved in stimulating adjacent nerves to sprout. In fact, there is good reason to believe that some factor or factors, carried down nerves by axoplasmic transport, are involved in sprouting (Aguilar, Bisby, Cooper and Diamond, 1973).

The major focus of the investigations described in this thesis is the further testing of the concept that the initiation of collateral branching of intact nerves after adjacent ones are cut is a consequence of the elimination of fast axoplasmic transport in the sectioned nerves. Furthermore, by measuring this nerve sprouting quantitatively, it was possible to investigate whether new sprouts grow randomly both in number and in location, or conversely whether they are subject to some control mechanisms, involving the target tissue, which operate to maintain a characteristic density of innervation.

II. HISTORICAL INTRODUCTION

1. Functional Significance of Axonal Branching

Although the axons of almost all neurons branch to some degree, there are only a few quantitative studies on the functional significance of axonal branching. A neuron acts as a unit, in that the nerve impulse usually invades all branches of the axon; the extent of axonal branching, therefore, is one significant measure of the influence a neuron exerts over its target. For sensory nerves it measures the proportion of the total sensory surface "represented" by a single neuron. In the mammal the extent of branching in a motoneuron axon is given by the ratio of the total number of muscle fibres in a muscle and the number of motor axons in the nerve that innervates it. The ratio gives the size of the motor unit, the average number of muscle fibres innervated by each motoneuron, assuming each muscle fibre is singly innervated. It varies widely from muscle to muscle in the body, and appears to reflect the functional requirements of that muscle. By having relatively few motor units of large sizes (approximately 1,000 muscle fibres each) the massive leg muscles can achieve a restricted but adequate range of powerful contractions, appropriate for support and movement of the animal. The small muscles of the finger, however, are controlled with precision by many axons, each of which, however, branches to innervate only 5 to 10 muscle fibres (Buchthal, 1958).

A similar correlation between the number of axonal branches and neuronal function exists in the sensory system and in the central

nervous system. The tips of the fingers, used to feel and discriminate textures, have a much higher density of sensory innervation (that is, number of axons per unit area of skin) than does the skin of the back (Arthur and Shelley, 1959; Sinclair, 1967). Although this consideration does not directly indicate the degree of branching of individual axons, it is probable that some axons must branch extensively to "represent" a large surface (on the back). In the Central Nervous System (CNS), the importance of a neuron in a circuit may depend largely on the number of target neurons it contacts by axonal branching. The influence that one neuron exerts on an individual target neuron depends in part, on how many times it branches to make synaptic contacts on it (Eccles, 1964; Rall, Burke, Smith, Nelson, and Frank, 1967). (The location of the synapses is also important.)

As a generalisation, it would seem that the more branching there is, the more important is the individual neuron, but the less precise and refined the overall control. When the latter qualities are required, branching of individual neurons is reduced, and more neurons are harnessed to work cooperatively. Even a modest change in the branching patterns of these neurons, therefore, could possibly have an important overall influence.

2. External Influences Modifying Circuitry During Development.

Even though the functional significance of axonal branching can be inferred, it is still unclear what determines its extent and pattern. One possibility is that this branching is probably largely specified by the genetic

information within the cells, the expression of which minimizes possible errors in connections between nerve cells and their targets. However, there is evidence to indicate that external influences play a large role in the determination of the final details of a circuit, and probably, therefore, in the extent of branching achieved by the individual neurons in it. Some circuits need an appropriate amount of incoming sensory activity for their continued maintenance (Wiesel, and Hubel, 1963). Other circuits depend on hormones for their successful development and maturation (Gorski, 1971). Still others appear to require some unidentified stimulus from their end-organs in order that their functional characteristics can be established (Miner, 1956). A few examples will help to make these points clear.

a) One preparation which has shown the need for sensory input on the maturation and maintenance of circuitry is the mammalian visual system. Kittens' eyes do not open until about ten days after birth, and therefore in this animal there was a good opportunity to determine which of the "adult" characteristics of the visual system are already specified genetically before birth, and to what extent the genetic information can be modified by visual stimulation. The experiments of Hubel and Wiesel on new-born kittens and on adult cats revealed that approximately 80% of their cortical neurons in both age groups respond to an appropriate visual stimulus represented in exactly corresponding locations in the visual fields of their eyes, or to both simultaneously (Hubel and Wiesel, 1962; Hubel and Wiesel, 1963). Many of the visual connections concerned with "binocularity" therefore, appear to be specified before birth, and do not depend on visual experience. However, if one pair of eyelids

was sutured closed at birth, and reopened only after three months, visual inputs to that eye failed to influence any of the neurons in the visual cortex, even after a year of subsequent visual experience (Wiesel and Hubel, 1963). From these results, and others (Hubel and Wiesel, 1965; Wiesel and Hubel, 1965) it can be concluded that although many neural connections in the visual system of the brain appear to be present at birth, an appropriate visual experience is necessary for their subsequent maintenance.

b) The role of testosterone in initiating the differentiation of hypothalamic neural circuitry is a good example of how extra-neuronal (hormonal) influences can modify nervous development (Gorski, 1971). The behaviour of the hypothalamic neurons which control pituitary release of gonadotrophic hormone is characteristically different in the two sexes. However, a single injection of testosterone to females or castrated males within the first few days after birth, results in the masculine pattern of pituitary "drive" (and indeed in male sexual behaviour) in the mature animal. In the absence of the hormone, the feminine "drive" develops, seemingly entirely under genetic direction, in rats of both sexes. Although the neurons mediating male sexual behaviour have not yet been adequately identified, and the direct effect of testosterone on them is not understood, the hormone almost certainly influences development of hypothalamic circuitry. Presumably, therefore, it could well affect the branching patterns of the processes of the young neurons in the hypothalamic region of the brain, and possibly in other areas also. Recently, Raisman has reported at a meeting (although not yet in published form) that he finds, when examining the hypothalamus

with the electron microscope, a higher number of non-amygdaloid synapses on dendritic spines in the pre-optic area in normal female rats compared to males. Furthermore, castration of male rats one day after birth, increases the number of dendritic spine synapses in the same area to the level found in normal female rats. However, this does not occur if the castration is performed later than seven days after birth. Furthermore, injection of testosterone into female rats during this same critical period reduces the number of dendritic spine synapses in the pre-optic area of these animals to that of the normal male rat.

c) The experiments of Miner (Miner, 1956), and later of Jacobson and Baker (Jacobson and Baker, 1969), indicate that during early life the skin can influence the development of central circuitry. Light touching of the skin of the frog with a fine brush evokes specific localizing reflexes, in that the animals respond by wiping the stimulated spot with the appropriate limb. Miner questioned how these specific reflexes developed, and suggested two probable explanations; either the outgrowing cutaneous fibres selectively seek out their proper ("predestined") terminal sites in the integument, or they grew out randomly to the skin, and acquired their positional specificity after they make contact with the skin. In the latter case the central reflex connections would develop on the basis of the later-acquired positional information, and not, as in the first explanations, *de novo*. To test these possibilities, Miner performed skin rotation in young tadpoles before the limbs had developed, and tested their cutaneous localization reflexes after metamorphosis. She dissected out a large transverse area of skin, and replaced it with the dorsal-ventral orientation reversed. In

this way, the dorsal nerves came to innervate ventral skin, and vice versa. After metamorphosis, stimulation of the transplanted skin (rotated 180°) evoked completely misdirected reflexes. When she now stimulated the belly skin (which was originally dorsal skin of the tadpole) the frogs responded by wiping their backs; and when she stimulated their backs (which was originally belly skin) they responded by wiping their bellies. These results indicate an ability of a target-tissue to specify at least some of the functional characteristics of the central circuitry underlying these reflexes. Again, it seems probable that appropriate branching with development of functional connections must have been induced in the spinal neurons.

3. Effects of Target Factors on the Growth of a Neuron

It appears, therefore, that afferent activity, hormonal interactions, and unidentified factors from the target tissue, can all modify the development of neural circuitry. It is not clear, however, how these modifications are produced at the cellular level but it seems very likely that altered neuronal growth and axonal branching are involved. Particularly relevant to the present study is the influence that an end organ can have on nerve growth. In his classic papers on the genesis of epithelial innervation, Ramon y Cajal described a growth pattern of the sensory nerves that suggested the operation of an important influence from their target-tissues (Ramon y Cajal, 1919). He observed that the incoming fibres often grew relatively long distances to reach the epithelial tissues, but only after arriving at them did

the nerves start sprouting collateral branches, each growing to a territory devoid of nerves. This sprouting eventually stopped, and Ramon y Cajal noted the absence both of any vast aneuritic spaces, and of any excessive collection of nerve fibres. He concluded from these observations that there are growth promoting influences emanating from the target tissue, and that these influences ultimately become offset or neutralized in some way by other factors released from the nerves themselves. From Ramon y Cajal's description it appears that the postulated target-tissue influences may be conveniently separated into two kinds. One attracts the nerve to the target; at the target, another influence stimulates the nerve to sprout branches.

4. Attractive Influences

Before discussing the factors that cause sprouting, the still controversial attractive powers of the target need brief consideration. During development it appears that nerve fibres, both in the periphery, and in the CNS, grow relatively long distances, by-passing many other neurons on the way, to connect with specific groups of cells. How much of their growth is genetically predetermined and how much is influenced by their immediate environment is still largely an unanswered question. Detwiler showed that limb buds can attract nerves to grow towards them (Detwiler, 1936). A salamander forelimb (normally supplied by nerves arising from the brachial segment of the spinal cord) when grafted to the trunk region, attracted trunk nerves to it; a limb grafted to the head became exclusively innervated by the cranial nerves. While

such experiments suggest that the end-organs can attract nerves to grow to them they do not give any clues as to the nature of the attractive force. In Detwiler's experiments this influence appeared to be largely unspecific, and could cause any nerve to grow to the limb-bud. Possibly any rapidly-growing peripheral tissue might, as a consequence of the increased metabolic activity, release side-products which have this effect (Speidel, 1941).

After the embryonic stage is over, there are many recorded instances in which regenerating nerves grow back precisely to their original location, indicating a specificity of attractive influences. When motor nerves are crushed or cut they tend to regenerate not only to the same muscle that they originally innervated, but to the same location on each muscle fibre (Miledi, 1960; Frank, Jansen, Lomo and Westgaard, 1975). Similar evidence exists for the visual system. In lower vertebrates there is a very orderly projection of the retinal ganglion fibres from the eye to the optic tectum in the brain (Gaze, 1970; Jacobson, 1970). Even though the nerve fibres regenerating after section grow randomly through the scar, after making contact with the optic tectum they appear to be capable of sorting themselves out, and the original orderly projection is largely restored. The great ability that these fibres have in finding their way back to their original location can also be demonstrated by cutting the optic nerve, and rotating the eye 180° (Attardi and Sperry, 1963). In these cases examination of the regenerating fibres, both by histological and electrophysiological techniques, revealed again that the optic nerves appeared to grow back to their precise location in the brain; as a consequence

these animals perceived the outside world upside down, and exhibited totally misdirected visuo-motor reflexes in response to stimuli in their visual field. These examples demonstrate that the influence target cells may have on the growth of regenerating nerves can be quite specific, and contrast with the limb-bud experiments mentioned above.

Nevertheless, any end-organ influence, manifested either during embryonic development or during the later more selective stages of nerve regeneration, might well be effective through one common mechanism acting at the level of the growing neuron. Among the many proposals that have been put forward, the two mechanisms that have received general recognition are (1) contact guidance, proposed by Weiss (Weiss, 1955), and (2) chemotropism, proposed by Ramon y Cajal, and subsequently revitalized and modified by Sperry (Sperry, 1965). The contact guidance theory proposes that there are mechanical factors in the immediate environment which preferentially align the growing tip of a nerve, and so facilitate its continued extension in a particular direction. This theory helps to explain why regenerating nerves succeed in reaching their end-organs more often when directed into a degenerating nerve stump, than if this precaution was not taken, (a result much appreciated by neurosurgeons). The other hypothesis, chemotropism, supposes the existence of a mutual chemoaffinity between particular chemicals liberated by the target tissue, and some features of the nerve tip which "recognizes" them, a process which may be not unlike antigen-antibody interactions. The continual secretion of these chemicals, specific for each end-organ, must create a concentration gradient which is detected by the "recognizing" elements, and so ensures the guidance of the nerve to the proper location.

There is good evidence that both contact guidance and chemotropism operate. Both mechanisms involve physio-chemical interactions between nerve fibres and their environment, but only chemotropism offers the subtle range of specificity required to explain the complexities of development in the nervous system.

5. Collateral Sprouting at the Target During Development

The phenomenon which is more relevant to this present thesis, is that of local nerve sprouting when the growing fibres arrive at the end organ. As mentioned above, Ramon y Cajal originally suggested (for the situation obtained in primary development) that the skin, for example, produces a local stimulus which causes nerve branching. In a series of visual studies on the terminal branches of living nerves growing into the tadpole tail during its primary innervation, Speidel made observations which supported Ramon y Cajal's idea (Speidel, 1932, 1935, and 1941). The skin of the tadpole tail is transparent, and Speidel was able to see that the majority of the new cutaneous fibres grew directly towards the skin, and then continued to grow as the tail grew larger by sending out collateral branches. A few aberrant branches sprouted into the deep tissues away from the skin, and presumably because these would be inappropriate as cutaneous receptors, were apparently subjected to continual remodelling. Some branches growing deep into the tissue would suddenly change their direction and turn superficially to establish connections with the skin; others retracted variable distances to change their direction, then continued to grow

towards the skin. Still other deeply-growing nerves eventually stopped and degenerated completely.

Although the studies of Speidel and Ramon y Cajal revealed that such collateral sprouting occurs almost entirely at the end-organ, the suggestion that it is provoked by a stimulus from the target-tissue is only conjecture. A later study by Fitzgerald on the primary innervation of the epidermis of the pig's snout contains more convincing evidence on this point (Fitzgerald, 1961). In this organ the number of dermal axons present at birth remains constant, but they are continually providing branches which grow up to the epidermis through the epidermal ridges to exist there simply as free nerve endings. Fitzgerald observed that these endings increased in proportion to the increase in number of epidermal ridges after birth, which strongly suggests that the epidermis is the source of a stimulus for the dermal axons to sprout collateral branches.

6. Regulatory Effects of Neurons on End-Organs

If the degree of branching of a neuron has functional significance, as mentioned earlier, then the sprouting at the target must be controlled in some way. It would seem that the amount of the stimulus provided, and the length of time that it is available, must be the principle determinant. These two factors, (the amount and the duration of stimulus) could be specified by genetic information within each target cell, however, one or both could be regulated by other factors, for example, something released by growing nerve fibres themselves. It seems un-

likely that the duration of the sprouting stimulus action is specified genetically. In experiments in which the target-tissue (for example, a limb) was prevented from receiving a nerve supply, and then after varying times grafted to a new site where nerves were present, it was found that innervation occurred with the same pattern and to the same extent as it would have had if it had developed normally (Piatt, 1942). There was no critical period during which the target stimulus was available.

There is good evidence that nerves can act as "trophic" regulators as distinct from their role in regulating moment-to-moment activity of the target. The maintenance of some sensory structures is under control of their sensory nerves. If the nerves to the tongue are cut, the taste buds disappear; then reappear after the same nerves have regenerated (Guth, 1958; Zalewski, 1969). Some lower vertebrates, such as salamanders, have a remarkable capacity to regenerate severed limbs. This regeneration process, however, is dependent on nerves, and the limb-bud stops regenerating (or this is greatly reduced in rate) if its nerve supply is sectioned (Singer, 1952, 1974). In some mammals, a muscle that has been minced appears to be capable of regenerating and reforming a complete muscle, but only if a nerve supply is available to it (Hsu, 1974). There is also evidence that neurons in the central nervous system are necessary for the proper maintenance of some end-organ structures on their target neurons, such as dendritic spines (Valverde., 1967).

Although the neural mechanisms involved in these trophic regulatory influences are not understood, the two most likely candidates are impulse activity (with concomitant transmitter release and effector

cell activity), and trophic substances released from the nerve terminals. Because some of these trophic phenomena are attributed to sensory nerves, where the impulse normally travels away from the innervated tissues and not towards them, impulse activity would seem to be an unlikely candidate. Nevertheless, it can not be ruled out completely because antidromic impulses invade the nerve terminals via axon reflexes. More conclusive evidence that tends to rule out the involvement of impulse activity in some trophic regulatory processes comes from denervation experiments, in which nerves were cut at different distances from the end-organ (Miledi and Slater, 1970). The onset of denervation changes was in fact directly correlated with the length of the remaining nerve stump attached to the end-organ; the longer this length, the longer the onset of denervation changes. This correlation is consistent with the idea that trophic substances are available in an axon "pool" and are released from the terminals to act on the target tissue. These results also suggest the possibility that these chemicals are flowing down the axon, and indeed the rate of their transport can be calculated by dividing differences in stump length by differences in the time of onset of denervation changes. Parker found that after cutting the nerve trunk supplying the lateral line organs in the catfish, these organs, which extend from the head to the tail along the trunk in these animals, degenerated in a particular manner. The ones closest to the site of nerve section degenerated first, followed by the next most proximal ones and the wave of degeneration travelled down the trunk at a rate of 20-30 mm per day (Parker and Paine, 1934). Similar experiments on mammalian skeletal muscle give rates of flow of postulated trophic substances

of about 360 mm per day (Miledi and Slater, 1970).

It is now well established that the substances made in the cell body travel down nerves by two separate systems; one is by slow flow, usually at 1-2 mm per day, the other system is called fast axoplasmic transport, by which some substances move at a rate up to 500 mm per day in some mammals (Jeffery and Austin, 1974). The estimated rates for trophic substances, as calculated by the method mentioned above, are in the range of fast axoplasmic transport. It seems very likely, therefore, that nerves exert their regulatory influences by continually releasing chemical substances that are made in the cell body and carried down the axon by fast axoplasmic transport.

7. Collateral Sprouting in Adults After Partial Denervation

Nerve sprouting occurs not only during primary development, but during adult life as well. Recent studies on the innervation of skeletal muscle indicate that the motor nerve endings are not static, but are gradually turning over, with new endings continually being formed as others degenerate (Barker and Ip, 1966; Tuffery, 1971). However, the most effective way to demonstrate the phenomenon of collateral sprouting of adult nerves is by partially denervating a given target-tissue. When this is done, the remaining intact nerves sprout branches in what is usually assumed to be an attempt to restore the original innervation pattern. This "denervation sprouting" appears to be a widespread phenomenon, occurring certainly in all vertebrates at any stage of life, and in virtually all nerves, peripheral and central, somatic and autonomic,

motor and sensory (Edds, 1953; Murray and Thompson, 1957; Moore, 1974).

Possibly, the clearest demonstration of denervation sprouting was that of Speidel, who used the techniques mentioned earlier, of direct microscopic observation of cutaneous nerves in the transparent skin of the tadpole tail (Speidel, 1933, 1935, 1941). He observed that 3 days after sectioning one of these nerves, the axons from a nearby region started sprouting collateral branches. These new sprouts were quick to restore the initial innervation pattern (about 14 days). Weddell, Gutmann and Guttman using physiological and histological techniques, obtained similar evidence of collateral sprouting for the sensory nerves to the rabbit hindlimbs (Weddell, Gutmann and Gutman, 1941). Immediately, after cutting the sural nerve, Weddell et al. found that the heel region was totally unresponsive to painful stimuli, as measured by reflex withdrawal of limb on pinching the skin. Using histological techniques they confirmed that, within one week after sural nerve section there was a total absence of normal myelinated nerves in the skin of the heel region. However, some two weeks after nerve section the anaesthetic region began gradually to reduce in size, and in one case eventually completely disappeared by 20 weeks. Weddell et al. interpreted their results as being due to collateral sprouting of sensory fibres of the adjacent peroneal and tibial nerves, since it was impossible in their experiments for the sural nerve to have regenerated in that time. When they subsequently examined the sural area histologically, they detected many new growing fibres in that region, which supported their interpretation.

In a series of experiments on partial denervation of skeletal

muscle, Edds, Weiss, Hoffman, Van Harreveld and others, quantitatively demonstrated, both histologically as well as functionally, that motor nerves were also capable of collateral re-innervation (see review, Edds, 1953). Weiss and Edds obtained indirect evidence that collateral sprouting occurred as early as one week after partial denervation of the hindlimb muscles in the rat (by sectioning the ventral spinal root L5) (Weiss and Edds, 1946). They could detect changes in the response from the adjacent intact nerves to these muscles: maximal stimulation of neighbouring segmental nerves (L4 and L6) contributing to the sciatic plexus caused a larger tension from the hindlimb muscles than occurred in the corresponding muscles on the other side of the animal, when the same contralateral segmental roots were stimulated. Normally, the two sides are symmetrical in this regard, so the "control" side represents the experimental side before surgery was performed. The increase in the tension response continued for six to eight weeks, until the total tension evoked by stimulating the intact roots, L4 and L6, equalled that produced when all three roots, L4, L5 and L6, were stimulated together on the control side of the animal. This increase in tension could of course be attributed to hypertrophy of the remaining innervated muscle fibres on the experimental side, and not to sprouting of the intact nerves into the denervated muscles. However, Edds and Hoffman, working independently, used techniques to stain nerve fibre and motor end plates, and showed that sprouting of the intact axons had indeed occurred (Edds, 1950; Hoffman, 1950). As early as one week after ventral root section, they could see new branches sprouting from intact intramuscular axons, at distances no

further than a millimeter back from their terminals. On an average, an axon could increase the number of its terminal branches to support almost three times the normal number of end plates.

Sprouting of intact autonomic fibres also occurs after partial denervation of their target tissue. Murray and Thompson showed that after sectioning preganglionic roots T1-T3, 90% of the fibres to the superior cervical ganglion degenerated, including the nerves responsible for pupil dilation (Murray and Thompson, 1957). Consequently, many of the ganglion cells became hypersensitive to the pre-ganglionic transmitter, acetylcholine; this hypersensitivity, however, declined after one month. At this time stimulation of the remaining pre-ganglionic nerves to the ganglion, T4-T7 (which normally have no influence on the pupil), now evoked pupil dilation. One explanation for these results is that the intact fibres had sprouted and made connections with ganglion cells not previously innervated by them. Pursuing this idea with light microscopic techniques, Murray and Thompson could detect collateral branches sprouting from the remaining fibres. However, these studies could not reveal whether these actually made new functional connections.

This work was confirmed by Guth and Bernstein, who also extended the findings by allowing the originally interrupted nerves T1-T3 to regenerate back to the ganglion (Guth and Bernstein, 1961). It took these nerves six months to find their way back to the ganglion, and recover their original function. It is a surprise that at the time T1-T3 regenerated and recovered function, nerve T4 lost its newly-gained influence over the pupil. No morphological study was performed so it is unclear whether the endings of T4, which presumably had sprouted,

persisted but became non-functional or whether they had regressed.

Although not as easy to detect as in the periphery, sprouting also occurs in the adult mammalian central nervous system. The first clear demonstration of this was the now classic experiments in Liu and Chambers (Liu and Chambers, 1958). After chronically sectioning all but one of the dorsal roots on the left side of cats, they showed by histological techniques that after six months, the spinal ramifications of the remaining root extended further up and down the cord, than did the corresponding root on the opposite side. In a comparable study, Goodman and Horel showed that retinal projections to the ventral part of the lateral geniculate nucleus, and to the nucleus of the optic tract, sprouted after these regions were partially denervated by chronic section of the visual cortical efferent fibres (Goodman and Horel, 1966). Both of these pioneering studies were based on light microscopy, and therefore could not indicate whether new synapses formed as a result of the collateral sprouting.

Raisman, using electron microscopy, showed that collateral sprouts in response to partial denervation, can indeed form new synaptic connections in the central nervous system (Raisman, 1969). After lesion of the medial forebrain bundle, Raisman found that the fimbrial fibres to the septal nuclei sprouted to form new synapses on some of the vacated sites on the cell soma. Normally fimbrial endings occur only on the dendrites of these neurons. Although morphologically they looked normal, no test were performed to see if these new synapses were functional. However, similar studies by Gottman and co-workers suggest that new collateral sprouts in the hippocampus do make functional contacts (Steward, Cottman

and Lynch, 1973). Some other studies demonstrating the existence of collateral sprouting in the CNS are (Bernstein and Bernstein, 1971; Lund and Lund, 1971; Moore, Bjorklund and Stenevi, 1971).

8. Collateral Sprouting in Adults Without Denervation

The exact cause of "denervation sprouting" is not clear. When a nerve is cut the distal portion of the axon undergoes Wallerian degeneration. As the axon begins to fragment, the surrounding Schwann cells start to proliferate, and invading macrophages from the blood remove the cellular debris. On the assumption that these non-neural cellular responses are triggered by products of nerve degeneration, it has long been thought that the stimulus for the nearby intact axons to sprout was of similar origin. Weiss and Taylor investigated the influence of degenerating nerve fibres by forcing nerves to regenerate into a Y-shaped arterial sleeve, which had one branch filled with degenerating nerves, and the other branch left untouched (Weiss and Taylor, 1944). After the nerves had regenerated through the arterial sleeve, they found no significant difference in the number of regenerating fibres growing into the debris-filled branch as compared to the "empty" one. These experiments certainly did not support the concept of a sprouting stimulus from the degenerating nerves. Other attempts to induce nerves to sprout, using "products of degeneration", have similarly failed (Weiss, 1934). This inevitably gives rise to the suspicion that other factors may be responsible for the sprouting observed after partial denervation. Further evidence in this direction comes from recent experiments that

report nerve sprouting without nerve degeneration, and from the experiments of this thesis.

Duchen and Strich investigated the effects of injecting sub-lethal quantities of Botulinum toxin into the gastrocnemius muscles of mice (Duchen and Strich, 1968). This substance causes paralysis by preventing the release of acetylcholine from the nerve terminals. Using the electron microscope (E.M.), they observed that the nerve terminals in the paralysed muscle looked normal. However, using a silver impregnation technique to stain nerve fibres, they found that the extensive outgrowth had occurred from the intact intramuscular axons. Interestingly, these new outgrowths failed to make functional contact, and regressed when the paralyzing action of the toxin wore off. Similar findings of sprouting after botulinum toxin poisoning has also been reported by Watson in the sternomastoid muscle of the rat, again with no evidence for the formation of functional contacts, (Watson, 1969).

In a study of the sympathetic innervation of the iris, Olson and Malmfors obtained evidence suggesting that increases in the size of the target tissue would cause these nerves to sprout (Olson and Malmfors, 1970). They first established the pattern of the normal sympathetic innervation to the iris by using the Falck-Hillarp method (a technique for histochemically visualizing catecholamines in adrenergic nerves). Next they transplanted a piece of iris to the anterior chamber of the eye, and using the same techniques, demonstrated that the intact sympathetic axons to the host iris sprouted and innervated the transplant. To exclude the complications of degenerating nerve products, they denervated the iris three months before the transplantation. In these

cases they found that, after sprouting had occurred, the density of innervation in the transplant was much the same as that for the intact host iris. Olson and Malmfors also noticed that other tissues, such as intestinal smooth muscle or arterial smooth muscle, had a similar ability to cause the sympathetic nerves to sprout. However, the density of innervation in these other tissues was different from that of the iris, and seemed rather to resemble the density these tissues have in their normal state. This important study suggests, (1) that adult nerves can sprout under some influence of the target tissue, and (2) this influence varies in effectiveness from one tissue to another.

The experiments of Aguilar, Bisby, Cooper and Diamond (1973) on the hindlimb of the salamander, which this present study extends, indicate that factors travelling down axons in the fast transport system may regulate nerve sprouting at the level of the target tissue. They first confirmed earlier work by Stirling on the segmental innervation of the salamander hindlimb, using physiological methods to measure touch-receptive fields in the skin. The innervation by the segmental nerves is perfectly symmetrical from one side to the other, which allows one side of the animal to act as the control for the other. As Stirling had shown, sectioning the 16th nerve on one side causes the two adjacent nerves, 15 and 17 to sprout and completely take over the denervated area (Stirling, 1973). Aguilar et al. treated the 16th nerve with a dose of colchicine that they showed interrupted fast axoplasmic transport, without killing the nerve, or interfering with electrical impulse activity. When the spinal nerve fields were investigated some 1-3 weeks after colchicine

treatment, the 16th nerve appeared completely normal and indistinguishable from the control side. However, the 15th and 17th nerves appeared to have sprouted just as though the 16th nerve had been cut. Since the only detectable difference in the 16th nerve after colchicine was the interruption of fast axoplasmic transport, Aguilar et al. concluded that factors flowing down nerves by fast transport were involved in the regulation of peripheral nerve sprouting.

9. New Hypothesis To Explain Why Nerves Sprout Collateral Branches

Aguilar et al. proposed an hypothesis to explain all nerve sprouting: the target tissue manufactures a substance that stimulates nerves to sprout; the nerves however release factor(s) from their endings, carried there in the fast transport system, which in some way neutralizes or offsets the effects of the growth-promoting substance. Sprouting therefore will continue until the effects of the nerve factor(s) balance that of the target stimulus. This hypothesis is a more general form of one suggested by Ramon y Cajal to explain sprouting during primary development, and in a sense, it represents a negative feedback control system to regulate the number of nerve endings in the target. Clearly, this hypothesis explains the collateral sprouting of intact nerves when adjacent ones are cut, since the neutralizing nerve factors transported down the axons will be eliminated in the cut nerves. As a consequence the pre-existing balance between these neural factors and the growth promoting substances from the target is disturbed, resulting in a net increase in the growth promoting substances. This increase then causes

the intact nerves to sprout until the new nerve terminals can release enough of the neutralizing factors to restore the original equilibrium. The other examples of nerve sprouting, in addition to that which occurs during primary development or after partial denervation, are the sprouting of motor nerves after botulinum poisoning (Duchen and Strich, 1968; Watson, 1969) and the sprouting of intact sympathetic fibres to the iris after a transplanted iris was placed into the anterior chamber of the eye (Olson and Malmfors, 1970). These cases can also be interpreted in terms of the new hypothesis. It is conceivable that in addition to blocking the release of acetylcholine, botulinum toxin would also prevent the release of the neutralizing nerve factors, and as with partial denervation or colchicine treatment, the level of the target-tissue growth substance would thereby increase, and cause the nerves to sprout. Similarly, the sprouting of the intact sympathetic nerves into the transplanted iris can also be attributed to an increase in the local level of growth promoting substances. In this case, however, the increase is not brought about by a decrease in the nerve factors but instead can be attributed to the additional growth promoting substances from the transplanted tissue itself.

III. GENERAL STRATEGY OF THE PRESENT STUDY

The major objective of this study is to examine further the new hypothesis to explain collateral sprouting proposed by Aguilar et al. (1973). There are two alternative possibilities which could account for the effects they obtained. One is that the colchicine, in addition to blocking fast neuronal transport, might have caused a small amount of degeneration confined to some nerve terminals within the area of the treated 16th nerve. Conceivably, this small amount of degeneration could then act as a stimulus for the adjacent nerves to sprout. Another possible explanation for the observed sprouting after colchicine is that the drug had a direct action on the target tissue which in some way initiated local nerve sprouting.

To test these possibilities, and to gain further insight into the mechanisms involved in sprouting, it was necessary first to devise a reliable quantitative technique with which to measure the density of nerve endings within a nerve field. Therefore, a detailed study was made of the quantitative organization and density of the mechanosensory endings in the normal salamander hind-limb skin. This same technique was then used to test for terminal degeneration after colchicine treatment. The results indicate that the colchicine was not causing any such degeneration. The possibility that sprouting was a result of a direct action of colchicine on the target was investigated by measuring the quantities of the drug which actually arrive at the target. The results again are consistent with the new hypothesis.

Finally, by quantitatively measuring the nerve sprouting in response either to partial denervation or to colchicine treatment, some hints were obtained about the nature of the control mechanism which operates to regulate nerve fields at the target.

IV. METHODS

1. Animals

All experiments were performed on male or female adult salamanders (Amblystoma tigrinum, see fig. 1). These amphibians normally live in the mid western region of the United States (Indiana, Wisconsin) and were bought from Mid West Reptile, a supplier in Indiana. The animals usually weighed anywhere from 20-35 grams and the head to tail length ranged from 10-20 cm. Because these salamanders could only be bought during the summer months (May-September) stock supplies of 500 animals were stored on a farm in Brantford, Ontario during the winter. When at the University, the animals lived in plastic containers, filled with moist moss to simulate their natural environment, and kept in an environmental room at 15°C and 75% humidity. The feeding and caring of the animals was done by a trained technician, using standardized procedures.

2. Surgical Procedures

a) Anaesthesia

All animals were anaesthetized before any surgery was performed. This was accomplished by placing the animals in a container filled with a one liter solution of 0.2% MS-222 (ethyl-m-aminobenzoate methanesulfonate, Sandoz) for 30 minutes. At this time the animals showed no movement or reflex responses to mechanical stimulation, such as pinching the tail. Normally, the animals remained in this state for approximately 60-90 min. If an animal showed signs of recovery before

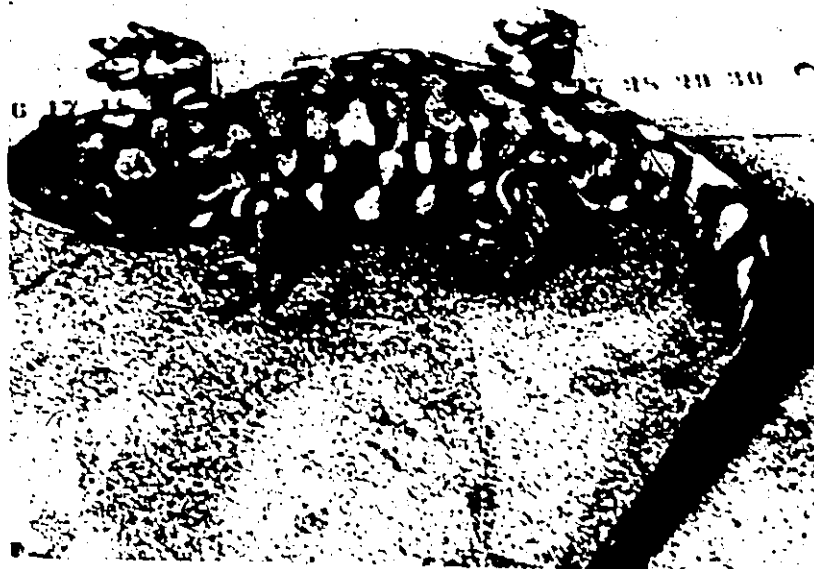


Fig. 1: Amblystoma tigrinum
This is a typical representative of the species of salamander used in these experiments. The scale in the background shows cm (numbered) and mm.

the surgical procedures were finished, a cotton pad, soaked in 0.5% MS-222, was applied over the head and thorax region, and proved adequate to re-induce a suitable depth of anaesthesia. Recovery from the anaesthetic seemed to occur without any noticeable side effects.

b) Decerebration

An incision was made over the occipito-atlantoideal joint, the occipito-atlantoideal ligament was exposed and cut, then the upper part of the cranial vault was removed. A section was performed at the level of emergence of the 10th cranial nerves, the brain was removed with forceps and the cranial cavity filled with cotton.

3. Nerve Section

First a medio-lateral incision was made just in front of the right ilium. Then, using watch makers forceps, a trough was made in the muscle until nerve 16 was exposed; this was carefully freed from the surrounding connective tissue. The nerve was cut at a distance of about 1 mm from the midline, leaving a distal stump of about 10 mm before the point where the 16th nerve joins the plexus formed with the neighbouring 15th and 17th nerves. The central stump of the nerve was tied off with 8-0 silk thread to minimise the chances of its regenerating. The wound was closed with 2 or 3 sutures of 6-0 silk thread.

4. Colchicine Application to Nerve 16

A few mm length of nerve 16 was carefully exposed as in (3) above. The trough made by the exposure route through the overlying muscle to the nerve trunk was filled with a solution of colchicine (BDH Ltd.) in

amphibian Ringer Solution (composition: mM NaCl 111, KCl 1.9; CaCl₂ · 2H₂O 1.1, MgSO₄ · 7 H₂O 1.6; NaHCO₃ 2.4). Colchicine concentrations ranged from 50 mM-100 mM. The control experiments were performed in exactly the same manner, except that Ringer solution alone was used without the colchicine. After exposing the nerves to these solutions for 30 min., the trough was washed out thoroughly with Ringer solution, and the wound closed with sutures.

5. Radioactive Colchicine and Scintillation Counting

(a) ³H-colchicine application to nerve 16

The application of the ³H-colchicine was no different from the usual application technique, as described in Methods, Part 4. To make up the 50 mM solution, 0.08 mg of ³H-colchicine (NEN, Specific activity 5.0 Ci/mM) was added to 9.92 mg of ordinary colchicine (BDH) and dissolved in 0.5 ml-amphibian Ringer (see Methods Part IV, for composition).

(b) Determination of radioactivity in skin

The animals were anaesthetized and decerebrated as described in Methods, Part 2. Dorsal and ventral strips of skin, 6-8 mm wide, and 2 mm long were dissected from both hindlimbs, placed on a small white card, and divided into a proximal and distal portion. Each of these samples was then prepared for liquid scintillation counting by processing them through a sample oxidizer (Inter-Technique Oxymat). The radioactivity ³H₂O was collected, dissolved in scintillation fluid (composition: 700 ml Dioxane, 300 ml Toluene, 20 g. Naphthalene, 9 g 2,5-Diphenyloxazole (PPO) and counted for 10 min. in a Beckman LS 230

Liquid Scintillation Counter.

6. Dissection for Nerve Recording

To dissect the nerves for impulse recording the salamanders were first anaesthetized with 0.2% MS-222 and decerebrated as described in Methods, Part 2b. The nerves to the hindlimb were usually exposed just after decerebration while the animal was still under the effect of the anaesthetic. First the overlying skin was removed from segment 14 to 18. Then the underlying muscles of the hip region were removed and the nerve trunks of 15, 16 and 17 were exposed on both sides. The muscle over the ilium was next cut away and the exposed bone was cut as close to the spinal column as possible, carefully avoiding the cutting of the 16th and 17th nerves lying beneath. The ilium was pulled away from the body, then the femur was cut close to the origin of the limb, and the bone removed. With fine watchmaker forceps, the nerve trunks were dissected free of connective tissue to the region where they branch to form the limb plexus. These nerves were cut as near to the vertebral column as possible and a thread was tied round the central end of the distal stump.

After the spinal nerves were mapped, as in part 8(a), it was often necessary to dissect free the cutaneous femoral posterior nerve (CFPN), a branch of the sciatic nerve. This was done by dissecting out the sciatic plexus (Fig. 2) and cutting the extensor nerve, the femoral nerve, the pudendal nerves, and the main sciatic nerve below the branch of the posterior cutaneous femoral nerve.

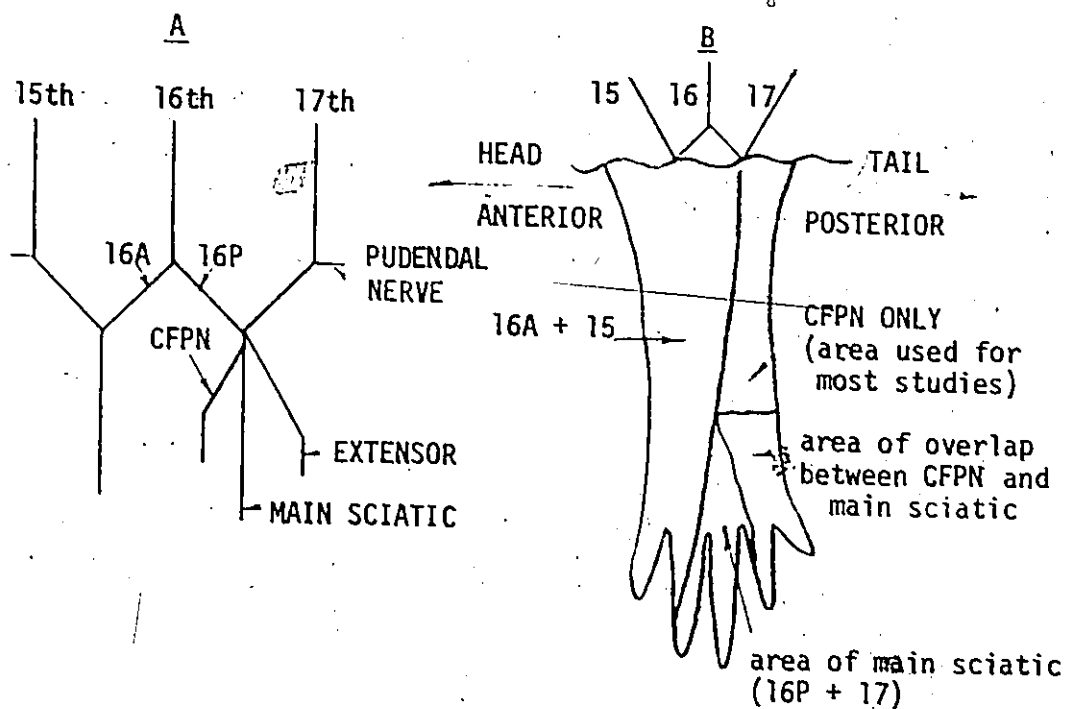


Fig. 2: The Nerves of the Sciatic Plexus and its Innervation.

a) Schematic diagram of the sciatic plexus. The 16th nerve divides into two branches; 16a (anterior) joins with the 15th nerve and 16p (posterior) joins with the 17th.

b) The area of skin on the dorsal surface of the limb innervated by each major nerve branch is indicated. The cutaneous femoris posterior nerve (CFPN) innervates a strip of skin roughly 5 mm wide on the posterior portion and runs distally to innervate the posterior one and a half toes. The 'main sciatic nerve' as described by Francis (1934) overlaps with the CFPN as shown. The pudendal nerve innervates the trunk skin, the extensor nerve and other branches of the main sciatic nerve innervate the ventral surface of the limb. None of the latter nerves are shown in this figure.

7. Recording Techniques

Nerve impulses were recorded from the entire nerve trunk with fine platinum electrodes, which served also to raise the nerve trunk from the body wall. When the recording was for a short time, less than 1 min. the nerves were lifted into air. For longer recording (up to 10 hr) the nerves were covered with aerated light mineral oil (liquid paraffin). All signals from the recording electrode were amplified by a Tektronics 5103 storage oscilloscope, filtered (L.f. 60 Hz, H.f. 3 KHz), and displayed on the screen with a resolution of 10-50 μ V/cm. The nerve activity was also monitored by feeding the output from the oscilloscope through a loudspeaker. The oscilloscope was triggered by a WPI pulse generator and the sweep speed was normally 2-5 msec/cm. The apparatus and the animal were connected to a common ground. Fig. 3 represents a schematic diagram of the recording set-up.

8. Stimulation of Touch Receptors

(a) Nerve fields

To obtain gross estimates of the area of innervation of a nerve branch, the skin of the hind limb was stimulated by touching lightly with a fine nylon bristle, while recording from the nerve. The area of skin that gave rise to impulse activity in the nerve was then traced out on a representative drawing of the limb (see Fig. 2).

(b) Mechanical stimulator

A more precise method of stimulating the touch receptors was used to make quantitative measurements of the density of nerve endings within a nerve field, of the size of an axonal receptive field, and of the

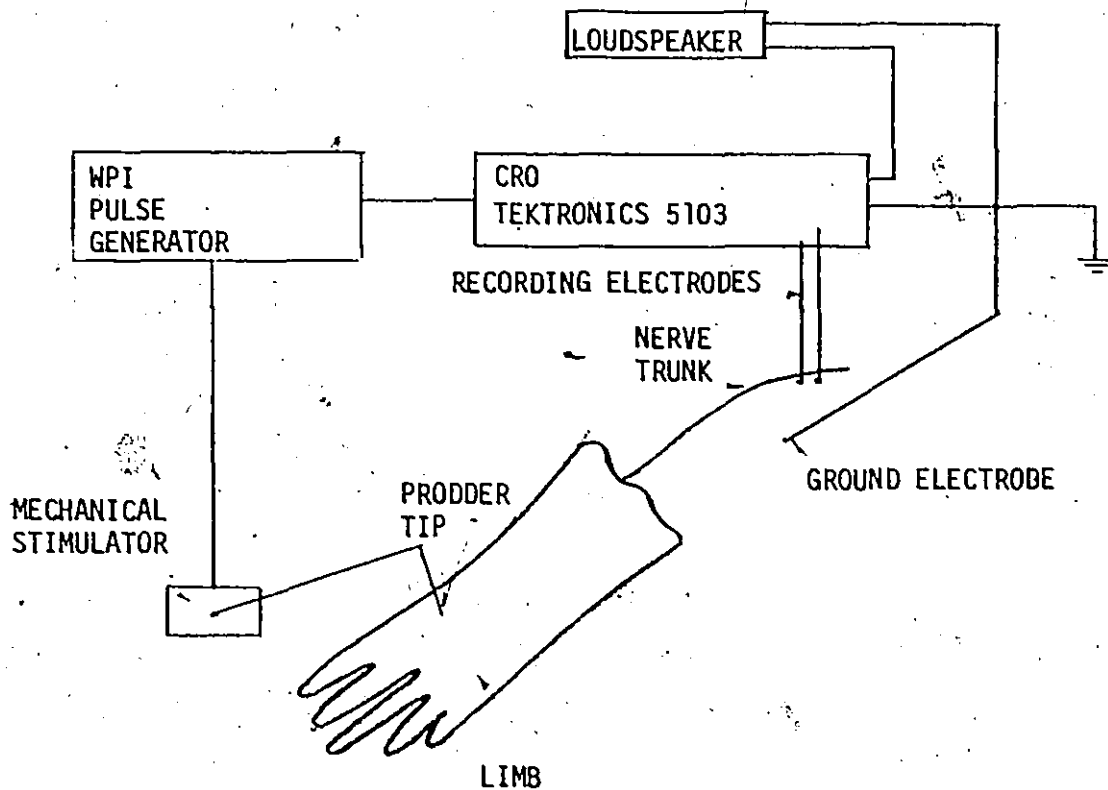


Fig. 3: Schematic Diagram of the Equipment Used in the Mapping of the Touch Receptive Fields.

For the mapping of gross nerve fields, the skin was stroked lightly with a bristle, and the evoked action potentials were amplified, relayed through a loud speaker, and displayed on a cathode ray oscilloscope (CRO) which was set in free run mode. The WPI pulse generator was used for the investigation of individual touch receptors; an electrical pulse was applied to the mechanical stimulator (piezo electric crystal). The WPI was also used to trigger the CRO. The apparatus and preparation were grounded to a common point.

size of the receptive field of a receptor. These measurements were made with the aid of a mechanical stimulator which was specifically designed for this purpose. Among the many points considered in the design, it was essential for the stimulator to have the following features:

(i) The spread of the mechanical stimulus should be minimized to achieve a high degree of resolution.

(ii) The stimulus should have a fast rate of rise, which remains essentially linear over the range used.

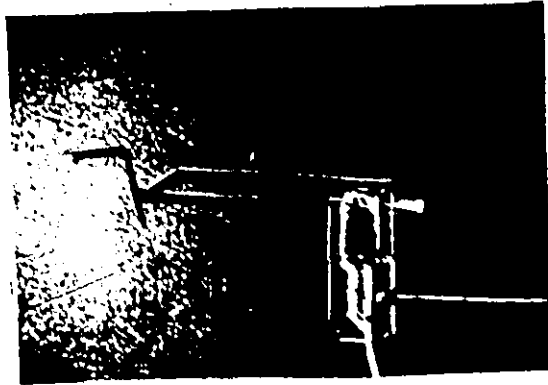
(iii) The rate of rise should be continuously variable over a range from 0-10 μ /msec.

(iv) The stimulator should not be so large as to obstruct the field of view, particularly since it was often necessary to stimulate the skin with two stimulators situated less than 100 μ apart. The stimulator was also required to be easily mounted on a manipulator.

(v) Finally, the stimulator should be simple and relatively easy and inexpensive to construct.

The mechanical stimulator used in all experiments is shown in Fig. 4. The major component of this stimulator is the piezo-electric crystal taken from an Astatic 12-U phonograph cartridge. The crystal was fixed at one end to a perspex holder while the other end was left free. When a voltage is applied across the crystal, the crystal bends and the free end deflects. This deflection was magnified by glueing a 3.5 cm long triangular shaped (for rigidity) aluminum section to the end of the crystal; the apex of the triangle was attached to the crystal and a small hollow rod, to hold the prodder tip, was glued to the base. Viscous damping was used to eliminate unwanted vibration due to this

A



B

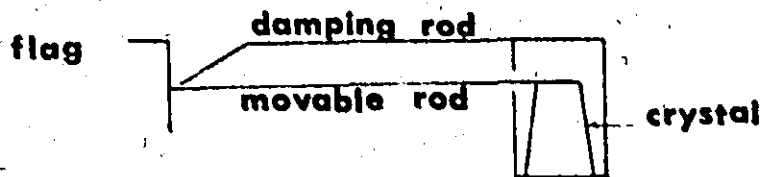


Fig. 4: A Photograph of the Mechanical Stimulator used in these Experiments

The black piezo electric crystal can be seen attached to a transparent perspex holder. At the free end of the crystal is a 3.5 cm aluminum extension. A flag used for calibrating the stimulator is attached to a hollow tube at the end of the extension. At the other end of this tube is a fine tungsten tip used to stimulate the skin. Another long extension is attached to the perspex holder at one end and runs parallel to the moveable extension. This second extension is used in conjunction with the viscous damping arrangements (see text for further details. A schema of the stimulator is shown in (B).

long cantilever arrangement. A fixed bar was attached to the perspex body and extended 3.5 cm parallel to the movable extension but separated from it by 1 cm. At the very end the immovable bar was bent, so as to come within 2 mm of the end of the movable extension. This 2 mm gap was filled with silicone grease which proved to be sufficient to damp out all unwanted movement without significantly reducing the rate of rise of the stimulator deflections. The prodder tips used to stimulate the skin were made from fine tungsten wire, one of which was mounted in a small aluminum tube which fitted tightly into the hollow rod at the base of the triangular aluminum section attached to the crystal. The sliding fit between the aluminum tube and the hollow rod made it easy to exchange one tungsten tip for another. The tungsten rods were electrolytically etched to any desired diameter, but the size most frequently used in these experiments ranged from 10μ - 100μ .

Monophasic square wave voltages were applied to the stimulator from a WPI isopulser model PC-3. The amplitude of the pulse varied from 1-100 volts and the pulse duration varied from 8-400 msec depending on the situation. A Tektronics TM503 PG505 pulse generator was sometimes used to vary the rate of rise of the stimulator. The whole prodder was mounted on a Zeiss sliding micromanipulator so that it could be moved accurately in horizontal steps of 25μ , parallel to the skin. The angle of placement of the prodder tip was critically aligned to minimize the lateral movement as the prodder deflected vertically. The most effective way to achieve this was to align the stimulator such that the line from the end of the tungsten tip to the centre of the crystal was parallel to the plane of the skin (see Fig. 5).

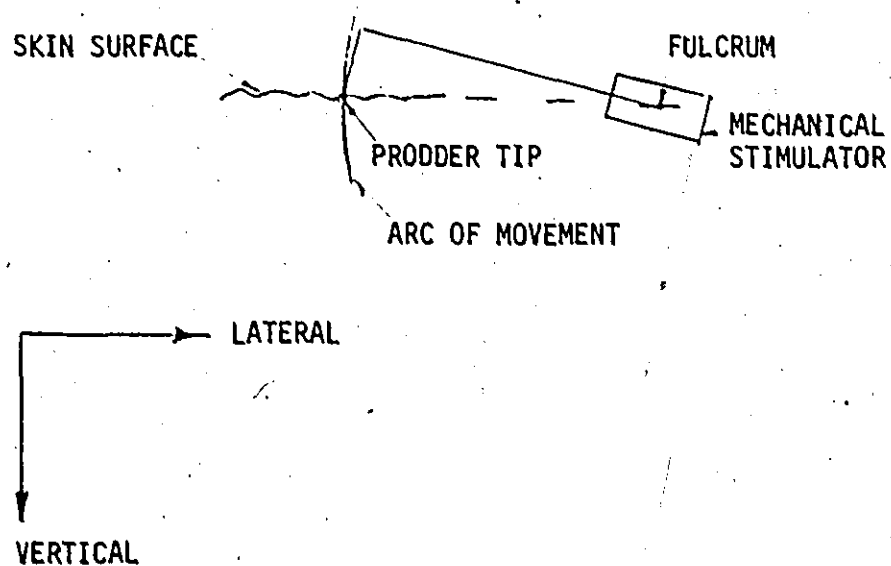



Fig. 5: Alignment of Stimulator.

This diagram indicates the arc of movement of the prodder tip when the line joining the prodder tip to the fulcrum of the stimulator is parallel to the surface of the skin to be stimulated. This arc maximizes the vertical displacement and minimizes the lateral movement of the prodder tip.



The actual displacement of the prodder tip was measured with the aid of a standard photocell, in the following manner. First the tungsten tip was replaced by a small flag assembly of similar mass. This flag was then carefully positioned between the emitter light and receiver cell of the photocell, which were critically aligned so that a small movement in the flag perpendicular to the beam resulted in a voltage change from the receiver cell which was displayed on Tektronics 5103 oscilloscope. The photocell was calibrated by moving the flag a known amount and recording the voltage output. There was a direct linear relationship between displacement and voltage output over the range used in these experiments (see Fig. 6).

Fig. 7 shows various characteristics of the prodder input-output relationship. Fig. 7 (a) shows a typical output voltage response to an applied square wave input. It should be noted that the time to peak of the response is 10 msec, and the rate of rise to that peak was sufficiently linear for the experiments in this thesis.

Fig. 7 (b) shows 4 different input-output responses. These records illustrate that response amplitudes rise linearly with the applied voltage. In addition the time to peak of the output appeared to be independent of the amplitude of the applied voltage and so by varying the amplitude of the square wave voltage applied to the crystal it was possible to vary the rate of rise of applied displacements on the surface of the skin. Fig. 8 shows that the relationship between the input amplitude and the rate of rise of mechanical movement was stable for long periods; the figure shows the input-output relationships for the same prodder tested twice a week for a month.

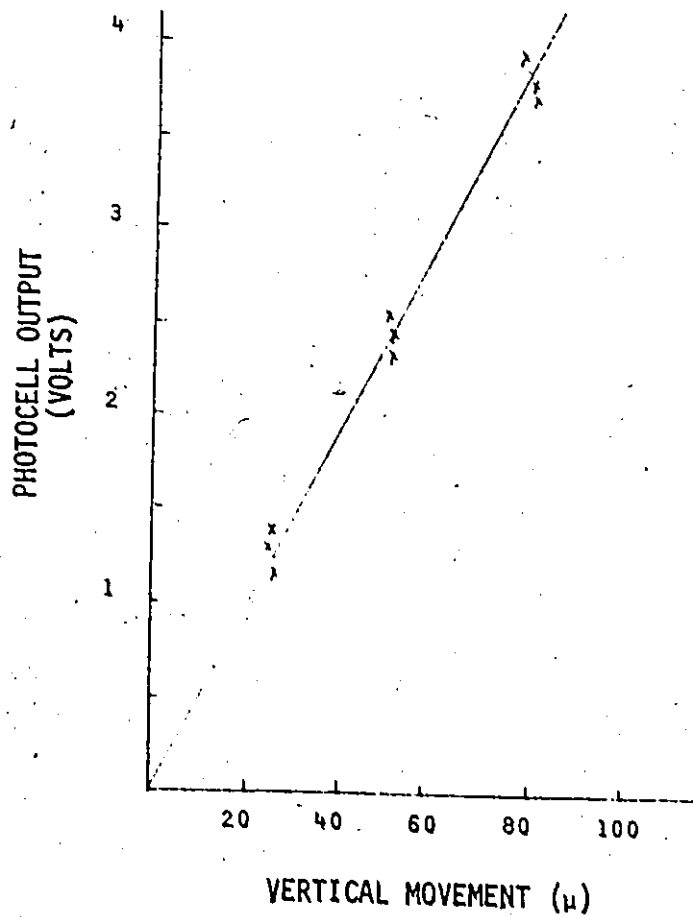


Fig. 6: Calibration of the photocell.

Different known vertical movements were applied across the photocell while recording the photocell output voltage. One volt output from the photocell equals 20-25 μ vertical movement of the prodder tip.

Fig. 7: Prodder Movements

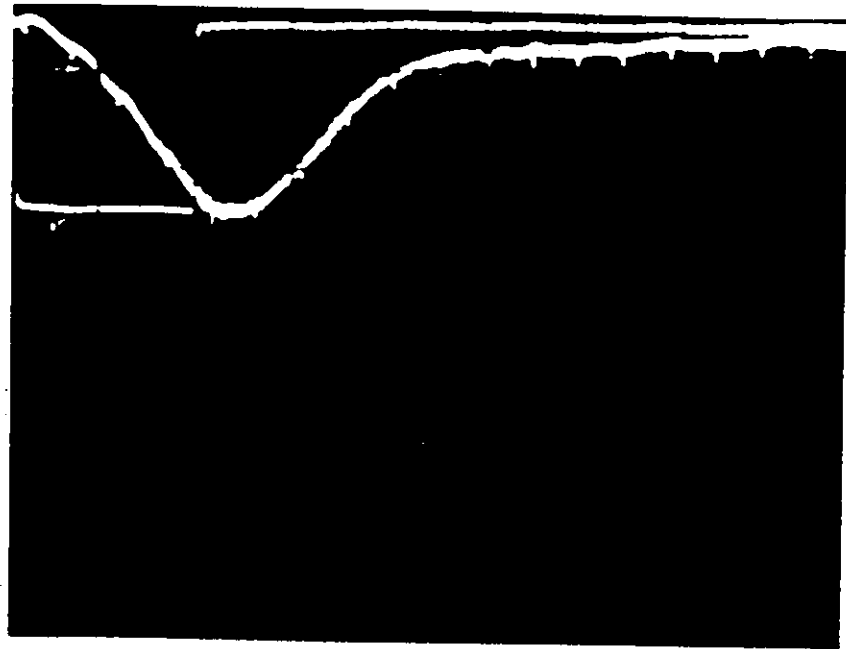
The output response of the photocell (which is proportional to the mechanical movements, see Fig. 6) is shown for different amplitudes of input voltages to the crystal. (In this figure, and in figures 9, 10 and 18, increasing input voltages and photocell output voltages are shown as downward deflections of the trace.) A is a single response to one applied pulse. B shows four different responses superimposed. As the voltage pulse increased, the mechanical displacement (photocell output) increased. The vertical scale is 25 volts for the applied voltage and 0.25 volts for the photocell output voltage.

A

photocell
output

applied
voltage

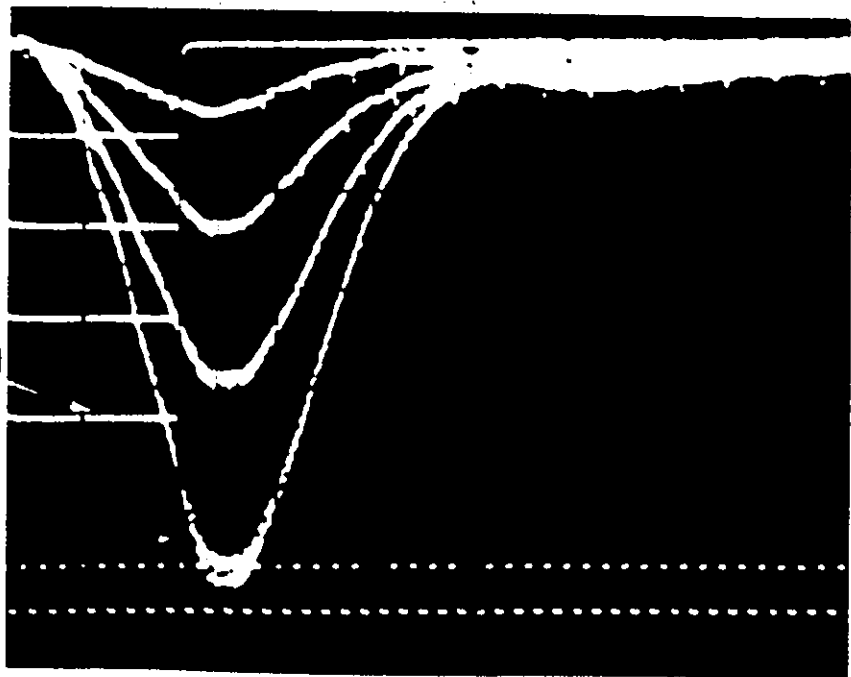
5msec



B

max voltage used
in these expts.

response to
max voltage



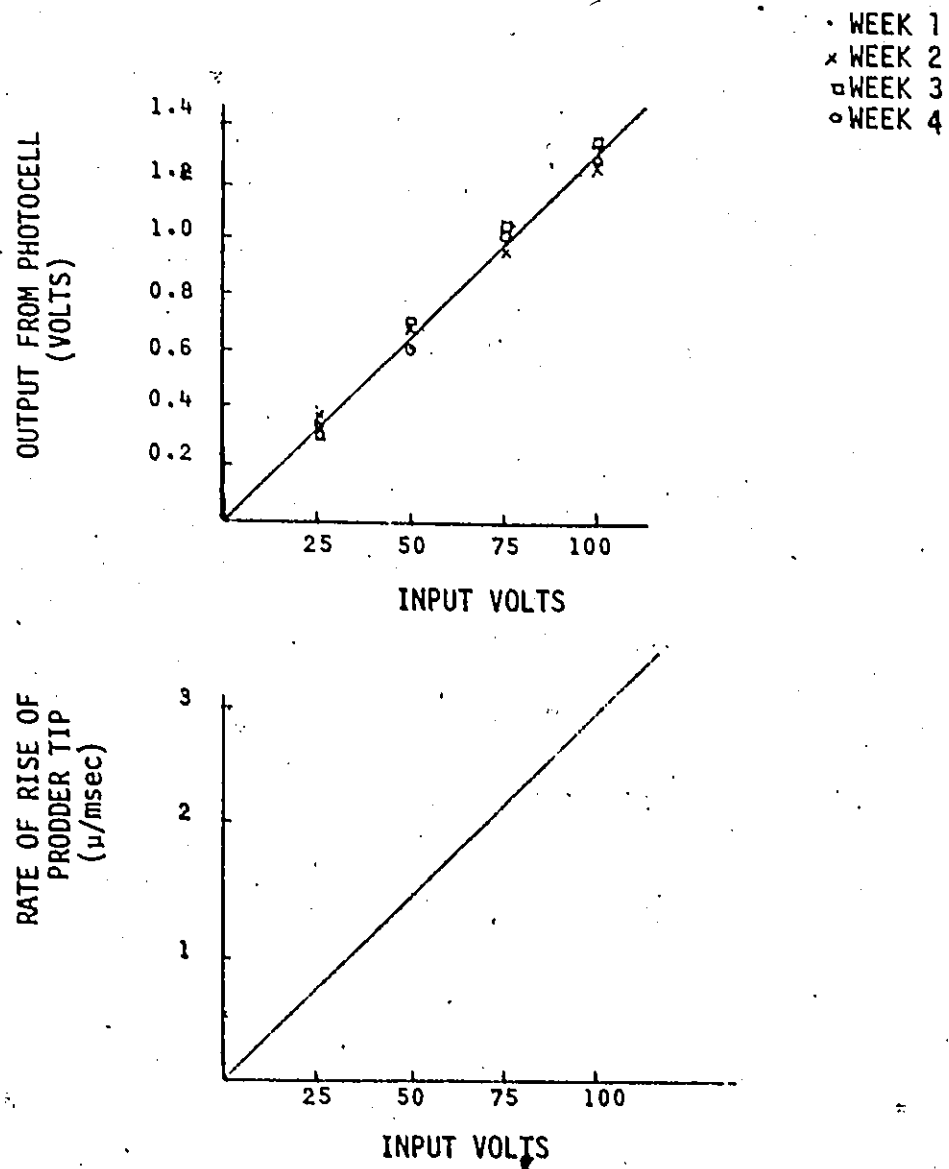


Fig. 8: Variation in Prodder Movement Characteristics with Time.

a) This graph represents the calibration of the prodder over a one month period. The input voltages are plotted against the output voltages from the photocell.

b) The relationship between the amplitude of the applied voltage and the rate of rise of the mechanical movement of the prodder is shown. The points for this graph were derived from figures 6 and 7.

Fig. 9 shows the output for a stimulus of long duration; the displacement was maintained for as long as the input was applied. Fig. 10 shows the effect of different rates of rise of the input voltage on the rate of rise of the mechanical movement.

9. Histological Techniques

(a) Light microscopy

The skin with the underlying nerve was placed in a small flask containing about 5 ml of 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3) for 2 hr; the specimen was embedded in Spurr epoxy resin. 1 to 2 μ thick transverse sections were cut, stained with 1% toluidene blue in 1% borax, mounted and covered with a coverslip.

(b) Electron Microscopy

Nerves were fixed and embedded as above. The ultrathin sections were cut using a Reichert microtome with a diamond knife, and subsequently stained in 25% methanolic uranyl acetate (5 min) followed by Reynolds' lead citrate (1963) and examined with a Philips 300 electron microscope. The light and electron microscopy were both done in collaboration with Dr. A. Parducz; my role was primarily concerned with producing the specimens, assisting with the subsequent processing, and with the appropriate guidance, examining the finished slides and photographs.

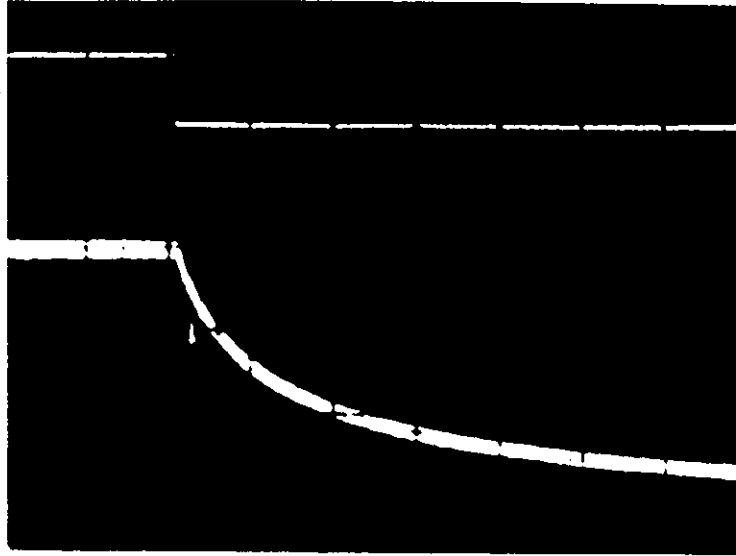


Fig. 9: Relationship between Mechanical Displacement of the Prodder (photo cell output) and a Maintained Applied Voltage to the Crystal.

The displacement remains fairly steady for as long as the voltage is applied (approx. 400 msec). The vertical scale is 25 volts for the input voltage and 0.5 volts for the output of the photocell. The horizontal scale is 50 msec. At times greater than that indicated by the arrow, the rate of rise of the mechanical pulse is less than the minimum rate required to activate the receptors (see Fig. 13).

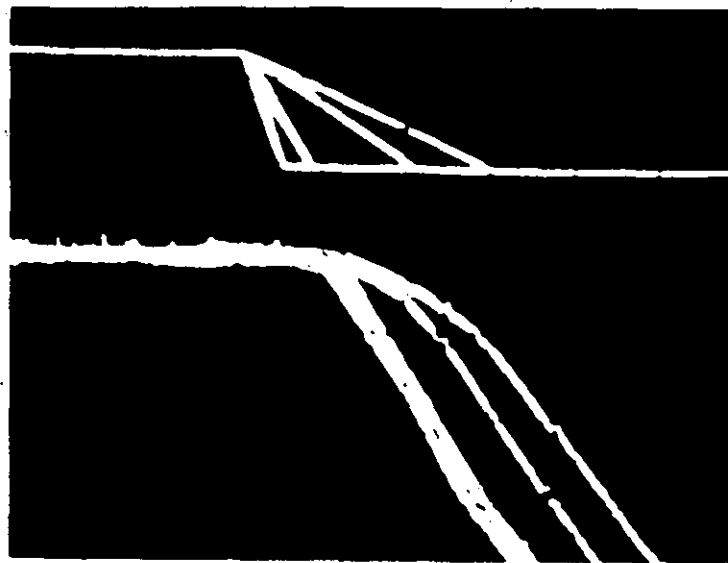


Fig. 10: The Response of the Mechanical Stimulator to Different Rates of Rise of the Input Voltage.

The different rates of rise of the applied input voltage does not affect the rate of rise of the output movement. However, the slower rates of rise of the applied voltage do affect the time of onset of the mechanical movement. The vertical bar is 50 volts for the input voltage and 0.1 volts for the output of the photocell. The horizontal bar is 2 msec.



V. DENSITY AND ORGANIZATION OF SKIN INNERVATION

1. Introduction

The main objective of this part of the study was to develop a reliable method for investigating the density of mechanosensory endings of a segmental nerve in the skin of the salamander hindlimb. This was required for the quantitative study on sprouting which is the principal aim of the investigations.

Theoretically the density and organization of nerve endings in the skin could be explored by histological techniques, but several reasons indicate that an electrophysiological approach would be more appropriate. Electrophysiological methods were already being used successfully to map the area of innervation of the segmental nerves to the hindlimb. The only major requirement was a more selective and precise mechanical stimulus. Even if the appropriate staining techniques could have been developed, it was suspected that the terminal branches and the mechanosensory endings would be near or below the limits of resolution of the light microscope, and therefore would be detected only with great difficulty, or not at all. The degree of resolution for the extracellular nerve recordings (the signal to noise ratio) is always adequate to resolve single action potentials from mechanosensory nerve trunks in this preparation. Furthermore, in this study it was important to know which segmental nerve a particular axon belonged to. This would be a difficult task if a histological approach were used, especially in the highly pigmented salamander skin, whereas this problem is easily overcome

electrophysiologically by recording separately from each of the segmental nerve trunks, while exciting the axon selectively by way of its sensory endings. Finally, the objectives of the research relate to functional characteristics of mechanoreceptors, such as the receptive field of a single axon, or of an individual touch receptor, and the determination of whether new sprouts become functional. These questions cannot be answered by histological techniques alone; ideally a combined approach is needed, involving morphological as well as physiological methods. Such an approach is currently being developed in this laboratory.

2. Results

(a) The Nerve Field

Most of these studies were performed on a portion of skin innervated exclusively by the cutaneous femoris posterior nerve (CFPN). The precise area was defined by mapping the field of innervation while successively cutting off all other obvious branches of the sciatic nerve (see Methods, Part 6). In addition, the branches which were cut were also mapped to define the area of overlap between these branches and the CFPN field. The results are shown in Fig. 2. The advantage of the CFPN over most of the other nerves is that it contains very few muscle afferents and therefore is relatively free of spontaneous activity (see part c, page 56). By restricting the investigation to the area indicated in Fig. 2, it was also easy to be certain that one was dealing with the total nerve supply to the region. The CFPN is found to be made up of axons only from the 16th and 17 segmental nerves, and the exact contribution from each varies from animal to animal.

(b) Selective activation of individual nerve axons

The first question that arises is can individual axons be excited by stimulating individual points on the skin, or frequently do many axons overlap and supply the same area of skin. In an attempt to answer this question, random points on the skin were stimulated with a 10 μ diameter stylus attached to the mechanical stimulator [described in Methods, Part 8(b)] while extracellular recordings were made from the segmental nerve trunk (as described in Methods, Part 7). At each point on the skin the rate of rise of the mechanical stimulus was increased in steps of 0.1 μ /msec to a value that evokes an action potential every time that the stimulus was applied. (The mechanoreceptors respond to a 'velocity stimulus' - see below, Fig. 12). Then the velocity of the mechanical stimulus was increased further until a second stimulus-tied action potential was evoked, or until the limit of the stimulator was reached, which is 10 μ /msec. The results of over 50 points are summarized in Fig. 11. At only 50% of the points tested could 2 axons be excited. At only 10% of the points tested the difference between the stimuli needed to evoke the first and second action potentials was less than 1 μ /msec. Differences in stimuli greater than 1 μ /msec were well within the resolution of the technique, and only if the difference between two stimuli was less than 0.2 μ /msec would it have been impossible to resolve. It can be concluded then, that individual axonal excitation can be readily resolved by stimulating different points on the skin. Furthermore, the probable maximum of 10% "overlap" of axons at single points was not likely to interfere with the analysis, as will become apparent in the text to follow.

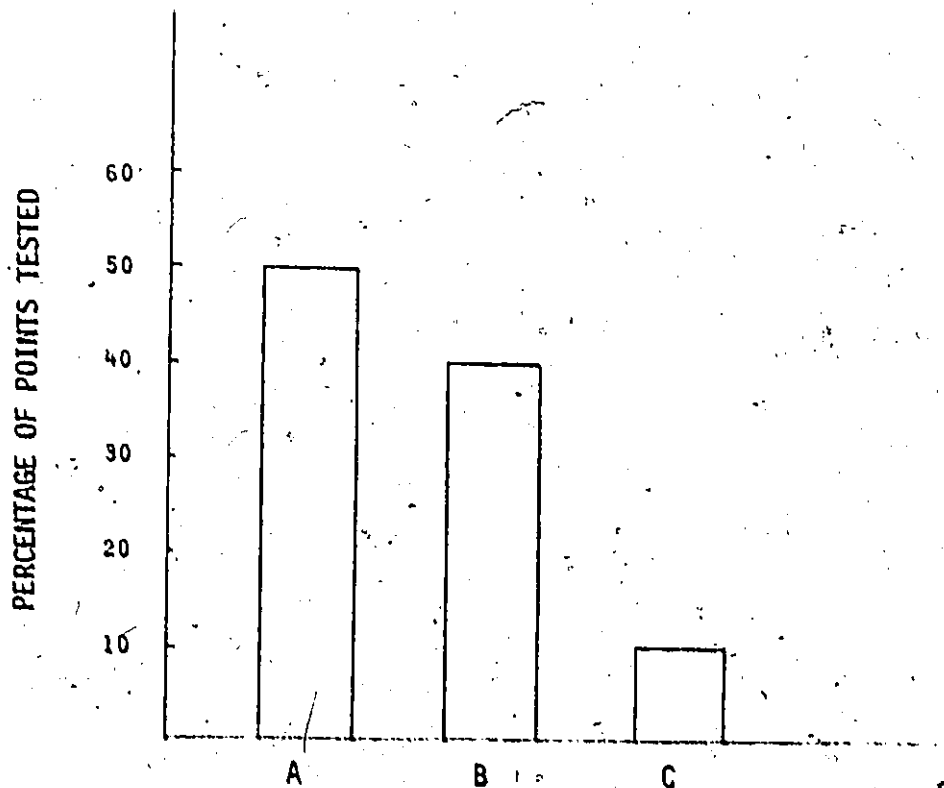


Fig. 11: Activation of Individual Nerve Axons.

At each point on the skin the rate of rise of the mechanical stimulus was increased in steps of $0.1 \mu/\text{msec}$ to a value that evoked an action potential every time that stimulus was applied. The velocity of the mechanical stimulus was increased further until a second stimulus tied action potential was evoked, or until the limits of the stimulus was reached. Column A represents the proportion of the points tested that responded with only one action potential. Column B represents the proportion of points tested at which the difference between the two stimulus needed to evoke the first and second spikes was greater than $1 \mu/\text{msec}$. Column C represents points where the differences between the two stimuli was less than $1 \mu/\text{msec}$.

(c) Receptor characteristics

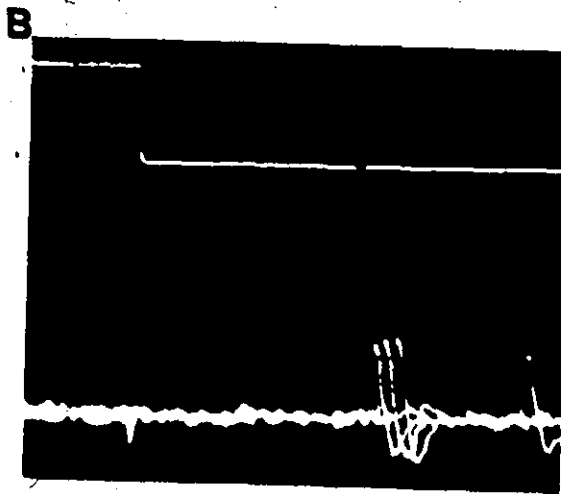
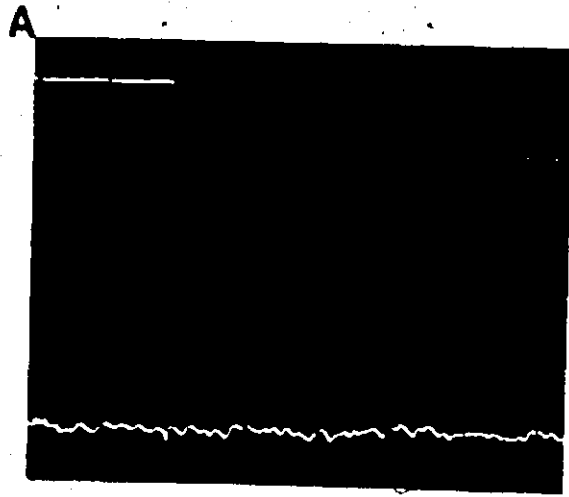
In the section above it was shown that individual axons could be excited by stimulating different points on the skin with a certain kind of mechanical deformation. That is, the test involved only one type of mechanoreceptor (a velocity sensitive one). In general, mechanoreceptors can be broadly divided into two classes based on their physiological characteristics. One class consists of those receptors which discharge only one or occasionally two impulses along their axons during the application of the stimulus, provided that the velocity of its rise is above a certain critical value, and they do not fire during the maintained plateau. These are known as "rapidly-adapting" receptors (Adrian, 1928). Occasionally such receptors fire when the stimulus is removed (an "off" response). At the other extreme are mechanoreceptors that often display an erratic resting discharge in the absence of any applied stimulus, and whose impulse frequency is enhanced by the mechanical stimulus and continues throughout its application. When the stimulus is withdrawn from those mechanoreceptors which do show a 'resting' discharge, there is often a brief pause in the impulse train which then soon returns to the resting value. These are the "slowly-adapting" receptors. The concept of "rapidly-adapting" and "slowly-adapting" receptors was first clearly stated by Adrian. Other classifications which are now used are "velocity detectors", for units that respond only during the application of the stimulus, and "displacement detectors" for units which respond to both phasic and static displacement (Brown and Iggo, 1967; Burgess and Perl, 1974).

The types of touch receptors present in the CFPN field were characterized by selectively stimulating different points on the skin with different types of mechanical stimulation. The results of this study indicate that all the mechanoreceptors in this field appear to be rapidly adapting, as shown in Fig. 12 (a-c). Each record shows the voltage input to the mechanical stimulation (Top trace), and the electrical activity of the sensory-nerve to the skin (lower trace). There was no response in (a) to a subthreshold stimulus, nor was there a resting discharge in these receptors. In (b) the stimulus was increased to the "critical" level, and it elicited a response to each of 4 stimuli, one being clearly delayed behind the other three. The maximum output from the stimulator was used in (c) and it was maintained for about 400 msec. Only one action potential was propagated, and it seemed no different from that evoked by the threshold stimulus in (b). Fig. 13 shows that altering the duration of the applied stimulus makes no difference to the sensory response. In most cases short duration pulses of 9 msec were used. All receptors investigated gave similar results, and this formed the basis for the conclusion that all receptors stimulated with the prodder in this study were rapidly adapting.

Several tests indicate that the action potentials detected were indeed single units and not compound potentials from a bundle of axons of similar threshold. They include: (1) all units were of approximately the same height, (2) stimuli just below the critical level evoked action potentials once every two or three times they were applied; in this case, when the action potential appeared it was a constant height

Fig. 12: Action Potentials Evoked by Stimulation of Skin with the Mechanical Prodder.

Top trace; the applied voltage to the crystal (increasing voltages are shown as downward deflections). Bottom trace; extracellular recording from the nerve. A, shows the absence of response to a sub-threshold stimulus ($0.75 \mu\text{/msec}$). There was no spontaneous discharge in these receptors. B the stimulus was increased to just the critical stimulus ($1.5 \mu\text{/msec}$). Four sweeps of the oscilloscope trace are superimposed, and show the variation in latency at this stimulus. The delayed spike, which occurred 4 msec after the earliest response, was a common occurrence when the stimulus was just threshold. The time taken for the delayed spike would include a significant increment due to the membrane conditions uniquely associated with subthreshold activation (Hodgkin and Huxley, 1952). C response to the maximum output from the stimulator ($3.0 \mu\text{/msec}$). There is still only one action potential. Vertical scale is 25 volts for the top trace and 50 μV for the bottom.



2msec



A scale bar indicating a duration of 2msec, consisting of a horizontal line with vertical end caps and a vertical line extending downwards from the center.

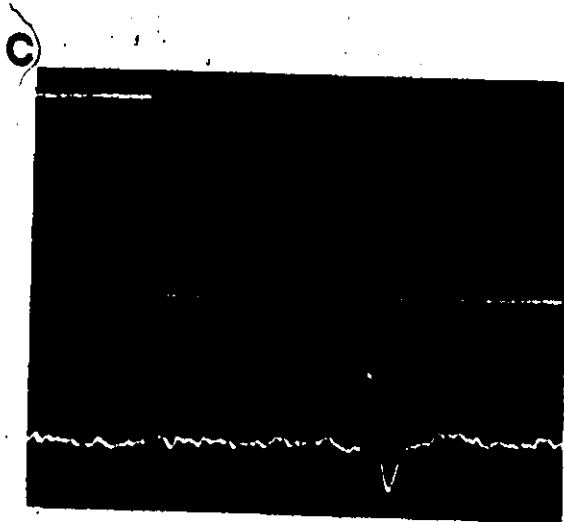
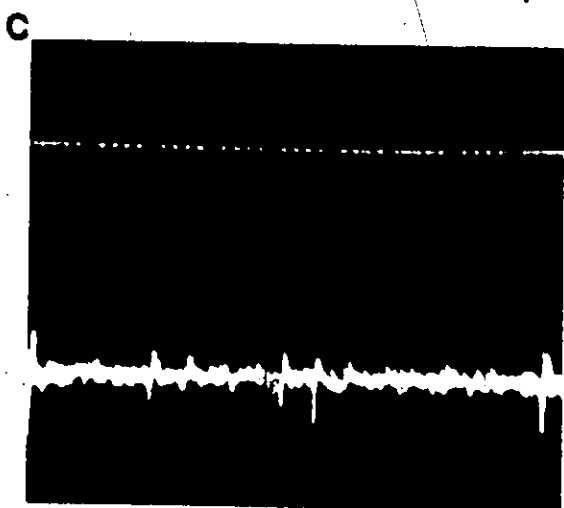
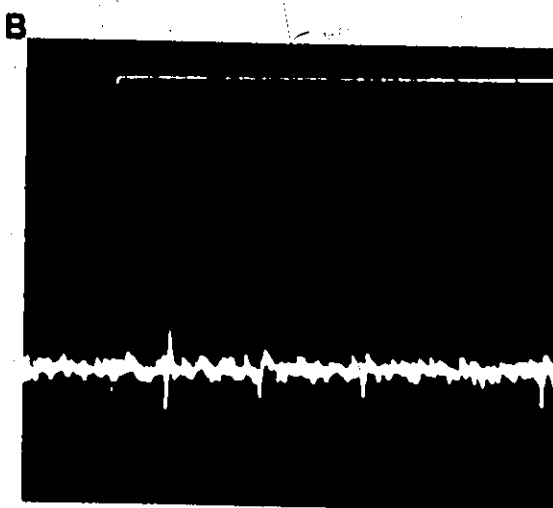
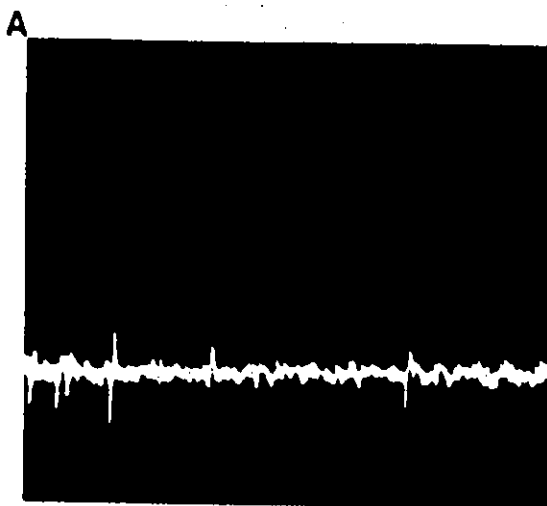


Fig. 13: The Effects of Altering the Duration of the Mechanical Pulse on the Response of the Touch Receptors.

Top trace; input voltage to crystal (increasing voltages shown as upward deflections in this figure as in Fig. 15, 16, 21 and 22), bottom trace; extracellular recording from the CFP nerve. A shows short duration pulse of 10 msec and $1.5 \mu/\text{msec}$ rate of rise. In B the pulse is maintained for about 400 msec. From Fig. 9 it is known that the mechanical displacement would virtually reach a maximum rate of rise within approximately 10 msec; after that time the displacement would have increased very slowly for approximately the next hundred msec of the applied voltage. In C the spontaneous activity is shown when no stimulus is applied. The vertical is 50 volts for the applied voltage and 20 μ volts for the nerve recording.



10msec
|

and when it failed there was no response, (3) the receptors could also follow suprathreshold stimuli at high frequencies, up to 10 Hz. At these high frequencies, every time the response appeared it had a constant height and latency (ie. the time from the onset of the applied pulse to the appearance of the spike). In those cases when it failed the trace was flat.

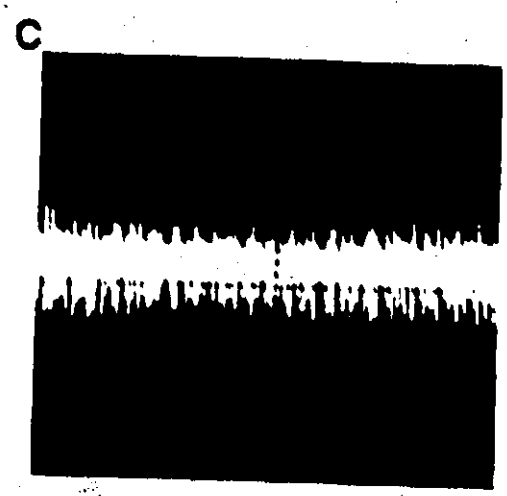
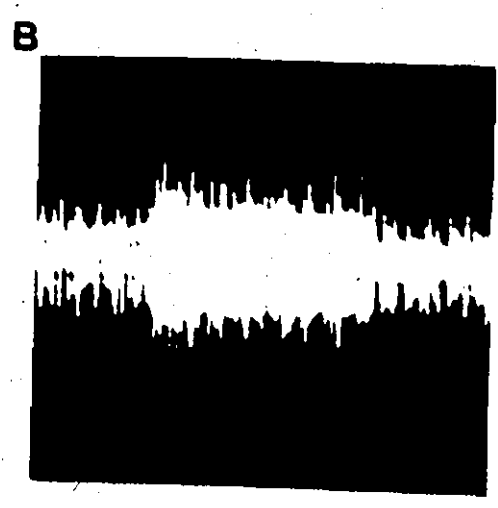
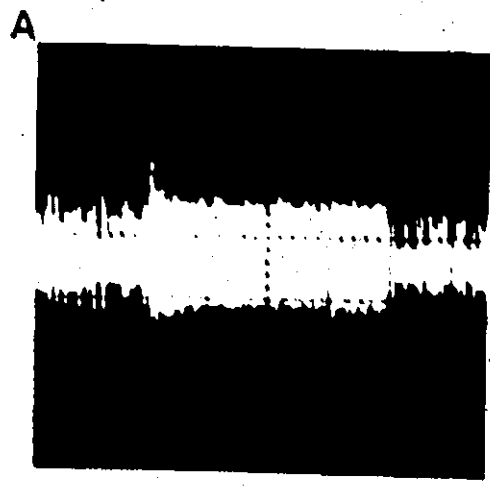
Although there are no slowly adapting mechanoreceptors in any part of the dorsal skin of the hindlimb a slowly adapting discharge could be evoked in the mixed nerve by a very large stimulus. This result was also obtained when the overlying skin was removed, as shown in Fig. 14. Clearly, the gross stimulus used in these circumstances was exciting receptors associated with the deeper tissues, presumably muscles and tendons. Such proprioceptors are known to be slowly adapting. They were not investigated in the present study.

(d) Estimate of length of terminal branches

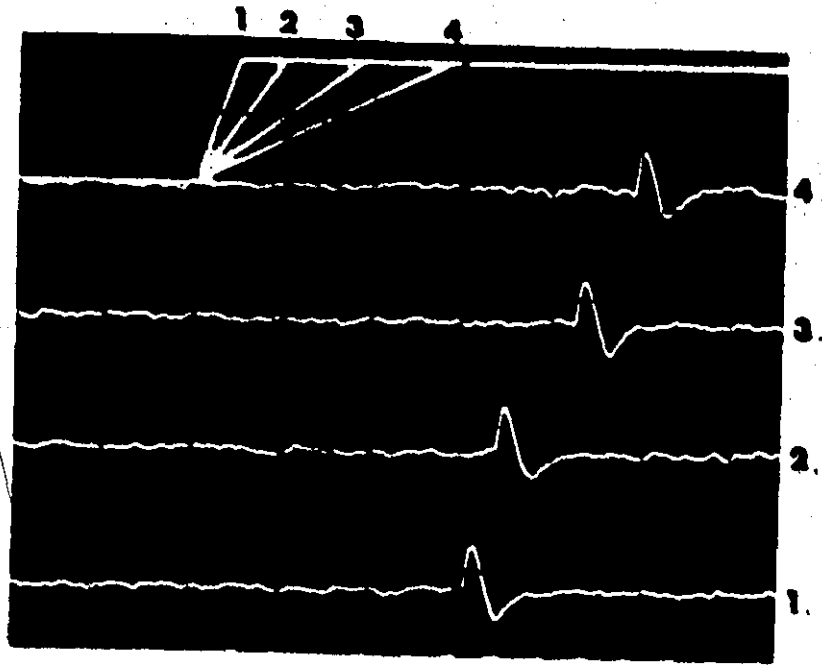
It should be noted (see Fig. 7) that the increasing amplitude of the stimulus, illustrated in Fig. 12, caused an increased rate of rise of the mechanical pulse; it is this velocity component which activates these skin receptors. Fig. 15 shows that as the velocity of the displacement increases, the latency of the spike decreases. (Compare to Fig. 10 which shows how the mechanical stimulus is affected by such changes in rate of rise of the applied voltage). There are at least 4 factors which affect the latency of the response: (1) the time it takes for the mechanical displacement to reach the critical velocity, (2) the "setting-up" time for impulse initiation, (3) the conduction velocity of the impulse in the terminal branches (referred to as terminal

Fig. 14: Test for Slowly Adapting Receptors

A very large stimulus (a probe of 10 mm² flat surface of contact) was applied to the surface of the skin covering the hindlimbs. In A the stimulus is applied to the limb (as indicated by the white bar). In B, the same stimulus was applied to the limb but the overlying skin had been removed; it evoked a similar pattern of impulses in the CFP nerve. The stimulus was clearly exciting receptors associated with the deeper tissues, presumably muscle, tendons and possibly joints. Part C is a record of the spontaneous activity in the CFPN with the skin removed and in the absence of an applied stimulus.



0.5 sec
| 2x10⁻⁵ volts



2 msec

Fig. 15: Effects of Different Rates of Rise on the Recorded Action Potentials.

The top trace is composed of 4 superimposed applied voltage inputs of different slopes (1-4). The bottom 4 traces are the corresponding recorded action potentials. Note, that the slowest rate of rise of applied voltage, 4, causes the longest latency response (the top trace of the action potential series). See Fig. 10. Vertical scale is 25 volts for the applied voltage 50 μ volts for the recorded action potentials.

velocity), and (4) the true axonal conduction velocity. The contribution of some of these components was estimated by recording simultaneously the latency of one response at different distances along the axon from the receptor (see Fig. 16), and then plotting the results on a distance vs. latency graph as shown in Fig. 17.

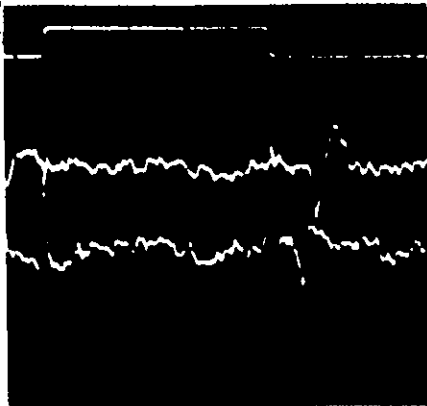
Because of the short length of nerve stump available, it was not possible to make more than 2 simultaneous records. The relatively limited amount of accuracy involved in measuring the exact distance between the two recording electrodes, allows this method to serve only as a crude estimate of the conduction velocity. The range was usually found to be 5-10 metres/sec, based on the slope of the straight line (Fig. 17).

The intercept of the line on the latency axis at 0 distance, in plots such as that of Fig. 17 gives the combined delay due to the other 3 factors mentioned above. By comparing the rate of rise of the mechanical movement of the stimulator tip (see Fig. 18), for a stimulus which was just sub-threshold for evoking an action potential, to the maximum possible rate (which was approximately 2 times the former value) one can detect a constant latency of 2.5-3 msec. between the application of the electrical stimulus, and the beginning of a movement (see Fig. 18). This time represents the lower limit of the time required for the mechanical stimulus to reach the critical velocity in order to initiate an action potential. After subtracting this lag time from the total delay, represented by the latency intercept value at 0 distance, the remainder (3 msec) represents the total of the "setting up" time plus the terminal conduction velocity time. The techniques used in this investigation cannot separate these latter two events. However, studies from other

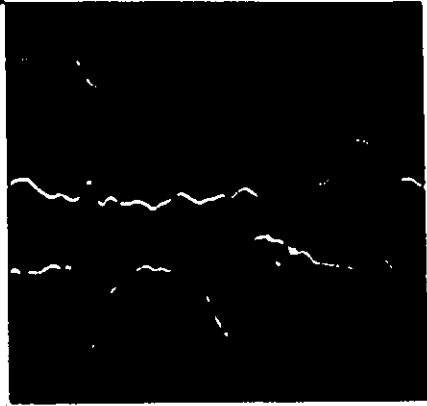
Fig. 16: Conduction Velocity

A, B and C each show records of a single action potential recorded at two different positions simultaneously along the nerve. On the right are expanded traces of the portion which include the response, indicated by the white bar on the left hand figures. The scatter indicates variability in the time for setting up the impulse. The difference in latency between these spikes at the two positions was very constant, and equalled 0.6-0.7 msec (see Fig. 17). The horizontal bar is 2 msec for the figures on the left and 0.5 msec for the figures on the right. The vertical bar is 50 volts for the applied voltage and 20 μ volts for the nerve recordings.

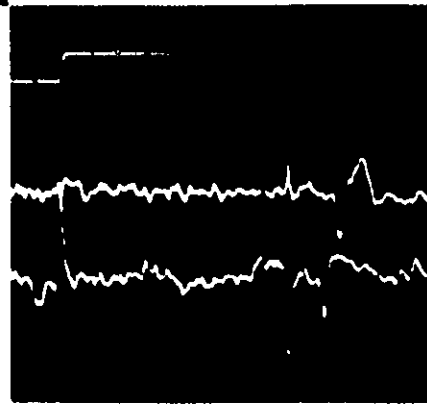
1a



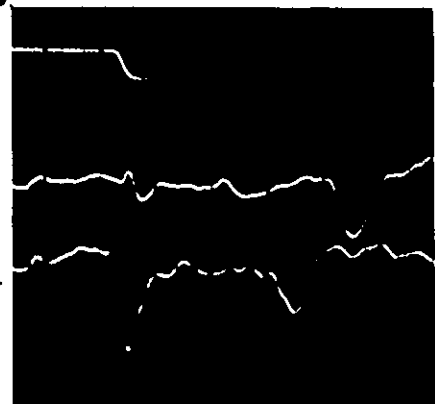
1b



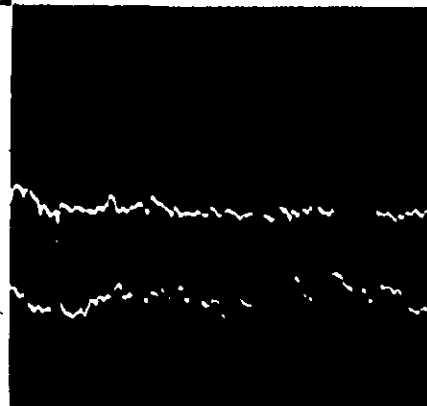
2a



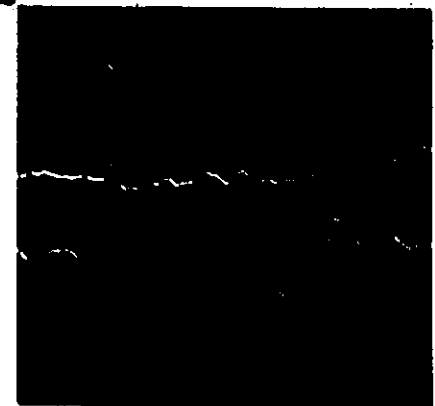
2b



3a



3b



T

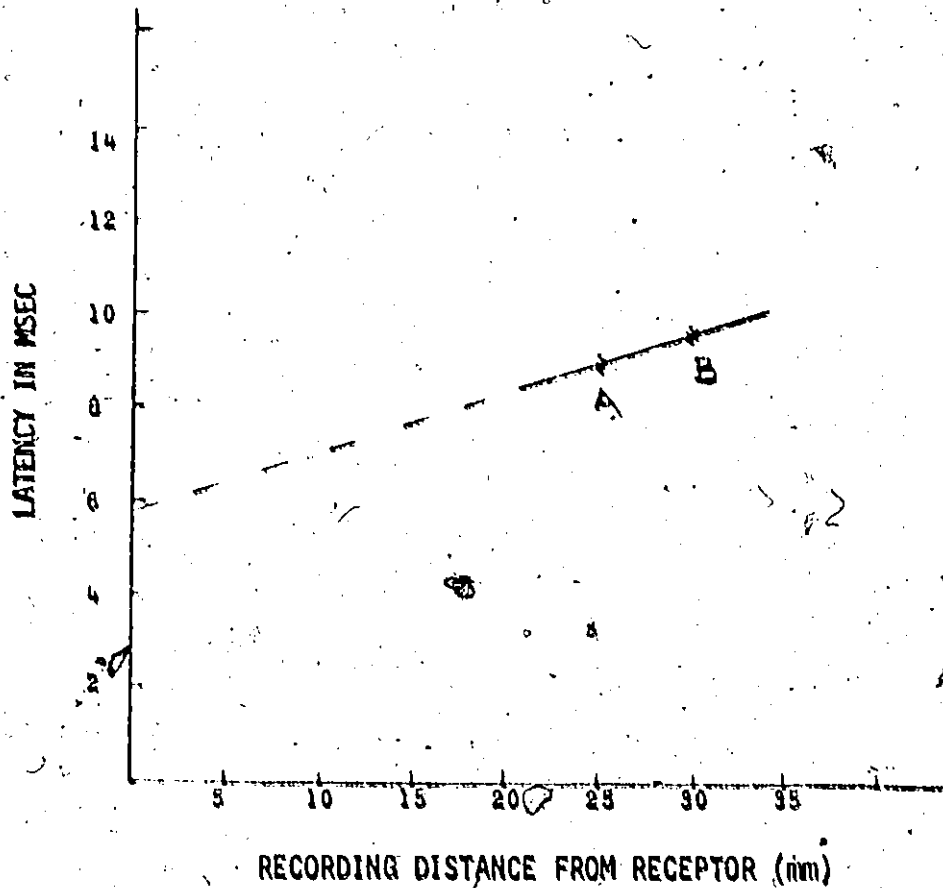
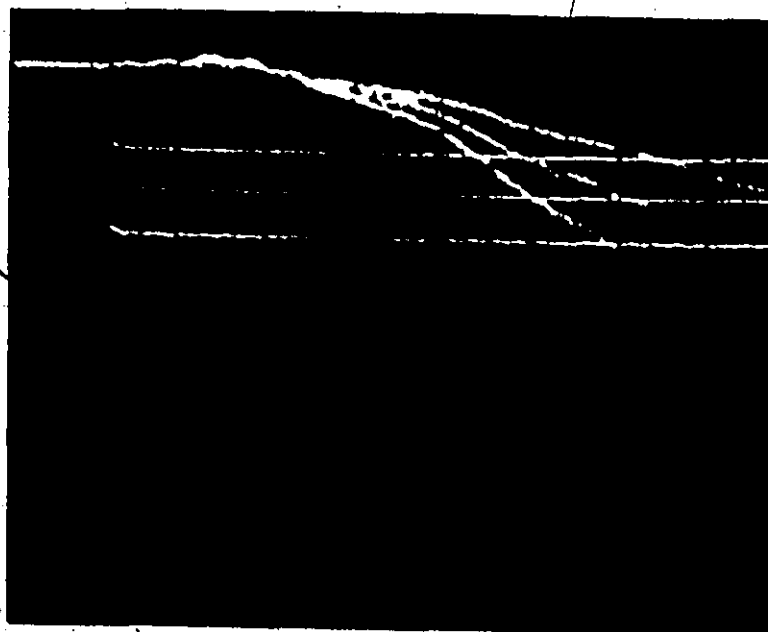


Fig. 17: Estimation of 'Setting Up' Time and Terminal Conduction Time.

A and B refer to the pair of impulses for the different records in Fig. 16. The inverse of the slope of the straight line gives the axonal conduction velocity (approximately 7 m/sec). There was little variation in the axonal conduction velocity for each trace. The latency intercept for each curve represents the combined delay due to mechanical lag of the prodder, 'setting up' time for the receptors and terminal conduction velocity (see text).



2 msec

Fig. 18: Comparison of the Movement of the Prodder for Different Input Voltages; Expanded Time Sweeps of the Output Movement.

It can be seen that there is a lag of 2.5-3 msec before differences can be detected between any of the traces. The top trace was just subthreshold for excitation of the receptors and the bottom trace was twice that value. (i.e. twice the rate of rise). The vertical bar 50 volts for the input voltage all 0.5 volts for the output of the photo cell.

rapidly adapting mechanoreceptors indicate that the lower limit of the setting up time is likely to be approximately 2 msec (Gray and Sato, 1953; Catton, 1958). This would then allow roughly 1 msec for the conduction along the terminal branches. If one assumes a value for conduction velocity in the unmyelinated terminals, then one can calculate the length of the terminal branch from where it leaves the parent axon, just below the dermis, to the site of the receptor in the skin which is probably at the base of the epidermis (discussed below). The terminal branches are unmyelinated in the skin and roughly 0.5μ in diameter (Parducz, personal communication). Conduction for unmyelinated axons of this size is in the range of 0.5-1.0 m/sec (Maruhashi, Mizugushi and Tasaki, 1952). From this, the length of the terminal branches can be calculated to be roughly 500-1000 μ m long. This estimate, admittedly a crude one, does give an approximate measure of how far these branches meander through the skin to make connection with the receptive point.

(e) Morphology of the salamander skin

In several instances in the literature, functionally-identified mechanosensory sites have been correlated with the presence of morphologically distinct structures in the skin (Quilliams, 1966). It was natural to ask if there were morphological structures which could be correlated with the rapidly adapting receptors found by physiological means to be in the salamander skin. If such structures could be found, then any physiological estimate of the density of mechanoreceptors in the skin could be confirmed by an independent morphological study.

Fig. 19 shows a light microscopic section of the salamander skin used in this study, prepared as outlined in Methods, Part 9(a). The

outermost layer of 3-4 cells makes up the epidermis which is roughly 50 μ thick. Immediately below this, a thin band of collagen separates the epidermis from the dermis. The blood vessels are in the dermis and so are the prominent large secretory glands whose opening ducts pass right through the epidermis to enable their contents to be discharged directly to the outside. A larger band of collagen fibres separates the dermis from the deeper skeletal muscle, and it is only below this collagen layer some 200-300 μ down from the skin surface, that one can detect any nervous tissue with the light microscope. In the section shown, a bundle of myelinated axons lies below the deep sub-dermis collagen layer. Several hundred of these sections were examined serially, and no specialized receptor cell-nerve ending complex was observed; it was presumed that the mechanoreceptors in these salamanders were free nerve endings, too small to be resolved with a light microscope. However, recent electron microscopic examination of the skin suggests that there may be some possible organization of the individual sensory endings which modifies this conclusion.

Fig. 20 is an electron micrograph of the epidermis. This section shows 2 or 3 profiles of unmyelinated nerve fibres in close contact with a particular cell in the epidermis. This cell is quite distinct from the surrounding cells, and is very similar in appearance to a "Merkel" cell, described by numerous authors in specialized mechanosensory structures especially prominent in the mammal (Smith, 1967; English, 1974; Burgess, English, Horch and Stensaas, 1974; Iggo, 1974). Although this ultrastructural investigation is still in its early stages, the evidence so far suggests that the cutaneous mechanoreceptors in the salamander are

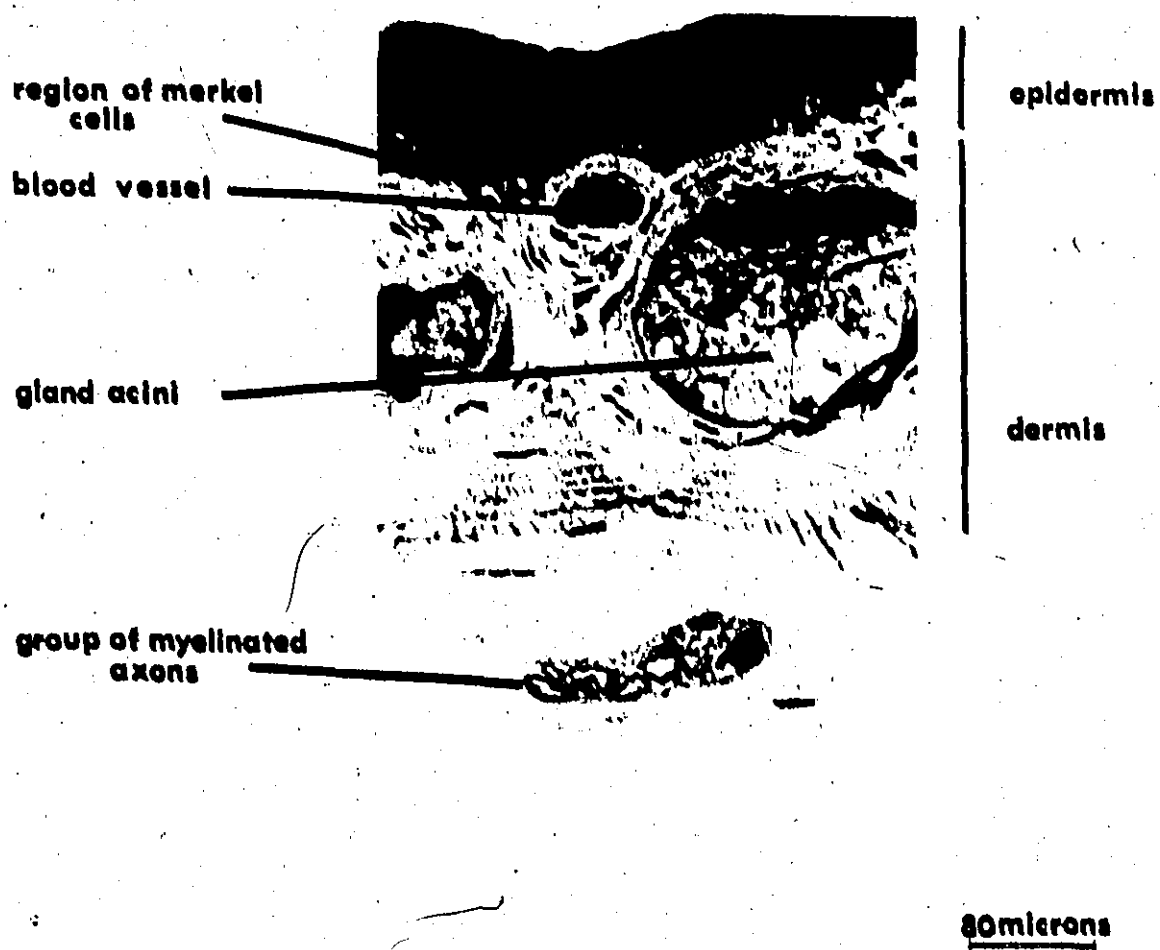


Fig. 19: A Light Microscope Section of the Salamander Skin.
The skin was processed as described in the Methods
Part IV, 9(a).

Fig. 20: An Electron Micrograph of a Portion of the Epidermis

This figure shows a nerve fibre (N) in close contact with a Merkel cell (M). These Merkel cells are found in the bottom layer of the epidermis, just above the basal lamina between the epidermis and the dermis. Notice the dark staining granules in the Merkel cell cytoplasm adjacent to the nerve fibres.
Scale: 2 cm = 1 micron.





in fact Merkel cell-nerve fibre complexes, and not simply free nerve endings.

The exact function of the Merkel cell is unknown and although it has largely been assumed to be implicated in mechano-transduction, this has never been demonstrated. One could also speculate that there exists a trophic function of these cells; perhaps they attract growing nerves to the epidermis (reasons for this suggestion will become clearer in the results below).

3. Axonal Receptive Fields

It was shown in the preceding sections that by selectively stimulating the skin at different points, one could activate individual axons which supply mechanoreceptors. These points all appear to be rapidly adapting (possibly they are Merkel cell-axon complexes).

At this point in the investigation it seemed possible that a study of the receptive fields of individual axons could be used to answer the main questions of this thesis, namely, does colchicine cause scattered degeneration of endings, and what is the quantitative nature of nerve sprouting? It was reasoned that, if colchicine was causing terminal degeneration, the individual axonal fields on the average might be smaller. After sprouting had occurred, the individual axonal fields of the sprouted nerves should be larger, and their shapes might offer further hints at the underlying mechanisms of sprouting. A study of axonal receptive fields was therefore conducted.

a) Method

In this study the CFPN field (see page 36) was used. In order to monitor the total mechanosensory output from this area, the discharge along both the 16th and 17th nerves was recorded simultaneously. The method devised for measuring individual receptive fields of axons did not require the nerve to be dissected down to single sensory units. Such a dissection would not have allowed many of the present investigations on the density and organization of the innervation, which were only possible when the total mechanosensory output from the skin into the nerve could be monitored. The technique used to measure the individual axonal fields was that of occlusion, which involved the simultaneous use of two separate stimulators (each one having a tip diameter of 10μ) to define the limits of the field under investigation.

As will be discussed in more detail below, the skin has localized spots of very high sensitivity to mechanical stimulation. One of these spots, which was found by a systematic search, was used as a reference point over which the reference prodder was positioned. The second prodder was positioned about $50-100\mu$ from the first. This 'roving' stimulator was then moved away from the reference point in steps of 50μ for each of 8 different directions, 45° apart (see Fig. 23). At each new location the second prodder was tested to see if it was still in the same axonal field as the first. This was done by activating the two stimulators separately with a suprathreshold stimulus (3-4 times that of the sensitive point); each prodder evoked an action potential on its own (except in rare instances when the second one was a totally insensitive spot). If one of these action potentials was in the 16th segmental nerve, and the other

In the 17th, then the two prodders were clearly in separate axonal fields. However, when both stimulators evoked spikes in the same segmental nerve (either the 16th or the 17th) the occlusion technique had to be used because only in a very few cases could the individual spikes be distinguished, with certainty, on the basis of their size, shape and latency. The occlusion technique consists of activating one stimulator a few msec before the other. When the two stimulators are inside the same axonal field, the second fails to evoke an impulse because the axon excited by the first is still in its refractory period. On the other hand, if the two stimulators are in different axonal fields, then two action potentials will be recorded. As the second stimulator is moved progressively away from the fixed reference, one in steps of about 50μ , it passes fairly abruptly (i.e. over a distance of $50\mu\text{m}$) from one field to the next. Often 2 separate axonal spikes were evoked at the boundary point between axonal fields by the second prodder alone, one spike being occluded by use of the reference prodder 50μ . Beyond this point the roving prodder evoked only 1 spike, which was not occluded. These transition points were recorded for each of the 8 directions radiating from the reference point, and the field was reconstructed by joining them up. By recording the total sensory output, one can easily map the neighbouring axonal fields, although the task is laborious.

Figs. 21 and 22 illustrate this technique. Fig. 21 shows the electrical recordings obtained when two stimulators were in the same field. When each stimulator was activated alone, one action potential resulted; when they were excited together there was still only one spike, indicating that both points were supplied by the same axon.

Fig. 22 shows the results when the prodders were in different axonal fields. Now the two stimulators, when activated simultaneously, gave two spikes which summated. An example of 3 adjacent fields which were all in the same segmental nerve is shown in Fig. 23.

b) Results

The preliminary studies which have been made of such axonal fields indicate a variation in their size from 0.5 mm^2 to 4 mm^2 . The interesting finding is, however, that each individual axon innervates its own territory of skin, and there is very little overlap between axonal fields. That is, the skin is organized essentially as a mosaic of individual axonal fields. However, many more fields will have to be mapped to be absolutely certain of the invariance of this pattern of innervation. It should be noted that the occlusion technique was only useful because there was so little overlap between axonal fields. If overlap had been extensive this method would have been more difficult to use.

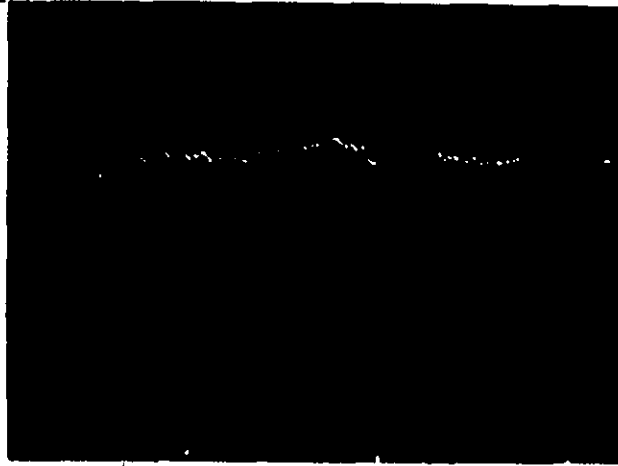
c) Limitations of axonal field studies

There are two practical limitations which enormously limit the extent to which the axonal receptive fields can be used to answer the quantitative question about whether colchicine was causing terminal degeneration in the treated nerve, and also to study quantitatively the response of nerves after sprouting. One has to do with the variation in field size. In general, these experimental animals survive, and remain in good condition, for approximately 12 hours after the beginning of the experiment, with the initial dissection and segmental field mapping taking about four hours of this. In the remaining 8 hours, there remains

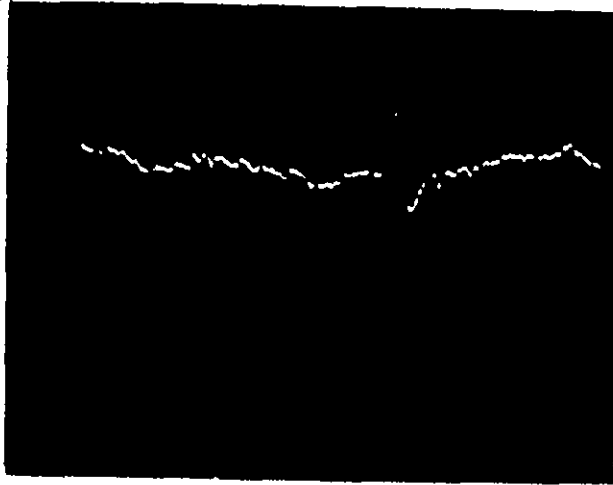
Fig. 21: Two Prodders in the Same Axonal Field

Top trace; the applied voltage to the crystal. Bottom trace; extracellular nerve recording from CFPN. A shows an action potential evoked by a stimulus from one prodder, and B shows an action potential evoked by the second prodder. C shows that when both prodders are activated together there is still only one spike. Therefore the two stimulators are in the same axonal field. The relative refractory period (when the second stimulus could not evoke a second response from a single axon) in these receptors can be as long as several hundred msec.

A



B



C

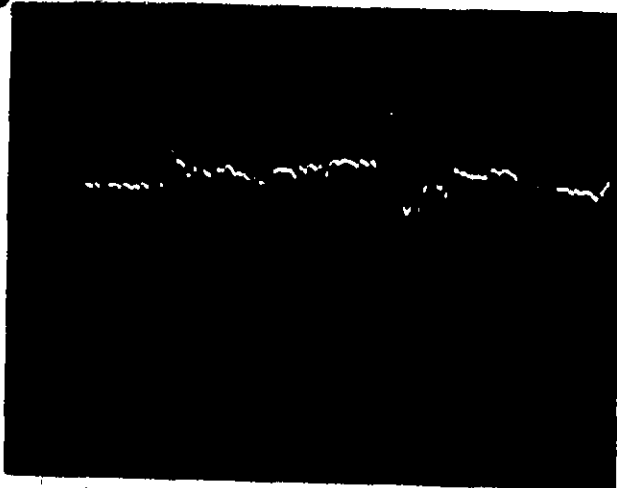
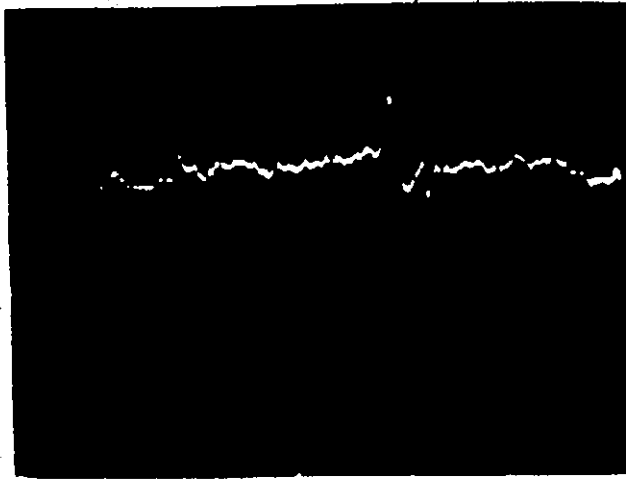


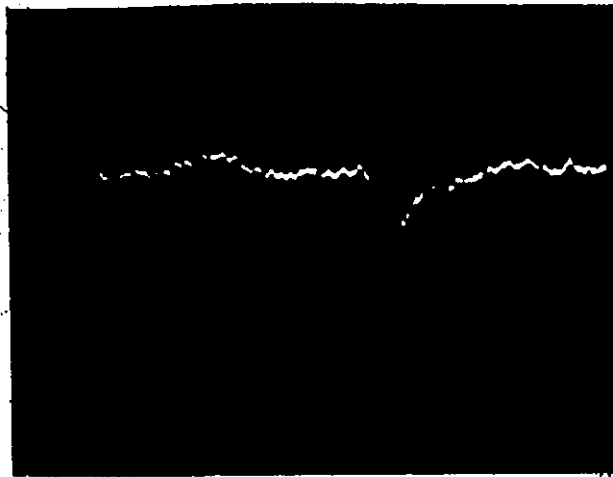
Fig. 22: Two Prodders in Different Fields

This figure is similar to Fig. 21 except that here the activation of both prodders together (C) evoked two action potentials which summated. The interval between the onset of activation of the second prodder was less than the relative refractory period for these receptors shown in Fig. 21.

A

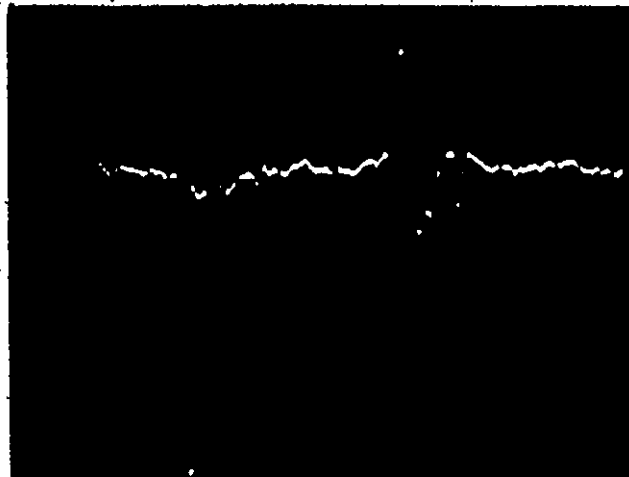


B



2msec
|

C



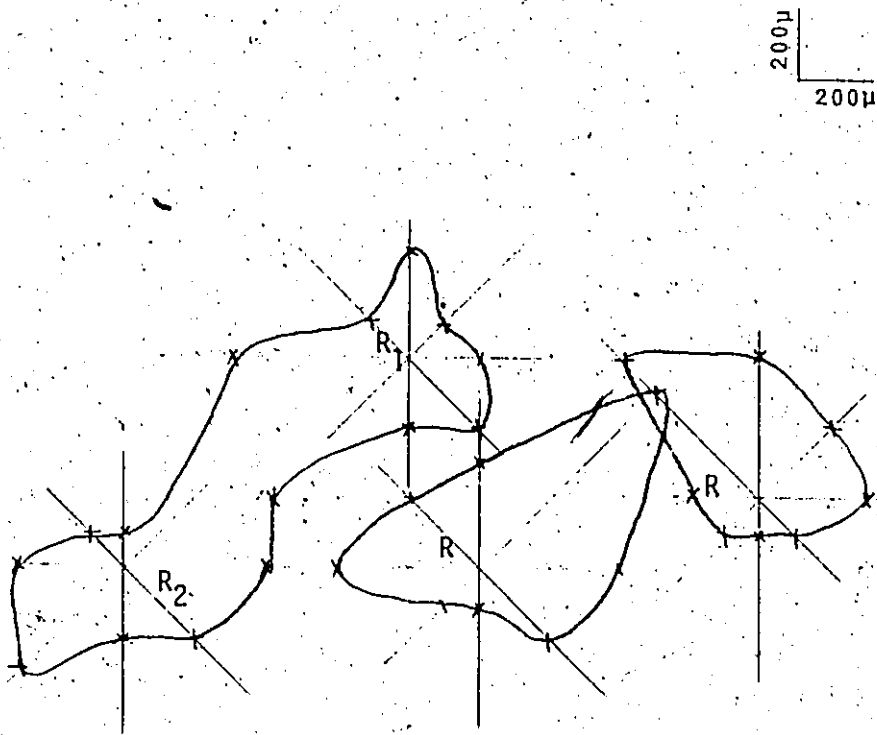


Fig. 23: Map of Three Adjacent Axonal Fields in the Same Segmental Nerve.

The diagram demonstrates the technique of moving in eight different directions to map out the area of each field. Notice that there is very little overlap between these fields. In each case the fixed (reference) prodder was kept at the original low threshold spot, (marked R) and the points marked X show where the second 'roving' prodder reached the edge of the field (see text). The relatively large field on the left required a second relocation of the reference prodder for its complete mapping.

only enough time to measure 5-6 axonal fields on each side of the animal. The results of axonal field measurements in a few animals shows such a wide variation (8-fold), that even if the average increase in axonal field size was 100% one would need at least 10 different axonal field measurements from each side of the animal to show any significant difference. Furthermore, if the basic hypothesis (that there are inhibitory nerve factors transported down axons which prevent adjacent axons from growing into their territory) is correct, then after partial denervation or colchicine treatment, only those axons neighbouring an affected axonal territory would be expected to sprout; ones surrounded by axonal fields of the same (untreated) spinal nerve presumably could not. This, then, would require an even larger number of fields to be mapped than first considered necessary on the basis of size variation alone.

In addition to these problems, there is an important objection which cannot be answered by axonal field studies. After colchicine treatment, it is conceivable that there is degeneration at the ends of a few terminals (the size of the axonal field remaining relatively unchanged), but that this 'dying back' does not reach the parent axon. This postulated degeneration could affect just a few endings in each axonal field. If this happened, then the sprouting that occurs after colchicine treatment could still be argued as resulting from "products of degeneration" (the classical hypothesis for denervation sprouting). To examine this important possibility, a method of investigating the individual receptors in an axonal field was developed. There is another argument against axonal field studies as a way of answering the quantitative questions of nerve sprouting. When a segmental nerve sprouts and increases its territory

of innervation, it is assumed that an individual axonal field would enlarge its area by sprouting new branches. However, it is conceivable that an axonal field could enlarge without there being any increase in the number of individual endings. In this case the individual terminals would rearrange themselves to cover a greater area, and the distance between each terminal would then increase. Because of all these objections, and in order to examine the various possibilities mentioned above, it was necessary to develop a method of investigating the number and distribution of individual receptors in an axonal field.

4. Touch Receptors in the Skin

a) The critical stimulus and its variation

In studying the problem of distribution of touch receptors within an axonal field, it is essential to know if all the individual receptors associated with that axon can be resolved, even though there may be a wide range of sensitivities among them. A detailed systematic map of sensitivity to mechanical stimulation was made of every point in a complete area within an axonal field, in an effort to answer this question. A 50μ diameter prodder was moved in steps of 50μ along the skin, and at every point the rate of rise of the mechanical stimulus was increased in steps of $0.1 \mu/\text{msec}$ until an action potential was evoked in one of the two nerves supplying the region (16th or 17th). This stimulus is referred to as the 'critical stimulus'; it represents the stimulus just large enough (that is, with just fast enough rate of rise) to evoke an action potential every time the stimulus is applied. While this critical stimulus would also appear to represent the "threshold

stimulus" for that point on the skin, the term threshold is used with a more restricted meaning later. The accuracy of the measurement of the critical stimulus was estimated by making repeated measurements many times at the same point on the skin. The stimulator was placed on a sensitive spot, and the applied voltage was gradually increased in successive steps until a spike was evoked 5 times consecutively; this value was recorded. A few minutes later, the stimulator tip was lifted vertically off the skin and then lowered again, and the critical stimulus redetermined as before. This procedure was repeated 10 times at that same point on the skin. This test was performed

on a few sensitive spots. The results showed that all critical stimulus values for any one spot were within 20% of the mean for that point, and this variation is taken to represent the error in the measurement of critical stimulus in these experiments.

The results of the systematic survey are represented in Fig. 24 both as a histogram giving the percentage occurrence of any given range of stimuli, and as the corresponding cumulative graph relating percentage of receptors excited to the stimulus velocity. In part c of this figure the experimental data is also shown in the actual two dimensional array used to obtain it. This study shows that there is a continuous range of sensitivities within an axonal field; the majority of spots are in the high sensitive range and the rest tend towards the low sensitive range.

Do these results mean that there is an individual receptor under each point tested, and that these receptors have differing sensitivities, or conversely, do the apparently higher threshold points indicate prodder

Fig. 24: Detailed Survey of Critical Stimuli Within an Axonal Field.

a) A histogram of the results, giving the percent occurrence of given ranges of stimuli. The range of the low threshold group is $0.57 \mu/\text{msec} - 0.93 \mu/\text{msec}$.

b) The same data is plotted as the corresponding cumulative graph, where each bin represents the sum of that bin plus all other bins to the left of it. This curve relates the percent of receptors excited to the stimulus velocity. Sturge's rule (Daniel 1974) was used as a guide for the interval width of the histograms in A and B as well as all the subsequent histograms in later figures. The actual interval widths were chosen to take into consideration both the sensitivity of the stimulator and the error involved in measurement of critical stimuli (see text)

c) Represents the actual values of critical stimuli found for all skin points in the dimensional array used experimentally. The range of critical stimuli for the low spots (L) medium spots (M) and high spots (H) are shown on the histogram in A. Notice that neighbouring every M spot one can locate an L spot.

locations at varying distances from the receptors, which are low-threshold? (Or is the true situation a mixture of these two extremes?) To answer these questions it is necessary to consider some of the physical consequences of mechanically deforming the skin by the methods used in these experiments.

(b) The simple hypothesis

In considering the data of the detailed survey of mechanical sensitivity, at every point in a given area, which in fact was within a single axonal field, it is interesting to note that the first bin of the histogram is the highest, and the height of the bins to the right of it falls off in an approximately exponential fashion at increasing stimulus strengths. This shape is what one would expect if there were only one population of the receptors, all of roughly the same sensitivity. The stimulus strength of the first bin would indicate the range of sensitivity of these receptors and its height would represent the frequency with which the prodder lands on one of these receptors. The bins representing the higher stimulus strengths indicate the frequency with which the prodder lands to one side of the receptors; the strength of stimulus needed would depend on exactly how far away the prodder was from the nearest receptor. Additional data in support of this possible model of receptor organization comes from inspection of the two dimensional array of data from this experiment (see Fig. 24). One notices that neighbouring every "medium spot" is a "low spot". This suggests that when the prodder was located at the former positions, the larger stimulus was needed in order to activate the neighbouring "low spot".

The simple hypothesis, then, is that the mechanoreceptors are all of fairly uniform sensitivity, sufficiently spaced out for a prodder of 10μ tip diameter to have a good chance (say 60-70% probability) of 'missing' them in any random survey. The data upon which this hypothesis is based was obtained from a detailed survey of touch receptors within only one axonal field. Before subjecting this model to critical testing, attempts were made to discover whether other axonal fields had these same characteristics. As mentioned earlier (in the discussion of axonal field studies), the task of making a series of systematic surveys within different axonal fields was considered impracticable, because it was too time consuming and labourious. Furthermore, such a study would give information about only a few axons in a segmental nerve, and, as stated before, the main objective of this study was to have a reliable method of measuring the density of nerve endings within the entire segmental nerve field. Therefore, a method had to be devised that would enable testing of the endings of many different axons.

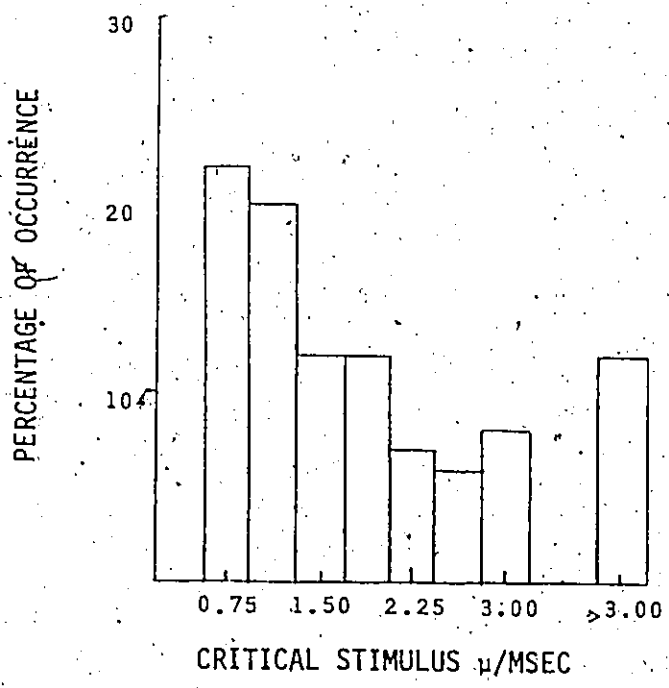
(c) Distribution of touch receptors in CFPN field

The method used to sample the touch receptors of many axons within the CFPN field was to make a random survey of the sensitivity to mechanical stimulation within a large area (approximately 5 mm x 3.5 mm), using the same devices described in the systematic survey. The 'critical stimulus' (see page 86) was measured for 75 random points, each point being located at least 400μ away from the preceding one. The results are represented in Fig. 25 both as a histogram, giving the percentages of occurrence of any ranges of stimuli, and as the corresponding cumulative graph relating the percentage of the population activated to

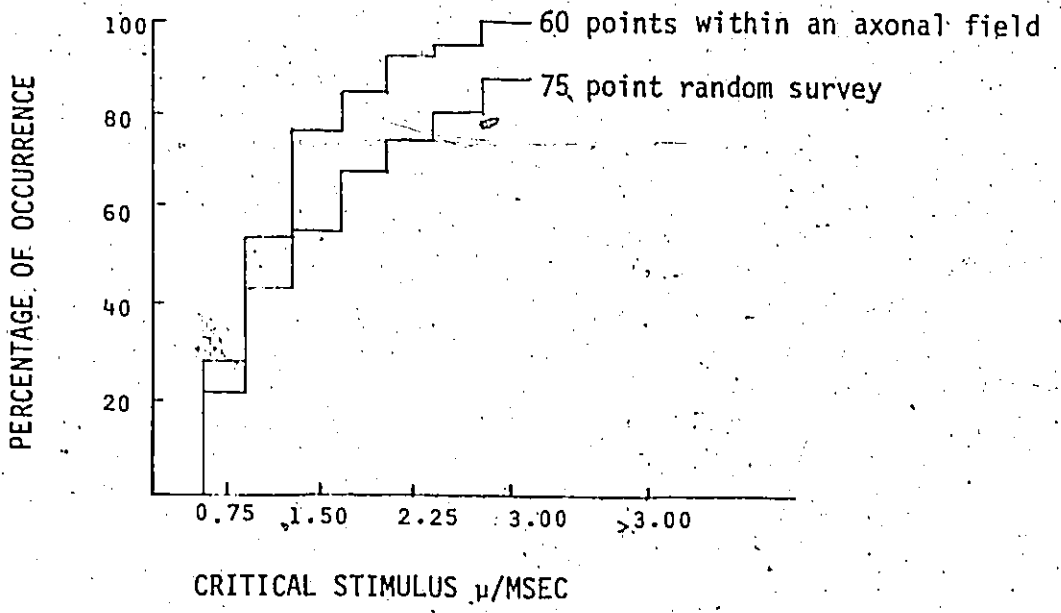
Fig. 25: Random Survey of Critical Stimuli of the Dorsal Skin Covering the Hindlimb

A. The results are plotted as a histogram giving the percent occurrence of any given range of stimuli. The range of low threshold group is $0.57 \mu\text{/msec} - 0.93 \mu\text{/msec}$. B. The same results are plotted as the corresponding cumulative graph where each bin represents the sum of that bin plus all other bins to the left of it. Superimposed on this cumulative graph is the results of the detailed survey of critical stimuli within an individual axonal field (Fig. 24) from a different animal. There was no significant difference ($P > 0.2$ based on the Kolmogorov-Smirnov test) between these two curves.

A.



B.



the stimulus velocity.

It is interesting to note that the results of this random survey look very similar to the results of the systematic survey within an individual axonal field (compare Figs. 24 and 25). In fact, there is no statistical difference ($P > 0.2$, based on the Kolmogorov-Smirnov test, see Conover, 1971) between the results of these two experiments, which were carried out on the hindlimb skin of different animals. This suggests that the distribution of receptors or, in terms of the hypothesis, the chance of landing on a receptor, is the same for individual axonal fields as it is for the entire CFPN field. For this to be acceptable, however, the random survey must be genuinely representative of the entire distribution of receptors in the CFPN field.

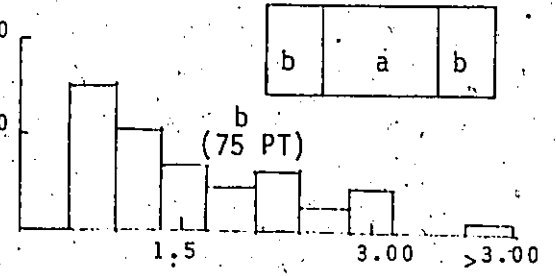
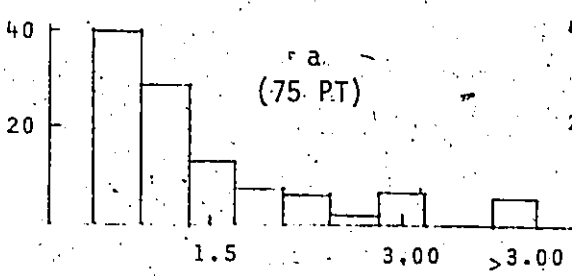
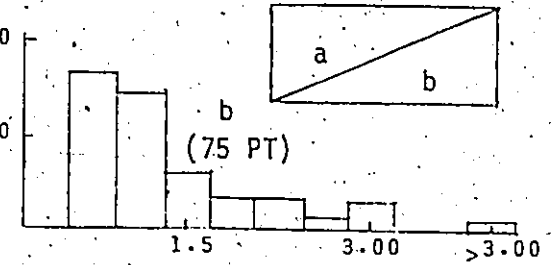
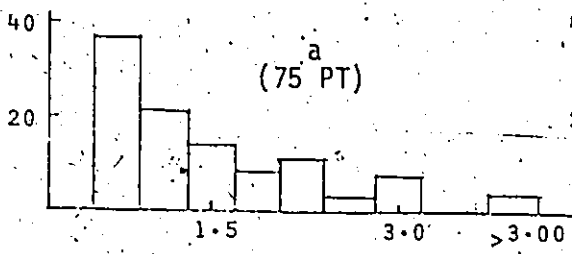
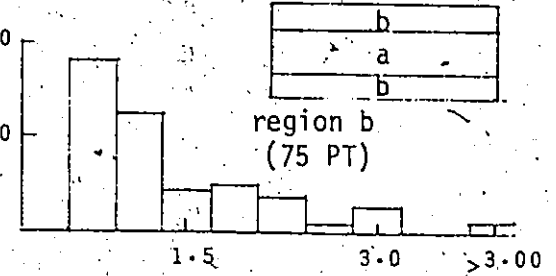
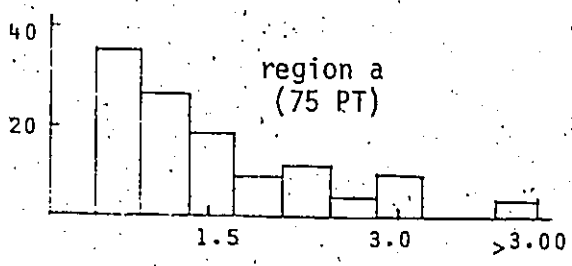
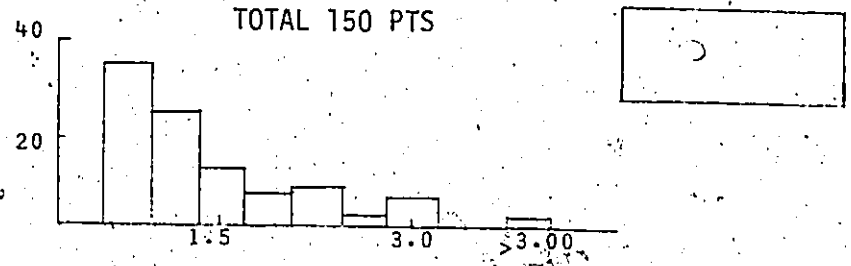
(d) Experiments to show that the results of the random survey represent the true distribution within the CFPN field

The following test was made to see if the results of the 75 point random survey represented the true distribution of receptors in the CFPN field. First, a large area of about 7 mm x 5 mm was surveyed at 150 random positions, each position being roughly 400 μ from the preceding one. Then, the skin area was subsequently divided up, into several different compartments, each including 75 points. The histogram and the cumulative frequency curves were constructed for each compartment, and compared to those of the other compartments, and to the one constructed for all 150 points together. The results are shown in Fig. 26. There is no significant difference ($P > 0.2$, based on the Kolmogorov-Smirnov test) between any of these histograms or curves, and therefore it can be concluded that a 75 point random survey is an

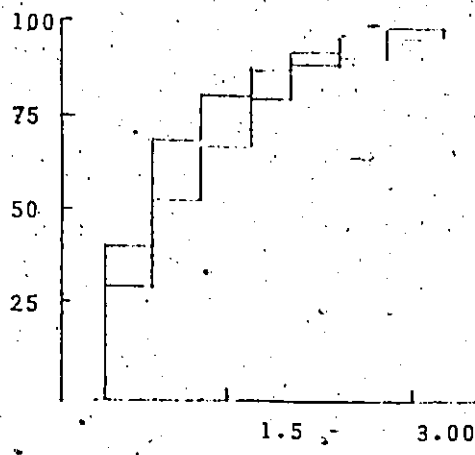
Fig. 26: Investigation of Gross Distribution of Sensitive Spots.

150 random points were tested, and the resulting values of critical stimuli were grouped by regions, as shown by the rectangles on the right of figure A. In A the total 150 points were divided into equal parts in a variety of ways, each part having 75 points. The various combinations were plotted as histograms (see Fig. 24) and compared. In B the cumulative curves corresponding to the extreme values from all 7 histograms in A were constructed. There was no significant difference ($P > 0.2$, by the Kolmogorov-Smirnov test) between these two curves.

A



B



acceptable sample of the total population.

(e) Uniformity of distribution

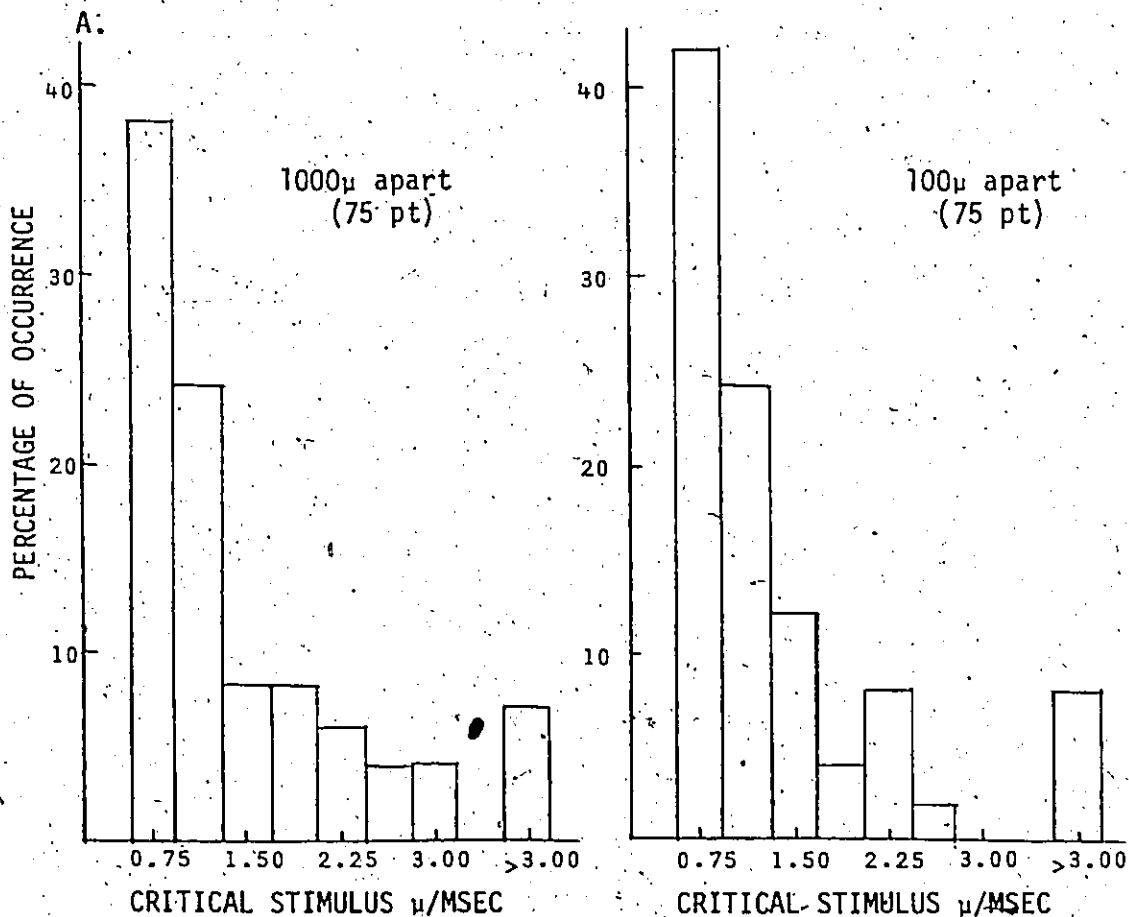
These results indicate that the distribution of receptors is grossly uniform over the large area described above. However, the above test does not rule out the possibility that the receptors occur in clumps, and that these clumps are uniformly spaced. (This highlights one of the problems in discussing uniformity; one can reduce the area considered to a value at which the uniformity must break down).

To test the extent to which the area under consideration can be reduced, while still giving the result of uniformity of distribution of the receptors, 75 points were sampled 1000μ apart and the results were compared to those of 75 points sampled within the same area but spaced only 100μ apart. The two histograms, together with the cumulative frequency curves, are shown in Fig. 27. No significant difference ($P > 0.2$, based on the Kolmogorov-Smirnov test) exists between them, and it is concluded that the distribution is uniform over an area of at least 0.60 mm^2 . In addition, Fig. 28 shows that there is no significant difference ($P > 0.2$) between 75 point random surveys in the CFPN field from one side of the animal to the other; i.e., the bilateral symmetry of nerve fields extends also to the exact distribution of individual receptors within them.

Fig. 27: Comparison of Distribution of Critical Stimulus in Small and in Large Skin Areas.

A) The results of two 75 point random surveys one over an area of 55 mm^2 and the other over an area of $.60 \text{ mm}^2$ are plotted as histograms.

B) The same results are plotted as the cumulative curves corresponding to the histograms in A and compared as in Fig. 25B. There was no significant difference ($P > 0.2$) between the two curves.



B.

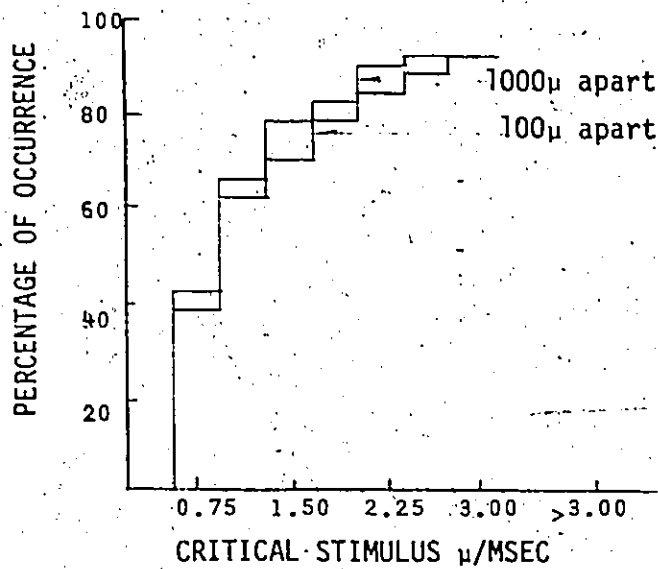
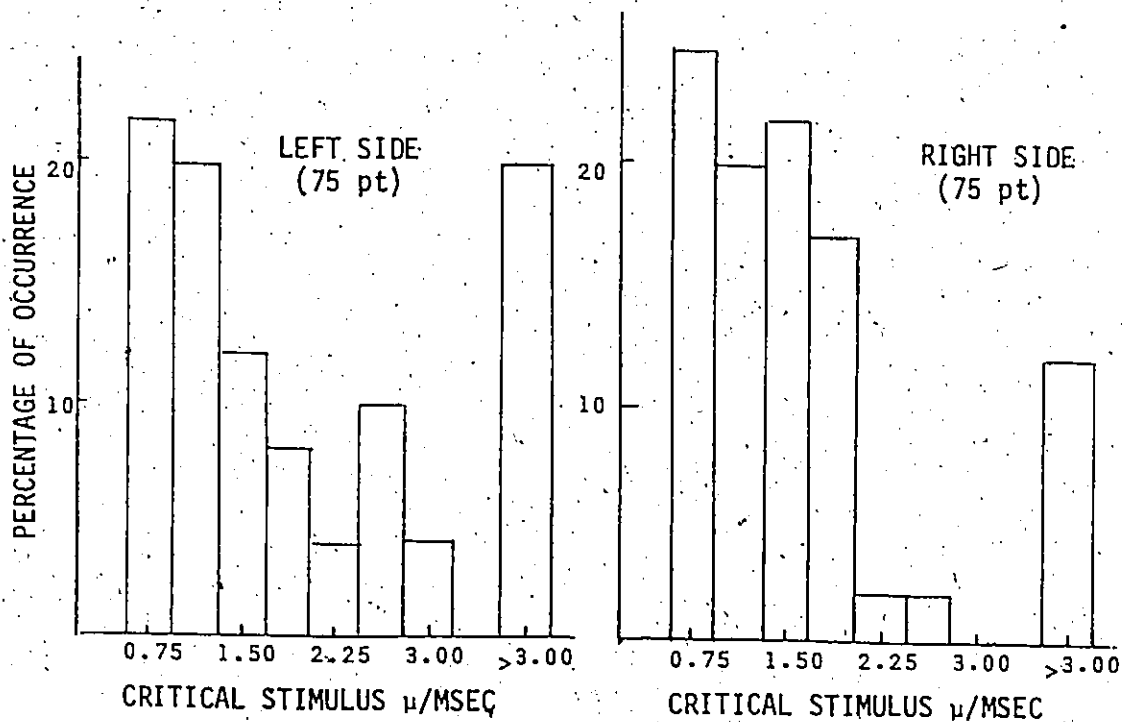


Fig. 28: Comparison of Distributions of Critical Stimulus from One Side of the Animal to the Other.

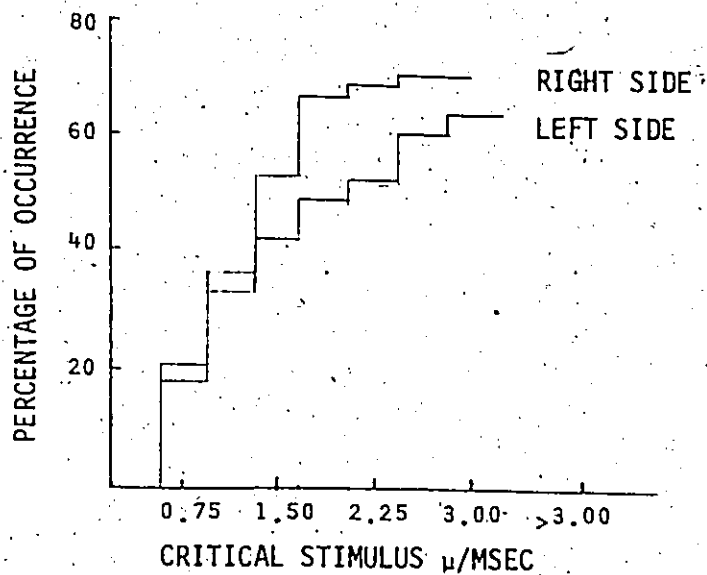
A) The histogram representing a 75 point random survey in the CFPN field is shown for both right and left sides.

B) The same results are plotted as the cumulative curves corresponding to the histograms. There is no significant difference between the two sides ($P > 0.2$) (c.f. Fig. 25B).

A.



B.



The distribution of receptors in the area of skin in which these tests were made refers to the total of the receptors belonging to both the 16th and the 17th nerve components of the CFPN trunk. Since the action potentials evoked by stimulating the receptors are recorded separately from each segmental nerve (see Methods, Part 7), the distribution of receptors can be obtained for both the 16th and 17th components. This method of sampling 75 random points in the CFPN area therefore allows the distribution of receptors belonging to each segmental nerve to be measured, and so the procedure satisfies the principle objective of this chapter. This, as stated at the onset, is to have a reliable method of measuring the distribution of nerve endings of a segmental nerve within the nerve field.

f) Location of nerve endings

It should be mentioned that "random" in these experiments means that the spacing between points was arbitrarily chosen to be 400μ , without regard to any morphological features such as the blood vessels or openings of secretory glands (see Fig. 29). A detailed study was made to investigate if the skin was more or less sensitive when the stimulator was placed over the blood vessel or a secretory gland opening. After surveying roughly 300 different points on and off blood vessels and secretory glands, it was concluded that these structures were in no way correlated with the mechanical sensitivity. Fig. 19 shows a cross section through the skin, as seen through the light microscope. The blood vessels

Fig. 29: Photomicrograph of the Skin Through the Dissecting Scope.

The bar is 250μ for A and 50μ for B. The openings of the secretory glands can be seen as dark spots surrounded by blood vessels arranged in an approximately polygonal array. From Fig. 19 it can be noted that these glands and blood vessels are actually in the dermis, four-five cells below the surface of the skin. In B, faint outlines of some of the essentially transparent epidermis cells can be made out.

6

104

A



B



104

6

and the secretory glands lie in the dermis, below a layer of 4-5 epidermal cells, through which the secretory duct passes to open on to the surface. When focusing with a dissecting microscope on the skin (see Fig. 29), one sees that these epidermal cells appear transparent and that the blood vessels and the ducts of the glands appear quite distinct. Since there was no detectable difference to mechanical sensitivity when the stimulator was over a blood vessel or a gland-duct, and taking account of the small size of stimulus needed to evoke an action potential in the nerve ($0.75 \mu\text{msec}$), it was suspected that the nerve endings that are activated by the mechanical stimulator are in this top 4-5 cell-thick epidermal layer. Recent evidence from Dr. Parducz of this laboratory indicates that there are only unmyelinated nerves in the skin and that in the epidermis ~~the~~ nerve fibers are always found close to, or actually in contact with, Merkel cells. These cells are commonly found in close relationship to nerve endings in presumed mechanosensory structures elsewhere in the vertebrates (English, 1974; Burgess and Perl, 1974; Iggo, 1974), and it can be speculated therefore that the associated nerve terminals represent the mechanosensitive endings here as well. The Merkel cells are always observed in the deepest layer of the epidermis, about 1/2-1 cell thickness above the region which contains the blood vessels and glands.

g) Testing of hypothesis to explain the distribution of sensitivity

Now, pursuing this study further, it is possible to test the hypothesis which explains the distribution of sensitivity to mechanical stimulation of the receptors in the CFPN area (see page 36). The major assumption of this hypothesis is that, when the prodder is located between receptors, higher stimuli are required to deform the skin in the

appropriate manner so that the nearest receptor (one of a uniformly sensitive population) will be activated.

It is possible to apply a theoretical treatment which, to some extent, describes how the skin will deform around the prodder as the stimulus is increased. The surface of the skin may be considered a thin diaphragm which receives mechanical support from the cells underneath it. As a force is applied to the surface of the skin, the cells underneath the prodder transmit the force laterally to the neighbouring cells so that the load becomes distributed in a radial manner from the site where it is applied (that is, the location of the prodder). This type of support is quite similar to that sometimes encountered in mechanical engineering, referred to as contact or Herzian stress, which describe the stress distributions in two adjoining objects, such as wheels and rails, balls and bearings, or mating gear teeth. Equations relating the deformation of a surface to the applied load have been worked out for many different contact situations and can be found in most handbooks of mechanical engineering (Roark, 1965). Fig. 30 shows an example of one of these contact situations, which in many respects is analogous to the present situation in the skin.

Fig. 30: Contact Stress Situation Used to Simulate Vertical Displacement of the Skin During the Prodding Experiments.

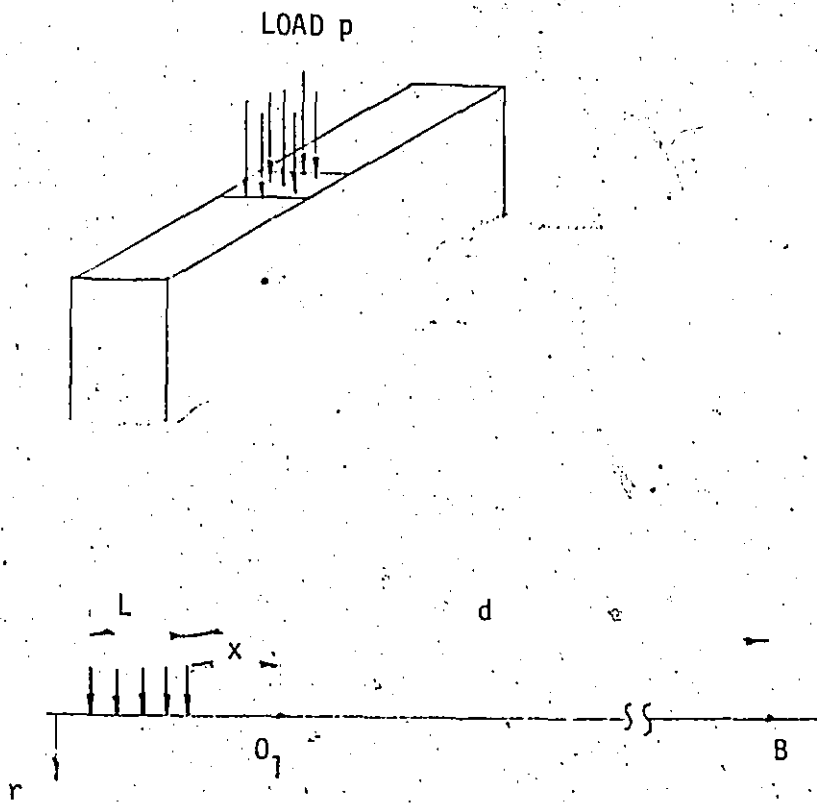
A) The stress situation of a load applied to a semi-infinite slab is shown diagrammatically. The relationship between the vertical movement of point O₁ to the applied load P is

$$r_1 = \frac{2P}{\pi E} \left\{ (L + x_1) \text{LN} \left(\frac{d}{L + x_1} \right) - x_1 \text{LN} \left(\frac{d}{x_1} \right) \right\} + \frac{PL(1 - \mu)}{\pi E} \quad (1)$$

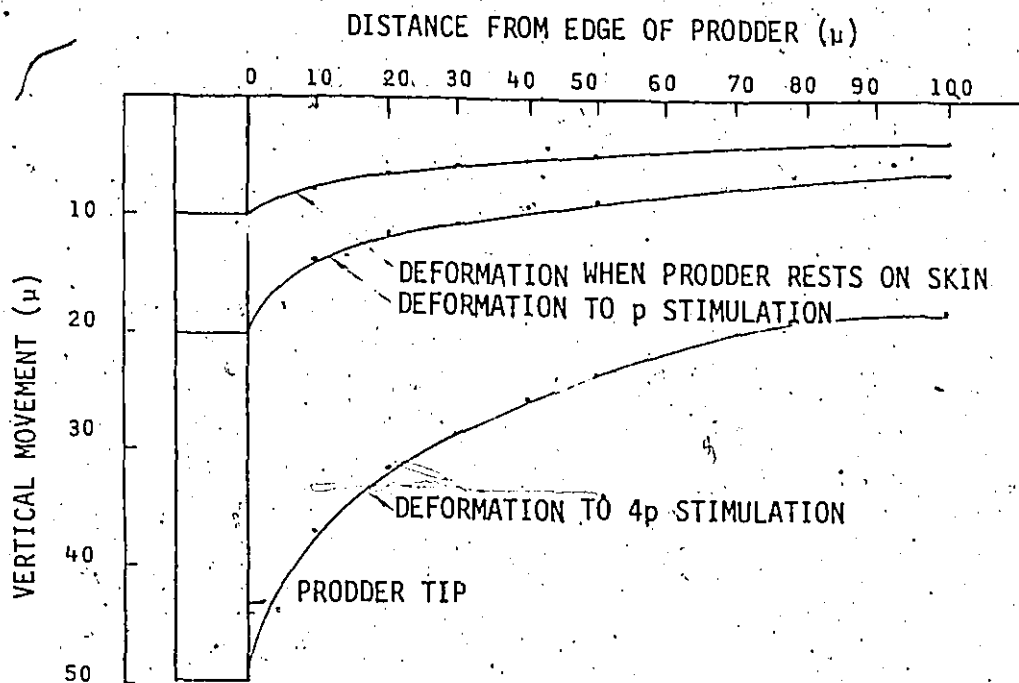
The explanation of the symbols of this expression, and the limiting conditions of this equation, are discussed in the text.

B) Equation 1 was solved to give the profile of vertical displacement for two different values of P. The values in equation 1 were chosen such that when the prodder was at rest on the skin surface, the tip indented the surface of the skin by 10 μ . The vertical displacement underneath the prodder (i.e. when X = 0) could be estimated from the calibration curves in Fig. 7 and 8. The range of movement to activate the low threshold receptor is about 10 μ and therefore by increasing the load by 4 times P, the displacement at X = 0 would then be approximately 40 μ from the resting level. Inspection of the profile of the vertical displacement reveals that when a 4P stimulus is used, the vertical displacement at a distance of 100 μ from the edge of the prodder (X = 100 μ) is approximately the same as that caused by a P stimulus at the origin, when X = 0. The significance of this finding is discussed in the text.

A.



B.



The equation relating the applied load to the deformation of the material surrounding the prodder is:

$$r_1 = \frac{2P}{\pi E} \left\{ (L + x_1) \text{LN} \frac{d}{L + x_1} - x_1 \text{LN} \frac{d}{x_1} \right\} + \frac{PL(1 - \mu)}{\pi E} \quad (1)$$

where

r_1 is the deflection at point O_1 in Fig. 30.

P is the applied pressure ($\frac{P}{L}$)

p is the applied load per unit length

L is the width of the applied load
(for the salamander skin this represents the prodder diameter).

O_1 is a point on the surface outside the area of the applied load.

x_1 is the distance the point O is from the edge of the applied load.

B is a point on the surface remote from the applied load.

E is the modulus of elasticity

μ is Poisson ratio

d is the distance that B is from the edge of the applied load.

and LN is the natural logarithm

By solving this equation for different values of x , one can obtain the profile of deformations of the surface of the skin as shown in Fig. 30.

It may be helpful in understanding this equation if the limiting situations are discussed briefly: (1) when $x = 0$ (ie. the spot at the very edge of the prodder) the vertical deflection, r_1 is maximum, (2) when $x = d$ (the "remote" point) the vertical deflection, r_1 , is minimum, although not quite zero because this function is exponential, (3) as L approaches 0 the deflection, r_1 , at point x approaches 0 (In other

words, the effect on the surrounding skin becomes negligible. This point can be appreciated by considering the case of applying a very sharp, thin pin to the skin. The pin would most probably penetrate the surface without deforming the surrounding skin), (4) as L increases, so does the deflection r , at x .

Now, in using this equation to simulate the prodding experiments in this study, the values $\frac{2}{\pi E}$ and $\frac{(1-\mu)}{\pi E}$ are held constant and adjusted so that when no stimulus is applied the prodder indents the skin by about 10μ . The values of the dimensions in the equations are selected to equal those of the prodding situation. Since a stimulus of 4 x low receptor threshold activated 80% or more of the total population, the solution to this equation was used to compare how much the profile of deformation changes as the stimulus is increased by a factor of 4. The solutions are drawn to scale in Fig. 30. This figure demonstrates that, as the stimulus is increased 4 fold, the surrounding skin becomes deformed for a distance as much as 80-100 μ further than it does for the smaller stimulus.

Therefore, if the situation of prodding the skin can be correctly represented by the theory of contact stresses, then the curves in Fig. 30 give an approximation of how the skin deforms as the stimulus is increased. Using this information one can predict that, if the prodder was 80-100 μ to one side of the receptor, it would activate this receptor if the stimulus were increased by 4 times the value needed to excite the receptor when the prodder was directly over it. This distance is probably over-estimated, because the skin receptors are rapidly adapting and sensitive to the velocity of deformation (see page 56); this theoretical treatment only considers the steady-state deformations without any regard to the

visco-elastic property of the skin, which would tend to slow down the rate of deformation.

h) Test of the theoretical treatment

It was possible to test the above theoretical prediction in a few experiments. A receptor lying near the edge of a selected axonal field was located; then the prodder was moved further out of the field and a large stimulus was applied in an attempt to activate the same receptor. In many cases, as in the one illustrated in Fig. 31, a lateral movement of 50μ from the low-threshold point required the stimulus to be increased by 4 times to activate the same receptor. This is in very good agreement with the theoretical treatment. The experiment is also consistent with the hypothesis that explains the distribution of sensitivity to mechanical stimulation within the CFPN area.

i) Spacing of receptors

This hypothesis assumes that there is only one population of receptors in the skin. This can be tested by using the data on the distribution of these receptors (presented earlier in this chapter) to predict the mean spacing and receptive field size of the receptors. Since this prediction would be based on the "one population" idea, a result actually obtained from a detailed systematic survey of the skin, which conformed to a predicted value, would also be good support for the hypothesis.

The results of the 75 point random survey, presented in Fig. 25 indicate that the prodder landed on a receptor (that is, the first bin of the histogram in Fig. 25) in 20% of the trials. Since this was a random survey, it can be concluded that 20% of the area sampled is covered by these receptors. In addition, the results of the test on

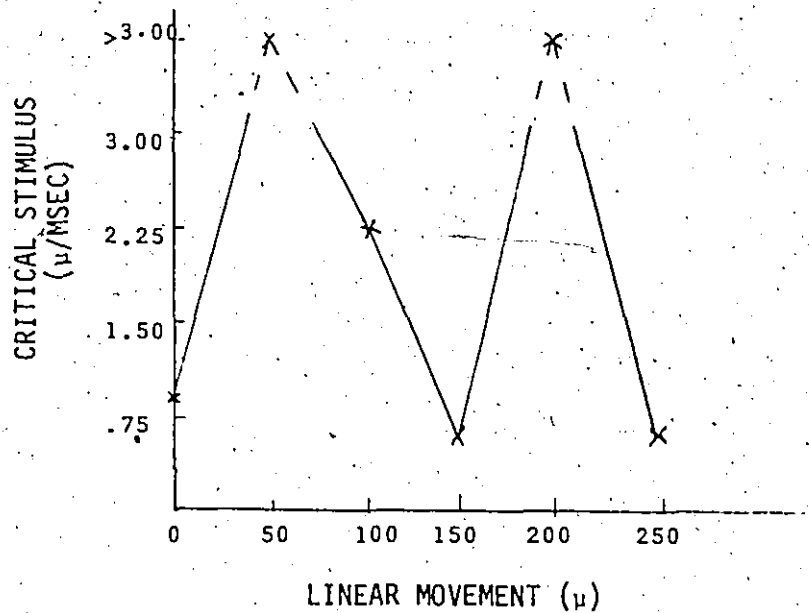


Fig. 31: The Relationship Between the Critical Stimulus Needed to Evoke an Action Potential and the Distance of the Prodder from a Selected Low Threshold Point On the Skin.

The 0 point was located by a systematic search and then a prodder was moved in a straight line in 50 μ steps. At each point the critical stimulus was tested. This figure can be usefully considered in relation to the systematic mapping which is illustrated in Fig. 33.

page 97 indicate that these receptors are uniformly distributed over the surface. If these receptors are considered to be in square arrays, a distance A apart, and uniformly distributed over the surface, as shown in Fig. 32, then the fraction of the square $A \times A$ occupied by receptors must be

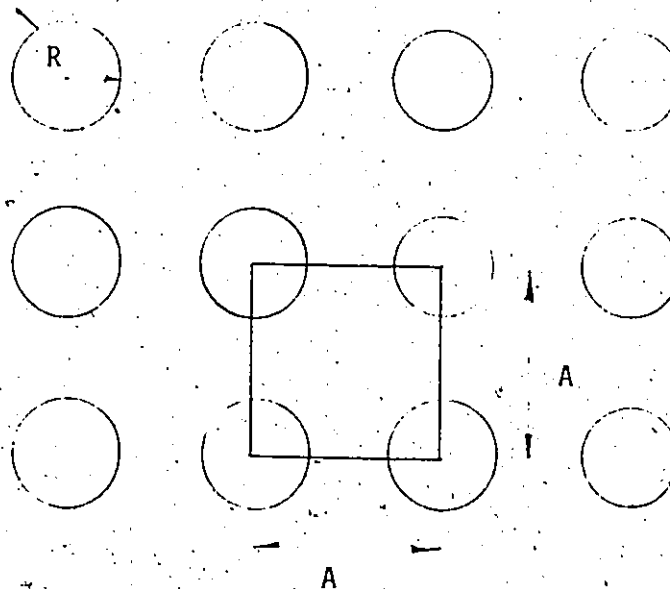
$$\frac{\pi R^2}{A^2} = 0.20 \quad (2) \text{ if}$$

R is the radius of the field of the receptor (assumed to be circular). It turns out that a different array of receptors, eg. hexagonal, does not greatly affect the calculated result. The section above on the profile of skin deformation (see Fig. 30) also indicated that a receptor could be activated 50μ away with a stimulus of 4 times that which is needed when the prodder is used directly on top of a receptor. (The stimulus needed to fire a receptor when the prodder is directly over it will be referred to as T , the true "threshold" stimulus.) Another way of saying this, is that the radius of the receptive field (R) of a receptor for a T stimulus increases by 50μ when a $4T$ stimulus is used.

Referring back to the 75 point random survey (see Fig. 25) one notices that 80% of the surface of the skin is receptive to a $4T$ stimulus. According to the "one population" hypothesis, the receptors would still be the same distance (A) apart for a $4T$ stimulus, and the only change is that the receptive field of the receptors is increased by 50μ . Therefore the fraction of the area $A \times A$ in Fig. 32 now receptive to a $4T$ stimulus is

$$\frac{(R + 50)^2}{A^2} = 0.80 \quad (3)$$

RECEPTIVE FIELD OF A
LOW THRESHOLD RECEPTOR



AREA OF $A \times A$ OCCUPIED
BY RECEPTOR IS:

$$1/4 \pi R^2 \times 4 = \pi R^2$$

R = RECEPTIVE FIELD RADIUS OF
A RECEPTOR

Fig. 32: Idealized Model of Receptors in the Skin.

This schematic diagram shows the receptors in a square lattice arrangement with the centres of their circular receptive fields a distance A apart. The fraction of the square A x A occupied by the receptive surface is

$$\frac{\pi R^2}{A^2}$$

Now, by substituting equation 2 into equation 3, it is possible to solve for the two unknowns, A and R.

A is, then, 200μ , and R is
 50μ

Therefore, the predicted radius of the receptive field of the receptor, assuming only one population of receptors, is 50μ and these receptors are spaced on the average 200μ apart.

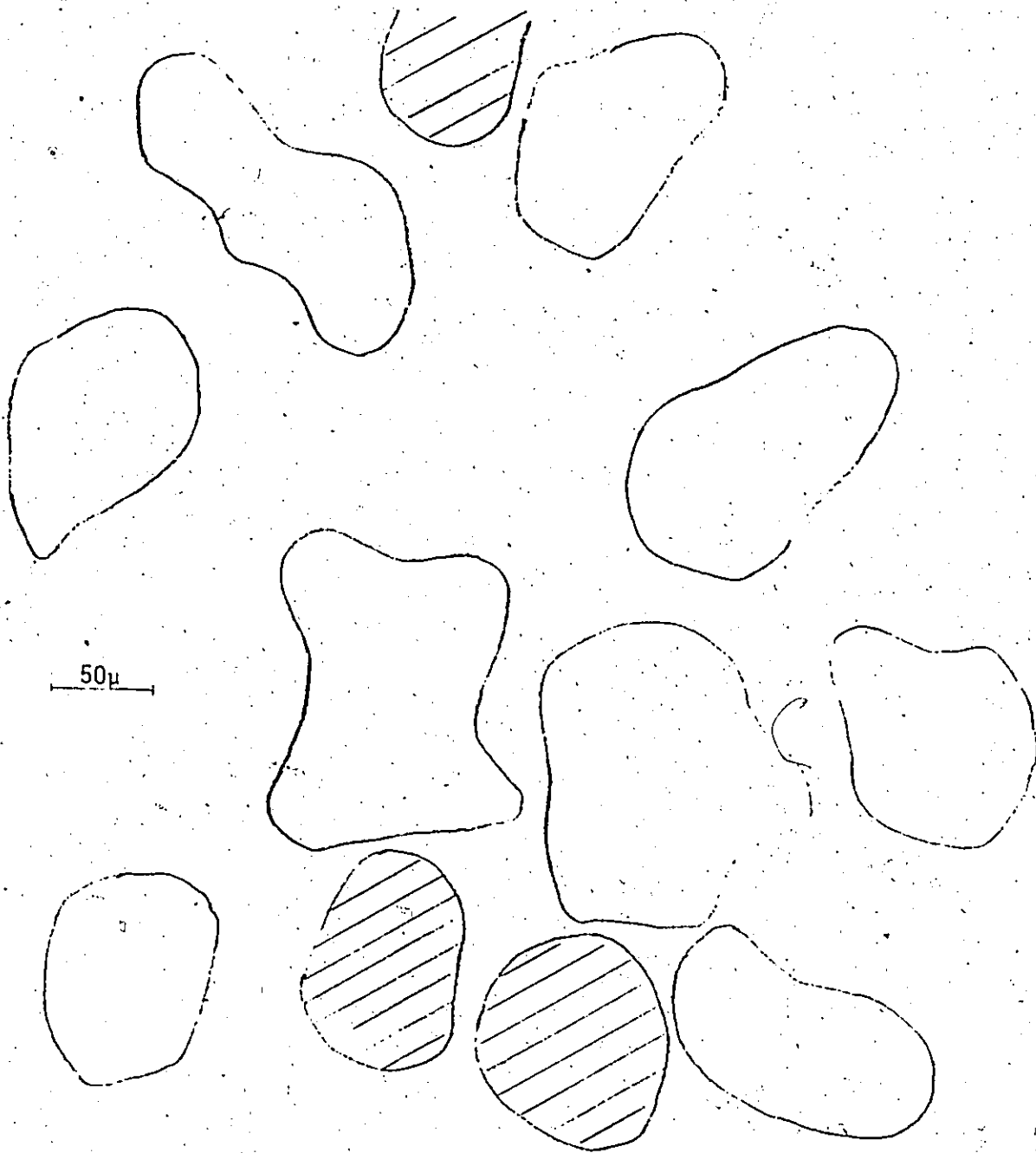
j) Experimental findings for receptive field size of receptors and their spacing.

A few experiments were attempted to provide a direct test of the validity of the above approach. On four separate occasions over 100 points were sampled for their sensitivity to mechanical stimulation in steps of 50μ over a large rectangular area of skin. The values of the 'critical' stimulus for each point were marked on a grid map of the skin. From this grid all points whose critical stimulus was within the T range (ie. high sensitive points) were marked, and enclosed within a continuous line. The results are shown in Fig. 33. These highly sensitive areas were seen to be grouped in roughly spherical arrays. Their mean radius was about 50μ and they were separated from each other by approximately $150-200\mu$. These admittedly crude results are consistent with the predicted values. The hypothesis which explains distribution of receptors is supported by this test.

A natural corollary of this hypothesis is that if a 250μ prodder was used to sample the skin, every point sampled should cover at least one receptor. These experiments are currently underway.

Fig. 33: Receptive Fields of Touch Receptors.

Over 100 skin locations were systematically surveyed in a square of 10×10 . The distance between successive points was 50μ . After the 100 critical stimuli were found, all points whose critical thresholds was within the mean low threshold range were circled, and neighbouring low points were enclosed by a line drawn by eye. As can be seen, the results of this systematic survey reduced to localized spots of roughly 50μ in radius with centre to centre distances about 150μ to 200μ apart. These localized areas of higher sensitivity to mechanical deformation presumably represents the individual receptive fields of the low threshold receptors.



5. Discussion

In this chapter a reliable method was developed for investigating the density of mechanosensory endings of a segmental nerve in the skin of the salamander hindlimb. This method consists of making a random survey of the skin, testing the sensitivity of each point to mechanical stimulation. When the results of such a study are plotted as a histogram, showing the percentage of occurrence of any given range of stimuli, the distribution is skewed, the highest percentage of occurrence is the lowest stimulus strength range and the height of the bins to the right of it gets progressively smaller at higher stimulus strengths. The hypothesis put forward in this chapter to explain this type of distribution is that only one population of mechanoreceptors exists in the skin, and that they are all of roughly the same sensitivity. The stimulus strength of the first bin (from the left) indicates the range of sensitivity of these mechanoreceptors and the height of the first bin would represent the frequency that the prodder locates one of these receptors. The bins representing higher stimuli indicate the frequency that the prodder lands to one side of these receptors; the strength of stimulus needed depends on exactly how far away the prodder is from the nearest receptor. The evidence presented in this chapter lends good support to this hypothesis.

The morphological studies by Drs. Parducz and Leslie in this laboratory (personal communication) also tend to support the hypothesis that there is only one population of mechanoreceptors in the skin. (Some of their results were discussed in this chapter, page 71 and page 72). The nerve endings in the epidermal layer of the skin always appear to be associated with Merkel cells, cells known to occur in identified mechanosensory structures in other preparations (Munger 1965, Smith 1967, Iggo and Muir 1969, Burgess, English, Horch and Stensaas 1974). The actual location of these Merkel cells is always in the deepest layer of cells in the epidermis, just above the basal lamina separating the dermis from the epidermis. If the Merkel cells are in fact a component of the mechanoreceptors, then sensitivity to touch for each receptor could well be the same in any one animal, since they are approximately the same depth in the skin. Furthermore, these Merkel cells appear to be sufficiently far apart (as judged from their relative sparsity in serial EM sections) that it is easy to conceive of the prodder being sited between two Merkel cells and therefore needing a higher stimulus to activate one of them. These morphological studies therefore support the physiological results described in this chapter. Direct evidence that the Merkel cells are in fact a part of the mechanoreceptors must come from a combined morphological and physiological study on the same skin. Such experiments are currently underway.

Because there is no comparable study on the organization of mechanoreceptors in the salamander, it is interesting to compare the present results to those described for a closely related species, the frog, especially since the morphology of the frog skin appears very

similar to that of the salamander (Parducz, personal communication and Whitear, 1974; 1975). Both the skin of the frog and the skin of the salamander have rapidly adapting touch receptors, and the sensory axons conduct action potentials with similar velocities (Maruhashi, Mizuguchi and Tasaki 1952, Catton, 1958, Rubin and Syrocki, 1936). However, the frog skin differs from the salamander in that it also contains slowly adapting mechanoreceptors. Further differences between these two species exist in the receptive fields of individual axons. The sizes of the axonal fields reported for the frog are much larger than those found in the salamander, (eg. 1 mm^2 - 30 mm^2 compared to 0.5 - 4.0 mm^2 , (Adrian, Cattell and Hoagland 1931, Maruhashi, Mizuguchi and Tasaki 1952, Lindblum, 1958, Verveen, 1963), however, the stimuli used to define the receptive fields in the frog were about 30 times the strength used in the present experiments; presumably the spread of stimulus could be in part responsible for the larger size fields. Another difference between the axonal fields in the frog and those in the salamander is that in the former the axonal fields overlap extensively, whereas the few fields measured in the present experiments showed only slight overlap. Again, this could be a function of the size and the spread of the stimulus used in the different experiments. On the other hand, the non-overlapping fields in the salamander might indicate a genuine difference between these two species.

It can be concluded that although the morphology of the two skins appears similar, the functional organization of the innervation between the frog and the salamander has some important differences. It is not

easy to suggest what advantages the one may have over the other. Although in theory a higher information content is available when fields overlap, this could be offset by the much smaller size of the fields in the salamander, which allows good spatial resolution of the stimulus. What is surprising is the lack of slowly-adapting receptors in the salamander.

VI. MECHANOSENSORY FUNCTION AFTER COLCHICINE TREATMENT OF NERVES

1. Introduction

Aguilar et al. (1973) found that it was possible to treat one of the segmental nerves to the salamander hindlimb with a dose of colchicine that eliminated, or reduced, fast axoplasmic transport without causing noticeable degeneration in the treated nerve trunks, or any detectable reduction in its area of innervation; however the adjacent segmental nerves sprouted just as though the treated nerve had been sectioned. This study was interpreted as evidence that some factor (or factors) is carried by fast axoplasmic transport, which is involved in the regulation of nerve fields.

There are at least two other possible explanations why the nerve sprouting occurred. One is that colchicine could have a direct action on the skin itself, which might in some way evoke terminal sprouting. There is some evidence, for example, to indicate that traumatizing skin can lead to nerve sprouting (Speidel, 1941).

The second possibility is that the colchicine treatment, in addition to blocking fast axoplasmic transport, might cause a small amount of degeneration confined to the terminal region of a few axons in the treated nerve. This scattered effect might not cause sufficient 'dying back' to affect the main region of the axons in the nerve trunk, and this would not have been detected in the morphological and electrophysiological studies made by Aguilar et al. (1973). These authors also showed that the nerves treated with colchicine in the doses that they used

were themselves not capable of sprouting in response to adjacent nerve degeneration. Nevertheless a hypothetical small amount of terminal degeneration could be conceived of as a stimulus for the adjacent nerves to sprout, a mechanism therefore in accord with the long-held view that 'denervation sprouting' is caused by products of degenerating nerves.

2. Results

a) Systemic Action of Colchicine:

i) Test for selective effects in the skin of the treated limb.

The question is: Does colchicine preferentially affect the skin of the treated limb, compared with that on the opposite side? The investigation therefore focused on the measurement of colchicine in the skin itself. Only if there was significant differences between the two limbs could the hypothesis of direct action be supported.

Animals were treated with 50 mM - 75 mM radioactive colchicine, as described in the Methods, part 5a. Following this treatment, strips of dorsal and ventral skin, 6-8 mm wide, were removed from both hindlimbs and divided into proximal and distal halves. The amount of labelled material in the skin was measured for each portion by liquid scintillation counting (see Methods, Part 5) and expressed as counts per minute (cpm) per mm^2 of skin. Sixteen animals were used in all, and all received colchicine on the same day. Two of them were investigated each day, beginning on the day after treatment, for seven days; two more provided results at 14 days after treatment. The results are presented in table I.

Table 1: Radioactive Material in Skin after Labelled Colchicine Application to One 16th Nerve.

The cpm minus the background are expressed per mm² of skin for different animals treated with ³H - colchicine at different times. E is the experimental side. C is the contralateral untreated side.

TABLE I

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DAYS AFTER TREATMENT	ANIMAL		DORSAL SKIN		VENTRAL SKIN	
			PROX REGION	DIST REGION	PROX REGION	DIST REGION
1	³ HC - 6	E	8.28	7.24	8.36	8.34
		C	9.64	8.14	8.25	8.43
	³ HC - 5	E	12.8	12.7	11.2	11.4
		C	12.1	10.2	10.0	10.3
2.	³ HC - 23	E	8.6	7.3	7.0	8.1
		C	8.3	7.2	7.7	8.0
	³ HC - 12	E	8.4	7.5	6.6	6.5
		C	9.1	7.4	8.5	8.8
3.	³ HC - 9	E	3.8	3.3	3.9	3.3
		C	4.2	3.6	3.0	2.8
	³ HC - 26	E	9.2	7.1	8.8	8.1
		C	9.8	8.1	8.9	8.2
4	³ HC - 25	E	3.7	3.6	3.6	3.3
		C	4.1	4.2	3.8	3.7
	³ HC - 34	E	7.0	6.3	7.0	5.5
		C	5.3	5.1	6.1	5.1
5	³ HC - 31	E	6.0	5.8	7.4	7.8
		C	6.0	6.0	6.3	6.1
	³ HC - 39	E	10.7	9.0	10.3	8.5
		C	9.4	8.8	9.2	9.4
6	³ HC - 48	E	7.1	6.7	5.4	5.4
		C	7.0	6.1	5.8	5.2
	³ HC - 46	E	9.8	9.4	6.3	5.0
		C	8.6	7.5	5.8	6.1
7	³ HC - 47	E	8.5	6.9	7.0	5.7
		C	7.3	5.9	5.0	6.0
	³ HC - 40	E	7.6	7.1	7.1	6.9
		C	7.4	6.6	7.1	7.5
14	³ HC - 10	E	5.3	4.3	4.6	4.1
		C	4.5	4.4	4.8	4.3
	³ HC - 19	E	9.6	8.0	7.5	6.5
		C	9.4	7.9	7.7	6.8
mean ± S.E.M.		E	7.9 ± 0.61	7.0 ± 0.57	7.0 ± 0.52	6.5 ± 0.5
		C	7.6 ± 0.58	6.7 ± 0.45	6.8 ± 0.52	6.7 ± 0.5
			P > 0.2	P > 0.2	P > 0.2	P > 0.2

It is clear from these results that there were no significant differences ($P > 0.2$) between amounts of labelled material in the skin on one side of the animal compared to the other side. Furthermore, there is very little difference in the skin counts for the 1 day animals compared to the animals examined 14 days after treatment. There is, however, almost a two-fold variation in the amount of radioactivity between animals; this is not surprising, considering the relatively crude method used to apply the drug to the nerves which involved no special precautions to restrict the colchicine only to the nerve. Nevertheless the results clearly show that no preferential localization of labelled material appeared in the skin of the treated limb compared to the untreated one.

ii) Quantitative estimate of colchicine in the skin

If it is assumed that the labelled material actually is all colchicine, it is possible to calculate how much of it is present in the skin. As stated in the Methods, Part 5, the radioactive colchicine (specific activity 5Ci/mMole was diluted with ordinary colchicine to give a final specific activity of 40 mCi/mMole). The results shown in Table 1 indicate that there was on the average about 10 cpm/mm^2 of skin. The counting efficiency of the technique used to determine the radioactivity was calculated to be roughly 65% from a quench correction curve and so the radioactivity could then be expressed in terms of disintegrations per minute (dpm). The skin samples were approximately 0.5 mm thick, and so the actual amount of radioactivity in the skin was about 8 dpm/mm^3 .

Since 1 mCi equals 2.2×10^9 dpm (Wehr and Richards, 1967) and the specific activity used in these experiments was 40 mCi/mM, it is

possible to calculate the amount of colchicine (assuming all the label is colchicine) in a mm^3 of skin.

$$\begin{array}{l} 1 \text{ mCi} = 2.2 \times 10^9 \text{ dpm} \\ \text{Sp. Act} \quad 40 \text{ mCi/mM} \end{array}$$

$$1 \text{ dpm} = \frac{1}{2.2 \times 10^9 \times 40} \text{ mM}$$

As described above, on the average the counts in the skin were about 8 dpm

the amount of colchicine corresponding to this value

$$\frac{8}{2.2 \times 10^9 \times 40} \text{ mM}$$

which is the quantity of colchicine in 1 mm^3 of skin. From the histological sections of the skin (see Fig. 19) the average cell is estimated to be approximately 20μ in diameter. Therefore the total number of cells per mm^3 of skin would equal 2.5×10^5 . Assuming an equal distribution of colchicine throughout the skin there would be 3×10^{-10} mM colchicine per 10^6 cells.

The amount of colchicine needed to arrest cell division is in the region of 6×10^{-10} mM/ 10^6 cells (Taylor, 1965).

It seems very unlikely therefore that this small amount of colchicine would have any effect at all on the skin cells. Even so, the results indicate that there was no greater accumulation of colchicine on the treated side, relative to the other, and that sprouting of nerves in one limb cannot be attributable to a direct action on the skin of that limb.

b) Test for functional degeneration after colchicine

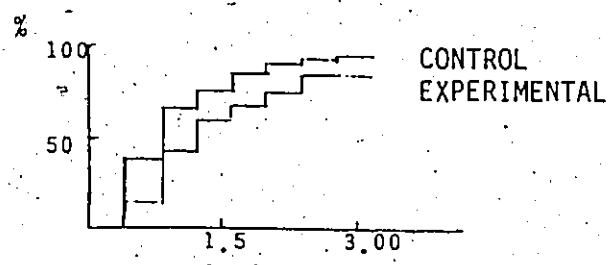
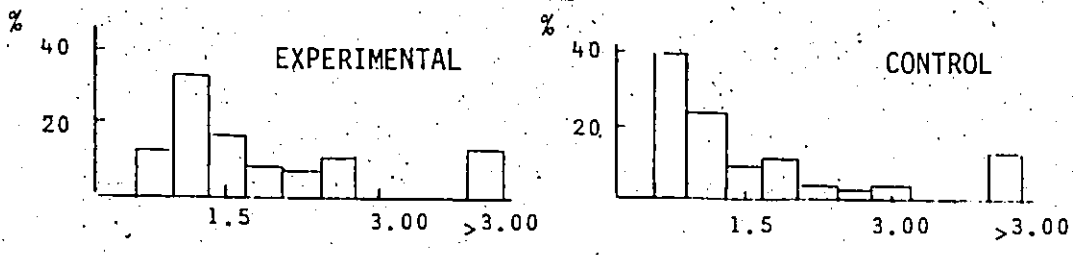
By using the techniques for analysing the distribution of individual touch receptors in the skin described in Part III, it was possible to investigate whether nerves treated with colchicine showed any functional loss of the touch receptors associated with it. As was shown, the receptors are uniformly distributed in number and sensitivity, and since they are symmetrical in this regard from one side of the animal to the other, the non-operated limb can be used as a control for the experimental one. Furthermore, as shown above, the amount of colchicine which reaches the skin is the same on both sides.

The question is: Do the receptors in the skin of the treated limb change in number and/or sensitivity, compared to those of the control side? Fig. 34 shows the results from three different animals, each one examined at a different time (6, 9 and 14 days) after the 16th nerve of one limb was treated with 75 mM colchicine. In each part of the Fig. 34 the distribution of touch thresholds of the receptors of the 16th treated nerve is compared to that of the control nerve on the other side of the animal. There is no statistical difference ($P > 0.2$) based on Kolmogorov-Smirnov test) between the results on one side of the animal to the other. These histograms can be compared to those in Fig. 35. In this case 3 different animals were examined at different times (5, 6 and 7 days) after the 16th spinal nerve was sectioned in one limb. As for the colchicine experiments, the experimental nerve is compared to its control on the other side of the animal. It is note-worthy that after 5 days there was little or no functional change in the touch receptors of the sectioned nerve. However changes in the distribution of the critical

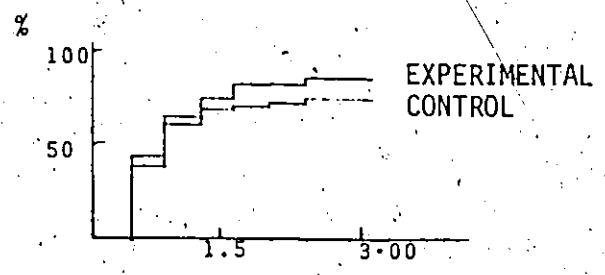
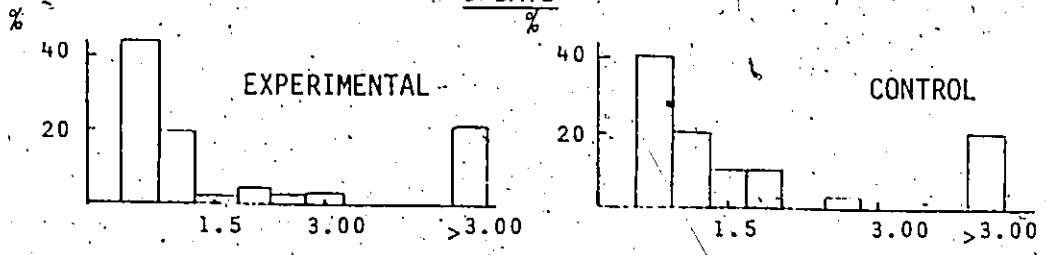
Fig. 34. Mechanosensory Function after Colchicine.

The sensitivity of the 16th nerve field to mechanical stimulation, after the 16th nerve trunk was treated with colchicine, was tested for 3 different animals, each one examined at a different time (6, 9 and 14 days) after treatment. The distribution of critical stimuli for a 50 point random survey is compared to that of the control 16th nerve field on the other side of the animal. There was no significant difference ($P > 0.1$) between the results for the two sides. (cf. Fig. 35).

6 DAYS



9 DAYS



14 DAYS

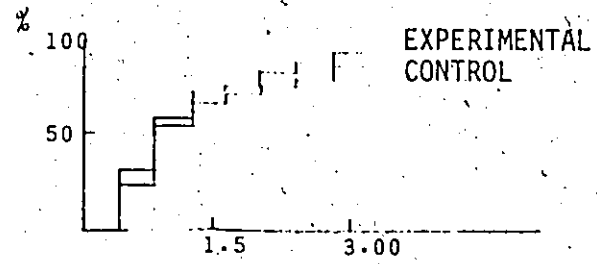
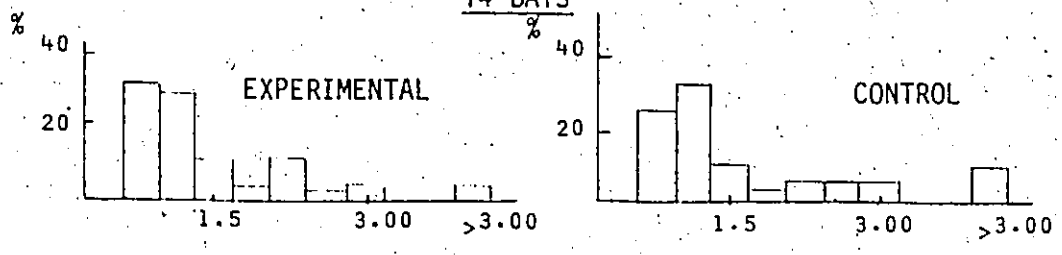
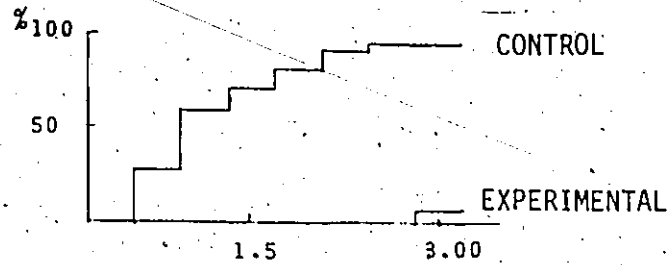
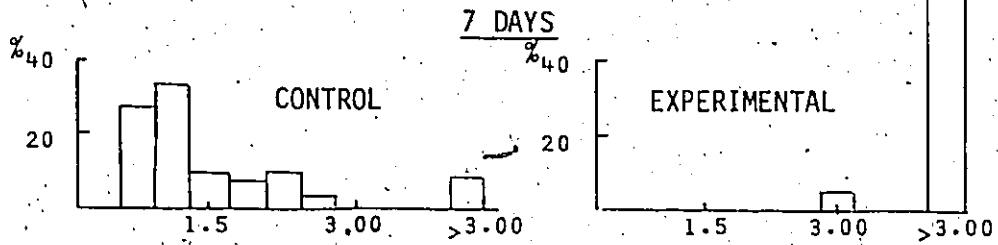
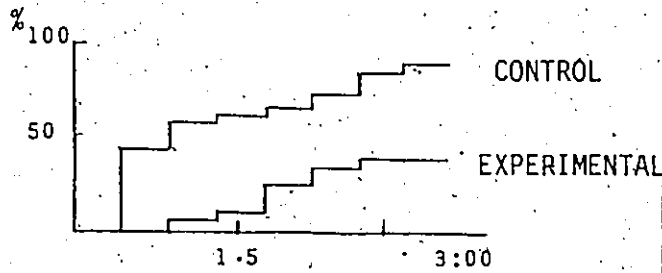
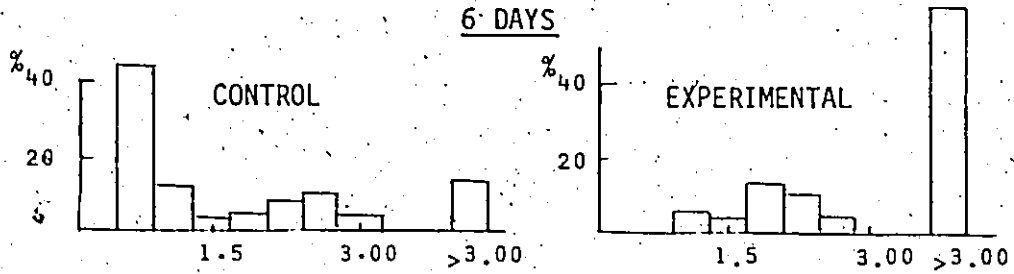
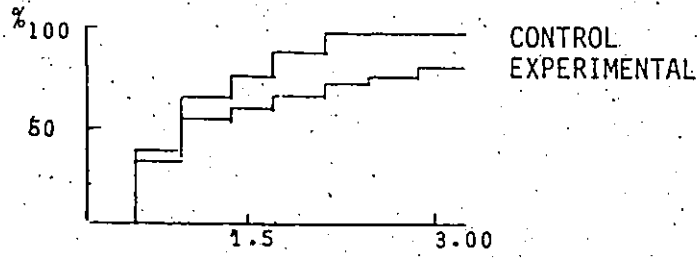
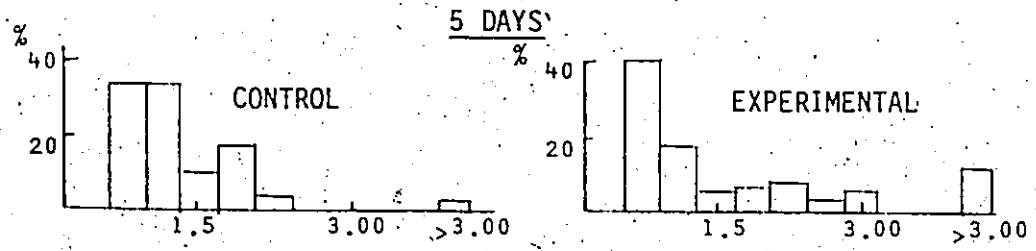


Fig. 35: Mechanosensory Function After Nerve Section

The sensitivity of the 16th nerve field to mechanical stimulation after 16th nerve section was tested for three different animals, each one examined at a different time (5, 6 and 7 days) after treatment. The records were made from the distal position of the sectioned 16th nerve. The distribution of critical stimuli for a 50 point random survey is compared to that of the control 16th field on the other side of the animal. There was no significant difference ($P > 0.2$) between the results on the two sides, 5 days after nerve section. However, on day 6 there was a significant reduction ($P < 0.005$) between the distribution on both sides. By day 7 only very few receptors remained functional in the skin.



stimulus could be clearly seen on day 6; there were many fewer low threshold spots and many more in the higher stimulus range, thereby completely changing the shape of the histogram from its usual skewed distribution as shown in the contralateral control limb. By day 7, about 80% of the touch receptor population was not detectable, that is, there was no response to very large stimuli applied to the skin at these points. It is also worth noting that the control histograms in these 3 animals are not statistically different ($P > 0.2$) from the 6 histograms in Fig. 34, giving further evidence that colchicine was not having a generalized systemic effect on mechanosensory function.

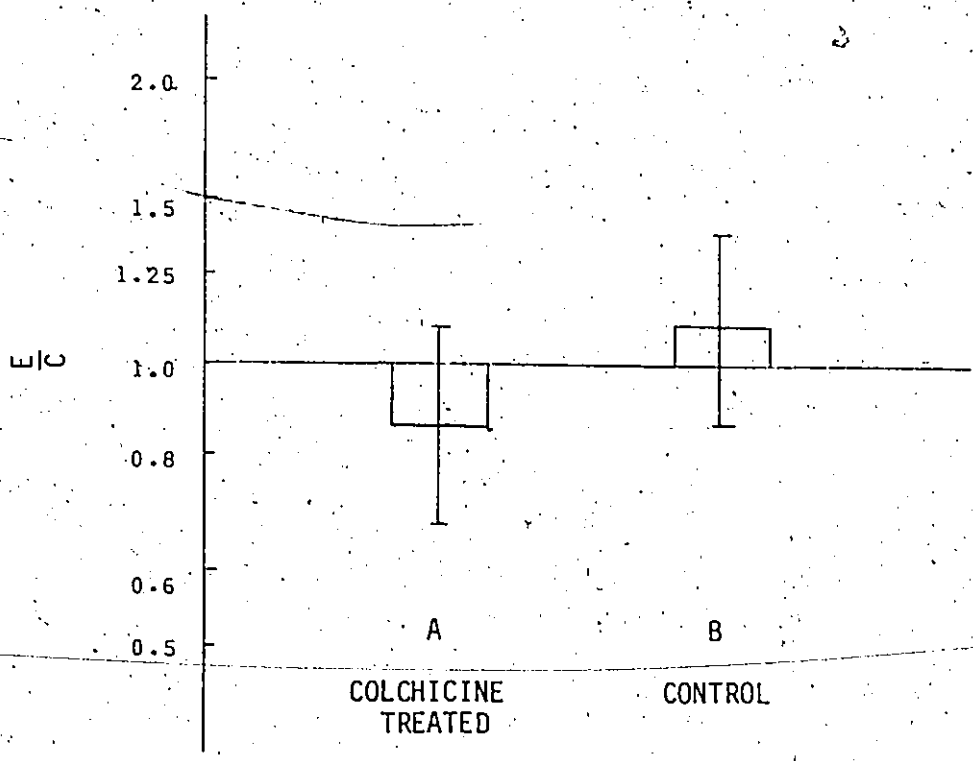
The mean difference between the number of low threshold skin receptors (the first bin to the left on the histograms), of the colchicine treated nerve compared to that of the contralateral control one for seven animals is shown in Fig. 36. These results indicate that colchicine-treated animals exhibit the same side-to-side variation as the normal untreated animals, and again there was no statistical difference ($P > 0.2$) between the groups of animals treated with colchicine and the normal untreated groups.

c) Colchicine toxicity

In most cases, the dose of colchicine used in these experiments had no detectable effects other than to interfere with neuronal transport, reduce the number of microtubules in the treated nerves, and cause sprouting of adjacent ones (Aguilar et al. 1973). However, in one group of animals, colchicine produced peculiar side effects. These salamanders were the same species normally used in these experiments (Amblystoma tigrinum) and were obtained from the same supplier.

Fig. 36: Low Threshold Receptors in Nerves After Colchicine Treatment

In 8 animals the 16th nerve was treated with 75-100 mM colchicine for half an hour, and 6-21 days later the same nerves were used to test the mechanosensitivity of the appropriate nerve field. There was no significant difference ($P > 0.2$) between the frequency of locating a low threshold spot belonging to the treated nerve compared to that on the untreated contralateral control nerve. Furthermore, these treated animals did not differ significantly ($P > 0.2$) from the untreated control animals.



Apart from the increased sensitivity to colchicine, the only other detectable differences between the two populations of animals was a slight variation in the skin pigmentation. The pigment in the toxic population was black with yellow spots whereas the pigment of the usual group of salamanders was dark green with yellow spots.

The first behavioural sign of toxicity showed up 5-6 weeks after colchicine treatment; instead of the normal walking gait, these animals exhibited a type of swimming movement, as if they were in water, even in their normal 'dry environment'. Although the animals appeared to walk and behave normally in the 1st 4 weeks after treatment, examination revealed that they were very insensitive to touch.

When the sensory fields were mapped as early as 7 days after treatment by recording from the segmental nerves while stimulating the skin with a fine brush, it was discovered that the hindlimb skin was practically void of functional mechanoreceptors on both sides of the animals. However, the motor innervation, as measured by EMG recordings from different muscle groups while stimulating the same segmental nerves (Aguilar et al. 1973) appeared quite normal. In a few cases the forelimb skin was also examined, and it showed similar defects to those in the hindlimb skin. These results suggest a systemic action of colchicine affecting the skin receptors in all limbs (at least); but without noticeable effects on the muscles or motor nerves.

To test if there was an unusually increased amount of colchicine getting into the circulation, a group of these animals was treated with 50-75 mM ^3H -colchicine as described in part a (see page 123), and the skin was examined for radioactive material. The results are shown in

Table 2.

In comparing the results to those obtained for the animals in Part I of this section (see Table 1), it can be seen that there is more than a 10-fold increase in radioactivity per mm^2 of skin at day 1 in the animals which showed toxic side effects. Fig. 37 is a graph of radioactivity in the skin plotted against the days after treatment, for the two different groups of animals. It is not clear what biological variation can account for this difference, but it cannot be explained on the basis of differences in size or sex, since such differences did not exist.

It was found that the amount of colchicine circulating systemically could be reduced by making a small petroleum jelly trough around the nerve, so that the drug was only in contact with the nerve and its surrounding tissue. In a few experiments involving this technique, there was a reduction in the toxic side effects. However, no quantitative study was performed, and subsequent shipments of salamanders were of the original kind normally used in this study.

3. Discussion

The results of the ^3H -colchicine experiments indicate that the small amount of colchicine that gets into the circulation, using the present method of application to the nerve, leads to an even distribution of labelled material throughout the skin of the animal. It seems improbable, therefore, that the drug can be acting preferentially on the skin of the treated side to cause nerve sprouting. Furthermore, the amount of colchicine that actually gets into the skin is probably too

Table II: Radioactivity In Skin of Toxic Animals.

This table is similar to Table I. However, the animals used in this study showed toxic side effects (see text). Notice that the cpm/mm^2 of animals one day after treatment are 10 times the values for comparable animal in Table I.

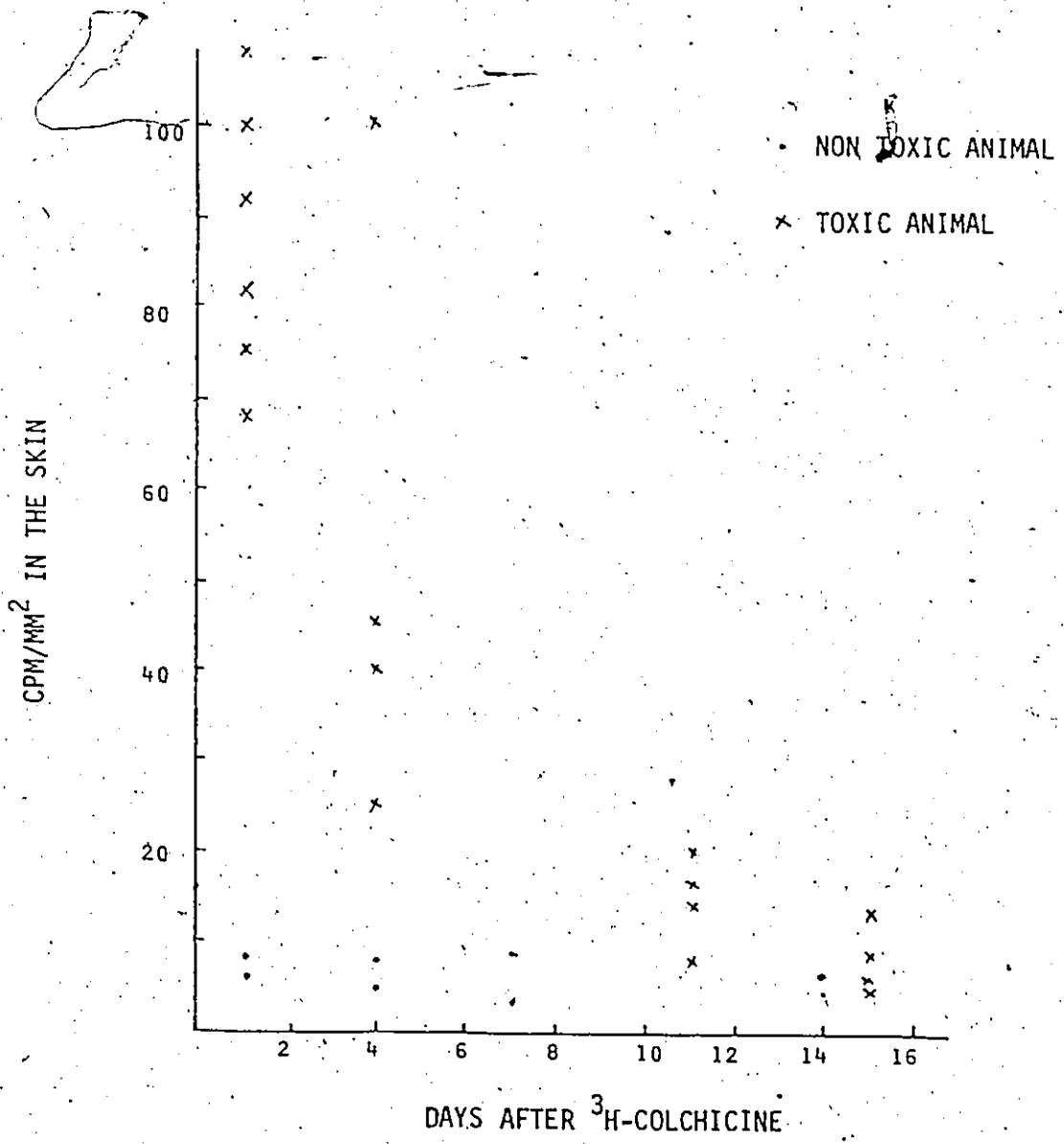
TABLE II

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DAYS AFTER TREATMENT	ANIMAL	COUNTS IN SKIN	
		EXP CPM/MM ²	CONTR. CPM/MM ²
1	³ HC - 146	89	78
	³ HC - 147	101	118
	³ HC - 148	80	74
	³ HC - 149	63	69
	³ HC - 150	74	115
	³ HC - 151	106	96
	MEAN ± S.E.M.	85 ± 6.6	91.7 ± 8.5
4	³ HC - 152	45	51
	³ HC - 153	26	21
	³ HC - 154	40	42
	³ HC - 155	141	67
	MEAN ± S.E.M.	63 ± 26.3	45 ± 9.6
11	³ HC - 84	4.4	8.3
	³ HC - 85	11.1	19.4
	³ HC - 86	15.9	25.3
	³ HC - 87	14.7	15.1
	MEAN ± S.E.M.	11.5 ± 2.6	17 ± 3.6
15	³ HC - 88	6.7	6.7
	³ HC - 89	9.6	7.1
	³ HC - 90	12.8	4.3
	MEAN ± S.E.M.	11.1 ± 1.9	6.0 ± 0.62

Fig. 37: Comparison of Radioactive Colchicine in the Skin of 'Toxic' and 'Non-toxic' Animals.

The skin counts for the two groups of animals, those of Table I (dots) and those of Table II (x's) are compared at different times after treatment. In the 'non toxic' animals (dots) there was a constant level of radioactivity from day 1 to day 14; the 'toxic' group (x's) initially had 10 times the level of radioactivity in the skin compared to the 'non toxic' group, but the level fell to the level of the 'non toxic' group by day 15.



small to have its usual "traumatic" (ie. anti-mitotic) effect on this tissue.

It is interesting that the animals which showed toxic side effects had a 10-fold increase in the amount of colchicine in the skin at day 1 after treatment, and also had a large loss of functional touch receptors, without any noticeable effect on the motor terminals. This result is very reminiscent of the "vincristine neuropathy" described in humans after receiving that drug in cancer chemotherapy (Dr. A. Upton, personal communication). These patients suffer a variable patchy loss of touch sensation; however, the sensory compound action potential is normal (recording in the peripheral nerve trunk), and only with special techniques can slight changes be detected for the motor innervation. It appears that there may be a direct action of these anti-mitotic poisons on the sensory terminal, which in some way interferes with its function, without necessarily affecting the sensory axon itself.

In this connection it has been reported that colchicine applied locally to the campaniform sensilla, a specialized mechanoreceptor in the cockroach hindlimb, completely disrupted its mechanotransduction properties, without interfering with the ability of the sensory axon to conduct impulses (Moran and Varela, 1971). The only detectable difference the investigators could find in these structures was a marked reduction in the number of microtubules seen by E.M. of the sensory ending itself. Perhaps in the present experiments colchicine acted in a similar fashion in those animals which showed the toxic side-effects, but this has not yet been tested.

The experiments on the functioning mechanoreceptors of the colchicine-treated nerves indicate that this drug was not causing degeneration of the nerve terminals, since it is hardly conceivable that this would happen without changes in the threshold for their excitation (compare to the results of nerve section). It seems therefore that the sprouting after colchicine treatment [see Aguilar et al. (1973) and Part 5 of this thesis] can be confidently attributed to the action of the drug in blocking fast axoplasmic transport.

The results after nerve section also support this hypothesis. There was no detectable change in receptor function five days after nerve section, even though sprouting of adjacent nerves is already underway by this time (see Aguilar et al. 1973). Since the nerve was cut about 25 mm from the sensory endings, the degeneration occurring at 6-8 days would suggest that axoplasmic transport of some essential material would be unlikely to have a rate greater than 3-4 mm per day. This upper limit (assuming no "stock pile" at the endings) is consistent with the receptor function being more dependent on a slow rather than fast transport, since the latter rate can be as high as 50-60 mm per day in these nerves (J. Fried, personal communication). The finding therefore that no functional loss occurred after colchicine is a further indication that the latter affects fast transport preferentially, and that this is the cause of the adjacent nerve sprouting.

VII. QUANTITATIVE MEASURE OF NERVE SPROUTING

1. Introduction

The hypothesis to explain why nerves sprout, for which these experiments were designed to test, proposes that a stimulus from the target-tissue acts on the nerves to initiate collateral sprouting, and in addition, that nerves secrete factors which neutralise the effects of this target stimulus in some way. Sprouting is presumed to continue until a balance is reached between the effects of the target stimulus and those of the neural factors. When these neural factors are reduced, either by colchicine treatment or by partial denervation, the adjacent nerves sprout. If it is assumed that the nerves secrete their substance from the terminals, that the transport velocity of these substances down the axon is constant, and that there is a good "safety factor" in the sense of abundance of substance available, then the balance between these neural factors and the target stimulus should be restored when the total number of endings (those not affected by the experimental treatment, plus the newly sprouted ones) quantitatively match the number of functioning terminals before colchicine treatment or partial denervation. When this matching is achieved sprouting should cease. The need for more neural factor in individual axons to supply the increased number of terminals could be met by an increase in the amount per unit length of axon. Alternatively there could always be an excess of factor available at the terminal portions of the axons, but its

rate of release is limited to the number of nerve endings. The methods of measuring the density of nerve endings in the skin (see Part III) made it possible to test certain features of the hypothesis by determining the number of endings in the skin after sprouting.

Another reason why the density of innervation had to be measured in this study on sprouting is that the segmental nerves in the animals used for these experiments often did not increase their area of innervation after sprouting, as was the case in the earlier experiments (Aguilar *et al.* 1973). In all animals the major nerve to the limb is the 16th segmental nerve, and the adjacent segmental nerves, 15 and 17, partially innervate the anterior and posterior portions of the limb respectively (see Fig. 2), overlapping with the 16th nerve. In the animals used in the earlier experiments there was almost always a region of the dorsal hind-limb skin between the 15th and the 17th nerve fields which was exclusively innervated by nerve 16. After colchicine treatment or partial denervation of the 16th nerve, the adjacent nerves would sprout, and extend towards each other to take over the 16th area. However, only in a relatively small number (about 5%) of the animals used in the present experiments was there an area which was innervated exclusively by the 16th nerve; the 15th and the 17th fields almost always abutted upon each other in the pre-operative situation. When the 16th nerve of these animals was cut or treated with colchicine, the adjacent nerves sprouted within their own territory, but did not increase their area of innervation. This was true even when two spinal nerves were cut, and only the 15th or the 17th nerve remained in the limb

(Cooper, Diamond, Macintyre and Turner, 1975). This apparent skin specificity is being investigated at the present time, and will not constitute a part of this thesis.

2. Methods

As was shown in Part V, a quantitative measure of the density of mechanosensory nerve endings in the skin can be obtained by making a random survey of the skin to point mechanical stimulation. When the results of such a survey are presented as a histogram (see Fig. 25) giving the percentage of occurrence of any given range of stimuli, the height of the first bin (from the left) indicates the frequency with which the prodder was sited on the most sensitive receptors. These receptors are referred to as the low-threshold receptors. The evidence presented in Part V indicated that these low threshold receptors are the only mechanoreceptors in the skin. When the prodder was located to one side of a mechanoreceptor a larger stimulus was needed. The strength of the stimulus is a function of the distance the prodder is from a receptor. The frequency with which the prodder location was to one side of a receptor is indicated by the bins to the right of the low threshold bin in the histogram.

Since most, if not all, the mechanoreceptors in the hindlimb skin are the low threshold receptors, sprouting is measured as the change in the frequency with which the prodder locates a low threshold receptor in a random survey before and after an adjacent segmental nerve is either sectioned or treated with colchicine. (Before refers to the results obtained from the contralateral control limb, which re-

presents the pre-operative state of the experiment, since the distribution of receptors is bilaterally symmetrical from one side of the animal to the other). The frequency with which the prodder locates a low threshold receptor is a measure of the density of these receptors.

Most of the experiments in this section were performed on the area of skin supplied by the CFP nerve (see page 36); simultaneous recordings were made from the 16th and 17th nerve. A few of the earlier studies, however, were made on a more anterior portion of the skin innervated exclusively by the 15th and 16th segmental nerves (see Fig. 2). There were no noticeable differences in the organization of mechanoreceptors between the CFPN area and the more anterior area.

3. Results

a) Quantitative Sprouting After Colchicine Treatment of Adjacent Nerves.

Fig. 38 compares the density of innervation in the two hindlimbs of an animal after 100 mM colchicine was applied to the right 16th nerve for 30 min. (see Methods, Part IV). The density of low threshold receptors for the colchicine treated 16th nerve is certainly not less than that of the control nerve, again indicating that colchicine is not causing any functional loss in these receptors (see Part VI). [The small increase in number falls within the statistical variation (eg. compared to Fig. 37) from animal to animal]. However, there is a clear increase in the density of low threshold receptors in the 15th nerve on the treated side, indicating that this nerve had sprouted. Furthermore, this increase was quantitative, that is it virtually matched

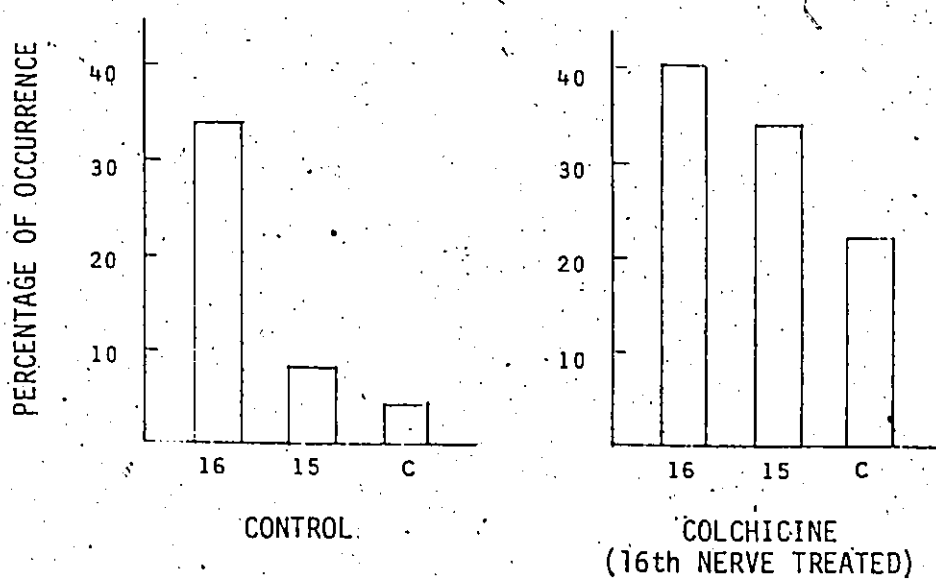


Fig. 38: Sprouting After Colchicine Treatment

In a single animal the touch receptor density in a region of skin shared by the 15th and the 16th nerve was investigated after the 16th nerve on one side was treated with colchicine. There was no loss in the population of receptors feeding into the treated 16th nerve, compared to the control 16th nerve (column A). In this animal the number of touch receptors associated with the 15th nerve was only a small proportion of the total. However, on the treated side the 15th nerve now supplied an extra population of receptors almost equal to the number associated with the 16th nerve (column B). The third column, C (referred to as coincidence) indicates the amount of overlap between the fields of the low threshold receptors. Over 70% of the newly-sprouted 15th nerve endings coincided with the existing 16th nerve endings on the treated side.

the number of 16 receptors present. The third column, referred to as coincidence, measures the overlap of low-threshold receptive fields belonging to the axons of one segmental nerve with those belonging to the axons of the adjacent segmental nerve. Normally this percentage is very low, in the region of 5%, as seen in the third column of the control side. Interestingly, 70% of the new 15th nerve endings coincided with the existing 16th nerve receptors, suggesting that they may grow preferentially towards the endings of the colchicine-treated nerve. In fact, from this data it is possible to deduce whether the new 15th endings are indeed growing to some preferred location in the skin or merely sprouting randomly.

i) Location of New Sprouted Endings

From the arguments presented in Part V, it is known that the low threshold receptors are uniformly distributed. Fig. 32 represents a schematic area of skin, with the receptors spaced uniformly within it. (For convenience a squared lattice configuration was chosen for these receptors, because it simplifies the following analysis. Other possible receptor spacing configurations have been tested, and do not greatly effect the following arguments.) The distance between two adjacent receptors is A , and therefore a square $A \times A$ on the skin contains the equivalent of one receptor. (This square joins 4 receptors, each occupying $1/4 \pi R^2$ of the square. Therefore 4 receptors would occupy πR^2 which is equivalent to the area of one receptor.) For there to be no overlap between the receptive field of a new receptor with an existing one, the newly sprouted ending must grow close to the

centre of this square; at any other location the receptive field of the new endings would overlap to some extent, that of the existing one. The truth of this statement follows from equation 2, Part V, and the results from Fig. 38.

$$\pi R^2 = xA^2$$

when R is the radius of the receptive field of the receptors

A is the mean distance between the centres of adjacent receptors

and x is the fraction of the square $A \times A$ which is occupied by receptors and also equals the frequency of locating a receptor in a random survey (see Part V).

The results in Fig. 38 give the value for the percentage of occurrence of the low threshold receptors, x in the above equation; this equals 35%. The mean spacing A can now be expressed in terms of the effective radius R and requires no further assumptions about size.

From equation 3 above

$$A = \sqrt{\frac{\pi}{.35}} R.$$

A approximately equals $3R$.

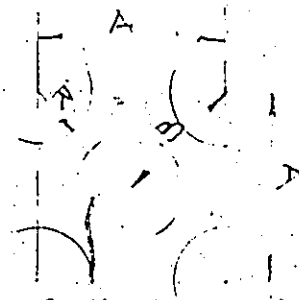
Therefore, the sides of the square in Fig. 32 equals $3R$. If the new ending was located in the middle of this square and did not overlap with the existing receptors, it is possible to calculate the distance B from the centre of the field of the new sprout to the centre of the closest existing receptor (see inset below). As can be seen above, $2B$ represents the diagonal of the square.

$$(2B)^2 = A^2 + A^2$$

$$B^2 = 1/2 A^2$$

$$B = 2.1 R$$

new
sprout



This means that when the new sprout was the farthest it could be from the receptors, at the centre of the square, its circumference would almost be touching the circumference of the existing low threshold receptor. If the sprout was located anywhere else within the square it would overlap to some degree with one of the existing receptors. The ratio of the actual area of this overlap to the area of the square $A \times A$, represents the value for coincidence. If the new sprout grew preferentially and exactly to the existing colchicine-treated endings there should be 100% coincidence, and the centres of the new sprouts and that of the existing endings would always coincide. If they grew preferentially away from the colchicine-treated endings they would have to end up near the centre of the square, and there would be no coincidence. If the new sprouts grew randomly they would occur in any position between the two extremes $B = 0$ and $B = 2R$; the average distance between the centres would be $B = R$ and this would give a coincidence of approximately 50% (in fact, when the centres of the new sprout and the existing receptor are a distance R apart the area of overlap of their receptive fields is roughly 45%). To summarize, 0% coincidence between the new sprouts and the endings of the colchicine treated nerve would indicate a preferential exclusion of the new endings by the existing ones, 45-50% coincidence would suggest that the location of the new sprouts was randomly achieved, and 100% coincidence would indicate a

preferential growth to the location of the existing endings.

In the experiments shown in Fig. 38 the percent coincidence was 70% which suggests that the new endings tended to grow preferentially towards the old sites.

b) Other Experiments

It is appreciated that the preceding analysis relates to a single experiment. Naturally further ones were attempted but no sprouting was detected. Four colchicine-experiments were tested only one week after colchicine treatment. Unfortunately at this stage of the work, it was not yet appreciated that the receptors took 3 weeks to mature to the level of sensitivity which would allow them to be excited by the range of stimuli used with prodder stimulation. (The bristle used for crude area mapping of nerve fields was effective because it constitutes a much larger stimulus.) Three other animals were tested during the winter (January and February) and, as will be discussed below, there is good reason to believe that collateral sprouting in these animals would have been unlikely because of seasonal variation (see page 157). Again, this knowledge was not available when these animals were tested.

In addition to all of these considerations, the technique of bathing the nerves in colchicine naturally resulted in the outermost axons of the nerve trunks being exposed to the highest concentration, and the ones in the centre of the trunk to the lowest. It was considered to be of prime importance not to kill any axons, and therefore it was inevitable that the usual treatment would presumably leave axons

in the centre of the trunk exposed to relatively smaller concentrations of the drug. In fact, from preliminary studies on quantitative measurements of axoplasmic flow it seems that only 30% of the material carried in the fast transport was blocked by the dose of colchicine used in these experiments. Therefore the sprouting from adjacent nerves after colchicine treatment would, on the average, only be expected to reach 30% at most of the total that would result from sectioning nerve 16. For this reason it seemed that a better method to investigate whether the number of newly sprouted endings would quantitatively match the pre-operative number was to study sprouting after partial denervation. In this way it would be certain that all the "treated" axons would be affected to the same extent, and this of course would be equivalent to total block of fast axoplasmic transport.

c) Quantitative Sprouting After Partial Denervation

Thirty animals were tested for quantitative sprouting at least three weeks after partial denervation produced by cutting the 16th segmental nerve. Quantitative sprouting was investigated by measuring the density of low threshold receptors as described in Part V. Fourteen of these animals died during the testing. This unfortunate occurrence was more frequent at the beginning of the experimental series but became less frequent with time. Two major factors which contributed to the initial lack of success were poor dissection (this improved later), and suffocation when the animals were submerged in light oil (see Methods, page 38). The latter was later overcome by bubbling the

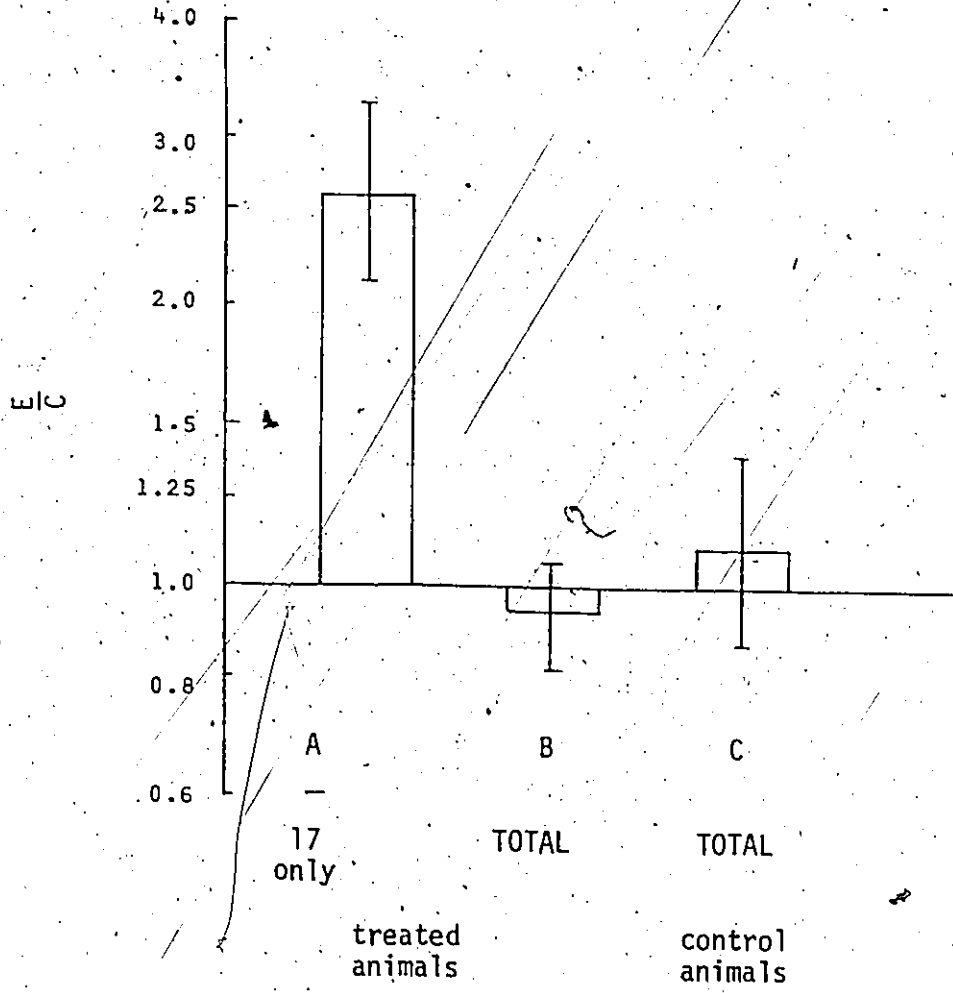
oil with oxygen throughout the experiment.

Of the remaining 16 animals, nine sprouted as indicated by the increased number of low threshold receptors in the adjacent nerve after partial denervation; seven did not. (These seven will be discussed in more detail below.)

The results from the nine animals that sprouted are shown in Fig. 39. This figure shows that a large increase in the number of low threshold receptors of the 17th nerve occurred after a partial denervation at least three weeks earlier. The mean increase of the operated side as compared with the opposite control one is over 100%, although there is some scatter. The variation in the relative amount of 16 and 17 axons present in the CFPN probably accounts for most of this scatter. In some cases the 16th nerve supplied the majority of endings to the area, and their elimination resulted in a large increase in the population of the 17 nerve endings. On the other hand, the increase in the latter was small when the 17th nerve already provided the bulk of the innervation of the area pre-operatively. The middle column in Fig. 39 which expresses the total receptor population on the experimental side of the animal (that is, those belonging to the 17th nerve) as a fraction of the total receptor population on the control side of the animals (those belonging to both the 16th plus the 17th nerves) indicates that the number of new sprouts on the experimental side quantitatively made up the number of receptors that had degenerated after nerve section. Furthermore there is no significant difference ($P > 0.2$) between the side-to-side density of these experimental animals compared to a group of untreated control animals shown in

Fig. 39: Quantitative Sprouting After Partial Denervation

The percent occurrence of 'low threshold' receptors feeding into the 16th and 17th nerves from a shared region of skin was measured, and the values compared between right and left limbs. Column A refers to a group of animals in which the right 16th nerve had been sectioned three weeks previously, and shows the right-left ratio for the number of 17th nerve touch receptor population only. An increase in 17th nerve receptors is clearly seen. Column B shows, for the same group of animals, the right-left ratio for the total receptor population (that is the 17th on the treated side, the 16th plus the 17th on the control). Column C shows right-left ratios for the total population of touch receptors in a control group of animals, with 16th plus 17th nerves in tact on both sides. There is no significant difference ($P > 0.2$) between column B and column C, indicating that the increase in 17th nerve receptors on the right side of the experimental group had quantitatively made up the loss due to the 16th nerve section (vertical bars equal S.E.M.). Note; these experiments were done during the summer months (c.f. Fig. 40).



column C.

d) Seasonal Variation

Returning to the seven animals that did not sprout, in two of these animals there was no 16th innervation in the CFPN, so this territory was not partially denervated after the 16th segmental nerve was cut, and the density of innervation was of course unchanged as a consequence. The results of the remaining five animals are presented in Fig. 40. In this case, it is clear that while there is no difference between the 17th nerve from side-to-side there is a large deficit in the number of low threshold receptors on the partially denervated side compared to the control. These animals were examined in the same manner as those that had sprouted; both groups contained animals from both sexes and there was no difference in size, weight and presumably age between the animals which had sprouted and those that did not. (Although the exact age was impossible to determine, both groups were adults, and using size as a measure of age they appear to be the same age.) Furthermore, there was no correlation between the amount of observed sprouting and the temperature at which these animals were kept.

However, a difference did exist between the time of year when the group that had sprouted and the group that did not, were examined. Table 3 lists the dates of operation and the dates of examination for each animal in the two groups. From this table it can be seen that four out of six animals operated on after the beginning of November, and examined before the end of February, did not sprout. Whereas, seven

Fig. 40: Seasonal Variation in Sprouting

The results of partial denervation in the salamander during the winter (c.f. Fig. 39). The percent occurrence of the 'low threshold' receptors feeding into the 16th and 17th nerves from a shared region of skin was measured, and the values compared between right and left limbs (ordinate). Column A refers to a group of animals in which the right 16th nerve had been sectioned three weeks previously; it shows there was no significant difference between the 17th touch receptor population on the two sides; i.e. no sprouting had occurred. Column B shows, for the same group of animals, the right-left ratios for the total receptor population (that is, 17th on the treated side, 16th plus 17th on the control side). There is a clear deficit of receptors on the treated side. Column C shows right-left ratio for the total population of touch receptors in the control group of animals with the 16th and 17th nerve intact on both sides.

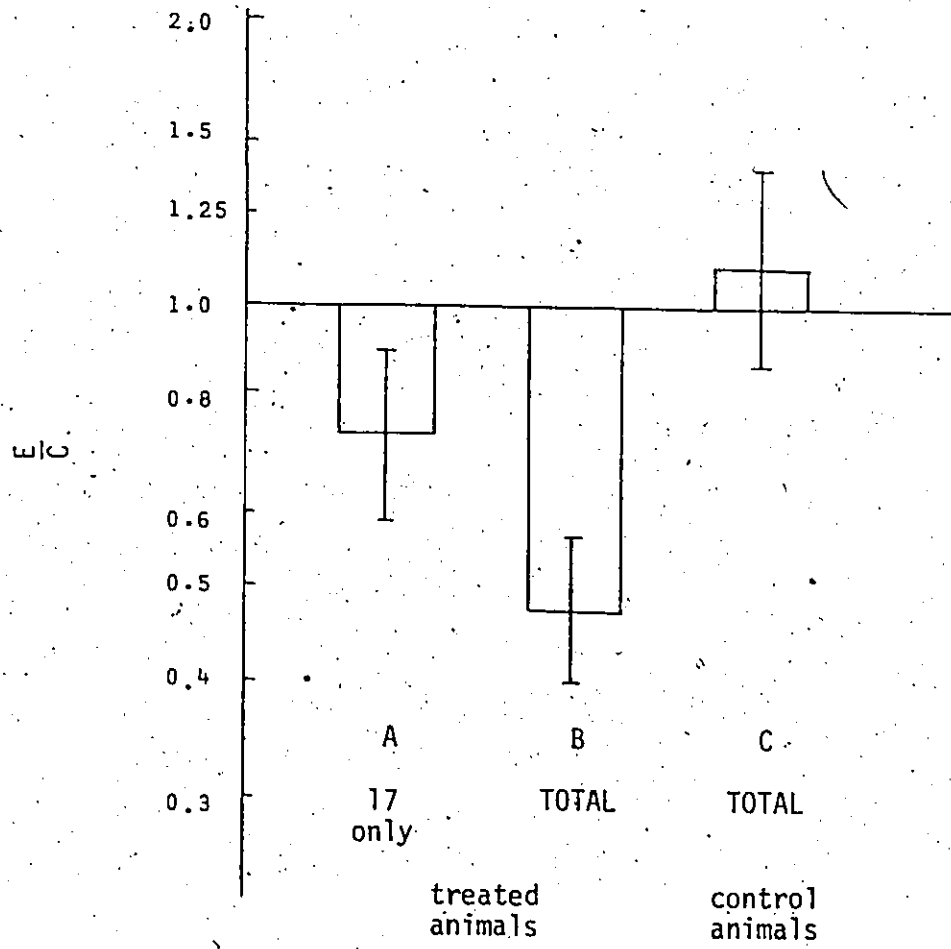


Table III: Seasonal Variation on Collateral Sprouting

The dates of operation (partial denervation) and dates of examination are presented for 14 animals. Notice that of the six operated on after Nov. 1 and examined before Feb. 28, four did not sprout after partial denervation. However, 7 of the 8 operated or examined outside of that time period did sprout.

* are the anomalous animals

TABLE III

	ANIMALS	OPERATION DATE	EXAMINATION DATE
SPROUTED	16-260	FEB 25 1974	MAR 25 1974
	16-241 *	JAN 18 1974	FEB 19 1974
	16-420 *	NOV 26 1974	DEC 15 1974
	16-263	FEB 25 1974	MAR 29 1974
	16-266	FEB 25 1974	APRIL 4 1974
	16-278	MAR 20 1974	APRIL 23 1974
	16-288	MAR 26 1974	APRIL 26 1974
	16-228	OCT 23 1973	NOV 22 1973
	16-209	AUG 20 1974	DEC 14 1974
NOT SPROUTED	16-262 *	FEB 25 1974	MAR 13 1974
	16-242	JAN 18 1974	FEB 27 1974
	16-434	NOV 28 1973	JAN 23 1974
	17-1	NOV 19 1973	JAN 13 1974
	16-393	NOV 1 1974	DEC 16 1974

out of the eight operated or examined outside of that period had sprouted, the only animal of this group that did not sprout was operated on in February but examined early in March. Similarly, one of the two animals that sprouted during the winter season was operated on in November and examined in December. Presumably, November and March represent months between the two extremes and it would be expected that there would be some scatter during this time, if indeed there is a significant difference in sprouting between the two seasons.

This data then suggests that collateral sprouting in the salamander is subjected to seasonal variation; it appears not to occur at all, or at a considerably reduced level, during the winter months. It should be noted that in the 'winter group' some of the intervals between the initial operation of nerve section, and subsequent testing, were up to 10 times longer than the interval needed to detect sprouting in the summer (Aguilar et al. 1973). A few experiments on nerve regeneration during the winter period indicated that these nerves were perfectly capable of regeneration and forming functional endings after being crushed or cut, and this regeneration occurred at rates comparable to those during the summer. Therefore, it would appear that these two processes, nerve regeneration and collateral sprouting, must be controlled in different ways. Presumably the cell body provides the 'drive' for an axon to regenerate which is triggered in an all-or-nothing manner at the site of section. The outgrowth of collateral sprouts from intact fibres is presumably under the influence of the target tissue, which provides the 'stimulus'; it may be postulated therefore that this peripheral stimulus (in the salamander) is absent

in the winter.

4. Discussion

a) Sprouting After Colchicine

The result that quantitative sprouting of the adjacent nerves occurred after colchicine treatment, at a time when there was no functional degeneration of the mechanoreceptors, is direct evidence in support of the hypothesis that factors carried by fast axoplasmic transport are involved in collateral sprouting. Although this is the result from only one animal, reasons were mentioned as to why the other seven animals did not show sprouting. Four out of the seven were tested too soon for new sprouts to have functionally matured, and the remaining three were tested during the winter season, when nerves do not usually sprout in the salamander. Finally, there are problems with the density measurements in these experiments. If the new sprouts were relatively sparse, because the fast transport in only 30% of the axons in the treated nerve were probably completely blocked by colchicine (see earlier discussion of this point), then it would be difficult to detect them.

b) Significance of Quantitative Sprouting

The finding that, after partial denervation, the new sprouts quantitatively made up the original density of innervation is also consistent with the hypothesis that target tissue and the nerves interact to regulate the density of the innervation of the end organ. It may be presumed that the stimulus from the target tissue is produced

at a constant rate but that the release of the neural factors is limited by the number of nerve endings at the target. An equilibrium will be reached when the supply of the neural factors has reached a level which will balance out the effects of the target stimulus. When this equilibrium occurs, sprouting ceases. Weiss (1937) and Litwiler (1938), working with transplanted limbs in the salamander, also observed that the density of innervation in transplanted limbs was normal, even though the number of axons supplying these limbs was reduced. Similarly, Olsen and Malmfors, studying the growth of adult adrenergic nerves, observed that after transplanting a nerve-free portion of an iris into the anterior chamber of the eye in rats, collateral sprouts emerged from the intact sympathetic nerves and innervated the transplanted portion of the iris (Olsen and Malmfors 1970). Furthermore, the density of innervation of the transplant after sprouting was similar to the density of innervation of the normal iris. Finally, Fitzgerald, studying the innervation of the pig snout during development, found that at a time when the skin was still growing and expanding the density of innervation remained constant, although the number of axons in the nerve trunk was unchanged (Fitzgerald 1961). All these observations suggest that each target tissue is capable of regulating its density of innervation. A similar conclusion was reached by Weiss (Weiss 1955).

c) Single Receptive-field Enlargement Rather than Collateral Sprouting?

It might be argued that these results of sprouting within the CFPN area could be explained by the receptive fields of a receptor becoming larger without there being an increase in the density

of receptors. However, consider the experiment in which the frequency with which the prodder located 15th nerve receptors increased from 10% to 30% after treating the adjacent 16th nerve with colchicine. For this increase to be accounted for by receptive field enlargements of the low threshold receptors, the receptive fields of the 15th nerve receptors would therefore need to have grown from an average radius of 50μ to a radius of approximately 85μ . The receptors are normally spaced 150μ to 200μ apart in the skin (see Part VI). Therefore the expected amount of coincidence (overlapping of receptive fields of 15th receptors) in this experiment should be 0. However, the results of this study indicated that the coincidence was 70%. Therefore there must be more 15th nerve endings. This is not surprising; in the animals, where enlargement of the gross fields of nerves 15 and 17 occurred after colchicine-treatment of the 16th nerve, the result could not have been due to enlargement of individual receptive fields of receptors but requires there to be more receptors.

Working with motor nerves, Edds observed that after partial denervation of muscle in the rat, the new sprouts quantitatively made up the terminals which had degenerated (Edds 1950, Edds 1953). Furthermore the new sprouts grew to the old endplates, presumably growing along the denervated Schwann tubes. Edds interpreted his results to mean that new sprouts compete for the vacated endplates. Only the successful ones mature; the unsuccessful ones regress. It is possible that a similar kind of phenomenon could also explain the quantitative sprouting in the present experiments although this would seem to require the existence

of some structural sites in the sensory system analogous to the end-plate. It is interesting, in this context, that the analysis of the sprouting after colchicine treatment suggests that the new endings may grow preferentially to sites where the terminals of the colchicine treated nerve endings are located. These sites could be the Merkel cells, which have been seen with the electron microscope to be associated with nerve endings in the epidermis. Located within these Merkel cells are membrane-bound granules whose function and chemical composition is unknown. It is conceivable that the Merkel cells secrete these granules to attract nerves to grow towards them. Burgess and his co-workers found that regenerating sensory nerves in the skin of the cat preferentially reinnervated their own Merkel touch spots (Burgess, English, Horch and Stenshaas 1974). There is also evidence from other preparations suggesting that regenerating nerves grow to their previous locations (Maturana, Lettvin, McCulloch and Pitts 1959; Attardi and Sperry 1963; Speidel, 1964; Frank, Jansen, Lomo and Westgaard 1975; Jansen and Nicholls 1972), although simple mechanical guidance may often be responsible for some of these findings.

Alternatively the new sprouts might have grown back to the same location as the existing endings in the present experiments because of mechanical reasons. As seen in Fig. 19, the secretory glands occupy a large portion of the dermis and are fairly close together. Perhaps the only places which would allow nerve fibres to pass through the dermis to the epidermis is between these glands.

One possibility which cannot be unequivocally excluded from these experiments is the unlikely one that the increase in low threshold touch receptors is not a result of sprouting but instead represents the activity of a population of non functional endings, which have become functional. The only direct proof against this would come from a combined physiological and morphological study, but there is some indirect evidence that suggests that this proposition is not responsible for the present results. In a few experiments the effects of direct electrical stimulation of points on the skin was tested; the results indicate that if indeed these non-functional endings are present, they are also electrically inexcitable. Furthermore, in these experiments the new sprouts took approximately 3 weeks to mature. This is consistent with other observations on terminal maturation of growing nerves. (Brown and Iggo 1963; Dennis and Miledi 1974). There seems no reason, therefore, not to believe that new growth was occurring in the experiments. The evidence quoted in favour of 'non functional' endings was that they were capable of 'turning on' and functioning normally by 2 days after partial denervation, i.e., too soon for normal growth to have been responsible. The question of whether non functioning synapses do, in fact, exist is still very controversial, and indeed experiments which have been reported to indicate their existence (Marotte and Mark 1970a; Marotte and Mark 1970b; Mark, Marotte and Mark 1972; Mark and Marotte 1972) cannot now be confirmed (Scott 1975).

VIII. FINAL DISCUSSION

The results of the present investigation supports the hypothesis previously put forward by Aguilar et al. (1973) for nerve sprouting after partial denervation. This hypothesis attributes the stimulus for sprouting to be growth substances secreted from the target tissue. Normally the effects of these growth substances are neutralized in some way by factors released from nerve endings. The observed sprouting after partial denervation results from the elimination in the sectioned nerve of these neural factors, thereby allowing the growth promoting substances to act on the remaining intact nerves. Similarly, treating nerves with colchicine mimics the effects of partial denervation by blocking the supply of the neural factors to the nerve endings and likewise causes the adjacent nerves to sprout.

The results of the quantitative study (Part VI) show that the present colchicine treatment did not cause a loss of mechanosensory nerve endings. The sprouting that resulted cannot therefore be attributed to products of nerve degeneration. Nor was it a result of a direct action of colchicine on the skin, which could have somehow initiated sprouting as a secondary phenomenon. It was shown that after the application of 50-75 μM radioactive colchicine to one 16th nerve, the amount of radioactive material in the skin was the same on both sides. Assuming that this radioactivity represented undegraded colchicine, and that it was uniformly distributed throughout the cells in the skin, there would be on the average 4×10^{-19} moles of colchicine within

each skin cell. This value is roughly 4 times less than the minimum amount of intracellular colchicine needed to block cell division (Taylor 1965). It is concluded, then, that neither products of nerve degeneration nor a direct action of colchicine on the skin are likely to be responsible for the sprouting after colchicine treatment. The most plausible explanation for this sprouting is the hypothesis of Aguilar et al. (1973) which was described above.

If this hypothesis is correct then it can also explain why the density of innervation of a target remains fairly constant in normal adult animals (Litwiler 1938; Weiss 1955; Fitzgerald 1961; Olson and Malmfors 1970). A constant density of innervation results from the establishment of a dynamic equilibrium between the continual release of both the sprouting stimulus from the target and the neutralizing neural factors. As a consequence of each ending releasing a substance which inhibits further nerve growth into its immediate vicinity, it would be expected that these endings should be uniformly spaced throughout the target. The present findings on the innervation of the salamander hindlimb skin (Part V) also support this concept. In only about 5% of the total area are there overlapping receptive fields of individual receptors, and the vast majority of them are uniformly distributed about 150-200 μ apart. Only in conditions like that induced by colchicine, which blocks the transport of the inhibitory factor to the nerve terminals, do nerve endings invade territories already occupied by existing endings. (The colchicine-treated nerves themselves do not sprout, as shown by Aguilar et al. 1973).

The evidence of the present thesis is against the generally accepted hypothesis that "products of nerve degeneration" act as a stimulus for adjacent nerve sprouting in the adult. Similarly the work of Olsen and Malmfors (1970) who showed that a nerve-free piece of iris transplanted into the anterior chamber of the eye evokes sprouting of host sympathetic nerves, and the work of Duchon and Strich (1968) in which motor axons, which had been treated with doses of botulinum toxin that prevent the release of acetylcholine but do not cause nerve degeneration sprouted collateral branches, also militate against the "products of degeneration" hypothesis. Another example of collateral sprouting in the adult, which was unlikely to have been stimulated by products of nerve degeneration, comes from studies by Yoon on the visual system of the goldfish (Yoon 1972). Yoon cut the optic nerve and separated one part of the tectum from the other with a dissolvable barrier, which prevented the regenerating optic fibres from growing over the entire tectum. As a result the entire innervation from the eye grew only to the half of the tectum to which it had access, where it formed the normal orderly projection, but now, interestingly, appropriately compressed in size. After a few months the barrier dissolved and the optic fibres sprouted into the vacated half of the tectum, in such a way as to maintain their same topographic order. While these experiments make important statements about specificity of nervous connections and plasticity of the nervous system, insofar as they relate to this thesis, these results are of special interest in that the nerve sprouting from one half of the tectum to the other

occurred at a time when the products of degeneration from the original innervation can be presumed to have disappeared (Turner and Singer 1975).

Finally, the evidence from the seasonal studies in this thesis, Part VII indicate that products of degeneration in themselves cannot initiate collateral sprouting. These studies show that while these salamander nerves will sprout collateral branches after partial denervation in the summer, there is a marked reduction in their ability to do so in the winter. While this lack of sprouting in the winter could be due to either the lack of stimulus or the inability of nerves to respond to it, the ability of nerves to regenerate and establish the original innervation during the winter makes it likely that it is the stimulus to sprout which is absent. Interestingly, it was clear from examination of histological sections of the cut nerves that Wallerian degeneration was occurring in both seasons. Therefore, an important question remains; what is the nature of this proposed stimulus which causes nerves to sprout?

One possibility which needs consideration is that there is an innate drive within nerve cells to attain some predetermined size and field (that is, the spread and density of its endings). The nerve cell could normally be prevented from reaching its potential size by inhibitory factors released from its own and its neighbouring nerve-endings. A reduction in these factors would then allow the cell to enlarge towards its potential size by sprouting, which would continue either until the new endings released enough inhibitory

factors to prevent further growth, or until the cell attained its predetermined size.

Although there is some evidence that a neuron can only sprout up to a particular size (Raisman 1975), this alone does not give any clues as to whether the stimulus to sprout originates within the nerve cell or in its outside environment. In fact the evidence from this present study (in particular seasonal studies in Part VII) and from the work of Olson and Malmfors (1970) on the growth of sympathetic nerves in the anterior chamber of the eye, as discussed above, strongly suggests that the stimulus for nerves to sprout originates outside the nerve cell.

There is good evidence for the existence of one such growth promoting stimulus which acts specifically on sympathetic nerves and embryonic sensory cells. This substance is referred to as nerve growth factor (or NGF), and is found in the target tissues of post ganglionic sympathetic nerves, as well as other regions (Levi-Montalcini and Angeletti 1968; Zaimis 1972). Recent evidence indicates that NGF is taken up by sympathetic nerve terminals and transported in a retrograde manner to the cell body (Stockel and Thoenen 1975).

Possibly the growth promoting substance from the end-organ hypothesised in this thesis and by Aguilar et al. (1973) could be NGF which would also be released from the target tissue and transported back to the nerve cell body. On this basis, one could propose an alternative explanation to that of Aguilar et al. to explain why nerves sprout after partial denervation or colchicine treatment. This

alternative proposal does not require the existence of any inhibitory neural factors. One merely postulates that growth promoting substances are not transported retrogradely along the cut or colchicine-treated axons, and consequently they build up at the tissue level and so act on the neighbouring intact nerves causing them to sprout. There seems no reason to suppose that the blocking of the retrograde transport in axons should also prevent these nerves in taking up substances from the periphery (at least for a while) but one could imagine (although not without difficulty) a stockpiling effect at the site of colchicine application or nerve section which would back up all the way down the axons to its terminals. The hypothesis proposed by Aguilar et al. (1973) does, however, offer an explanation for all known cases of sprouting (see page 28), while the alternative model can not adequately explain why nerves sprout after botulinum toxin, Duchen and Strich (1968).

In any case it can be concluded that the stimulus for intact nerves to sprout collaterals originates outside the cell, presumably from the target tissue. This concept has the added attraction of being consistent with the probable mechanisms which operate during tissue innervation during primary development (Ramon y Cajal 1919; Speidel 1941; Fitzgerald 1961). First a nerve grows out, relatively unbranched, to the end organ; this drive could be genetically determined. [This process could also apply for nerve regeneration where the drive to regenerate presumably originates within the cell body, but is triggered by the process of nerve section; presumably a signal travels from the cut end to the cell body (Cragg 1970; Watson 1974; Lieberman 1974).] When the

nerve arrives at the end organ, it is stimulated by locally manufactured substances to sprout collateral branches and make connections (c.f. Ramon y Cajal, 1919). This raises the possibility that collateral sprouting in the adult, as observed in the kinds of experiments mentioned above, and during primary development, depend on similar mechanisms. The implication here is that such mechanisms may be available to initiate sprouting and regulate nerve fields throughout the lifetime of the animal.

Value of Hypothesis is Supported by the Evidence of This Thesis

The findings of this thesis indicate that there is a control mechanism to regulate the size of nerve fields, which involves an interaction between the effects of growth promoting stimuli produced by the target tissue, and factors brought to the end organ by fast neuronal transport. From preliminary observations in this laboratory it is known that the block of fast axoplasmic transport by colchicine wears off in roughly 45 days, after which the transport in the treated nerves presumably resumes. It is not yet known whether the sprouted endings regress after the colchicine block wears off, but regression as a phenomenon has been observed by many workers in different preparations (Speidel 1941; Redfern 1970; Bajust, Lewis and Westerman 1973; Bennett and Pettigrew 1973; Fangboner and Venable 1974).

This control mechanism should not be thought of only in terms of the peripheral nervous system but could operate equally well in the CNS. Hubel, Wiesel and La Vay found that following monocular deprivation in monkeys during early life, either by eye removal or by lid suture,

the cortical projections of the lateral geniculate neurons connected to the open eye enlarged their territories at the expense of the territories associated with the other eye (Wiesel, Hubel and Le Vay 1975). Since the territories of these projections are of equal size at birth, the changes presumably reflect sprouting of one set of terminals and very likely a regression, or at least cessation of, growth of the other. Wiesel, Hubel and Le Vay also observed that the size of geniculate cells connected to the deprived eye were smaller than normal. This decrease in size could well be associated with reduced neuronal transport in the deprived axons, since such a correlation is suggested by other observations (Grafstein, Murray and Ingolia 1972). Since neurons with reduced neuronal transport cannot respond as effectively to a sprouting stimulus from the target (Aguilar et al. 1973) as axons with normal neuronal transport, only the normal axon would sprout.

IX. CONCLUSIONS AND CONJECTURES

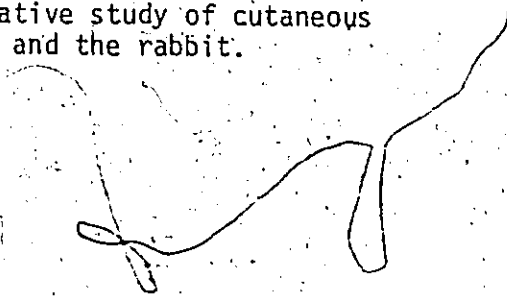
If the interpretations of the results of this thesis are correct, then there exists even in adult life a mechanism which the organism could utilise to regulate the fields of individual nerve cells. Since the size of the field of a neuron is an important measure of the influence which it can exert over its target, this mechanism could provide a basis for modifying the input/output characteristics of a neural circuit. Ultimately, such a change could be realized as a change in the behaviour of the organism.

The control of neural field sizes depends upon a postulated mutual interaction of two substances; one emanates from the target, and causes nerves to sprout and enlarge their fields, while the other is released from the nerve endings and serves to neutralize the effects of the target factor. Such a mechanism could clearly be useful in certain circumstances to help restore lost innervation subsequent to nerve degeneration. However, of more importance, this mechanism could also be used by the animal to bring about prolonged functional changes in behaviour such as occur during learning and conditioning. Learning and conditioning are used in this context in a general sense, to mean an increase in the probability of a specific output occurring as a consequence of the repetitive activation of a given input. This outcome could be achieved by having at least one neuron of the activated circuit increase its effectiveness over its target, and one of the most effective means for a neuron to do this would be to project more endings on to the target.

(Eccles 1964, Rail, 1967). Such a situation would be achieved by the mechanisms proposed in this thesis. However, in order for sprouting to be responsible for an increase in effectiveness of a neuron in a behavioural sense, there would need to be a link between the increased impulse activity caused by the repetitive application of the input, and sprouting. There has been at least one suggestion in the literature that increased impulse activity in motor nerves can lead to hyperinnervation of skeletal muscles in rats (Hoffman 1952), although this has not been confirmed. Chronic stimulation experiments are currently under way in this laboratory to investigate the effects of impulse activity on nerve field sizes in the salamander hindlimb.

If a link between impulse activity and nerve field sizes can be demonstrated, then the most active pathway of two (or more) projections sharing a target neuron would be expected to have the greater field size, and therefore the greater influence over that target. It is noteworthy that in the experiments of Wiesel, Hubel and Le Vay (1975) mentioned earlier, the cortical projections from the open eye have larger field sizes in layer IV of the cortex than do the adjacent ones from the closed eye. The mechanisms suggested by the experiments of this thesis might be involved in phenomena such as this, and indeed could provide a morphological basis for learning.

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