A PHYSIOLOGICAL AND MORPHOLOGICAL INVESTIGATION OF THE MERKEL CELL-NEURITE COMPLEX IN XENOPUS SKIN

BY

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

December, 1983

THE MERKEL CELL-NEURITE COMPLEX IN XENOPUS SKIN

DOCTOR OF PHILOSOPHY
(Medical Sciences)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: A Physiological and Morphological Investigation of the Merkel cell-neurite complex in Xenopus skin

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NUMBER OF PAGES: xiv, 287

Abstract

The overall objective of this study was to investigate the physiological and trophic interactions that can occur between sensory nerves and their targets or end organs, specifically those between cutaneous mechanosensory axons and the epidermal Merkel cells. Previous work has shown that in salamander skin, the Merkel cell-neurite complex forms the morphological basis of the rapidly-adapting, low-threshold touch receptor (Cooper and Diamond, 1977; Parducz et al., 1977), while in mammalian species, it is associated with slowly-adapting mechanoreceptors. The present investigation has been carried out using Xenopus frogs (Xenopus laevis), in which the Merkel cells are located around the visible openings of the cutaneous gland ducts. A voltage-controlled mechanical stimulator of 10 μm tip diameter was used to compare the mechanosensory thresholds when the stimulator was applied directly over the gland openings ("on" locations) to those when the stimulator was located between the openings. The most sensitive points were always the "on" ones, and the results indicated that these represented a single population of rapidly-adapting, low-threshold touch receptors. Therefore, the locations of these mechanoreceptors coincided with the positions of the epidermal Merkel cell-neurite complexes, suggesting the latter have a mechanosensory function in Xenopus laevis.

An attempt was made to clarify the role of the Merkel cell in

the mechanosensory process and in the trophic interactions believed to take place between the Merkel cells and the sensory nerves; this was done by following the development of mechanosensitivity when sensory nerves grow into nerve-free skin and observing whether there was any correlation with the appearance of the morphological features characteristic of the Merkel cell-neurite complex. These studies involved monitoring of the reinnervation of denervated skin, and the innervation of new skin that had regenerated in place of a portion previously excised. Merkel cells were shown to be present in both situations by using the fluorescent dye quinacrine as a marker for the Merkel cells and by EM examination. The development and maintenance of the Merkel cells seemed to be independent of nerves; they survived denervation, and they appeared in regenerated skin even in a totally denervated limb. Ingrowing sensory nerves eventually contacted these Merkel cells, which thus act as targets for these nerves. Preliminary results suggest that recovery of discrete low-threshold touch spots requires that contacts occur between the nerve endings and the Merkel cells. The mechanosensitivity develops gradually, however, concomitant with the gradual maturation of the Merkel cell-neurite complexes.

ACKNOWLEDGEMENTS

I wish to thank Dr. Jack Diamond for his continual enthusiasm, advice and encouragement throughout the course of this investigation and during the preparation of this manuscript.

I would also like to thank Dr. Colin Nurse for his advice and stimulating discussion thoughout this investigation.

I thank Drs. A. Dingle and R. Garfield who served as members of my supervisory committee for their comments and suggestions.

Special thanks are due to Michael Holmes, Lynn Macintyre and Bert Visheau for their assistance in various aspects of this project.

I wish to acknowledge the support of the Natural Sciences and Engineering Council of Canada, the Medical Research Council of Canada, and the Muscular Dystrophy Association of Canada. Portions of this work were supported by a grant from the National Institutes of Health.

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Section I

Introduction

Developmental neurobiologists have long been interested in how the terminals of afferent nerves acquire their specific sensory functions, and how these endings come to be appropriately distributed in the periphery.

Although sensory axons arise from probably only two morphologically distinguishable cell types in the dorsal root ganglion (Lawson et al., 1974), there are many different modalities of cutaneous sensation. These could result from the individual sensory neurons maturing according to intrinsic developmental programs that determine the functions of the receptive ends of their axons in the periphery. Alternatively, the fibres might grow out to the periphery and differentiate there according to local cues. The latter would seem to be especially likely if the endings became associated with particular peripheral structures; this association could then be responsible in some way for conferring on the terminal complex a characteristic physiological response. In general, sensory nerves can be considered to be of two types, those whose peripheral terminals are specialized to respond to specific stimuli in the absence of associated sensory cells and those whose endings develop specific sensory functions in

association with recognizable end organs (Munger, 1971; Iggo, 1976). If, as suggested above, local cues direct the differentiation of sensory endings, then the fibres subserving specific sensory functions in the absence of recognizable end organs or sensory cells would presumably require some specific influence(s) from the target tissue itself, perhaps at specific loci. Additionally, sensory cells or end organs could act as targets to which the fibres could become distributed appropriately, but this leaves unanswered the question of how the targets are initially distributed.

The overall objective of these studies was to investigate the physiological and trophic interactions that may occur between sensory nerves and their targets or end organs, specifically those between cutaneous mechanosensory axons and the Merkel cells of the vertebrate epidermis. The Merkel cell-neurite complex is generally regarded as a mechanosensory transducer (e.g., Iggo and Muir, 1969), although there is no direct evidence for this. The present study is an attempt to determine the role of the Merkel cell and the nerve ending in the functional and trophic interactions that might occur between these two components. For example, one would like to know whether the Merkel cell or the nerve ending is the actual transducer of mechanical stimuli, or whether the Merkel cell is even required for the characteristic physiological response of the complex. Another question of interest is whether the Merkel cell resembles other sensory receptors in terms of its trophic relationship with the sensory nerves; are sensory nerves required for the development and maintenance of the

Merkel cell, or does the Merkel cell appear independently and act as a target for the developing nerves? The approach used was to study the physiological and morphological basis of the normal mechanosensitivity in Xenopus skin and to examine the loss and recovery of this mechanosensitivity as well as the fate of the Merkel cells following nerve lesion and regeneration. The results should lead eventually to a further study in which the physiological and morphological findings described in this thesis will be extended to the level where the redevelopment of fine structural features may be correlated with the recovery of the characteristic mechanosensory function of the Merkel cell-neurite complex.

Section II

Background to the Investigation

The review presented in this section deals with some of the literature which bears upon the questions posed in the Introduction. The first part discusses the extent to which specific cutaneous modalities are associated with particular receptor structures. The second part deals with the trophic interactions that can occur between sensory axons and their targets or end organs.

I. Cutaneous sensations and receptors:

1. Sensory modalities:

Several distinct qualities of sensation can be evoked by stimulation of the skin: mechanical, e.g., touch, pressure, vibration; thermal, e.g., warmth or cold; and nociceptive or pain. Each of these modalities requires an appropriate or "adequate" stimulus (Sherrington, 1947). For example, a mechanical stimulus applied above a certain rate, which causes a particular minimum amount of deformation of the skin surface is an adequate stimulus for the sensation of touch. Similarly, the lowering or raising of local temperature beyond a minimum critical level is adequate for thermal sensation. The stimulus for pain sensation appears to be any potentially or frankly damaging

treatment of the skin, and can include intense mechanical and thermal stimuli (Sherrington, 1947).

It has become generally accepted that there are probably always specific nerve fibres which are responsible for each sensation. Descartes (1662) was one of the first to suggest such a relationship in the form of a mechanical displacement of the ends of nerve fibres in a sense organ causing a corresponding movement of the same fibres where they ended centrally in the brain. However, this concept was first clearly defined in Muller's Law of Specific Energies (1848), which proposed that the nerves belonging to each of the senses were specialized to react preferentially to certain stimuli and when activated a given nerve would always evoke a particular sensation; although any given sensory nerve might be activated by several kinds of stimuli applied to its terminals, it was always most readily excited by the stimulus appropriate to the sensation evoked by that nerve.

Since peripheral nerves are made up of fibres of different sizes, this feature has been studied as one basis for the different modalities. In general, each of the cutaneous modalities has been found to be served by a particular class of afferent fibres, the fibres being classified according to diameter and conduction velocity (Gasser and Erlanger, 1929; Heinbecker et al., 1933; Bishop, 1946); some modalities may, however, be represented by more than one class of fibres (see below).

The fibres of cutaneous nerves can be classified into three major groups, based on fibre diameter and conduction velocity; these

are the myelinated Aα and Aδ, and the unmyelinated C fibres (Gasser, 1960). The Aα fibres (6-12+ μm diameter, 36-72+ m/s conduction velocity) mediate tactile discrimination or pressure sensations. In the small myelinated Aδ (3-6 μm diameter, 15-30 m/s conduction velocity) and the unmyelinated C (0.4-1.5 μm diameter, 0.5-2 m/s conduction velocity) fibre range, touch, itch, pain and temperature are represented; the apparent difference between the two groups is that the A fibres contribute to sensations that exhibit more accurate localization, while those of the C fibres tend to be more diffuse (Bishop, 1959; Vallbo et al., 1979). The Aδ and C fibres are considered by some investigators to be rather non-specific compared to the larger myelinated fibres, since they will respond to a variety of different stimuli usually in the high threshold range (Adriaensen et al., 1983). Some of the Aδ and C fibres will respond only to either noxious mechanical or noxious thermal stimuli; others are classified as polymodal nociceptors and are responsive to thermal, mechanical and chemical stimuli (Iggo, 1960; Burgess and Perl, 1967; Perl, 1968; Bessou and Perl, 1968; LaMotte and Campbell, 1978; Vallbo et al., 1979; Lynn and Carpenter, 1982; Adriaensen et al., 1983).

In summary then, while at least some of the different cutaneous modalities are associated with particular classes of nerve fibres distinguished by their conduction velocity, and thus their size, there is a certain amount of overlap between the afferent fibre groups with respect to the functions they subserve and their size (e.g., the smallest Aδ conduction velocity is equivalent to that of some of the

unmyelinated fibres). Thus afferent fibre grouping by conduction velocity or size does not provide a totally adequate correlation with the different sensations elicited upon stimulation of the skin.

The next section which deals primarily with cutaneous receptors in higher vertebrates, discusses the likelihood that it is the characteristics of the nerve ending, and especially those of any end organ that might be associated with the ending, that are responsible for the physiological character of the sensory nerve.

2. Cutaneous receptors:

The sensory nerves serving the skin can be divided into two main groups, those whose peripheral terminals are specialized to respond to sensory stimulation in the absence of distinct (i.e., morphologically recognizable) associated cells, and those that terminate at recognizable end organs; these end organs presumably contribute to the receptive character of the whole terminal complex (Iggo, 1976; Munger, 1977; Diamond, 1982). The two main groups are usually referred to as free nerve endings and corpuscular receptors, respectively; the second group also includes the epithelial cell-neurite complexes (Munger, 1971; Iggo, 1977).

Free nerve endings and the more specialized sensory cell-neurite complexes, that is the Merkel cell-neurite complexes, are found in the epidermis, while in the dermis there are also free nerve endings, and in addition hair follicle receptors and encapsulated receptors, such as the Ruffini endings, Meissner's corpuscles and

Pacinian corpuscles (Munger, 1971; Iggo, 1977; Sinclair, 1981). The functional significance of receptor morphology will be discussed under the categories of free nerve endings, encapsulated receptors and epithelial cell-neurite complexes.

(a) Free nerve endings:

Nerve endings that terminate freely are generally those of the unmyelinated C fibres (Cauna, 1966). However, it is clear that small myelinated Aδ fibres can also terminate in this manner (Hensel, 1976; Kruger, et al., 1981; Munger and Halata, 1983). The term "free nerve endings" refers to those endings that are devoid of significant encapsulation or associated sensory cells; they are not truly "naked" since the terminal is usually associated with Schwann cell processes or the Schwann cell basal lamina (Kruger et al., 1981; Munger and Halata, 1983). Terminals of this sort are found throughout the dermis and in association with the hair follicles (Cauna, 1966; Munger and Halata, 1983; Halata and Munger, 1983). Additionally, some of the free endings penetrate the epidermis, losing their Schwann cell covering at the level of the epidermal-dermal basement membrane (Cauna, 1976; Kruger et al., 1981). These small myelinated Aδ and unmyelinated C fibres do indeed end freely in the epidermis and not in association with any specialized epithelial cell, such as the Merkel cells (see below).

Historically, the free nerve endings were considered to subserve mainly painful sensations (Adrian et al., 1931; Adrian, 1932; Maruhashi et al., 1952). It has, however, been shown that fibres in

this category of the small slowly conducting fibes (mainly the C fibres) can also act as mechanoreceptors, thermoreceptors and polymodal nociceptors (Iggo, 1960; Burgess and Perl, 1967; Hensel, 1976; Vallbo et al., 1979; Lynn and Carpenter, 1982). For example, Hensel (1976) has correlated the location of cold -responsive spots on a cat's nose with bulbous free nerve endings that penetrate the epidermis. In a study of the afferent innervation of the rat hind limb, it was found that a large proportion of the C fibre units, which are accepted as terminating freely, responded to both noxious heat and pressure; other C fibres acted as either cold thermoreceptors or mechanoreceptors (Lynn and Carpenter, 1982). In cats, low-threshold C fibre mechanoreceptors which respond to touch and cooling have also been reported (Iggo, 1960; Bessou et al., 1971). Additionally, A fibres which end freely have been shown to act as high-threshold mechanoreceptors (Kruger et al., 1981).

Given this information, one might argue that there is no requirement for end organs in the physiological responses, since free nerve endings can apparently subserve all modalities reasonably well. Nevertheless, many investigators have suggested that the role of the end organs associated with sensory nerve terminals may be to modify the adaptation of the receptor response to the stimulus or to provide for a better localization of the stimulus (e.g., Munger, 1971; Iggo, 1976; Sinclair, 1981). The following section will discuss some of the structural features of encapsulated receptors that appear to be important in the characteristic physiological response of these

receptors.

(b) Encapsulated receptors:

The encapsulated, or corpuscular, receptors are located in the dermis or sub-cutaneous layers, and are associated with the large myelinated A fibres (Bishop, 1959; Mountcastle, 1974; Vallbo et al., 1979). The following receptors are included in this category: in the mammal, Pacinian, Meissner's and Golgi-Mazzoni corpuscles, and the Ruffini endings; in birds, Herbst and Grandry corpuscles; and in reptiles, lamellated corpuscles. As discussed later, there do not appear to be any corpuscular receptors in the lower vertebrates, that is the amphibians and fishes, with the one exception of the lamellated receptor in frog skin described by von During and Seiler (1974). For the corpuscular receptors of the higher vertebrates, which all act as mechanoreceptors, investigators have attempted to correlate the physiological response with the morphological characteristics, in order to determine the contribution of the structural features to the response to mechanical stimuli.

A common structural feature of these mechanoreceptors, with the exception of the Ruffini endings and the Grandry corpuscles, is the presence of lamellar cells of Schwann cell lineage surrounding the sensory neurite (Munger, 1971; Iggo, 1976). Another interesting feature of the corpuscular receptors is the apparent specialization of the axon terminal region, with the elaboration of spur-like processes which project between the lamellar or satellite cells, or in the case

of the Ruffini endings come into contact with the collagen fibres in the inner core of the complex (Spencer and Schaumberg, 1973; Gottschaldt and Kraft, 1978; Gottschaldt et al., 1982). These axon specializations act to increase the surface area of the terminal region and probably increase the sensitivity of the receptors to mechanical stimulation. The role of the non-nervous components in association with the axonal specializations seems to be to convey more effectively mechanical stress to the nerve ending.

The contribution of the accessory structures in the response has been best studied in the Pacinian corpuscle. When pressure is applied to the outside of the corpuscle, the lamellae of the capsule are temporarily deformed, thus deforming the nerve terminal; however, the inner lamellae rapidly move back to their original position so that excitation of the terminal is not maintained, resulting in rapid adaptation (Hubbard, 1958).

The Ruffini ending, on the other hand, which responds to stretching of the skin (Chambers et al., 1972) is a slowly- adapting (SA) type II receptor, characterized by a resting discharge, uniform interspike intervals and a normal (Gaussian) pattern of interval distribution of the adapted discharge (Iggo, 1974). Encapsulation does not seem to be the structural feature that is important in the physiological response, since several examples of Ruffini endings have been described in which the capsules were absent (Halata and Munger, 1980). What does appear to be important though, is the association of the axon terminal and Schwann cells with bundles of collagen fibrils

which merge with those of the dermis; the collagen fibres, which are 'non-elastic', could provide an effective mechanism for transmitting the stretching of the skin to the axon terminal (Chambers et al., 1972; Gottschaldt et al., 1982).

The fact that all known receptors with pronounced capsules and extensive lamellation are rapidly-adapting, and that one without these features is not, suggests that the contribution of a capsule might be to control the amount of stimulus that reaches the nerve terminal, and thus modify the response of the nerve ending, as is the case in the Pacinian corpuscle. This is discussed further in section 4. However, as seen below, and as will be apparent from the results of this thesis, it is possible to have a rapidly-adapting mechanoreceptor that is not associated with such structural features. This would seem to indicate either that a rapidly-adapting response could be an intrinsic characteristic of certain nerve endings, or that for these particular receptors some other structural feature substitutes for the lamellated corpuscles in this regard.

(c) Epithelial cell-neurite complexes:

As described by Munger (1971) and Iggo (1977), these are cutaneous receptors which are found within the epidermis, usually in the basal layer, close to the epidermal-dermal junction, occasionally abutting the basement membrane. These complexes consist of nerve terminals in a particular association with a specialized epithelial cell, the Merkel cell (Merkel, 1875; Munger, 1965). These complexes

act as mechanoreceptors, but unlike the previously described receptors, there is no encapsulation of the nerve ending. This suggests that either the Merkel cell has a passive mechanical role similar to the capsular elements of the other receptors, or that the Merkel cell itself acts as the mechanosensory transducer, and then excites the nerve ending in some other way. As will become obvious in later discussion, this is a problem that has not yet been satisfactorily resolved.

Over a century ago, Merkel reasoned that since all the other special senses had specialized epithelial cells associated with the nerve endings, then the sense of touch, which was considered to be the fifth special sense, should also be equipped with similar terminal structures (Merkel, 1880). He had already examined touch-sensitive areas in the skin of numerous animal species and man; among the areas studied were the bill of various birds, the snout, lips, eyelids and ear skin of cats, pigs, cows and humans (Merkel, 1875). In the touch-sensitive spots, he discovered some distinctive cells upon which, or as he thought at the time, within which, the sensory nerves terminated as flattened discs. Since these cells were found in highly mechanosensitive areas and structures, e.g., the sinus hairs, Merkel believed them to be the special sensory cells of the skin associated with the sense of touch, and for that reason he called them "Tastzellen", or touch cells. It is also interesting to note that in addition to these touch cells he also observed free nerve endings and suggested that these were the "Temperaturnerven" (Merkel, 1875).

However, despite his belief that his newly-described cells were the touch receptors, there was no direct evidence then (or even now) to support this view, and the Tastzellen were subsequently termed Merkel cells so that a specific functional capacity would not be implied (Tretjakoff, 1902).

Merkel used osmium-fixed tissue to demonstrate the cells and the nerves, the Merkel cells appearing as pale cells somewhat larger than the surrounding epithelial cells and having lobulated nuclei. Other later investigators described similar cells using various histological techniques, including silver stains (Botezat, 1908; Boeke, 1932) and methylene blue (Dogiel, 1903; Vincent, 1913; Pinkus, 1927); however, due to the fixative (formalin) required for most of these methods, the structure of the Merkel cells was not always preserved, and the 'cells' often appeared as pale vacuoles associated with the clearly-stained nerve discs. The term "Merkel's discs" was used to describe the structures as they appeared in silver-stained or methylene blue-stained material, and refers only to the nerve terminal (Boeke, 1932).

It was not until the application of electron microscopy to the study of the sensory innervation of skin, that it was possible to show that there was indeed a specialized cell, different from the surrounding epithelial cells, associated with the Merkel's discs, or nerve endings (Cauna, 1962; Munger, 1965; Patrizi and Munger, 1966). Using EM, Merkel cells have been described in the skin of animals from all vertebrate phyla. In fishes, amphibians and mammals, the Merkel

cells are found in the epidermis (Munger, 1965; Parducz et al., 1977; Fox and Whitear, 1978; Whitear and Lane, 1981), while in reptiles and birds, cells with similar fine-structural features are located in the dermis (von During, 1974; Saxod, 1980). This difference in location tends to confuse the issue of the origin of the Merkel cell, as will be discussed later.

At the ultrastructural level, the Merkel cells can be distinguished from other epidermal, or dermal, cells by the presence of numerous cytoplasmic dense-cored granules (90-120 nm diameter) which tend to accumulate at the dermal side of the cell, usually at the site of nerve contact (Munger, 1965, 1971; Winkelmann and Breathnach, 1973). The granules, the contents of which are unknown, are thought to have a role in both trophic and physiological functions, although such involvement is still speculative (e.g., Munger, 1977; Winkelmann, 1977; Hartschuh and Grube, 1979; Andrews, 1981). Another interesting structural feature of the Merkel cell is the presence of spinous cellular processes which protrude from the Merkel cells like fingers and interdigitate with the surrounding keratinocytes (Smith, 1967); perhaps these spines have a function analogous to that suggested for the axonal spines on the terminal neurites of the corpuscular receptors discussed earlier, namely to convey more effectively mechanical stress to the nerve ending.

In mammals (a full description of the amphibian Merkel cell-neurite complex is given in Section 3b), the disc-like nerve terminal contains numerous mitochondria and also some clear

synaptic-like vesicles (Munger, 1965); the presence of a relatively large number of mitochondria appears to be a common feature of terminals which exhibit slowly-adapting physiological responses (Iggo, 1976), and thus require a large energy supply. Morphologically, synapses occur between the Merkel cell and nerve ending, often with "post-synaptic" densities at the nerve terminal membrane, and localization of dense-cored granules near the synaptic area on the Merkel cell side, as well as the presence of "pre-synaptic" dense projections on the Merkel cell membrane; on this basis, if indeed synaptic transmission were to occur, its direction would be from the Merkel cell to the afferent nerve. It is intriguing that in the amphibian at least, including the material examined in the present study, there appears to be a reciprocal synaptic relationship between the Merkel cell and nerve endings; that is, there are synaptic regions that are morphologically polarized in the direction of Merkel cell to nerve, and other ones (often adjacent) polarized from nerve to Merkel cell (Munger, 1977; Fox and Whitear, 1978). Such synaptic relationships seem to imply a two-way transfer of information. In other systems where reciprocal synapses occur, such as in the olfactory bulb (Shepherd, 1978), the retina (Dowling and Werblin, 1969) and the carotid body (McDonald and Mitchell, 1975), the synapses are thought to be involved in local inhibitory feedback control circuits during physiological activity; one could envisage that the existence of reciprocal synapses in the Merkel cell-neurite complex is involved in a similar type of feedback control, perhaps allowing for the

rapidly-adapting response of the receptor in amphibians (see below).

In mammalian hairy skin, groups of Merkel cell-neurite complexes are found in the basal epidermal layer of the touch domes, or Haarscheiben (Pinkus, 1927; Brown and Iggo, 1963; Smith, 1967; Munger, 1971). In the rat each epidermal touch dome can contain up to 150 Merkel cells (Nurse et al., 1983a) and is supplied by one or a few myelinated axons; from conduction velocity measurements these are of the Aα class (Gottschaldt et al., 1973), and they branch extensively to supply the Merkel cells (Iggo and Muir, 1969). Merkel cells are also found in a collar of several hundred cells arranged around the upper regions of the outer root sheath enlargement of the sinus hairs, or vibrissae (Patrizi and Munger, 1966; Halata and Munger, 1980; Gottschaldt and Kyau, 1982). In glabrous skin, the Merkel cells are usually present at the base of the epidermal ridges or rete pegs (Munger, 1965; Janig, 1971; Munger and Pubols, 1972). It is interesting that slowly-adapting responses to maintained stimulation of the overlying skin or associated hairs have been recorded in the nerve supplying all these locations in mammals (Smith, 1967; Iggo and Muir, 1969; Janig, 1971; Munger and Pubols, 1972; Gottschaldt et al., 1973; Horch et al., 1974). These receptors are SA type I receptors characterized physiologically as having no resting discharge, a quite variable interspike interval, and an interval distribution of the adapted discharge that is Poisson in nature; this pattern of discharge is attributed to the presence of multiple spike generation sites in these receptors, which contain large numbers of Merkel cell-neurite

complexes (Iggo, 1974).

To summarize then, in the skin of the higher vertebrates, there is a number of morphologically distinct receptors, which all act as mechanoreceptors. Some of these, like the Pacinian corpuscle or Ruffini endings, appear as highly organized structures in which the emphasis is on the transmission of the mechanical stimulus to the nerve ending. Others, like the Merkel cell-neurite complexes, are relatively simple, but in their fine-structural details it is clear that they are actually complex, in ways that suggest the presence of mechanisms designed for purposes other than the simple transmission of mechanical deformations. Additionally, free nerve endings, morphologically the simplest receptors of all, have been shown to respond to light mechanical stimuli, as well as to act as high-threshold mechanoreceptors and thermoreceptors. It seems that the presence of a complex or lamellated capsule results in a rapidly-adapting response to stimulation; the free nerve endings and unencapsulated receptors tend to display slowly-adapting responses. The previous discussion does not clarify the role of the Merkel cell in terms of its possible contribution to the physiological response. While it is conceivable that the Merkel cell could act to increase the surface area of the terminal neurite or to convey the mechanical stimulus to the terminal more effectively, it is also possible, as discussed later, that it has an entirely trophic role, to do, for example, with regulation of the distribution of the mechanosensory endings or with inducing in these endings their characteristic physiological mechanosensitivity. These

questions are addressed by the present study.

3. Cutaneous innervation in Amphibians:

In various species of amphibians that have been studied, the endings of the cutaneous nerves, which run along the undersurface of the skin, enter the deep dermal layers at numerous points between the granular and mucous glands found in the dermis. Within the dermis, some of the axons end freely, in expanded tips, while others end in association with the skin glands and blood vessels (Whitear, 1974; Spray, 1976). Spray refers to the expanded tip endings as encapsulated endings, but does not elaborate any further on the structure, or provide any references; presumably the 'encapsulation' refers to the Schwann cells that normally surround the terminals of these free endings. Other endings penetrate the epidermis and end freely with terminal expansions amongst the keratinocytes (Hulanicka, 1909; Nafstad and Baker, 1973; Whitear, 1974), or they end in association with the Merkel cells (Merkel, 1880; Hulanicka, 1909; Nafstad and Baker, 1973; Parducz et al., 1977; Fox and Whitear, 1978; Ovalle, 1979).

There do not appear to be many specialized receptors in amphibians. An early reference (Gaupp, 1904) indicates that there are only two types of sensory endings, one in the form of free nerve endings arborizing in the epidermis, and another which is more highly differentiated and is included in his description as a small group of cells in the corium surrounding the terminations of a medullated fibre; this second type is probably the same as the lamellated corpuscle

described much later by von During and Seiler (1974) in the dermis of the frog. This corpuscle consists of two to four layers of perineural cell processes surrounding the terminal portion of a large (8-12 μm diameter) myelinated axon. Although it has been assumed that this receptor represents a rapidly-adapting mechanoreceptor similar in function to the mammalian Pacinian corpuscle (von During and Seiler, 1974), the necessary combined morphological and physiological study to support this assumption has not yet been carried out.

In addition to these receptors described in frog skin, the epidermal Merkel cell-neurite complexes in salamander have been shown to act as low-threshold, rapidly-adapting mechanoreceptors (Cooper and Diamond, 1977; Parducz et al., 1977). The present results (that have been briefly reported, Mearow and Diamond, 1983) show that these complexes function in the same manner in Xenopus frogs. As discussed earlier, the actual role of the Merkel cell is not known, but the results of this investigation may be able to provide some new information in this regard.

In frogs, certain dome-like elevations in the epidermis (Holloway et al., 1976) and warts (Ogawa et al., 1981) have also been demonstrated to be sensitive to tactile stimulation. Unfortunately, there has been no good morphological description of these structures. Early light microscope studies described tactile elevations, which may be the same structures, but did not mention the presence of any specialized structures or cells, such as the Merkel cells (Hulanicka, 1909, 1913).

Physiological studies have demonstrated the existence of both rapidly- and slowly-adapting responses to mechanical stimulation in amphibian skin (Adrian et al., 1931; Hogg, 1935; Maruhashi et al., 1952; Lindblom, 1962; Holloway et al., 1976; Ogawa et al., 1981). The rapidly-adapting responses could well be associated with the Merkel cell-neurite complexes, that are the object of the present study, while the slowly-adapting response has been postulated to arise from some deeper dermal structures since the response was still present in some experiments where the epidermis was removed (Adrian et al., 1931). The latter point is briefly re-examined in the present study (see Results, Part IIB, section 1).

Clearly the Merkel cell-neurite complex is the only receptor consistently described in amphibian skin.

The Merkel cell-neurite complex:

Amphibian Merkel cells are very similar in structure to their mammalian counterparts. However, their distribution differs; in the salamander they are not densely grouped in special structures but are usually scattered singly throughout the epidermis (Parducz et al., 1977; Fox and Whitear, 1978), while in Xenopus the cells may occur in very small groups (of only two to four cells) associated with gland openings (Fox and Whitear, 1978; Nurse et al., 1983b; Mearow and Diamond, 1983). As in mammalian skin, the usual position of Merkel cells in anuran skin is immediately above the basal layer of the epidermis; in urodeles the cells are normally situated amongst the

basal layer cells, but only rarely in contact with the basement membrane (Fox and Whitear, 1978).

Nerve contacts with the Merkel cells are generally seen on the deep, or dermal, surface of the cell, but have been observed on all sides (Parducz et al., 1977; Fox and Whitear, 1978). Unlike the mammalian complex, the nerve does not form a disc-like ending, but rather, appears to form several terminations on one cell; physiological investigations in the salamander indicate that, at least functionally, the Merkel cells are innervated by a single axon (Cooper and Diamond, 1977; Scott et al., 1981). Each of these multiple terminations can form synaptic contacts with the Merkel cell. Fox and Whitear (1978) describe in frog and toad skin synapses with post-synaptic densities on the nerve membrane and pre-synaptic cytoplasmic densities at the Merkel cell membrane, which suggests that chemical information is transferred from the Merkel cell to the nerve ending. However, reciprocal synapses do occur between the Merkel cells and nerve terminals, and appear to be much more common in the amphibian than in the mammalian complex; for example, in the salamander, each of the two to four boutons on a Merkel cell has a reciprocal relationship with the cell (Diamond, unpublished observations). It is reasonable to suppose that such synapses play some role in either the physiological or trophic interactions that occur between the Merkel cell and nerve ending (Diamond et al., 1976; Scott et al., 1981).

Correlative neurophysiological and ultrastructural studies have shown that the Merkel cell-neurite complexes in salamander epidermis

act as rapidly-adapting mechanoreceptors (Cooper and Diamond, 1977; Parducz et al., 1977). This is in contrast to the mammalian situation where, as described earlier, the Merkel cells are associated with slowly-adapting mechanosensory structures (Iggo and Muir, 1969; Burgess et al., 1974). It is conceivable that the common occurrence of reciprocal synapses in amphibians, compared to mammals, may be the basis for the rapid adaptation of the receptors in the former. On the other hand, the numerous Merkel cell-neurite complexes of the mammalian touch dome could conceivably each act as rapidly-adapting impulse generators, which if they were to fire asynchronously (Horch et al., 1974; Iggo, 1976), could provide an apparently slowly-adapting response to deformation of the dome that, although maintained, contained small irregular fluctuations in frequency.

4. Mechanosensory transduction:

Sensory receptors act as transducers; that is, they convert one form of energy into another, in this instance into electrical energy, ultimately realized in the form of nerve impulses. Thus mechanoreceptors convert mechanical energy, such as that produced by stretching or compression of the receptive elements, into electrical energy. This section will discuss the available evidence as to possible roles of the various components of mechanoreceptors in the transduction process, to see if any clues emerge regarding the possibility of the Merkel cell acting as a transducer.

Goldman (1965) describes a hypothetical mechanoreceptor as

having several elements including a transformer, a transducer, perhaps an amplifier and an encoder. The transformer, which is considered to be a passive element, receives the mechanical stimulus and translates it into an apporpriate mechanical stress which acts directly on the transducer. The transducer is the element that actively converts the mechanical energy into another form, either electrical or chemical, which excites the impulse generator region of the afferent nerve, giving rise to an action potential. The transducer is believed essentially to be an integral part of a cell membrane, which when mechanically distorted produces a local non-propagated electrical depolarization; the depolarizations that have been studied to date in sensory receptors occur as a result of an increase in membrane permeability to sodium ions (Katz, 1950; Diamond et al., 1957; Julian and Goldman, 1962). The local potential, also termed the generator potential, varies with the size of the stimulus and also the rate at which it is applied (Katz, 1950; Gray and Malcolm, 1951; Julian and Goldman, 1962); when threshold is reached, the local potential gives rise to one or more action potentials in the afferent nerve fibre (Adrian and Gelfan, 1933; Katz, 1950).

The contribution of the different components of a receptor to the sensory transduction process vary with receptor types. For example, in the case of the muscle spindle and Pacinian corpuscle, the spindle muscle fibres and the Pacinian corpuscle capsule are accessory structures which act as transformers conveying the mechanical distortion of stretch and compression, respectively, to the nerve

terminal which functions as the transducer (Katz, 1950; Loewenstein, 1965). In contrast, lateral line organs and the cochlea, for example, contain ciliated mechanoreceptive elements, the hair cells. In both these systems the hair cells act as the transducer. Before these examples are discussed further, it is useful to consider the long-known phenomenon whereby mechanical stimuli applied quite unambiguously to the nerve directly, can produce excitation; in this instance there is no doubt that the nerve membrane must be acting as a transducer.

Such experiments wherein the nerve was stimulated by mechanical means were first carried out by Tigerstedt (1880) and Gotch and Macdonald (1896), who used a falling weight technique to stimulate frog nerve trunks; Blair (1936) did similar experiments using compressed air jets to provide the mechanical stimulus. The results of these early experiments indicated that the nerve excitation produced by mechanical stimulation was the same as that produced by electrical stimulation. For example, pressure-duration curves obtained for mechanical stimulation were similar to strength-duration curves obtained with electrical stimulation; the absolute refractory period for mechanical stimulation was similar to that of electrical stimulation (Adrian, 1928; Blair, 1936). In addition, in both situations, the rate of change or application of the stimulus was an important feature in producing excitation; a very gradually applied current or mechanical stimulus was not effective in producing impulses (Lucas, 1907; Adrian, 1928).

More recent experiments involving mechanical stimulation of

single axons (e.g., lobster giant axon, Julian and Goldman, 1962) and of bundles of axons from the frog sciatic nerve (Julian and Goldman, 1962) and cat peroneal nerve (Rosenblueth et al., 1955) have demonstrated the same results. In addition, however, these studies have indicated that the mechanical stimulus acts primarily to reduce membrane resistance; this reduction in membrane resistance is probably due to an increase in membrane permeability to sodium, and to a lesser extent, potassium ions (Goldman, 1965), resulting in membrane depolarization. This has also been shown to be the case in intact receptors (Katz, 1950; Diamond et al., 1957).

The nature of the transduction process in the Pacinian corpuscle has been extensively investigated; it has been shown that the nerve terminal is clearly the transducer in this case (e.g., Loewenstein, 1965). For example, by stripping away the capsule and most of the inner core, mechanical stimuli can be applied more or less directly to the axon terminal (Loewenstein and Rathkamp, 1958). The ending responds to the stimulus, but behaves more like a slowly-adapting ending, in that the generator potential is prolonged. When the terminal is then enclosed in an artificial capsule, the response becomes very similar to the normal response (Loewenstein and Mendelson, 1965). Hubbard (1958) showed by direct observation and measurement of the lamellae movements that the rapid adaptation of the Pacinian corpuscle could be an inevitable consequence of the mmechanical properties of the capsule.

The hair cell is a mechanoreceptor in which the nerve ending is

not the transducer. In this receptor, bending of the stereocilia on the apical surface of the hair cell in response to acoustic stimulation causes membrane distortion which results in depolarization of the hair cell; the signal is conveyed to the afferent nerve endings, presumably chemically by way of the synaptic contacts that exist between the hair cells and afferent terminals (Wiederhold, 1976), although the precise nature of the mechanism is not known (Hudspeth, 1983).

As a point of further interest, it has been noted that the terminal regions of the axons of various mechanoreceptors, such as the Pacinian and Herbst corpuscles or Ruffini endings, possess distinct spine-like processes. These spines, which contain bundles of microfilaments, articulate with the accessory structures of the receptors, and it has been suggested that these spines act to increase the mechanosensitivity of the terminal, and that they may be the actual transducer site (Spencer and Schaumberg, 1973; Gottschaldt et al., 1982).

Given this background , the question which arises is, what is the nature of the transduction process in the cutaneous receptors that have Merkel cells associated with them? There are obviously two alternatives; either the Merkel cell acts as the transducer (e.g., Iggo and Muir, 1969; Horch et al., 1974), or the nerve ending is the transducer (e.g., Gottschaldt and Vahle-Hinz, 1981); in the latter case the role of the Merkel cell could be to convey the mechanical stimulus to the nerve ending more effectively, or it could have an entirely trophic or modulatory role. These possibilities will be referred to further in the Final Discussion.

II. Nerve-target interactions:

1. Induction and maintenance of receptors:

In addition to the functional relationships that have been discussed, there is evidence that nerve endings and receptor cells can be involved in trophic interactions, i.e., those necessary for the differentiation and maintenance of both the sensory cells and nerve terminals. Additionally, if end organs were to develop independently of nervous influence then they could act as specific targets for the appropriate nerves.

It appears that the majority of sensory receptors in the somatosensory system are induced to develop by the arriving sensory nerves, and that they also show a dependence upon a sensory innervation for their continued existence, although the degree of dependence varies among species. In these instances, there is likely to be an information transfer from the nerve to the precursor cells, and in some instances subsequently to the receptor cells themselves. Structures falling into this category include: taste buds (Torrey, 1934; Fujimoto and Murray, 1970; Zahm and Munger, 1983); muscle spindles (Zelena, 1957; Zelena and Soukup, 1973); Pacinian corpuscles (Zelena, 1978, 1980); Grandry and Herbst corpuscles (Saxod, 1978, 1980; Ide and Munger, 1978); Meissner's corpuscles (Ide, 1977, 1982a, b); and the mechanoreceptors of the acoustico- lateralis system (Stone, 1933; Jones and Singer, 1969; Szamier and Bennett, 1973; but see Speidel, 1947a, b; Tweedle, 1977).

It is not clear whether the Merkel cells show this same sort of nerve dependence in mammals where the evidence is especially confusing (Palmer, 1965; Smith, 1967; Burgess et al., 1974; Benkenstein, 1979; English et al., 1980; Nurse et al., 1983a). However, based on the results of Scott et al (1981) in the salamander, and the results of this thesis in Xenopus, it would appear that the amphibian Merkel cells, at least, differ from most of the receptors mentioned above, in that they can differentiate and survive independently of nerves.

In order to illustrate the various types of sensory cell-nerve interactions, several examples will be discussed in more detail.

(a) Taste buds:

In the gustatory system, the sensory or taste cells act to transduce the taste stimulus and transmit the information to the afferent axon (Beidler, 1974). These sensory cells require sensory nerves both for their development and subsequent maintenance.

The taste bud cells have been classified into three or four histologically different types by various investigators (see Murray, 1973), though it is generally agreed that the basal cell is probably the precursor of the other cell types, which are the chemosensory cells and supporting cells. The chemosensory cells are characterized by their synaptic relationships with the sensory nerve endings, specific cytoplasmic dense-cored granules, numerous clear synaptic-like vesicles and apical microvilli that extend into the taste pore (Ide and Munger, 1980; Zahm and Munger, 1983).

During development of the taste buds, the organization of the appropriate cells into the buds begins at a time when nerve fibres are first seen to approach the gustatory epithelium. It has been observed that penetration of the sensory nerves into the epithelium at the site of the developing taste bud is necessary for the full differentiation of the taste bud and taste cells (Farbman, 1965; Fujimoto and Murray, 1970; Zahm and Munger, 1983). Interestingly, Farbman (1965) described the presence of large dense-cored granules in some cells, upon which the nerves eventually terminated, just prior to the arrival of the nerves in the epithelium and suggested that these cells and the granules played some role in attracting the nerves to the correct locations in the developing taste bud. Similar large granules were observed by Ide and Munger (1980) and Zahm and Munger (1983), but these were found in the supporting cells, and not in the chemosensory cells upon which the nerves terminate. However, these authors do suggest that the smaller dense-cored granules that are normally found in the mature chemosensory cells may play some trophic role in attracting the nerves to their final termination site.

In any case, the evidence indicates that sensory nerves are involved in the differentiation and development of the taste cells and taste buds. In addition, the nerves are necessary for the subsequent maintenance of these structures. Numerous investigations have provided evidence for the rapid degeneration of taste buds following loss of their innervation (Olmsted, 1920; Torrey, 1934; Zalewski, 1969; Fujimoto and Murray, 1970). For example, taste buds of the catfish

barbel degenerate within eleven days after loss of their innervation (Torrey, 1934). Similarly, in the rat (Farbman, 1965) and in the rabbit (Fujimoto and Murray, 1970), taste buds degenerate within seven to ten days following denervation. The fast rate of degeneration is related to the relatively rapid turnover of the individual taste cells. The lifetime of an individual taste cell is about ten days (Beidler and Smallman, 1965; Conger and Wells, 1969). The rate of degeneration of the taste buds is also proportional to the distance from the point of nerve section to the taste bud (Torrey, 1940; Cheal and Oakley, 1977), and degeneration of taste buds may also be induced by blockade of axoplasmic transport in the nerve with colchicine (Sloan et al., 1983). These results indicate that trophic factors supplied by the nerve are normally required for the maintenance of the taste bud cells.

(b) Pacinian corpuscle:

This mammalian receptor, in which the nerve is the actual transducer, the capsule acting to passively transfer the stimulus to the terminal, also exhibits a dependence upon sensory innervation for its development, and to a lesser extent for its maintenance. For example, if the nerve supply to the interosseous membrane of the lower leg is removed up to four days after birth in the rat, the corpuscles do not develop in the interosseous membrane (Zelena, 1980). If denervation is carried out after this time, the immature corpuscles that have already formed survive, but their further growth is inhibited (Zelena, 1980). This 'critical period' lasts only up to four days

after birth, by which time most of the Pacinian corpuscles in the rat have already differentiated. Denervation of the Pacinian corpuscles in adult rats results in some atrophy of the corpuscles, although the main structural features are preserved (Zelena, 1982).

The induction of Pacinian corpuscles involves specific interaction of sensory nerves with the appropriate, or competent, tissue (Ilyinsky et al., 1973; Chalisova et al., 1980). Experiments were carried out in which either a foreign sensory nerve or a motor nerve were grafted onto the denervated intestinal mesocolon of cats, where Pacinian corpuscles can be found normally. It was found that the sensory, but not the motor, nerve induced formation of the Pacinian corpuscles in the mesentery. However, if the nerves were grafted onto an area that did not normally contain Pacinian corpuscles, such as the mesentery of the omentum, no corpuscles were induced (Ilyinsky et al., 1973; Chalisova et al., 1980). These results indicate that the sensory nerve could induce differentiation of Pacinian corpuscles only in competent tissue.

(c) Lateral line system:

This third case provides another example of a system in which the sensory cells (in this instance the hair cells) act as the transducers of the stimulus; however, in this example the influence of the nerve upon receptor development and maintenance is not as obvious as in the cases described above.

The lateral line organs of fish and amphibians develop from an

epidermal placode which is located near the vagal ganglion. The placodal cells, once they have reached a certain stage of differentiation, migrate in particular pathways beneath the surface of the epidermis to their final location where they form the individual receptors of the lateral line system (Stone, 1933; Speidel 1948). The results of various experiments performed to determine the role of the lateral line nerve in the differentiation and development of the lateral line organs have been interpreted as indicating that the receptors develop independently of the nerve supply (Stone, 1933; Speidel, 1947a, 1947b; Tweedle, 1977). For example, Harrison (1903) showed that migration of the epidermal placode cells in tadpoles still took place after removal of the vagal ganglion. More recently, Tweedle (1977) observed that the lateral line organs could develop normally in salamander larvae rendered aneurogenic by surgical removal of all presumptive neural tissue, that is neural crest and neural tube, prior to the migration of the placodal cells.

The conclusion drawn from these experiments, that the lateral line organs can develop independently of nervous influence, is not entirely justified, however. Nerve fibres from the vagal ganglion were known to enter the placode prior to the migration of the cells and to maintain contact with them during their migration and dispersal (Stone, 1933; Speidel, 1948; Sato, 1976). In addition, Tweedle (1977) points out that, although there were no nerves present during the migration of the placodal cells in the aneurogenic larvae, there could have been early nerve contact with the placode prior to the removal of the neural

tissue. It seems possible then, that the vagal fibres could be exerting an early influence on the primordial placodal cells causing them to differentiate prior to migration. Cells influenced at this early stage could then continue to develop apparently independently of further nervous influence.

The lateral line nerve can, however, exert a trophic influence on the lateral line organs, the evidence varying depending upon the species studied. Lateral line organs in the catfish show signs of degeneration by four days after section of the nerve, and complete disappearance of the receptors by sixteen days (Brockelbank, 1925; Szamier and Bennett, 1973). In contrast, the lateral line receptors of amphibians survive for prolonged periods of time after section of the lateral line nerve; Speidel (1948) observed single organs still present up to nine months after denervation. In other cases, for example, the newt, degeneration of the receptors began about two months after lateral line nerve section; this process could be accelerated when the surrounding area was also denervated by cutting the cutaneous nerves supplying that area (Jones and Singer, 1969), suggesting the existence of a nervous influence from the surrounding cutaneous sensory nerves in addition to those normally innervating the receptors.

(d) Merkel cell-neurite complexes:

The final example, the innervation of the Merkel cells in the skin, does not fit easily into the above mentioned categories. The influence of the nerve upon the development and maintenance of the

Merkel cell is not well understood, mainly because of the great deal of variation in the experimental results obtained from different species.

For example, the results of mammalian studies indicate that cells termed 'presumptive Merkel cells' (identified by the presence of the characteristic dense-cored granules) appear in the epidermis prior to nerve contact. Nerves, however, are seen at this time in the dermis directly below the location of the presumptive Merkel cells. In fetal sheep (Lyne and Hollis, 1971) and rats (English et al., 1980), nerves were located in the dermal layers, some micrometers below the epidermis (English et al., 1980), when the presumptive Merkel cells were first observed in the epidermis. Merkel cells have been seen in the vibrissae of mice at fourteen to fifteen days of gestation, prior to the appearance of neurites associated with these cells at day seventeen (Call and Bell, 1979). However, nerves are present in the dermis prior to the appearance of the Merkel cells (Van Exan and Hardy, 1980), and those could play some role in the final differentiation of the Merkel cells.

It is not clear from these results whether the nerve influences the differentiation of the Merkel cells for example, by the release of diffusible agents acting at a distance. The experiment of denervating the skin at a very early fetal stage (prior to day sixteen in rats) to see if the Merkel cells would still develop in the absence of nerves has not yet been done.

Sensory nerves seem to be required for Merkel cell development in birds. For example, it has been observed that avian Merkel cells,

which occur in the dermis, appear several days after nerve fibres have ramified throughout the dermis (Saxod, 1978, 1980; Ide and Munger, 1978), and the Merkel cells were always seen in association with the nerves. Saxod (1978, 1980) carried out experiments using chickens and quail, in which various combinations of tissue and nerve grafts were examined; he demonstrated that the Merkel cells would not develop in the absence of nerves. Furthermore, he observed that only sensory nerves were capable of inducing the development of the Merkel cells.

In frog tadpoles, nerve fibres appear to be associated with the development of Merkel cells. These fibres were already present in the epidermis when presumptive Merkel cells were first seen in the developing labial ridges, and in some cases had already contacted these presumptive cells (the cells were still referred to as presumptive at this stage because they did not yet seem to have the full complement of dense-cored granules) (Tachibana, 1979).

However, in the salamander Merkel cells can certainly develop in the absence of innervation, as shown by Tweedle (1978) for aneurogenic larvae and by Scott et al (1981) for skin regenerating in nerve-free adult salamander limbs. The results of this thesis indicate that the same is also true of the Merkel cells in Xenopus frogs.

There are conflicting reports regarding the effects of denervation on Merkel cells. In the salamander (Scott et al., 1981) and Xenopus (this thesis), Merkel cells survive denervation, for as long as six to seven months. On the other hand, several studies have provided evidence for the degeneration and loss of Merkel cells

following denervation. For example, in opossum snout skin (Palmer, 1965) and cat touch domes (Brown and Iggo, 1963; Burgess et al., 1974; English, 1977), Merkel cell atrophy accompanied denervation. However, in rat touch domes (Smith, 1967) and vibrissae (Benkenstein, 1979), and in the glabrous skin of the cat nose (Hartschuh and Weihe, 1977) atrophic changes were minimal or absent and Merkel cells appeared to survive for several months after denervation. In contrary reports Merkel cells of the cat vibrissae have been reported to survive denervation on the one hand (Hartschuh and Weihe, 1979) and to degenerate following nerve section (Chelysev and Vinter., 1983) on the other.

A recent quantitative study throws light on the question. Nurse et al (1983a) demonstrated that denervation of rat touch domes results in a loss of about 60% of the Merkel cells present at the time of denervation. In order to account for the prolonged survival of some Merkel cells in the same dome, the suggestion was made that the surviving cells represent a stable sub-population, while the rest represent a labile population which is sensitive to denervation (Nurse et al., 1983a). Two populations of Merkel cells, some with and the others without nerve termination appear to occur in both the touch dome (English, 1977; Nurse et al., 1983a) and the vibrissae (Suzuki et al., 1979; Gottschaldt and Vahle-Hinz, 1981; Gottschaldt and Kyau, 1982).

The presence of both innervated and non-innervated Merkel cells in touch domes and vibrissae, and the apparent difference in lability of sub-populations of Merkel cells to denervation, could account for

the variability in the reported results regarding the influence of the nerve upon the development and continued survival of Merkel cells.

2. Merkel cells as targets:

It has been proposed that the Merkel cells in salamander skin act as targets for growing cutaneous mechanosensory nerves (Diamond, 1979; Scott et al., 1981; Diamond, 1982). If they are targets, then appropriate information must be expressed by the Merkel cell for eventual recognition by the nerves. This could be in the form of specific molecules, either on the Merkel cell surface, or released from the cell into the immediate environment. One line of evidence for this proposal comes from experiments in which new skin regenerated in place of a portion excised from a denervated salamander hind limb; the skin that regenerated eventually contained the normal number and distribution of Merkel cells. When sensory nerves were allowed to grow into the regenerated skin, normal mechanosensory function was established, and the Merkel cells were found on EM examination to be innervated. The location of the Merkel cells was significantly correlated with that of the physiologically defined individual touch spots. Because the new skin had regenerated in the absence of nerves, the regenerating nerve fibres could not have been following Schwann tubes (in the new skin) back to the Merkel cells (Scott et al., 1981), as has been suggested for the reinnervation of cat touch domes (Burgess et al., 1974; Horch, 1979; but see Horch, 1982).

Another line of evidence for a target role of Merkel cells

comes from studies of partially denervated skin (Diamond et al., 1976; Cooper et al., 1977). It was found that the denervated skin was reinnervated by sprouting of the adjacent intact nerves and that the density of touch spots was restored to control values; sprouting then ceased, functionally at least. Since, in a later study (Scott et al., 1981) denervated Merkel cells survived, they could act as targets for the nerve sprouts. Moreover, once innervated these Merkel cells seemed to lose their target character, and their distribution appeared to regulate the number of functional nerve endings in the skin. The findings of the present investigation (see Results) are consistent with the Merkel cell acting as a target in Xenopus too.

It is tempting to speculate that the dense-cored granules, which are a characteristic feature of the Merkel cells, may be involved in these trophic interactions with the nerves (e.g., Munger, 1977; Diamond, 1979; Zahm and Munger, 1983). Alternatively, they may contain a biogenic amine or peptide involved in mechanosensory function. As regards the latter, attempts to demonstrate the more common biogenic amines (e.g., dopamine, norepinephrine and serotonin) in these cells have failed (Smith, 1967; Iggo and Muir, 1969; Crowe and Whitear, 1978; Hartschuh and Weihe, 1979). Other granule-containing cells which are known to contain these monoamines or the less common ones that do not fluoresce with the conventional Falck-Hillarp technique (e.g., octopamine) can be selectively stained with neutral red (Stuart et al., 1974); preliminary results from this laboratory indicate that the Merkel cells of the salamander and rat may also selectively accumulate

this dye, raising the possibility that an unidentified amine may yet be present in the Merkel cell (C. Nurse, personal communication). Met-enkephalin-like immunoreactivity has been reported to be associated with the granules in rodent Merkel cells (Hartschuh et al., 1979), but not of other mammals (Hartschuh et al., 1983); the Merkel cells of other mammalian species have been recently shown to exhibit vasoactive intestinal peptide (VIP)-like immunoreactivity (Hartschuh et al., 1983). Quinacrine, which accumulates in a variety of neurosecretory cells, also accumulates in the Merkel cells (Crowe and Whitear, 1978; Nurse et al., 1983b). This dye is known to react with purines (Irvin and Irvin, 1954), and apparently binds to a component of the dense-cored granules in the cells it labels (Alund and Olson, 1979). The accumulation of quinacrine in the Merkel cells suggests that the granules may contain a purinergic compound, such as ATP or adenosine. These substances have not yet been tested to determine whether they play any role in trophic interactions between nerve fibres and their targets. Evidence to corroborate that the Merkel cell granules are indeed important in trophic interactions must await purification and identification of the granule contents. On the other hand, the granules might be involved in the modulation of the physiological functions of this complex, i.e., mechanosensation (see Final Discussion).

Section III

Rationale and General Strategy

The principal aim of this study was to investigate the contribution of the Merkel cell to mechanosensory function in Xenopus skin. This investigation was prompted in part by results obtained in studies of cutaneous nerve regeneration and sprouting in the salamander, and also by the great variability observed in nerve-Merkel cell interactions in other species.

The Merkel cell-neurite complex is generally thought to be involved in mechanosensory transduction, although there is no direct evidence for this. One puzzling feature of this complex is that it is associated with a slowly-adapting response in mammals and a rapidly-adapting response in birds and amphibians. The question that arises is, what could be the role of the Merkel cells in these responses, and in particular, do the Merkel cells function as sensory transducers? These cells could, for example, have some 'feedback' function, modifying the response to mechanical stimulation of the nerve ending, itself the basic transducer. This is an especially interesting possibility in the amphibian system where a reciprocal synaptic relationship between the Merkel cell and nerve ending seems likely.

The Merkel cells act as targets for growing nerves in the salamander; whether they do so in other species is not clear. As

targets their role might be primarily to distribute the sensory nerve endings appropriately (although this leaves the problem of how the Merkel cells are distributed in the first place).

Another possibility is that they may act to promote the maturation of growing cutaneous nerve endings into their characteristic mechanosensitive state.

The present investigation was undertaken, therefore, in an attempt to clarify the role of the Merkel cell, using Xenopus laevis for the study. Xenopus has a distinct advantage over the salamander (which was the subject of previous investigations from this laboratory) in that the location of the Merkel cells is known to be in close relation to the easily visible (with a dissecting microscope) gland openings; this special relationship does not exist in the salamander where the distribution of Merkel cells is apparently random and there are no obvious morphological cues as to their location. Of special importance, a new in vivo marker for the epidermal Merkel cells is now available, quinacrine, which can be recognized in the light (fluorescent) microscope. These features allow for a precise and quantitative investigation of the Merkel cell-neurite complex, both physiologically and morphologically, in a way not previously possible. It was, however, first necessary to determine the physiological characteristics of this complex in Xenopus, and this was especially important given the species variation mentioned previously. Having done this, the approach was to denervate one hind limb totally and allow one of the nerves supplying the limb to regenerate; the

contralateral limb was used as the control. The recovery of cutaneous mechanosensitivity was then followed physiologically, and its relationship to the Merkel cell population originally present was determined. In addition, the appearance of Merkel cells and the development of mechanosensitivity in regenerating skin was also studied in a similar way.

The final stage of this study (work still in progress) was to begin to correlate specific stages of the physiological recovery with ultrastructural features of the Merkel cell-neurite complex.

Section IV

Specific Aims

The specific aims of the study were to answer the following questions:

I. Are the epidermal Merkel cells (with their nerves) related to the mechanosensitivity of the skin, and if so, what is the physiological character of this sensitivity?

II. Do Merkel cells act as targets for cutaneous nerves?

a) Will Merkel cells survive denervation, and if so will they become reinnervated by regenerating nerves, and will the Merkel cell-neurite complexes develop normal mechanosensory function? b) Will Merkel cells differentiate in regenerated skin, and if so is this process nerve-dependent? c) If Merkel cells do differentiate in regenerated skin, will they become innervated, and will the Merkel cell-neurite complex develop mechanosensory function?

The answers to these questions give information regarding the possible role of Merkel cells in regulating the distribution of cutaneous mechanosensory nerve endings. In addition, the following questions were asked in an attempt to determine the role of the Merkel cells in the processing of mechanosensory information.

III. Does the disappearance of mechanosensory function that must occur after nerve section correlate with specific

alterations in the fine structure of the Merkel cell-neurite complex?

IV. Does the recovery of mechanosensitivity following nerve regeneration to denervated or newly-regenerated skin require that nerve endings contact the Merkel cells?

V. If so, are there stages of that recovery that can be correlated with detectable fine structural features of the Merkel cell-neurite complex?

Section V

Materials and Methods

1. Animals

All animals used in this study were male or female adult Xenopus laevis, obtained from Carolina Biological Supply Company (Burlington, North Carolina). The animals usually weighed 15-20 g and the nose-rump length ranged from 4.5-6.0 cm. They were housed at room temperature (20°C) in groups of 6-12 in large tanks filled with aged water and aquatic plants. The animals were fed with mealworms biweekly, and the tanks were also cleaned biweekly.

2. Anaesthesia

All surgical procedures were performed on animals anaesthetized by immersion in a 0.3% solution of MS-222 (ethyl-m-aminobenzoate methane sulfonate, Sandoz) for 15-30 minutes. At this time the animals showed no movement or reflex responses to mechanical stimulation such as lightly pinching the fore-limb. Normally, the animals remained in this state for 60-90 minutes. If an animal showed signs of recovery before recording procedures were completed a piece of tissue soaked in a 1.0% solution of MS-222 was placed over the head region; this was adequate to re-induce an appropriate depth of anaesthesia. Recovery from the anaesthetic occurred without any noticeable side-effects,

taking about 1/2- 1 hr.

3. Surgical procedure

In most experiments the right hind-limb of the animal was used as the experimental side and the left hind-limb served as the corresponding control. A few experiments were done with the reverse situation and served to confirm that no laterality of behaviour, anatomy or regenerative capacity was present in these animals.

(a) Nerve section or crush

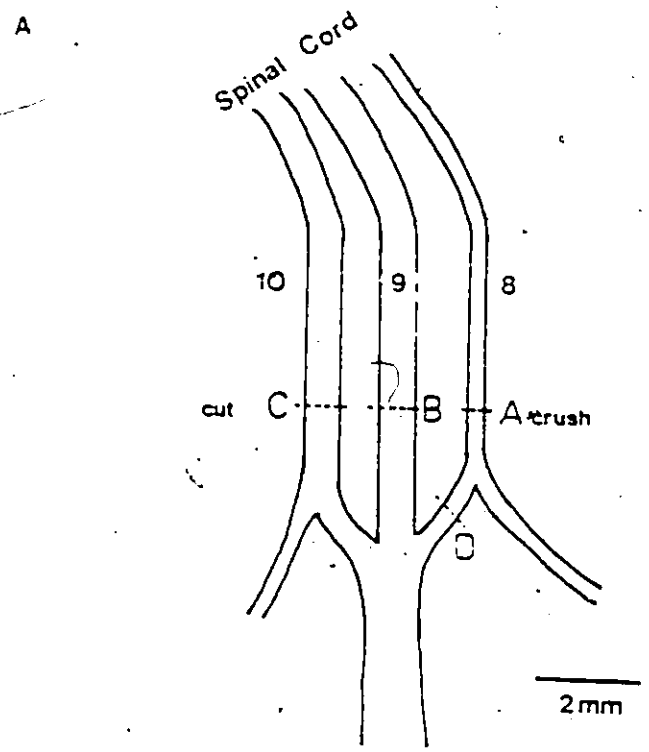
First a longitudinal incision of 1-1.5 cm was made to the right (or left) of the dorsal midline, just caudal to the right (left) ilium. Using blunt dissection, a trough was created in the underlying musculature of the area, until 3 major spinal nerves (8,9 and 10) constituting the lumbar plexus were exposed (Ariens Kappers et al., 1967). The nerves were then freed from the surrounding connective tissue. In the experiments where all the nerves were sectioned, each was cut about 5-10 mm above the point where the plexus begins (see Fig. 1). When nerve regeneration was not required, both the proximal and distal nerve stumps were tied off with 6-0 silk thread and buried in the overlying musculature.

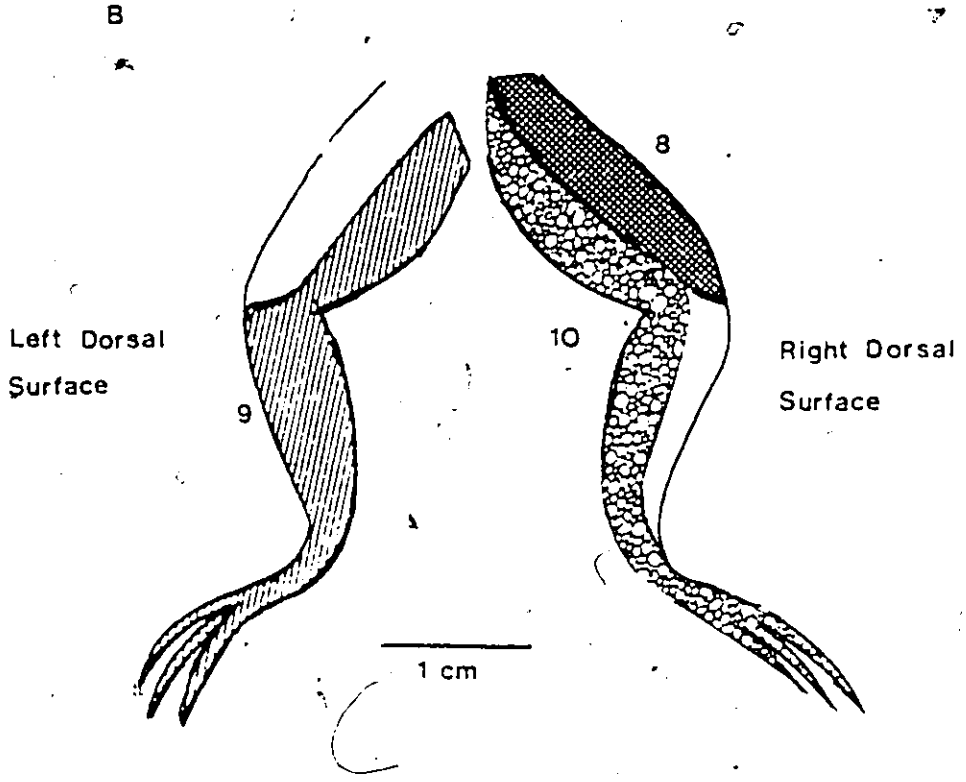
In the experiments where nerve regeneration was to be studied, 2 of the 3 nerves (usually 9 and 10) were sectioned above the plexus and tied off as above. The 3rd nerve (8) was then crushed, using flat-ground watchmaker's forceps, about 1 mm above the point where it

Fig. 1. Diagram of the lumbar plexus and nerve fields.

(A) is a schematic diagram of the lumbar plexus (nerves 8,9 and 10) showing the points where nerves 9 and 10, and a branch of 8, were sectioned (points B,C and D), and where nerve 8 was crushed (A). In the experiments where all three nerves were sectioned, nerve 8 was cut at point A.

(B) presents the fields of innervation of nerves 8,9 and 10. The fields of nerves 8 and 10 are shown on the right hind limb, and that of nerve 9 is shown of the left hind limb; they are shown separately since nerves 9 and 10 overlap extensively. Nerve 8 displayed the most constant field of innervation.


A
Spinal Cord
10
9
8
cut
C
B
A crush
D
2mm


B
Left Dorsal Surface
Right Dorsal Surface
8
9
10
1 cm

joins the plexus (see Fig. 1); a 1-2 mm length of the nerve bundle was compressed 3 times, each compression lasting 4-5 s. Immediate and persistent (several days) failure of impulse conduction through the crushed region verified the effectiveness of this procedure.

The incision was closed with silk sutures and the animals allowed to recover in individual tanks; the animals were swimming within 1/2 hr after the surgery.

(b) Dissection for nerve recording

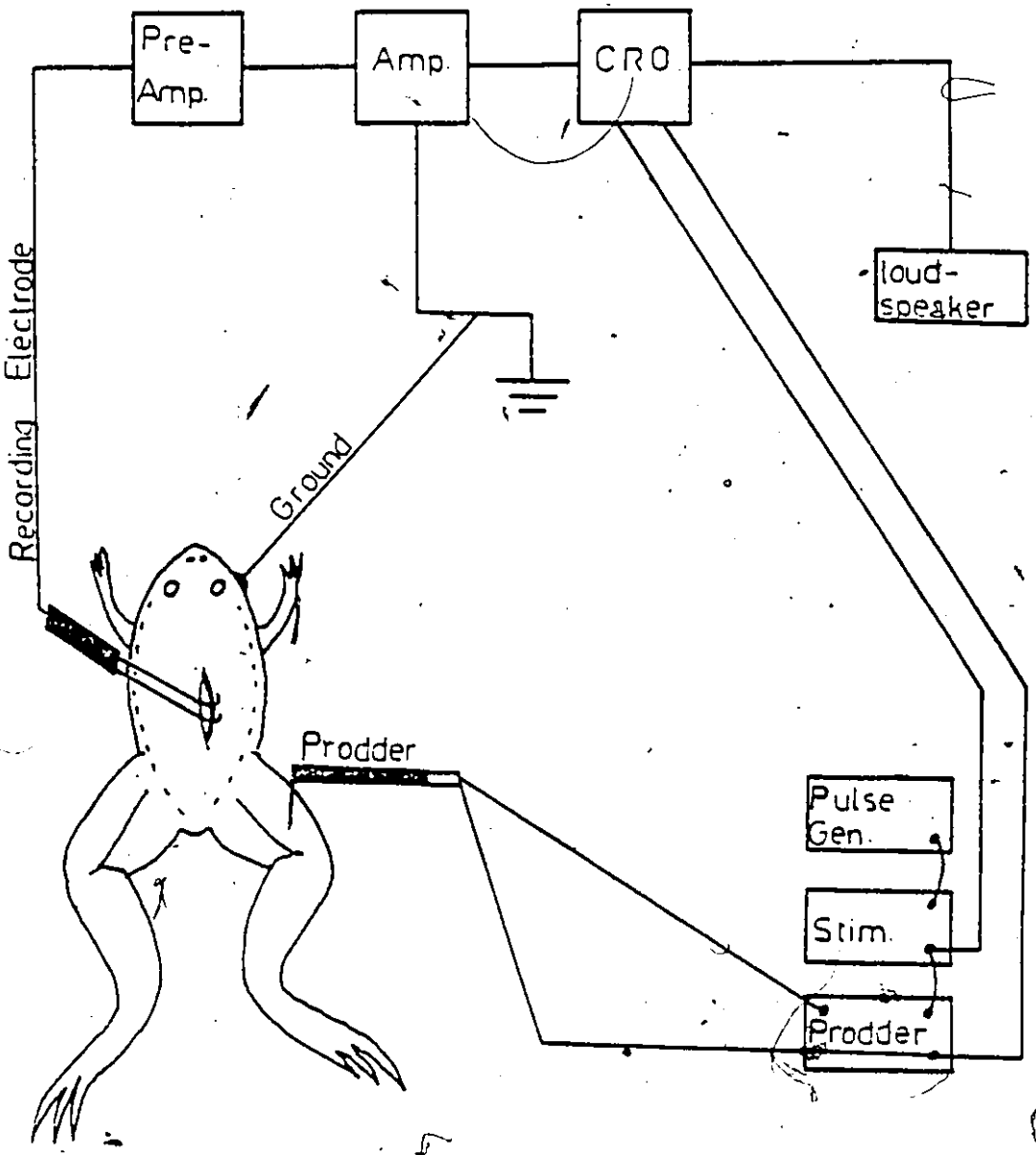
The animals were anaesthetized as described above. The dissection used to free the nerves for recording "in continuity" (see below) was similar to that described for nerve section or crush. However, more care was taken in freeing the nerves from the surrounding connective tissue, so as to minimize the possibility of inadvertently damaging the nerves; a 1 cm length of nerve was usually freed and used for recording purposes.

4. Recording techniques

Nerve impulses were recorded extracellularly from the spinal nerve trunk with fine platinum electrodes. Recording was usually done "in continuity"; for this the nerves were lifted onto the electrodes with a polished glass probe and then lifted away from the body cavity by the electrodes. Care was taken not to stretch the nerve during this procedure. To prevent drying, the nerve was coated with aerated light mineral oil and the raised portion on the electrodes was additionally

Fig. 2. Schematic diagram of the equipment used in the mapping of the mechanosensitive fields.

For mapping of gross nerve fields, the skin was stroked with a brush, and the evoked action potentials, recorded extracellularly with platinum wire electrodes, were amplified through the preamplifier and amplifier, and displayed on a cathode ray oscilloscope (CRO). A mechanical stimulator ("prodder") was used in the investigation of individual touch receptors; an electrical voltage pulse was applied to the prodder piezo-electric crystal by way of the pulse generator (Pulse Gen.) and stimulator (Stim.). The output from the prodder photocell (see Fig. 3) was displayed as a voltage deflection on the oscilloscope. The apparatus and preparation were grounded to a common point.


Pre-
Amp.
Amp.
CRO
loud-
speaker
Recording Electrode
Ground
Prodder
Pulse
Gen.
Stim.
Prodder

coated with petroleum jelly. Recording could be carried out for several hours under these conditions without any evident deterioration of the nerve.

In one set of experiments, recording was carried out to determine the time course of the loss of mechanosensitivity; this was done using the cut distal portion of the nerve, still attached to the skin. The nerve stump was lifted onto the electrodes and covered with petroleum jelly, which also served to hold the nerve stump in place.

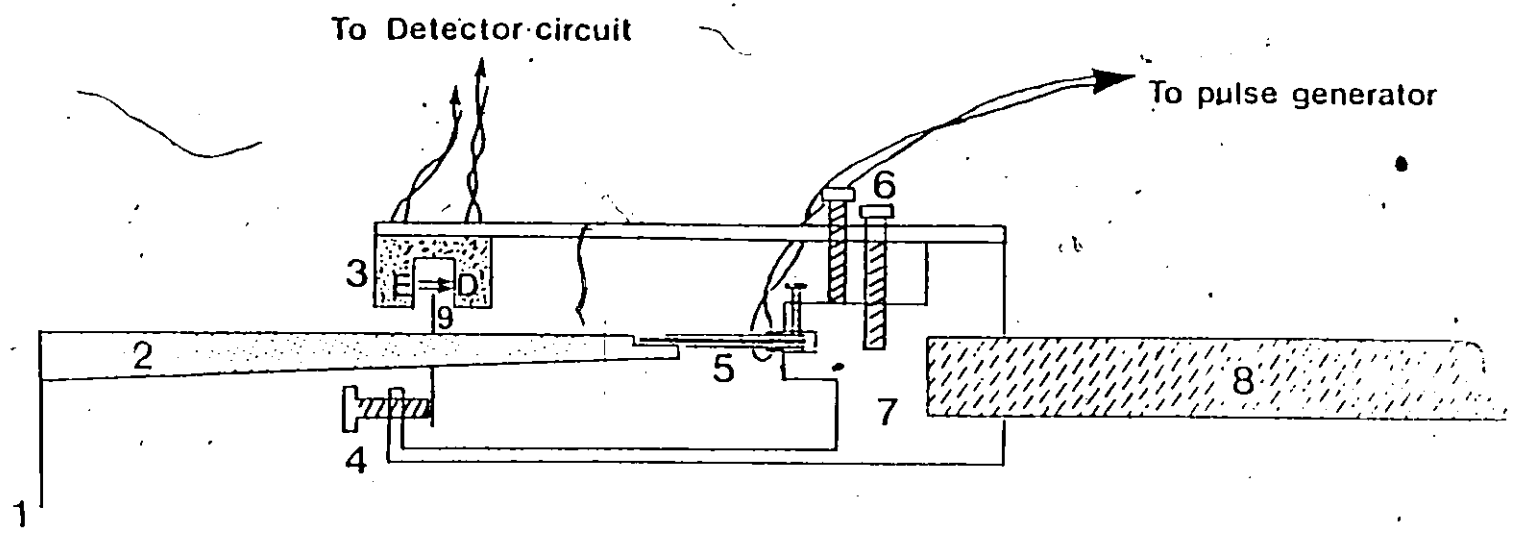
Signals from the recording electrodes were amplified using a Grass P15 preamplifier in line with a Tektronics 5A18N dual-trace amplifier, displayed on a Tektronics D-13 dual-beam storage oscilloscope. Impulse activity was also monitored as an audible signal, by relaying the oscilloscope output through a loudspeaker. The oscilloscope was triggered by a Devices Digitimer (type 3290) pulse generator. The apparatus and the animal were connected to a common ground. Fig. 2 represents a schematic diagram of the recording set-up.

5. Skin stimulation

Gross estimates of an area of innervation or reinnervation were obtained by lightly brushing the skin with a fine bristle. Once the extent of the nerve field being studied was determined, quantitative studies of the innervation were carried out using a voltage-controlled mechanical stimulator ("prodder") (Fig. 3). The major component of this stimulator was a piezo-electric crystal which is fixed at one end to an acrylic block. When a voltage is applied across the crystal, the

Fig. 3. A drawing of the mechanical stimulator.

A voltage was applied to the piezo-electric crystal (5); the vibration of the crystal was amplified by the aluminum extension bar (2) to which a tungsten wire tip (1) was attached. The deflection of the crystal caused a deflection of the arm and tip (which sits on the skin surface), which thus results in stimulation of a small region (determined by the diameter of the tip) on the skin. With the aid of a photocell (3) which was connected to the detector circuit, and then to the oscilloscope, prodder deflections were measured as voltage deflections; by moving the prodder a measured amount (vertically) and recording the voltage output, a calibration curve was constructed. The stimulator was attached to a support rod (8) which was held in a micormanipulator.



1 TIP

2 ALUMINUM ARM

3 OPTO-ELECTRONIC DETECTOR

4 VISCOUS DAMPING SCREW

5 PIEZO-ELECTRIC ELEMENT

6 RANGE ADJUSTING SCREWS

7 ACRYLIC BLOCK

8 SUPPORT ROD

9 VANE

crystal bends and the free end deflects. This deflection is magnified by an aluminum extension arm glued on to the free end of the crystal. Prodder tips made of tungsten wire electrolytically etched to the desired tip diameter were attached to the free end of the aluminum bar.

Monophasic square wave voltages were applied to the prodder from a Devices Digitimer 3290 pulse generator and isolated stimulator, which also triggered the oscilloscope (see Fig. 2). Alterations in the amplitude of the applied voltage were used to alter the rate of movement of the prodder tip, as shown in Fig. 4. Larger voltages (with the same pulse width) caused a larger displacement of the prodder tip, though the time-to-peak remained the same; this produced a faster rate of displacement of the prodder tip in a given time period. Ranges of velocity that were used varied from 0-30 um/ms, in increments of 0.1-0.2 μm/ms.

The actual displacement of the prodder tip was measured with the aid of a standard photocell. The aluminum arm of the prodder has a small metal 'flag' which projects between the 'Emitter' and 'Detector' components of the photocell. Movement of the prodder arm allows light to hit the photocell; this is seen on the oscilloscope as a voltage deflection. The prodder can be calibrated by applying a known deflection and determining the output of the photocell in volts (Fig. 5).

6. Mapping technique

The term "mapping" refers to the procedure in which a

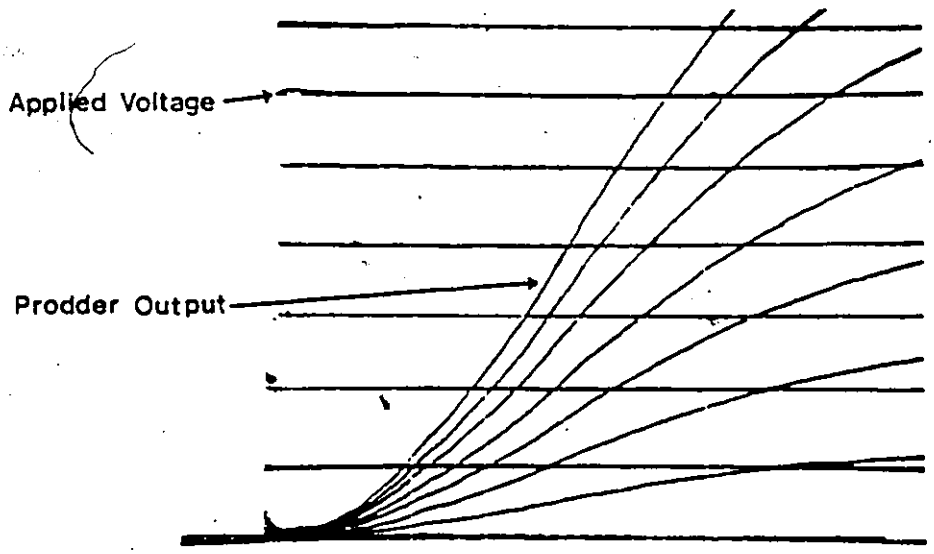
Fig. 4. Output response of the prodder.

The output response of the prodder photocell is shown for different amplitudes of input voltages to the piezo-electric element.

The input voltage is shown as the horizontal traces in (A) and the short horizontal pulses in (B). The length of the applied pulse in (A) was 20 ms and in (B) the pulse width was 2ms. The photocell output (the rising traces in each figure) increased with larger applied voltages, and thus a faster rate of displacement of the prodder tip occurred. The lowest applied voltage trace corresponds to the lowest prodder output trace, and so on.

The vertical scale is 5 V for the applied voltage traces, and 10 mV for the photocell output. The horizontal scale is 1 ms.

A


Applied Voltage
Prodder Output

B

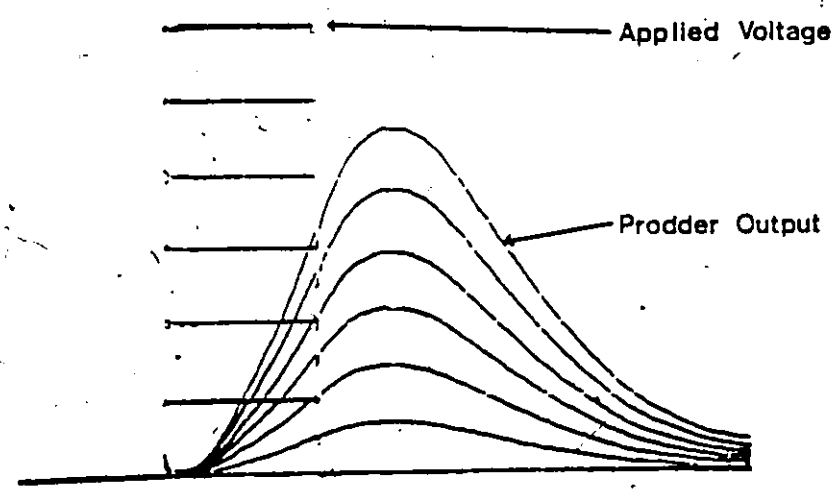
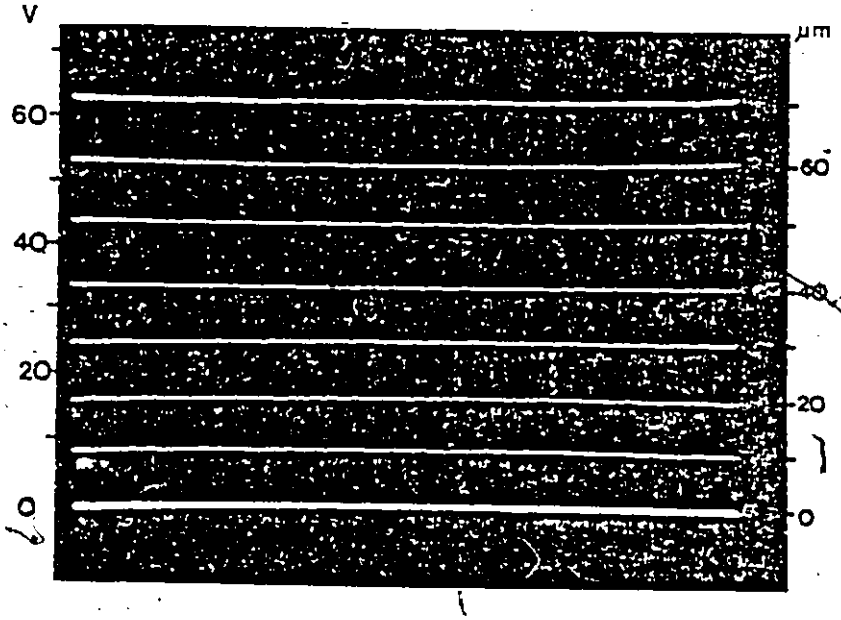

Applied Voltage
Prodder Output

Fig. 5. Method of calibration of the mechanical stimulator.

A. The prodder was displaced downwards in 10 μm steps (using a micromanipulator and a flat probe which was placed on the upper surface of the prodder tip), and the voltage output recorded; the voltage output recorded for each 10 μm step is displayed as subsequent traces in the figure, with 0 μm displacement showing no voltage deflection.

B. The voltage output was then plotted against the forced displacement (in μm); calibration curves constructed in this manner were then used in the experimental situations to determine the displacement of the prodder tip for the recorded photocell output. The displacements so determined were then expressed as the rate of rise (μm/ms).

A


V
60
40
20
0
μm
60
40
20
0

B

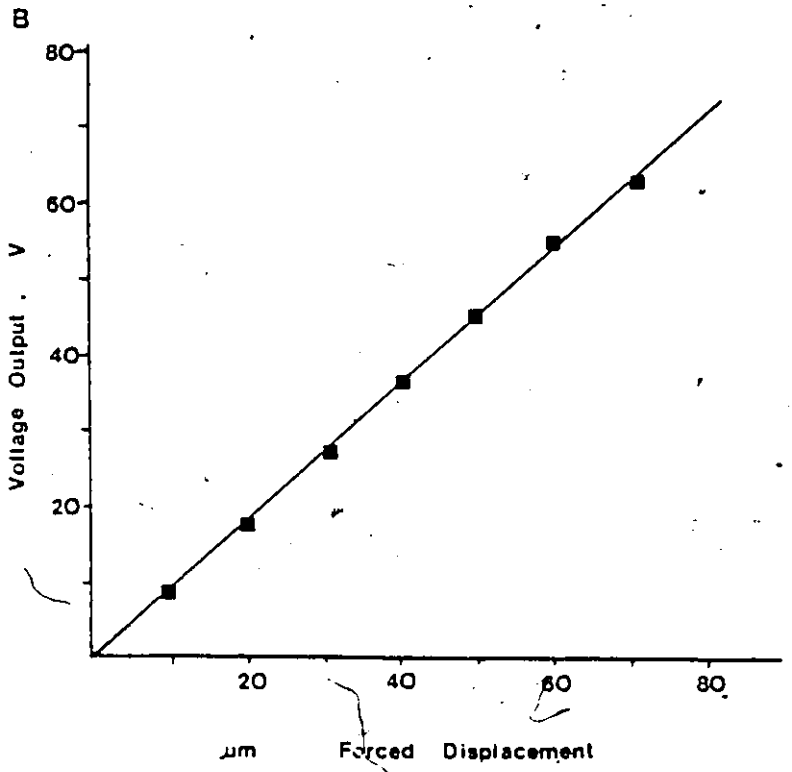

80
60
40
20
Voltage Output , V
20
40
60
80
μm Forced Displacement

Fig. 6. Electronmicrograph of collagenase treated skin.

In a number of experiments it was necessary to split the epidermis from the dermis; this was done by incubating pieces of skin in 0.1 mg/ml collagenase (in a balanced salt solution) for 30 minutes, and then mechanically peeling the epidermis from the dermis.

The electron micrograph shows a sample of skin that was split, but fixed with the epidermis overlaying the dermis; splitting generally occurred at the level of the basement membrane (bm). The epidermis (E) is to the top left of the figure, and the dermis (D) is to the bottom right.

Calibration bar - 4 μm


E
D
bm

certain small area of skin was physiologically examined to determine the distribution of low-threshold touch-sensitive spots. This was done by moving the prodder in 90 μm steps, with the aid of a micromanipulator and a square grid (14 sq x 14 sq) graticule set into one of the eyepieces of the dissecting microscope; at the magnification used (99x) each square was equivalent to 90 μm x 90 μm. Systematic mapping was done by recording the critical stimuli required to evoke an action potential, when the prodder tip was applied to the skin at constant (90 μm) intervals across the grid; in most cases 50-100 grid squares were examined.

7. Histological techniques

(a) Fluorescent identification of Merkel cells

Quinacrine dihydrochloride, a fluorescent dye, was used to visualize Merkel cells in fresh (unfixed) whole mounts of skin. In most cases, animals received an injection, into the dorsal lymph sac, of 10-15 mg/kg body weight of quinacrine dihydrochloride (BDH Chemicals Ltd.) dissolved in distilled water. In some instances, pieces of already excised skin were incubated in 10^{-5}M quinacrine in buffered amphibian Ringers solution (NaCl 111.0 mM, KCl 1.8 mM, glucose 10.0 mM, Hepes 10.0 mM, pH 7.4) for 5-10 minutes, and then washed with fresh Ringers solution for 1 hr at 4^{o}C.

Several hours after the quinacrine injection, the animals were anaesthetized in MS-222, and small pieces of skin (1-2 mm^2) were removed from the dorsal aspect of the hind limb.

When the background fluorescence from the blood vessels and glands in the dermis obscured visualization of the Merkel cells in the epidermis, it was necessary to separate the epidermis from the dermis mechanically after enzymatic treatment. The skin pieces were incubated for 1 hr at 4°C in a 1 mg/ml solution of collagenase (Gibco Laboratories). After incubation in the enzyme, the samples were washed in fresh 70% Hanks BSS, and, using fine-tipped forceps, the epidermis was peeled away from the dermis. EM examination of skin treated in this manner showed that the splitting generally occurred at or below the level of the basement membrane (Fig. 6).

The excised pieces of whole skin or epidermis were mounted epidermal side up in liquid paraffin on glass slides and then cover-slipped. These were examined for the presence of fluorescent cells using a Zeiss photomicroscope equipped with an epi-fluorescence condenser (III RS); the filters used were exciter BP 436/5, barrier LP 478 and beam splitter 460.

(b) Electron microscopy

Small areas (1-1.5 mm^2) of skin (which in most cases had been previously physiologically mapped) were excised and immediately immersed in cold fixative. The fixative consisted of 2.5% glutaraldehyde, 2% paraformaldehyde, 2% DMSO (dimethyl sulfoxide), in 0.1 M phosphate buffer, pH 7.4. The pieces were trimmed with a fresh scalpel blade, and allowed to remain in the fixative for 4 hrs to overnight at 4°C.

Samples were then washed with 0.1 M phosphate buffer and post-fixed in an aqueous solution of 2% OsO_4, which also contained 1% $K_2Cr_2O_7$ to prevent oxidation of the OsO_4. Following a wash with 0.1 M acetate buffer (pH 5.0), the samples were stained en bloc with 1% aqueous uranyl acetate. Dehydration was then carried out in a graded series of acetone solutions, and the tissues were embedded in Spurr's epoxy resin.

Ultramicrotomy was carried out using a Cambridge Huxley ultramicrotome. Semithin sections were cut either perpendicular or horizontal (en face) to the skin surface and stained with a 1% solution of toluidine blue. Because the Merkel cells are normally located near the gland ducts, semithin sections were cut until at least one gland duct was observed in the section, and then ultrathin sections were cut. This approach usually ensured that there was at least one Merkel cell in the sections to be examined. Usually 1 or 2 grids were produced, each with 5-10 section. Thin-sectioned regions were, generally, separated by 2-4 μm of semithin sections. However, serial sectioning was done in several instances to allow the examination of the extent of innervation on normally-innervated Merkel cells.

The grids were stained for 3-5 min with Reynold's lead citrate and then examined in Philips EM 300 or 301 electron microscope.

(c) Light microscopy

Pieces of freshly-excised skin were flattened on a cover-slip and fixed by immersion into chilled Bouin's solution for at least 4

hrs. Following fixation, samples were thoroughly washed in 70% ethanol and then dehydrated in alcohol and cleared in methyl benzoate overnight. For embedding, the skin pieces were placed in xylene for 1/2 hr and then 3 changes of Paraplast (Polysciences) for 1 hr each; the samples were oriented in the blocks and the paraffin allowed to harden. Serial sections (7 µm thick) were cut using an AO microtome; the sections were mounted on albumen-coated slides and allowed to dry overnight. Sections were stained using a modified Ungewitter's silver stain (Ungewitter, 1951; A. Foerster, personal communication). The slides were then coverslipped and examined with a Zeiss photomicroscope.

Section VI

Results and Discussion

Part I. Physiological characteristics of Xenopus cutaneous mechanosensitivity:

1. Nerve fields:

The hind limb of Xenopus is supplied by the three spinal nerves, 8, 9 and 10, which combine to form the lumbar plexus. Most of the experiments to be described involved nerve 8 and the area of skin that it innervates. The peripheral field of innervation of each of the spinal nerves was defined by recording the nerve impulses from the nerve trunks extracellularly and mapping the responsive area with a fine bristle while successively cutting all other branches of the lumbar plexus. An example of an extracellularly recorded response to brushing the skin is presented in Fig. 7. The fields of innervation for each of the spinal nerves are shown in Fig. 8. The 8th nerve innervates the lateral dorsal and ventral portions of the thigh. An advantage to using nerve 8 is that the degree of resolution for the extracellular nerve recordings, the signal to noise ratio, is better in this nerve than in the normally larger nerves 9 and 10. In all cases, the degree of resolution was always adequate to resolve single action potentials (see below).

Fig. 7. Response to brushing the skin.

This is an example of extracellularly recorded impulses in the 8th nerve, in response to brushing the skin. During the period indicated by the bar, a bristle was stroked across the thigh skin in one sweep.

Vertical calibration; 20 μV
Horizontal calibration; 0.1 s

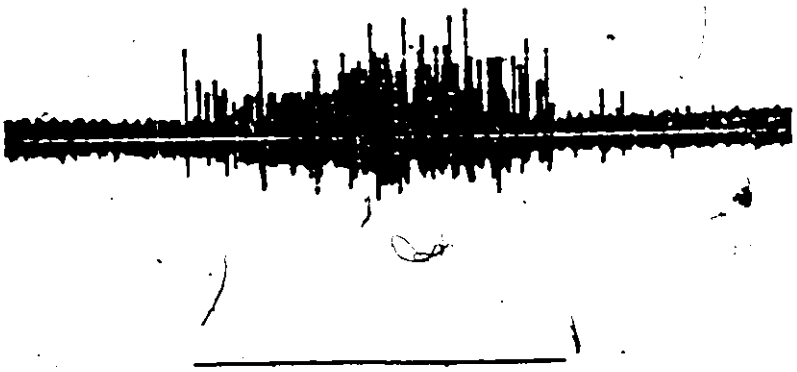
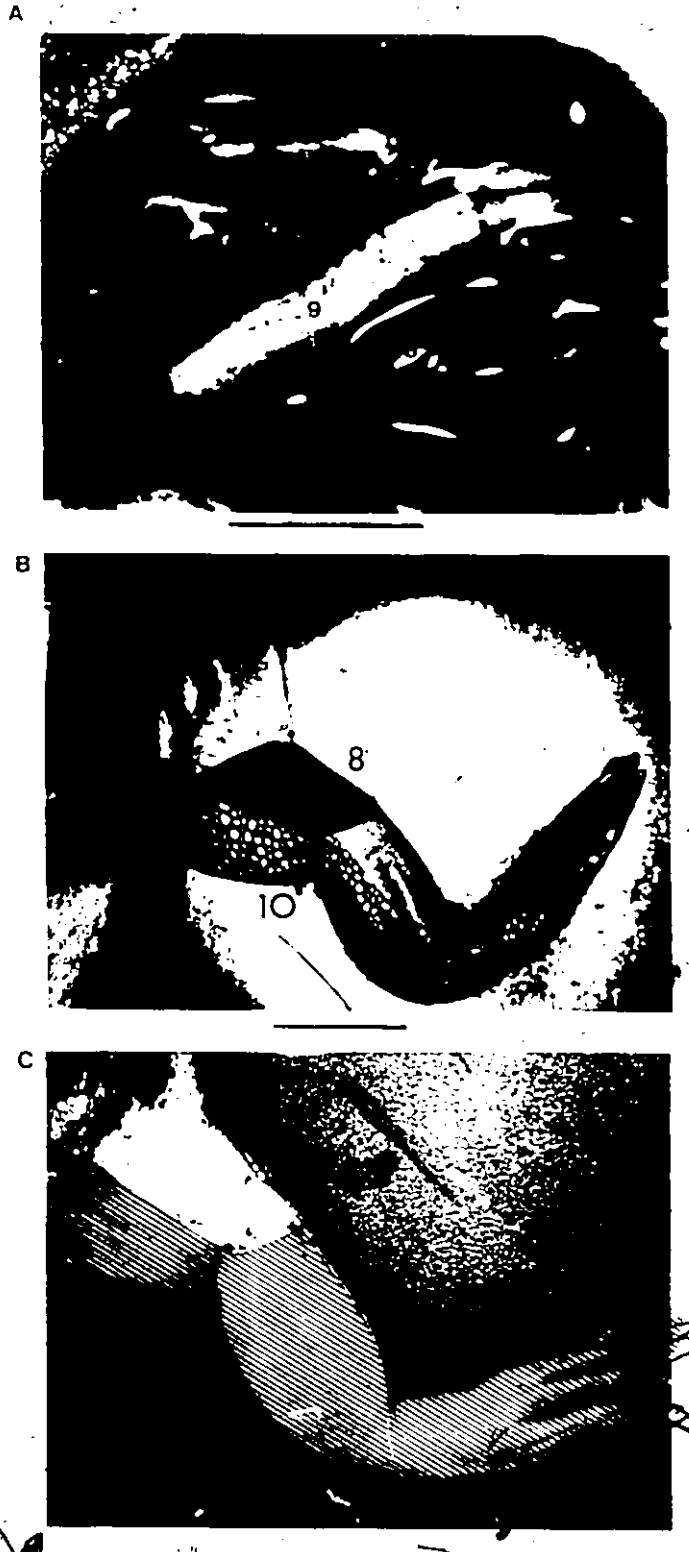


Fig. 8. The lumbar plexus and nerve fields.

(A) is a photograph of the right lumbar plexus in situ, showing nerves 8,9 and 10. The spinal cord is located to the top right of the photograph (but is not shown). The thigh is located to the lower left of the figure.
Calibration bar; 5 mm

(B) and (C) are photographs of the right hind limb showing the distribution on the dorsal aspect of the leg the low-threshold mechanosensory fields of nerves 8 and 10 (in B) and 9 (in C) (mapped as described in the text). The 9th and 10th mechanosensory fields are shown separately to reveal their overlap more clearly.

Calibration bar; 1 cm


A
9
B
8
10
C

2. Activation of single receptors:

In order to stimulate small punctate areas of skin accurately, a voltage-controlled mechanical stimulator ("prodder") was used (see Fig. 3 in the Methods), and the impulses evoked were recorded extracellularly from the nerve trunks. Initially, a 30-40 μm diameter prodder tip was employed in the mapping procedure (described below), but it was subsequently found that a tip of 10 μm diameter allowed a more precise localization of the stimulus.

The stimulation technique allows activation of individual axons. In a random survey of an area of skin, each point was stimulated with an intensity just large enough to evoke an action potential in response to every prod. These receptors are sensitive to the rate of rise of the stimulus and are thus classified as velocity detectors (see section 3). Then, the stimulus velocity was increased until a second, third, and even fourth stimulus-tied action potential was evoked, or the limit of the prodder was reached (this was approximately 20 μm/ms). The results of such a survey demonstrated (Appendix Table 1) that at every point tested 2 or 3 impulses could be excited; a fourth spike could be detected in about 50% of the points. It was found that almost always the second, third and fourth impulses could be shown to summate with the first one; in some cases summation occurred only with the third and fourth spikes. This indicates that the second, third and fourth spikes arose in axons separate from the first and were probably elicited by stimulus spread to adjacent receptors. It is possible, however, that one axon could serve more

than one receptor, in which case (if the receptor were adjacent to the one being stimulated) summation of impulses would not occur.

These results differed from the situation in the salamander where, using the same test, two axons could be excited in only 50% of the spots tested (Cooper, 1977). However, in the salamander, the 'touch spots' were shown to be about 150-200 μm apart, while in Xenopus, as will become apparent in later sections, the touch spots are more closely arranged, being about 50-100 μm apart; thus a greater proportion of the surrounding receptors might be activated at any given spot as compared with the salamander.

In any case, it was always possible to selectively excite a single axon with this technique, since the difference between the stimulus required to evoke the first and second action potentials was always greater than the resolution of the technique, which is approximately 0.13 μm/ms (see below). The critical stimulus was taken to be that which was just adequate to evoke one spike every time it was applied. Throughout this study the critical stimulus is also used to define "threshold". An increase beyond this stimulus was required to evoke the second spike, which for different positions of the prodder on the skin ranged from 0.6 to 10 times the critical stimulus. Only about 15% of second spikes were elicited with less than two times the initial critical stimulus; 60% of the second spikes required greater than 5 times this critical value (Appendix Table 1).

Several other tests indicate that the action potentials observed were single units and not compound action potentials from

small bundles of axons of similar thresholds. They were: i) the units were of approximately the same height; ii) a stimulus just below threshold could evoke a characteristic action potential of this height about once out of every three or four times it was applied; iii) at threshold, defined here as the smallest stimulus that would evoke an all-or-nothing spike each time it was applied, there was "jitter" (i.e., with repeated stimuli the spikes arose with slightly different latencies), but the individual spike amplitudes never varied (Fig. 9); iv) the receptors could follow suprathreshold stimuli at relatively high frequencies, e.g., 10 Hz. At these frequencies, every time the impulse appeared it had the same height and latency.

The conclusion that is drawn from these tests is that the technique allows individual axonal activation; that is, it seems likely that individual units are being activated by the mechanical stimulator. It does not, however, distinguish whether a single axon (or unit) branches to form more than one receptor termination.

3. Critical stimulus:

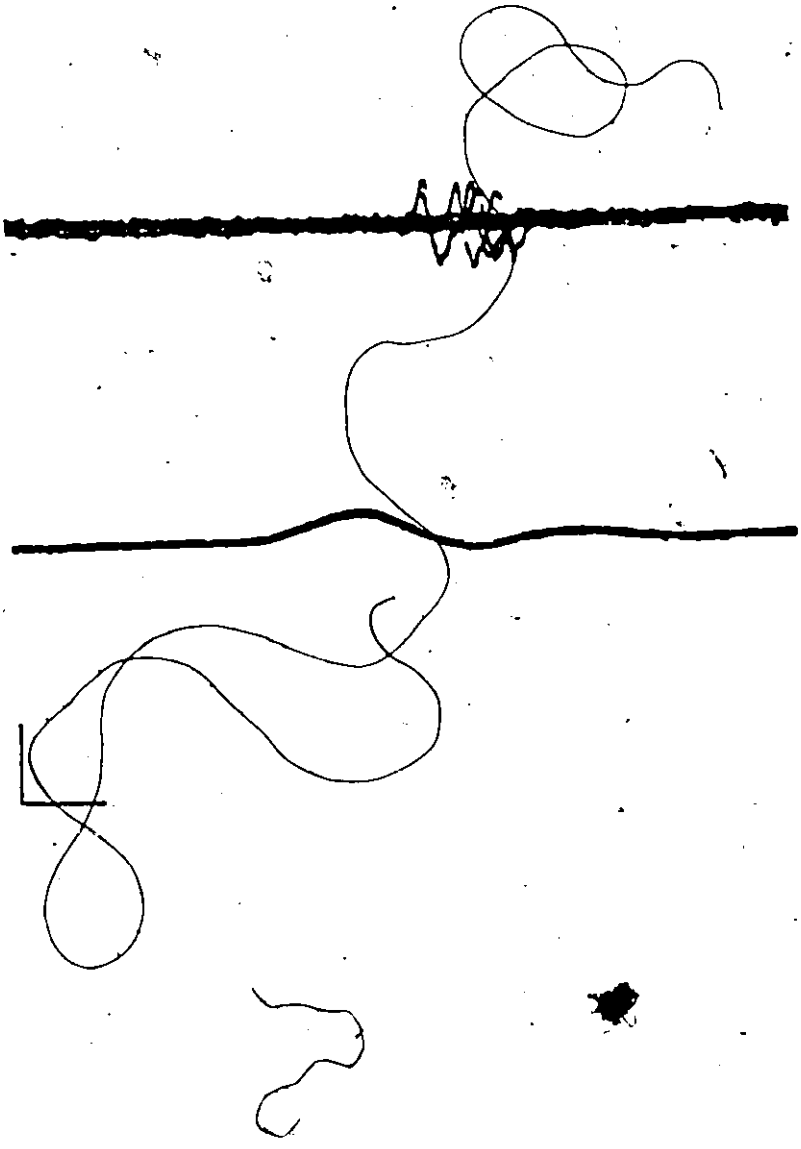
The critical stimulus, which here is taken to represent the threshold of excitation of a single sensory unit, has been defined above. The accuracy of measurement of this stimulus, in μm/ms, was assessed by making repeated measurements at the same point on the skin. The stimulator was placed at one spot so that it just touched the skin, and successive trials were made in which the applied voltage pulse was progressively increased until a spike was evoked each time out of five

Fig. 9. Variations in the latency of action potentials evoked by 'threshold' stimulation of the skin with the mechanical prodder (see text).

The top traces are the extracellular recordings from nerve 8. The bottom trace is the voltage output of the prodder photocell.

The threshold stimulus was applied 6 times, and the records were superimposed to show the variation in the latency of the responses.

Vertical calibration: top trace, 20μV; bottom trace, 10mV
Horizontal calibration: 2ms



trials; this value was recorded. The prodder was raised vertically and then lowered to touch the same spot, and the critical stimulus redetermined; this procedure was repeated ten times. A number of points were similarly examined. The results of this test are presented in Appendix Table 2. The values for any one spot were always within one "unit of resolution" of the mean; this unit was taken as the smallest increment in the rate of rise that could be measured with the stimulator and oscilloscope set-up available and its value was 0.13 μm/ms. This value was taken to represent the error in measurement of the critical stimuli in these experiments, i.e., the maximum resolution with which the stimulus could be determined.

4. Receptor characteristics:

In a previous section it was shown that individual axons could be stimulated by mechanical deformation of the skin. Mechanoreceptors such as these, that respond during the rising phase of the stimulus, are classified as rapidly-adapting receptors (Adrian, 1928). That is, they respond during the application of the stimulus, usually with only one or two impulses, and the response occurs only if the velocity of the applied stimulus is above a certain critical level; in contrast to slowly-adapting receptors, they do not fire during a maintained stimulus. Occasionally, rapidly-adapting receptors, for example the Pacinian corpuscle, also give rise to one or two impulses when the stimulus is removed; this is termed an "off" response.

The results of stimulating different points on the hind limb

skin of Xenopus with mechanical stimuli of varying duration demonstrated that all the cutaneous mechanoreceptors in this region are rapidly-adapting. Fig. 10 presents three examples of the rapidly-adapting response characteristic of these mechanoreceptors to short duration stimuli (2 ms pulse width). In addition, these receptors are very sensitive, acting as low-threshold receptors; for example, the critical stimulus in Fig. 10a is 0.63 µm/ms, in (b) 1.0 µm/ms and in (c) 1.63 µm/ms. The range of the response of most of the points tested was from 0.25-3.0 µm/ms. These stimuli can be compared to those required to excite the salamander touch spots (0.57-3.0 µm/ms, Cooper and Diamond, 1977) toad touch receptors (0.08-1.0 mm/s, Lindblom, 1965) and mammalian touch receptors (0.08-3.5 mm/s, Lindblom, 1965).

When a prolonged stimulus was used (as illustrated in Fig 11a, in which a 40 ms pulse duration was used) only one action potential was observed as found for the brief pulses; in this particular example four oscilloscope traces were superimposed and in two of them the applied stimuli were sub-threshold; the two remaining stimuli were at the threshold level (1.5 µm/ms rate of rise) giving rise to an afferent spike in each case. Fig. 11b shows the response to a 0.5 s pulse width stimulus of 1 µm/ms rate of rise; in this case there is an 'off' response, which occurred occasionally only with the long duration stimuli. In none of the points tested with prolonged stimuli did the impulse activity last for the duration of the stimulus application; response was observed only during the dynamic phase of the stimulation,

Fig. 10. Mechanical stimulation of Xenopus skin.

Figs. A-C show responses recorded extracellularly from nerve 8 (top trace in each pair of records) when a mechanical stimulus from the prodder was applied to the skin of the thigh. The bottom trace (in each pair of records) shows the voltage output of the prodder photocell.

Each pair of records (A-C) was taken from a different experiment. The arrows show the single spike obtained with each stimulus. These spikes were all-or-nothing, and with a threshold stimulus "jittered" in latency, as shown for the receptor in Fig. 9.

In (A), the critical stimulus was 0.63 μm/ms; in (B) it was 1.0 μm/ms, and in (C) it was 1.63 μm/ms.

Vertical calibration: top trace, 20μV; bottom trace, 10mV.
Horizontal calibration: 2ms

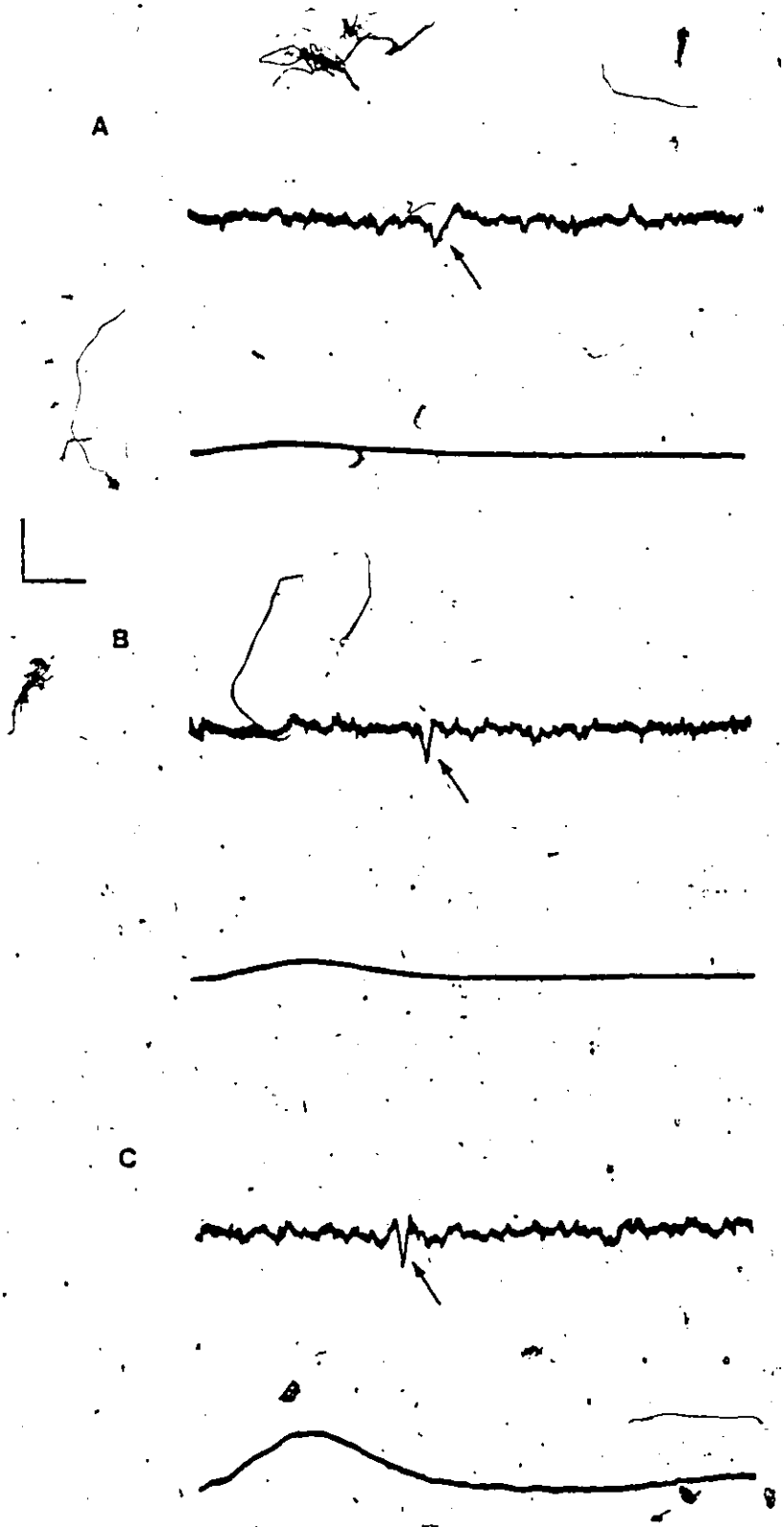

A
B
C

Fig. 11. Response to prolonged mechanical stimulation of Xenopus skin.

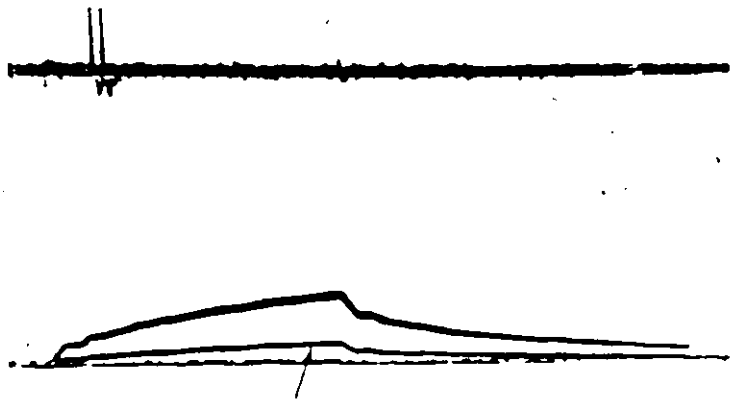
Figs. A and B show responses recorded extracellularly from nerve 8 (top trace in each pair of records) when a prolonged mechanical stimulus from the prodder was applied to the skin. The bottom trace in each pair of records shows the prodder output.

Each pair of records was taken from a different experiment. In (A) four stimuli were applied, two of which were subthreshold (arrow); the four records are superimposed. The top trace shows two action potentials; each threshold stimulus elicited one spike. The stimulus duration was 40 ms; the critical stimulus was 1.5 µm/ms.

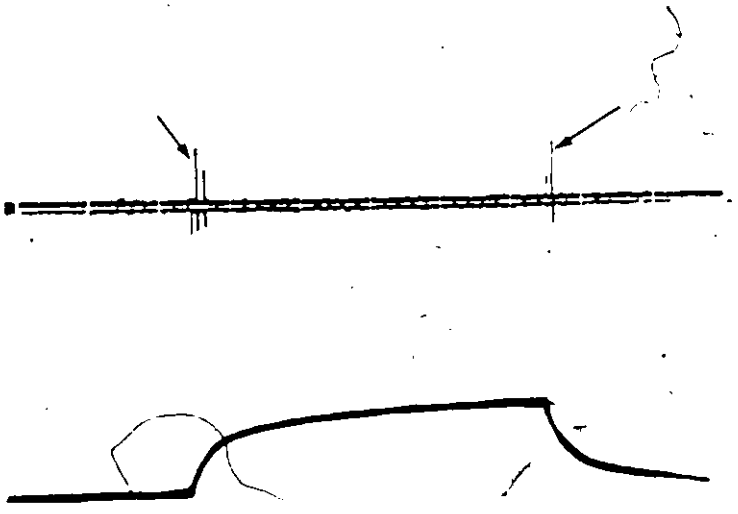
In (B) only one stimulus was applied; two impulses (arrow) were recorded during the rising phase of the stimulus, and one (arrow) was recorded at the termination of the stimulus (an "off" response). The stimulus duration was 0.5 s; the critical stimulus was 1.0 µm/ms.

Vertical calibration: top trace, 50µV; bottom trace, 50mV (A), 100mV (B)
Horizontal calibration: 10ms (A); 0.1s (B)

A



B



that is, all points were rapidly-adapting.

Although there were no slowly-adapting receptors found in the skin of the thigh, a slowly-adapting discharge could be evoked in the mixed nerve by a prolonged stimulus applied to the lower leg; this was not due to cutaneous receptors, since it was still present when the skin overlying the area being tested was removed. The absence of slowly-adapting receptors in the skin of the thigh was also demonstrated by the following experiment. A slit was made in the skin, distal to the area being tested, and a small piece of coverslip glass was inserted under the skin to isolate it mechanically from the underlying tissues. Application of a maintained stimulus, either by a long (about 1 sec) pulse to the prodder, or by allowing a small probe to rest on the skin surface, resulted in an afferent discharge only when the stimulus was applied, or removed, and not at all during the static phase of the stimulus.

As a point of interest, it should be noted that although there do not appear to be any cutaneous slowly-adapting receptors in the thigh and leg, there may be some in the back and belly areas. In the early stages of this investigation both back and belly skin were examined, and in two of the eight animals investigated slowly-adapting discharges were observed in response to maintained (100-300 ms pulse width) stimuli. However, these slowly-adapting responses, recorded from purely cutaneous nerves, could be evoked in only three to five of the tested spots (about 100) on these animals.

5. Axonal conduction velocity:

Conduction velocity of the axons associated with these rapidly-adapting mechanoreceptors was examined in five animals. The conduction velocity was estimated by recording with two sets of recording electrodes located at two different points along the nerve trunk. Fig. 12 shows two examples of the results obtained. The distance between the pairs of recording electrodes was 4 mm; the time taken for the impulse to be conducted between the two recording points (measured from spike onset) was 0.2 ms in the upper example, and 0.3 in the other. This allows an approximate estimate of the conduction velocity for these two axons, the values being 20 m/s in the first example and 13 m/s in the second case. The average conduction velocity, calculated from ten different measurements, was 14.5 ± 0.97 m/s. This value is comparable to values cited in the literature (15-35 m/s) for tactile afferent fibres of frogs, the probable diameter of fibres with these conduction velocities being in the range of 8-12 μm (Adrian, 1932; Maruhashi et al., 1952; Catton, 1958). The axonal conduction velocity for the rapidly-adapting mechanoreceptor axons in salamander is somewhat slower, with a range of 5-10 m/s (Cooper, 1977).

6. The mapping technique:

The number and distribution of individual receptors within the 8th nerve field were investigated by a mapping technique. The term "mapping" refers to the physiological examination of the defined area

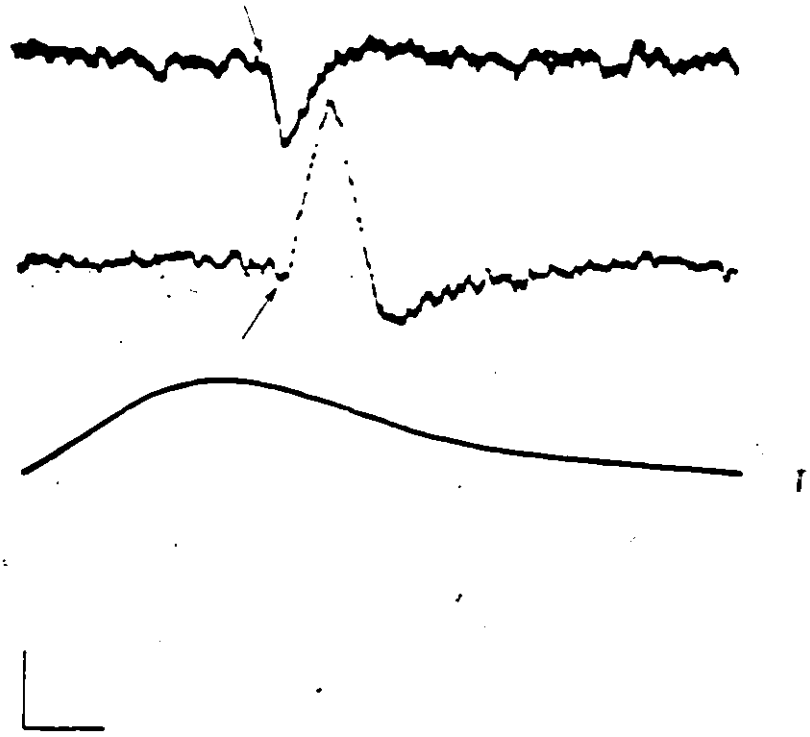
Fig. 12. Axonal conduction velocity.

A and B each show records obtained from two different animals of a single action potential recorded at two different positions (upper two traces) simultaneously along the nerve; The bottom trace in each figure is the prodder output.

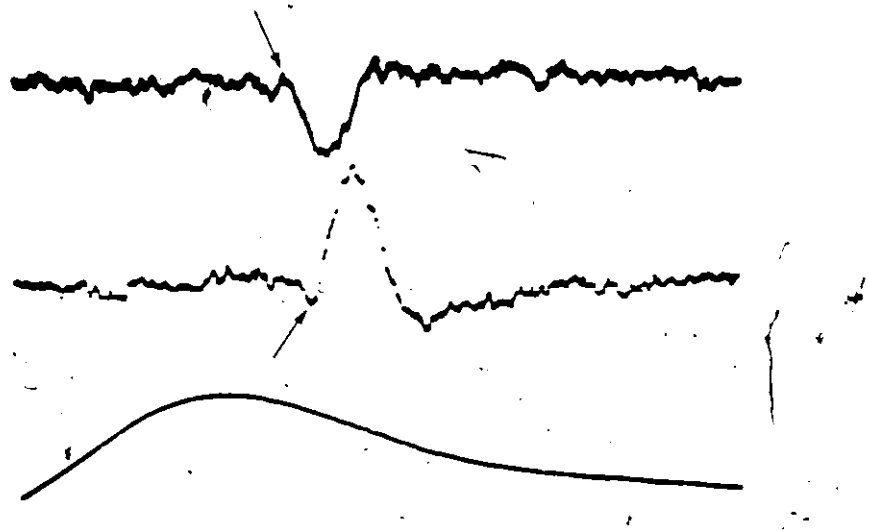
In each case the lower of the two nerve recordings was made at the more central location along the nerve, i.e., farther from the skin. The distance between the two recording electrodes in each experiment was 4 mm. The difference in latency (measured from spike onset) between the spikes at the two positions was , in (A) 0.2 ms and in (B) 0.3 ms. The conduction velocity calculated for the axon in (A) is 20 m/s and in (B) is 13 m/s.

Vertical calibration: top traces, 20μV; bottom trace, 10mV
Horizontal calibration: 2ms

A



B



of skin (usually less than a mm^2) with the prodder, to determine the distribution of the touch sensitive spots (touch spots). In the absence of any information concerning the location of the receptors (but see below) one can obtain information with this procedure that indicates the presence of a randomly distributed population of low-threshold mechanoreceptors. The mapping was done by moving the prodder in 90 μm steps, with the aid of a micromanipulator and a 14 by 14 squared graticule set into one of the eyepieces of the dissecting microscope that was used; at the magnification used (99x) each square was equivalent to 90 μm by 90 μm. In most of the experiments a systematic mapping was done, by recording the critical stimulus required to evoke an action potential in the middle of each consecutive square of the grid for a selected microsope field of the skin; the number of squares examined in any one map, or survey, varied from 40-100, but was usually greater than 50 . In one series of experiments to be described later (the "on" and "off" gland opening experiments), systematic mapping involved placing the prodder directly "on" a gland opening, or "off" to one side. In the present series the gland openings were not specifically selected for; that is , the prodder was moved in 90 μm steps, whether or not a gland opening was present at that point.

The results of the distribution of critical stimuli in a given area were then plotted as a histogram, giving the percentage occurrence of any given range of stimuli. The number of class intervals or bins used in constructing the histograms was chosen using Sturge's rule

(Daniel, 1974) as a guide; Sturge's rule is described by the equation

$$k = 1 + 3.322 \ (\log_{10} n)$$

where k represents the number of class intervals and n is the number of values in the data set under consideration. For the range of sample sizes used in the present experiments (n=40-150), the number of bins used is 6-8. The width of the intervals may be obtained by dividing the range of values by the number of intervals. The actual bin widths were chosen using this guideline, and taking into account the error involved in the measurement of the critical stimuli; i.e., the bins should not be less than about two times the smallest increment of stimulus amplitude (in rate of rise) that was resolvable.

The design of many of the experiments carried out in the later parts of this study was to measure quantitatively the loss or recovery of mechanosensitivity following a nerve lesion, by comparing the distribution of critical stimuli in an experimental situation with that of the control situation. In order to compare these two distributions, the histograms were plotted as cumulative frequency curves relating the percentage of receptors excited to the stimulus velocity (see Fig. 13). The two curves were then compared using a non-parametric statistical test, the Kolmogorov- Smirnov two-sample test for large samples ($n > 40$). A non-parametric test was used because it does not require any assumptions about the distribution of the sample or population, that is, whether or not it has a normal distribution; the usual parametric tests, such as the Student's t test, assume that the population is normally distributed (Siegel, 1956; Daniel, 1974). The

Kolmogorov-Smirnov two-sample test is a test of whether two independent samples have been drawn from the same population; it is concerned with the extent of agreement between two cumulative distributions. If the two samples have been taken from the same population, then the cumulative distributions should be fairly close to each other. If they are from different populations, then the difference between the cumulative distributions will be relatively large. The test determines the difference that is required to exist between the two distributions in order to reject the hypothesis that the two samples are from the same population; that is, it determines the difference that needs to exist before one can say that the experimental distribution is significantly different from the control distribution.

Fig. 13 presents two examples to illustrate the use of the test; in one case, Fig. 13 a, the distributions are very similar and there is no significant difference between the two curves. In the second example, Fig. 13 b, the actual difference between the two curves is greater than the difference calculated using the Kolmogorov-Smirnov test; the probability that these two samples are from the same population is less than 0.001, that is, they are significantly different ($P<0.001$).

7. Side to side variation:

Since the hind limbs of a single animal were to be used as experimental and control limbs, respectively, it was necessary to determine whether there was any intrinsic side-side variation which

Fig. 13. Use of the Kolmogorov–Smirnov statistical test (see text).

The results from two different experiments (A–C and D–F), in which the return of mechanosensitivity was being examined at different times after crushing the 8th nerve, are presented to illustrate the use of the Kolmogorov–Smirnov two sample test. The ordinate represents the range of critical stimuli (in μm/ms); the abscissa in A, B, D and E represents the percentage occurrence of tested points in each bin, while in C and F, the abscissa is the cumulative frequency.

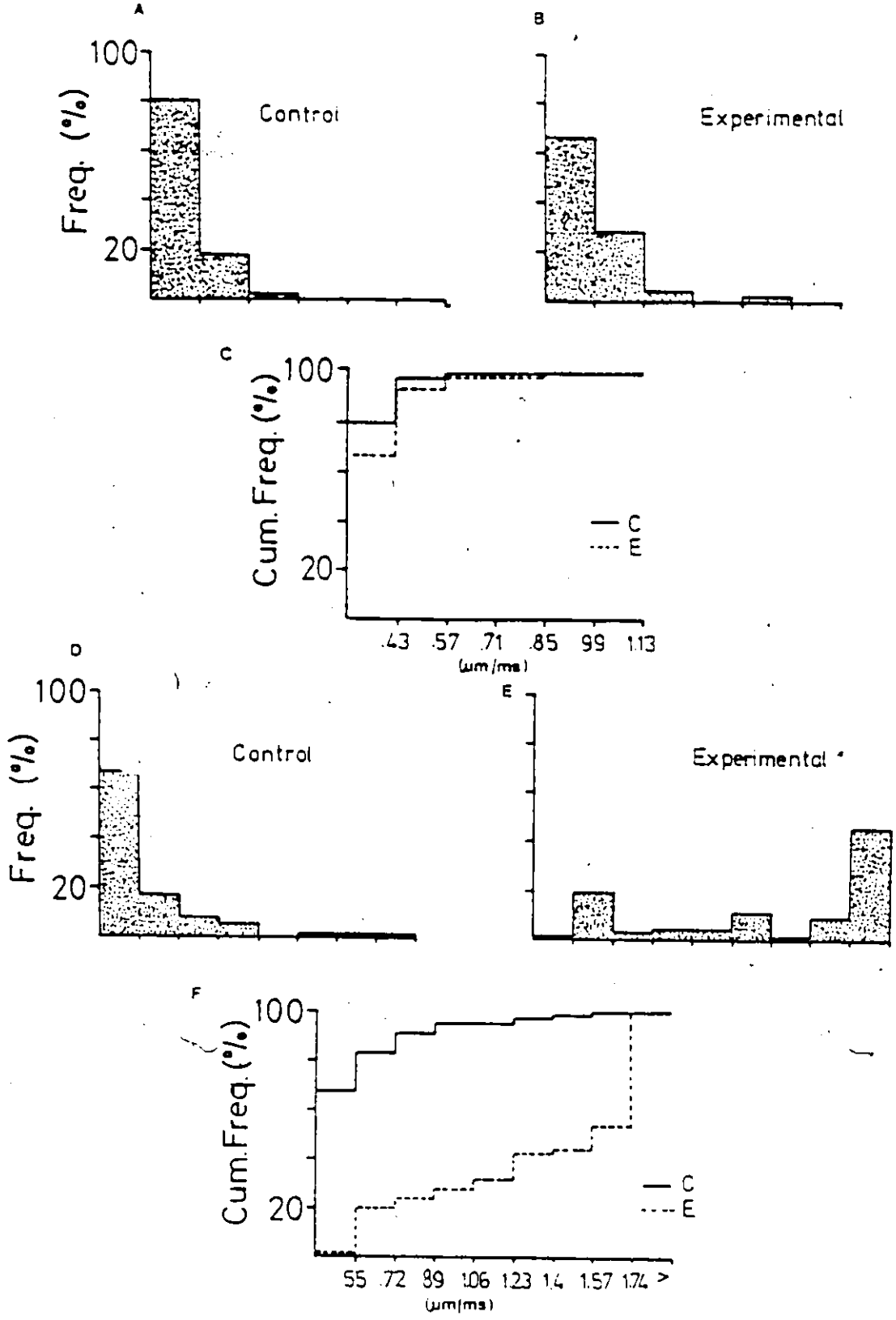
In (A) and (B) the distributions of critical stimuli (in μm/ms) for a physiologically mapped area of skin (see Methods) are presented as histograms; (A) is the control distribution and (B) is the experimental distribution from an animal examined 14 weeks after nerve crush. (C) shows the cumulative frequency curves constructed from the information presented in the histograms.

In (D) and (E) the distribution of critical stimuli from an animal examined 6.5 weeks after nerve crush is presented; the control distribution is shown in (D) and the experimental in (E). (F) presents the cumulative frequency curves constructed from the information in the histograms.

The Kolmogorov–Smirnov test determines the difference that must exist between the two cumulative frequency curves at any interval, if the two samples are to be considered significantly different. If the actual (measured) difference is equal to, or greater than, the calculated difference, then the two samples differ significantly.

In (A) the calculated difference (at P=0.1) is 18.5%; the actual maximum difference that exists between the two curves is 15% (obtained by subtracting the cumulative frequency value of E from C at each bin, or stimulus range), therefore the two sample distributions do not differ significantly.

In (B) the calculated difference (at P=0.001) is 33.2%; the actual maximum difference between the two curves is 67.7%, therefore the two sample distributions do differ significantly (P<0.001).


A
B
100
20
Freq. (%)
Control
Experimental
C
100
20
Cum.Freq.(%)
C
E
.43 .57 .71 .85 .99 1.13
(µm/ms)
D
E
100
20
Freq. (%)
Control
Experimental
F
100
20
Cum.Freq.(%)
C
E
55 .72 .89 1.06 1.23 1.4 1.57 1.74 >
(µm/ms)

might preclude such an approach. The mapping of the skin was carried out as previously described in six animals. The gross nerve field was determined by brushing the skin and recording from the nerve trunks, first on the right and then on the left side. Then the distribution of critical stimuli for several areas of skin was measured for each side. The location of these areas in the thigh was found not to make any difference to the threshold distributions. The results were plotted as histograms and the cumulative frequency curves were then constructed and are presented in Fig. 14 . Using the Kolmogorov-Smirnov test, there was no significant difference between the left and right sides in terms of this particular physiological profile.

It was therefore concluded that the use of the left hind limb as an internal control for the experimental right hind limb was permissible. Additionally, these control results, along with the controls of the subsequent experiments, demonstrate that there are discrete low-threshold, rapidly-adapting touch-sensitive spots in Xenopus skin.

8. Summary Discussion:

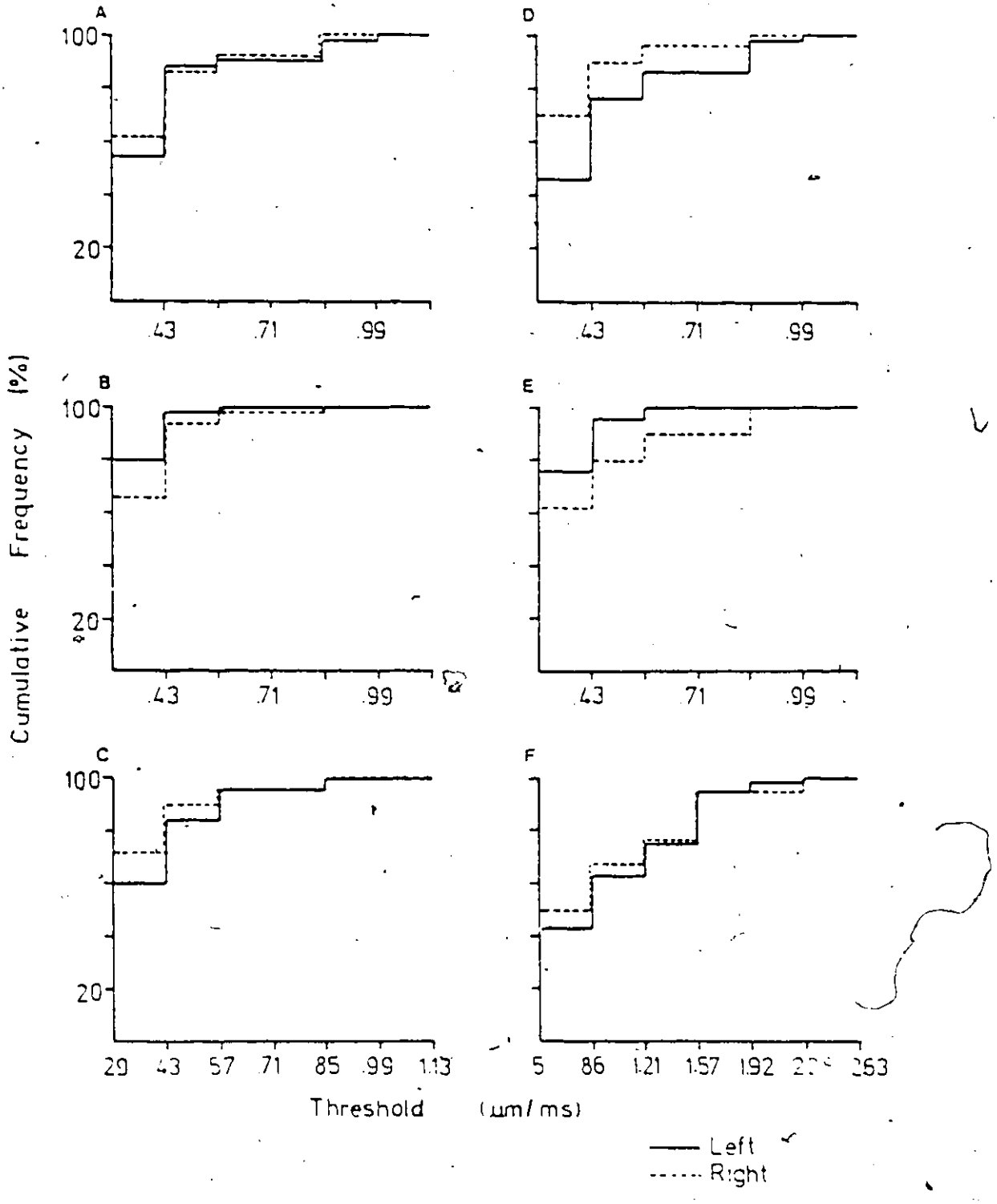
The results described in the foregoing sections show that there are discrete spots in Xenopus skin which respond to low- threshold mechanical stimulation, and which are rapidly-adapting. Moreover the relatively rapid means of generating the critical stimuli distribution curves for a given area of skin, i.e., its "mechanosensory profile", promised a good basis for the studies to be attempted later, in which

Fig. 14. Side to side comparison of distibution of critical stimuli in control animals.

The distribution of critical stimuli in an area of skin that was mapped as described in the text, for the right and left hind limbs of control animals, is presented. Histograms of these data were plotted (not shown) and cumulative frequency curves were constructed using the information from the histograms.

The cumulative frequency curves of critical stimuli distribution for the left (——) and right (- - -) hind limbs of six different animals are shown (A-F).

Using the Kolmogorov-Smirnov test, there was no significant difference between any of the pairs of curves ($P>0.1$), indicating that there was no significant side to side variation in the threshold distributions.


A
B
C
D
E
F
100
20
.43
.71
.99
29
57
85
1.13
5
86
1.21
1.57
1.92
2.53
Cumulative Frequency (%)
Threshold (um/ms)
Left
Right

nerve degeneration and regeneration would be followed physiologically by changes in these curves. However at this stage it seemed worthwhile to ask whether the data allowed a further result to be obtained, namely the normal distribution, receptive field size and organization of the low-threshold mechanoreceptors, as had been previously done for salamander skin (Cooper and Diamond, 1977).

The distribution of thresholds obtained in the normal, or control, animals indicate that there is a range of sensitivities in a given area with the majority of the points being highly sensitive and the rest being of lower sensitivity. There are several ways of interpreting these results. For example, it is possible that there is only one population of mechanoreceptors of uniform distribution and threshold; the spacing of these receptors would allow for the probability that a randomly-located small diameter prodder could be placed between them. A second possibility is that there are closely-spaced receptors with a variety of sensitivities extending over the range indicated in the results. A third possibility is that the situation is a combination of the first two (Cooper and Diamond, 1977).

The simplest hypothesis is the first, namely that there is 1 population of essentially similar touch receptors. According to this hypothesis, the height of the first bin (on the left) of the histograms indicates the frequency with which the prodder is placed directly over a receptor. The bins representing the higher stimulus strengths would then indicate the frequency with which the prodder is placed to one side of the receptors; the strength of stimulus required to evoke an

impulse would depend on how far away the prodder was from the nearest receptor.

Assuming such a single population of receptors, their spacing and the receptive fields can be estimated by i) an examination of the spatial arrangement of thresholds obtained in a systematic mapping of a selected region of skin, or ii) by use of an analysis described by Cooper and Diamond (1977).

(i) This approach is valid only if the resolution of the prodding technique and the spacing of the receptors allows successive prods (in the systematic mapping) to "find" the less sensitive area between the receptors. If the prodder is too gross, and the receptors too close, the receptive fields blend into one large, highly sensitive area. Several examples of control maps are shown in Fig. 15. Each square represents the placement of the prodder tip after a 90 μm movement of the tip along the surface of the skin. There seem to be relatively large areas of high sensitivity (the L points). At this point one cannot distinguish between few receptors with large receptive fields and numerous receptors with small receptive fields. The higher threshold points (M) represent areas where the prodder is located between the low-threshold receptors.

(ii) The analysis used by Cooper and Diamond (1977) to determine receptor spacing and receptive fields assumes that the receptors are distributed uniformly (they determined that this was approximately true of salamander skin). Let the receptors therefore be arranged in a square array in which the centre of the receptive fields,

Fig. 15. Spatial arrangement of several critical stimuli surveys.

A,B and C represent the actual spatial distribution of critical stimuli values recorded in surveys done in three different animals. Each square represents the location of the prodder tip on the skin, the prodder being moved in 90 µm steps; at each point skin was stimulated with the prodder and the critical stimulus required to evoke an action potential was recorded. L are the low-threshold points, for which the range of thresholds was 0.28-0.43 µm/ms. M are the higher-threshold points, for which the range of thresholds was 0.44-3.0 µm/ms.

A

L	L	M	L	M
M	M	M	L	L
L	L	M	L	M
M	L	L	L	L
L	L	L	M	M
L	L	L	M	M
L	L	L	M	L
L	L	M	M	M
L	M	L	L	L
M	M	M	L	L
L	M	M	L	M
M	L	L	L	L

90 μm

B

M	L	L
M	L	L
M	L	M
M	M	L
M	M	M
L	M	M
M	L	M
M	L	L
M	M	L
L	L	L
M	M	L
L	L	M

90 μm

C

L	M	M	L
L	L	L	M
L	L	L	L
L	L	M	L
M	M	L	M
L	L	L	L
L	M	M	L
M	L	L	L
M	L	L	L
L	L	M	M
M	L	L	L
L	L	L	M

90 μm

assumed to be circular and of radius r, are a distance D apart (see Fig. 16). In this treatment the frequency with which the prodder tip is placed within a single receptive field, that is the height of the first bin in the threshold histograms, measures the proportion of the area of the sampled skin that is occupied by receptive surface. For example, if the frequency of occurrence of the low-threshold points is 70%, then 70% of the total area sampled consists of receptive surface. One can estimate r and D by using the equations outlined by Cooper and Diamond as follows: the relationship between the frequency of locating a receptor and the receptive field can be written

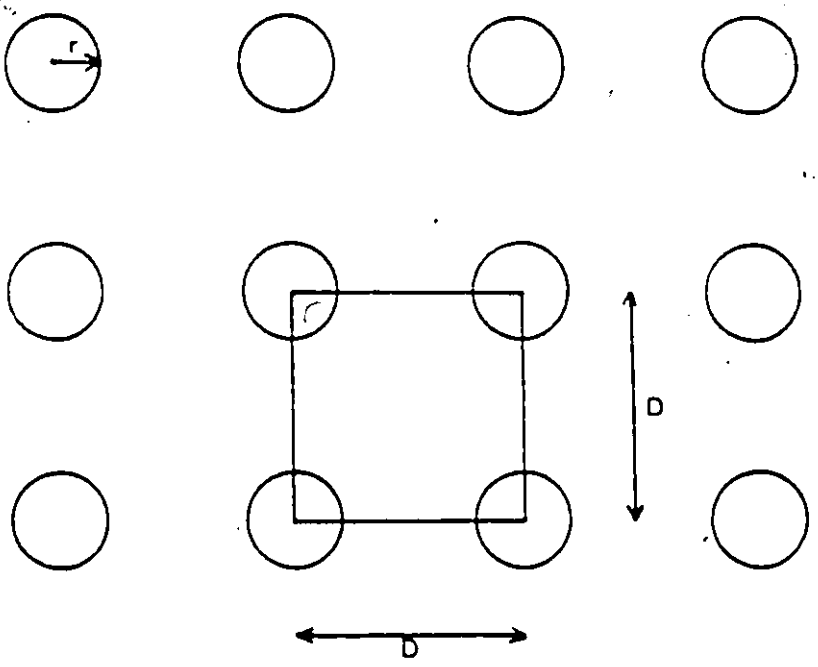
$$Ax = n\pi r^2$$

where x is the frequency with which the prodder tip occurs within the receptive field, r is the radius of the circular receptive field of a single receptor (which thus has a receptive field area of (r^2), n is the number of receptors in the sampled area, A is the area sampled (see Fig. 16). However, the value of r is actually related to the stimulus strength; when the prodder tip is located to one side of a receptor, a larger stimulus will be needed to excite that receptor than when the prodder is directly over it; i.e., the larger the stimulus, the greater the value of r.

The relationship between r and the stimulus strength can be determined by measuring the critical stimulus when the prodder is placed directly over a receptor and then at some measured distance away. This was done in the present study by first locating a sensitive spot at the very edge of the recorded nerve field; the prodder was then

Fig. 16. Idealized model of mechanoreceptors in the skin.

This schematic diagram represents the low-threshold mechanoreceptors in a square lattice arrangement (see text). The centers of their circular receptive fields are a distance D apart; r is the radius of the receptive field. The fraction of the square $(D \times D)$ occupied by receptive surface is $4 \times \pi(1/4\ r^2)/D^2 = \pi r^2/D^2$. (from Cooper and Diamond, 1977).


r
r
D
D

moved out from this point into the non-responsive skin (adjacent to that supplied by the nerve recorded from) in 90 μm steps. In a number (10) of such experiments, it was found that by moving out 90 μm from a receptor, the stimulus had to be from 2 to 5 times the critical value to excite the same receptor; that is, the receptive field increased by 90 μm when the stimulus was increased 2-5 times threshold (2-5T). Moving out from the edge of the field by another 90-180 μm generally required the maximum stimulus that could be provided by the stimulator, or it was not possible to excite the receptor at all. With this result, one can predict the average size of the receptive fields and the average distance between the receptors, using the equations presented in Cooper and Diamond (1977), and described below. The equations are as follows:

$$\pi r^2 = xD^2 \qquad (1)$$

$$\pi(r + L)^2 = yD^2 \qquad (2)$$

where x is the % skin area occupied by low-threshold receptive surface, L is the increase in radius of the receptive field when the prodder is moved one step, and is effective with a 2-5 times increase in threshold, and y is the new proportion of the skin area that is activated by this 2-5T stimulus. L is equal to 90 μm as indicated above; x and y are obtained from the cumulative frequency curves. D is the distance between receptors.

In the present study, values for x ranged from 30-80%, and values for y ranged from 80-100%. Using an average value of x as 55% and y as 90%, and solving equations 1 and 2 for r and D, the radius of

the receptive field is 317 μm and the distance between the receptors is then 758μm; these values seem rather large when compared to the actual maps of threshold distributions (Fig. 15). It appears that this analysis falls down, because the technique does not seem to resolve individual receptors. Indeed a casual examination of the typical data in Fig. 15 suggests that this would be the case; the mechanoreceptors in Xenopus skin are far more numerous and more closely arranged than in salamander skin, to which the analysis was originally applied.

In salamander skin this same approach and analysis indicated that the mechanoreceptors were approximately 250 μm apart with receptive field radius of about 75 μm. Particularly interesting was the finding in the salamander that although there were no apparently morphologically distinct skin surface structures, such as glands or warts, associated with the touch spots, there were specialized epidermal cells that could be observed with EM, the locations of which were subsequently correlated with the location of the touch spots (Parducz et al., 1977). These cells were the Merkel cells, cells that have been found in association with mechanoreceptive structures in other animals (e.g., Iggo and Muir, 1969; Munger, 1971). Since the skin of Xenopus does contain Merkel cells (Crowe and Whitear, 1978; Fox and Whitear, 1978) the obvious question was asked. Is the mechanosensitivity of Xenopus skin associated with the Merkel cells? The following sections deal with the investigations to answer this question and describe an experimental approach that obviated the difficulties just outlined, providing in the end the important answers

that were not available from the analysis described above.

Part II A. Anatomical basis of cutaneous mechanosensitivity in Xenopus:

1. Morphology of Xenopus skin:

Xenopus skin was examined using both histological and electron microscopic techniques. The multilayered epidermis is pierced by the openings of numerous glands, both mucous and granular , which are found in the dermis (e.g., Fig. 17). Also found in the upper dermal region are small capillaries and nerve bundles. Below the area containing the glands is a thick connective tissue layer, the corium, which consists of alternating bands of collagen fibres. Deep to the corium is another layer of loosely-arranged connective tissue which contains the major nerve bundles and blood vessels (Fig.17a).

The nerve fibres run from the deep dermis up to the upper layers of the skin in bundles oriented perpendicular to the surface of the skin. Fig. 18 presents two light micrographs of silver-stained paraffin sections which show nerve bundles traversing the corium and entering and ramifying through the upper layers of the dermis. Examination of serial paraffin sections showed that some of the nerve fibres in the dermis seem to end freely or in association with the glands or blood vessels; other fibres enter the epidermis and appear to end freely. At the EM level it was observed that a proportion of the intraepidermal endings terminated on Merkel cells. Axonal profiles were seen, however, away from the Merkel cells; whether these end as free nerve endings or in association with Merkel cells is not clear,

Fig. 17. Photomicrographs of a toluidine blue stained section of Xenopus skin.

(A) is a cross-section through a plastic section (0.5 µm thick) of Xenopus skin that was processed for EM, as described in the Methods.
E, epidermis; D, dermis; C, corium; GG, granular glands; MG, mucous glands; d, duct of the granular gland; bv, blood vessel in the connective tissue layer deep to the corium.
Calibration bar: 20 µm

(B) is a higher magnification of the gland duct area in (A). A longitudinally oriented cell (MC) located to the side of the duct was later shown with EM to be a Merkel cell.

Calibration bar: 10 µm

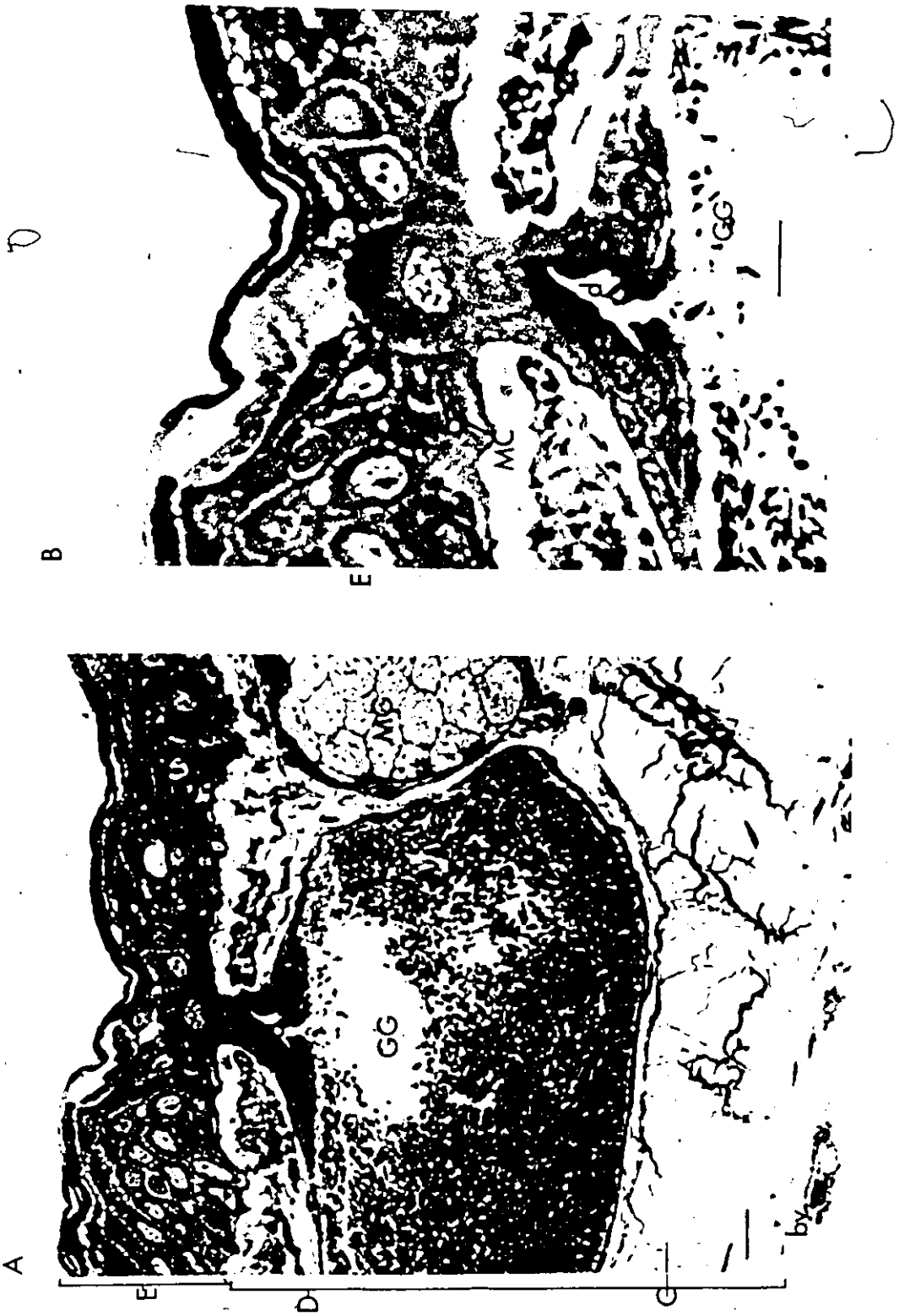

A
B
E
D
C
GG
MC
GG
by

Fig. 18. Photomicrographs of silver-stained paraffin sections of Xenopus skin.

(A) and (B) are photomicrographs of silver-stained paraffin sections of Xenopus skin (processed as described in the Methods), showing the vertical orientation of bundles of nerve fibres (n) coming up from the deep dermis. In (A), some of the fibres can be seen ramifying in the upper dermal layers. In (B), a thick bundle runs beneath one of the glands (g). The dark spots in the upper dermal region of (A) are pigment cells (p). The arrow points to the epidermal-dermal boundary. E, epidermis; D, dermis; C, corium.

Calibration bar: 20 μm


B
E


A
E
D

because their course was not followed with serial sections.

EM examination of the skin revealed that the Merkel cells are usually located around the gland ducts in the epidermis. In cross-sections of skin, one Merkel cell is usually seen on each side of a gland duct, about 2-3 epidermal cells away from the duct and one cell up from the basement membrane (Fig. 17b). In skin sectioned horizontal to the surface (en face), the Merkel cells were located in a ring around the gland openings (Fig. 19). From this viewpoint the Merkel cells appeared to be rather long and narrow crescent-shaped cells, ranging from 20-40 μm long and 5-10 μm wide. The cells contain the characteristic dense cored granules (90-120 nm diameter), which are generally more numerous in the part of the cell facing the gland duct. Bundles of tonofilaments are found throughout the cytoplasm. Cytoplasmic 'spines' were also observed protruding from the Merkel cells and interdigitating with the surrounding epidermal cells. Overall, the cytological features of Xenopus Merkel cells were very similar to the Merkel cells described in other animals.

Intraepidermal axons terminate on the Merkel cells. In the normally-innervated skin that was examined with the EM, every Merkel cell observed had at least one nerve ending contacting it (see Fig. 20a). In addition, on each Merkel cell at least one morphological synaptic contact was seen. The synaptic area was usually characterized by a "post-synaptic" dense bar at the nerve terminal membrane, and "pre-synaptic" dense projections on the Merkel cell membrane (Fig. 20a). The nerve terminal usually contained numerous clear "synaptic"

Fig. 19. Electron micrographs of Xenopus skin sectioned en face.

(A) and (B) are electron micrographs of Xenopus skin (processed as described in the Methods) sectioned en face (i.e., in the plane of the skin surface) to show the gland openings (GO) with the Merkel cells (M) curved around the opening. Notice the numerous characteristic dense-cored granules in the Merkel cells; in (A) these granules are concentrated in the region of the Merkel cell facing the duct, which is also the area of nerve (n) contact.
K, the surrounding epithelial cells, keratinocytes.

Calibration bar: 5 μm

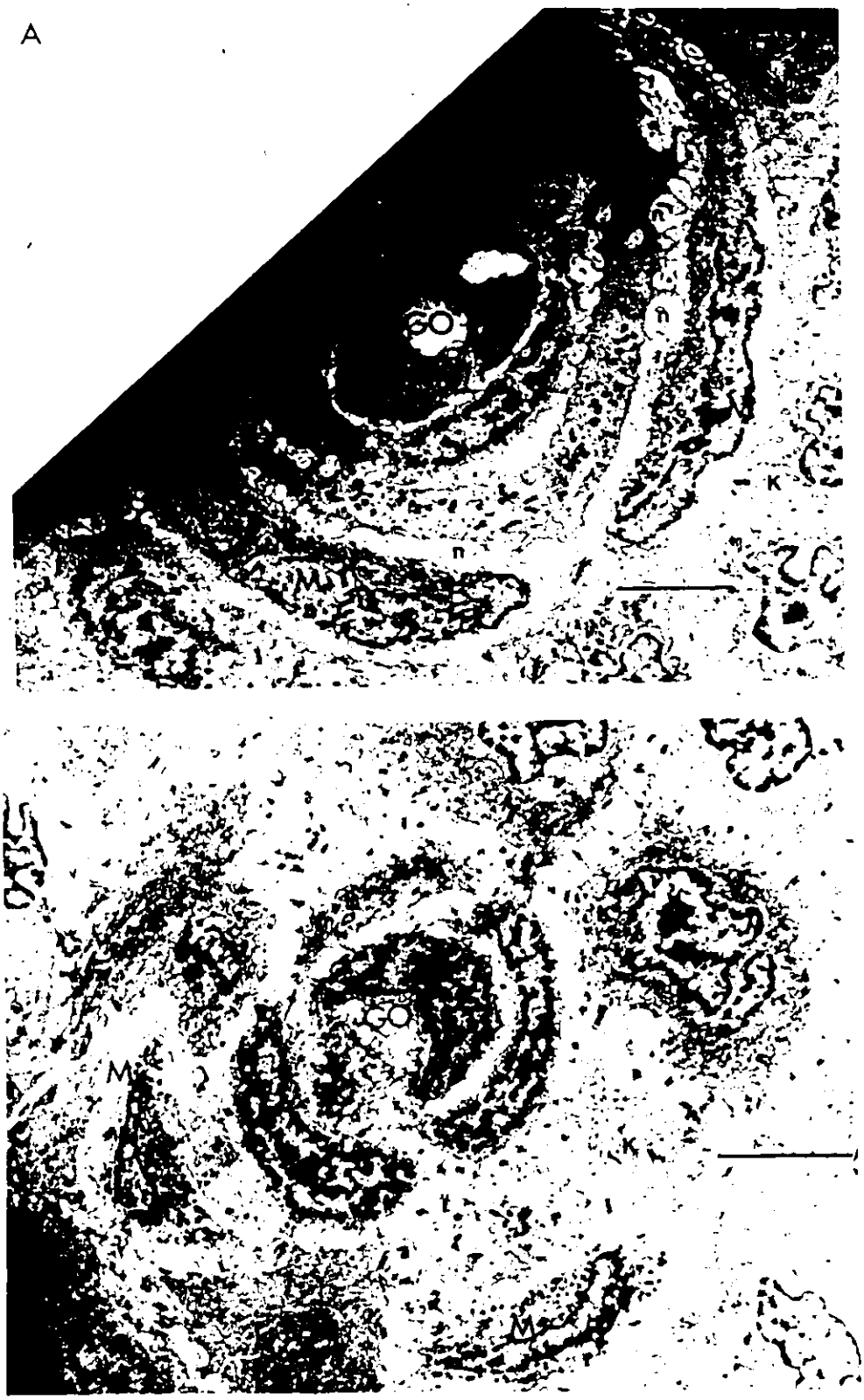

A
GO
n
K
n
M
B
GO
M
K
M

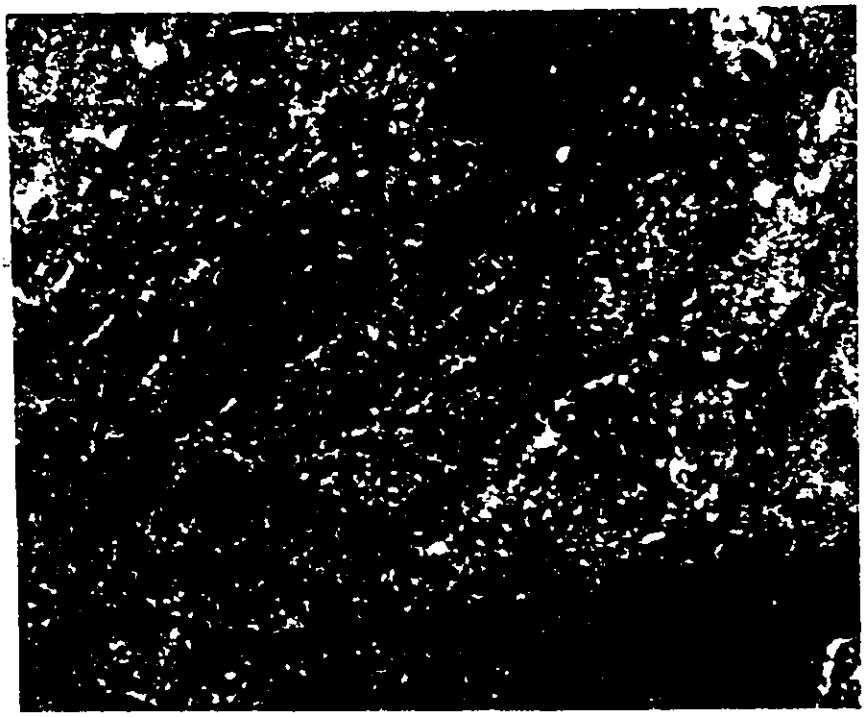
Fig. 20. Electron micrographs of Merkel cells in Xenopus skin.

(A) is an electron micrograph of normally-innervated Xenopus skin showing a Merkel cell. This particular section shows four nerve ending profiles (n) contacting the Merkel cell (MC); morphological synapses are visible on two of them (arrows). The nerve endings contain clear synaptic vesicles and several mitochondria.
K, the surrounding keratinocytes

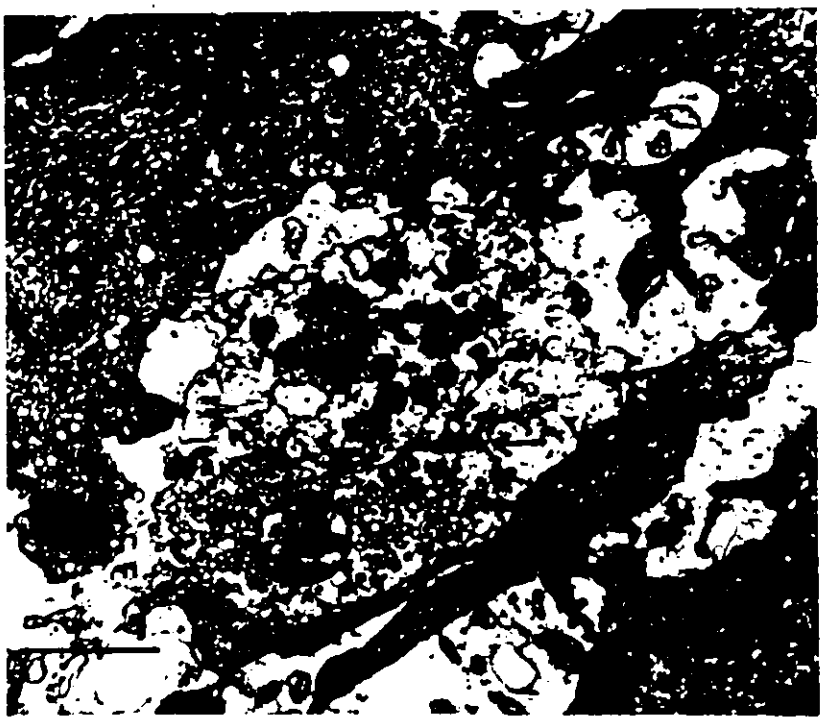
(B) is an electron micrograph of a portion of a Merkel cell (MC) from normally-innervated Xenopus skin. A nerve ending (n) with numerous clear vesicles (v) contacts the Merkel cell. Notice the "pre-synaptic" cytoplasmic densities on the Merkel cell membrane; the nerve ending membrane appears to have a "post-synaptic" thickening, and there is an area clear of vesicles just underneath this thickening (the arrow marks the contact zone). There is a profile suggestive of an "empty" dense-cored granule near the pre-synaptic densities in the Merkel cell (arrowhead). m, mitochondrion.

Calibration bar: 1 μm

A



B



vesicles (approximately 60 nm diameter) and some mitochondria.

When viewed in cross-sections of skin, the nerves were observed to form multiple terminations, which were often, though not always, on the dermal surface of the Merkel cell. When viewed en face, the nerve endings were usually seen on the surface of the cell facing the gland duct (Fig.19a).

A further, interesting feature of the Xenopus Merkel cell-neurite complex is that a morphologically reciprocal synaptic relationship was occasionally observed. The reciprocal synapse I observed in Xenopus skin was characterized by having a synaptic junction morphologically polarized from Merkel cell to nerve ending at one area of the terminal, and in the same terminal a short distance away, another junction polarized from nerve ending to Merkel cell. The occurrence of reciprocal synapses is discussed in more detail in Part IIIB, section 1.

2. The use of quinacrine to visualize the Merkel cells:

Merkel cells, as well as certain other dense core granule-containing cells such as mast cells and adrenal chromaffin cells, take up the fluorescent dye quinacrine (Olson et al., 1976; Crowe and Whitear, 1978; Bock, 1980; Nurse et al., 1983b). Work in this laboratory has provided direct evidence that Merkel cells are labelled with quinacrine, by EM examination of isolated individual quinacrine fluorescent cells (Nurse et al., 1983b). The use of quinacrine is a powerful technique since it allows quantitative

experiments involving the Merkel cells to be performed with relative ease, experiments which are not easily done when one has to rely on EM to identify the cells. The quinacrine fluorescent cells will now be referred to as QFCs.

In the present study, several different protocols were used to visualize the Merkel cells with quinacrine. First, already excised pieces of skin were incubated in vitro in 10^{-5} M quinacrine in amphibian Ringer's solution for 15 minutes. These were then washed in fresh Ringer's solution, mounted on microscope slides in liquid paraffin and examined with the fluorescent microscope for the presence of QFCs. In other experiments, animals were injected subcutaneously with quinacrine (10 mg per kg body weight) and then skin samples were removed at various times after the injection. It was found, however, that the background fluorescence was too high in samples removed 1-2 days after quinacrine injection to distinguish clearly the fluorescent cells in mounts of whole skin. The optimal time for observing QFCs in whole skin mounts was found to be 30 minutes to 1 hour after the injection; that is, skin removed and examined up to 1 hour after a sub-cutaneous injection of quinacrine displayed very little background fluorescence and the QFCs were clearly visible.

A typical example of the observed distribution of QFCs in a whole mount of adult Xenopus skin is shown in Fig. 21. In this micrograph, there are 8 clearly visible groupings of fluorescent cells, about 50-100 μm apart. The crescentic-shaped Merkel cells are arranged around the openings of the gland ducts. The gland duct itself is seen

Fig. 21. A fluorescent micrograph of quinacrine fluorescent cells (QFCs) in Xenopus skin.

This is a fluorescent micrograph of a whole mount of skin from an adult frog that was injected with quinacrine (processed as described in the Methods). Eight groupings of crescentic-shaped quinacrine fluorescent cells (QFCs) are shown around gland openings (marked G). The white arrowheads indicate the individual fluorescent cells. The large black areas are pigment cells.

The inset shows one grouping of fluorescent cells at slightly higher magnification.

Calibration bar: 20 μm


G
G
G
G
G
G
G
G
20μ

as a dark circular area bounded by the QFCs; several of the openings (marked G) are visible in the photomicrograph. Many of the QFCs are apparently crescentic-shaped as seen more clearly in the inset of Fig. 21; this was confirmed by examination of the electron micrograph (Fig.19) of skin sectioned en face, which shows two long cresentic Merkel cells curving around the gland duct. In cases where there are only one or two QFCs around the gland opening, the cells are 15 to 40 µm long; when there are three or more QFCs around the duct, they tend to be somewhat smaller, about 10-15 µm long. The QFCs are generally about 10-20 µm away from the duct opening, which agrees with the EM observations of the location of the Merkel cells.

The assumption made in these experiments is that the epidermal QFCs are identical with the Merkel cells; the validity of this assumption arises from several observations. The crescentic-shaped fluorescent cells occur only in the epidermis, confirmed by their presence in epidermis (but not in the dermis) of skin that had been enzymatically split. EM examination of the skin also showed Merkel cells only in the epidermis, in the same location and with the same shape as the fluorescent cells (compare Fig. 19 and 21). Although there are other cells in the skin that can take up quinacrine, e.g., mast cells, these are not present in the epidermis; thus there is no ambiguity as to the location and identification of the Merkel cells. As mentioned earlier, Crowe and Whitear (1978) also concluded that in Xenopus skin the quinacrine fluorescent cells are Merkel cells, and Nurse et al. (1983b) provided direct evidence on the identity of the

two. It seems safe to conclude then, that the QFCs seen in Xenopus epidermis in this investigation are indeed Merkel cells.

3. Side to side comparison of QFC distribution and density:

The quinacrine fluorescent technique for visualizing the Merkel cells offers an enormous advantage over EM examination of the skin, in that numbers and distribution of the Merkel cells can be easily quantified. This approach has been used in the present study to compare the effect of denervation on Merkel cell survival and also to monitor the appearance of Merkel cells in regenerating skin (see Part III).

Since the left and right hind limbs of the animals were to be used as control and experimental limbs, respectively, it was necessary to determine whether there was any intrinsic side to side variation in either the density or distribution of the Merkel cells. The distribution, that is the number of QFCs around each gland opening (GO), was determined by examining whole mounts of skin with the fluorescent microscope and recording the number of GOs with one, two, three or four QFCs around them, and then plotting this information as a histogram. The density of Merkel cells was determined by counting the total number of QFCs in the skin being examined and expressing the value as QFC per mm^2 skin area.

The results (from 14 animals) are presented in Fig. 22 and Table 1. There is no significant difference in either the distribution (using the Kolmogorov-Smirnov test) or the density (using the Student's

Fig. 22. Distribution of QFCs around the gland openings (GO) in Xenopus skin.

Areas of skin from the left and right hind limbs of control animals injected with quinacrine (10 mg/kg) one to two hours earlier were examined with the fluorescent microscope for the distribution of QFCs around the GOs. The histograms show the frequency of occurrence (% of the total observed) of GOs that were associated with 1, 2, 3 or 4 QFCs. Each pair of histograms shows the distribution of QFCs/GO in the left (L) and right (R) hind limb skin sample; the results from six different animals are presented. There was no significant difference between the left and right sides (using the Kolmogorov-Smirnov test, $P>0.1$).

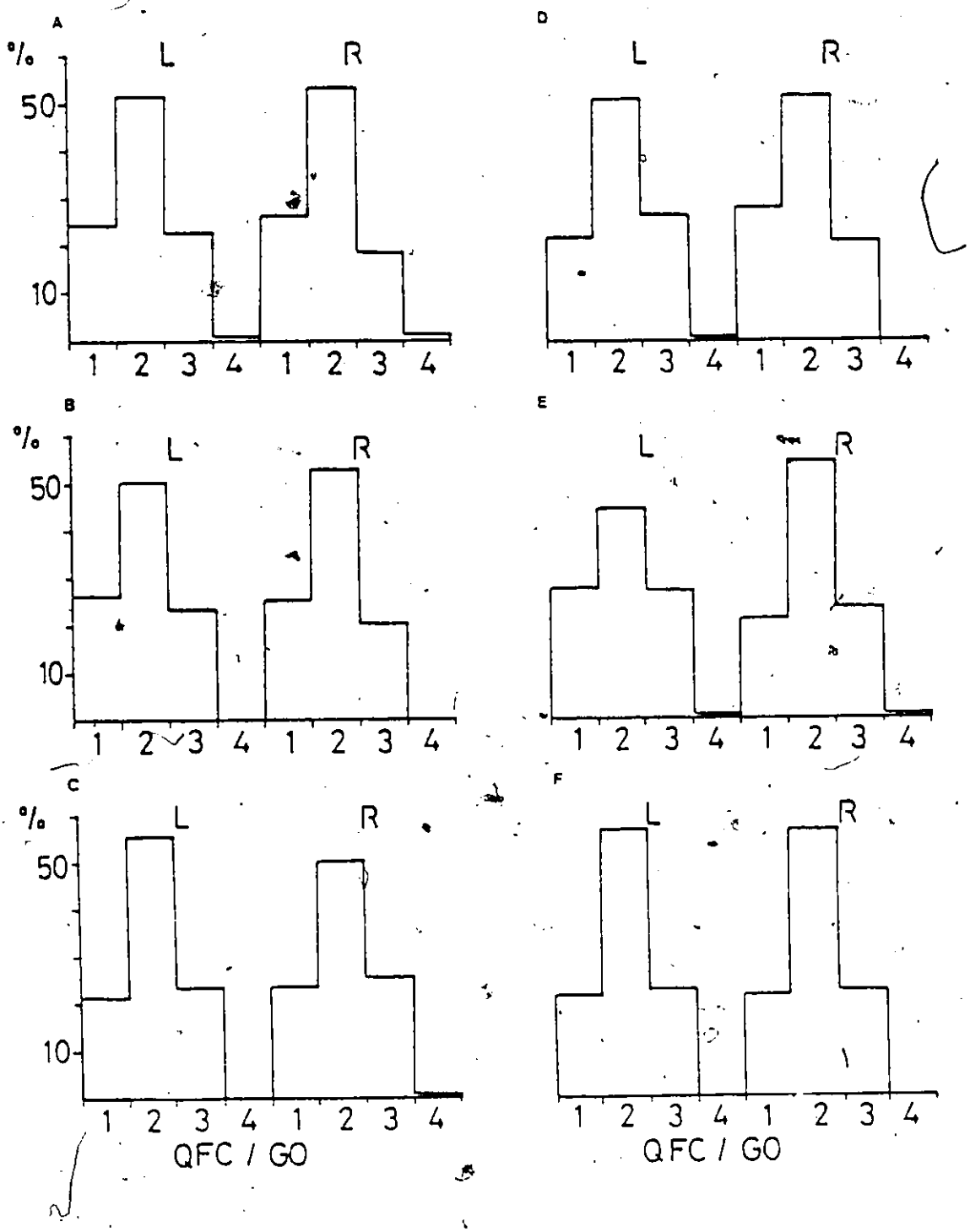

A
%
L
R
50
10
1 2 3 4 1 2 3 4
D
L
R
1 2 3 4 1 2 3 4
B
%
L
R
50
10
1 2 3 4 1 2 3 4
E
L
R
1 2 3 4 1 2 3 4
C
%
L
R
50
10
1 2 3 4 1 2 3 4
QFC / GO
F
L
R
1 2 3 4 1 2 3 4
QFC / GO

Table 1. Side to side comparison of QFC density in normal animals.

Animal #	QFC Density (QFC/mm^2 skin area) Left Side	Right Side	Ratio X_L/X_R
1	161.1	161.5	0.997
2	166.4	165.7	1.000
3	187.7	190.8	0.984
4	143.7	154.9	0.928
5	110.9	116.7	0.950
6	130.4	127.5	1.023
7	139.0	149.7	0.929
8	110.6	120.3	0.919
9	171.0	149.2	1.146
10	119.8	127.4	0.940
11	172.9	184.8	0.936
12	134.9	130.9	1.030
13	171.4	166.2	1.031
14	189.2	185.3	1.021
$\bar{X}$	150.6	152.2 *	0.988
SEM	7.2	6.7	1.65×10^{-2}
n	14	14	14
SD	27.08	24.9	6.17×10^{-2}
CV (SD/$\bar{X}$)	0.18	0.16	0.06 **

* There is no significant difference between the left and right side QFC density ($P > 0.1$, using the Student's t test).

** The coefficient of variation (CV) indicates that there is less variation between the left and right side of the same animal, than there is between animals.

t test) of Merkel cells, when right and left sides are compared. Although the density of QFCs between animals is rather variable, the side to side variation in any one animal is quite small,as can be seen from an examination of the ratio of QFC density in the left side to that of the right side (Table 1). Comparison of the coefficient of variation (SD/mean, Brown and Hollander, 1977) shows that the variation between animals is larger (0.18 for the left side, 0.16 for the right side) than the side to side variation in any one animal (CV = 0.06). Therefore, the left side can be used as a control for the experimental right limb with respect to this parameter, as well as the physiological parameters described earlier.

Part II B. Correlation of physiological and morphological observations:

1. Epidermal location of the low-threshold, rapidly-adapting mechanoreceptors:

The low-threshold rapidly-adapting receptors could be localized, in this instance to a particular part of the skin; these receptors were present only in the epidermis. In these experiments the epidermis was removed by scraping (as originally done by Adrian et al., 1931) or by enzymatic treatment of the skin with trypsin. The presence of the rapidly-adapting, low-threshold receptors was determined using the physiological mapping technique as described earlier. The pooled results of 6 such experiments in which trypsin was used to remove the epidermis are presented in Fig. 23. After removal of the epidermis, it is apparent that there were no low-threshold responses remaining in the dermis. However, high- threshold responses could be obtained in these same preparations. These may represent the response of dermal endings which are responsible for nociceptive reflexes (Adrian et al., 1931).

It was therefore concluded that the low-threshold, rapidly-adapting mechanoreceptors are confined to the epidermis, in agreement with other results obtained for the frog (Adrian et al., 1931) and the salamander (Cooper and Diamond, 1977).

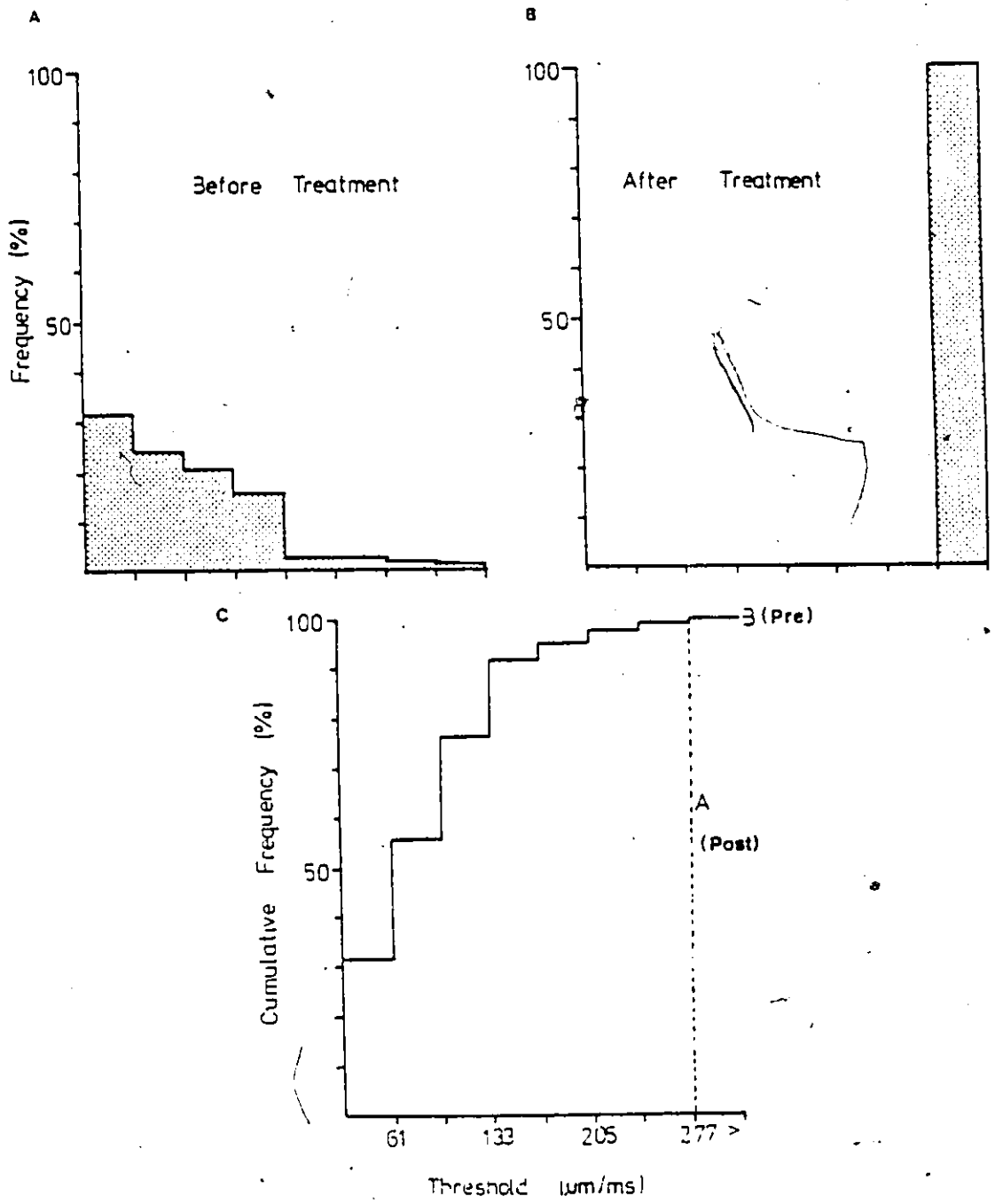
2. Coincidence of touch spot locations with the Merkel cells:

The preceding sections have shown that i) the low- threshold,

Fig. 23. Loss of low-threshold receptors after removal of the epidermis with trypsin.

The epidermis was removed from skin patches in situ, or from isolated skin-nerve preparations, following incubation of the area of skin with 0.1% trypsin. The treated area was mapped physiologically in a systematic manner with the prodder prior to, and after, the treatment, and the distribution of thresholds (pooled from six experiments) is presented as histograms. (A) is the distribution of critical stimuli before treatment, and (B) is the distribution of critical stimuli, for the same areas of skin, after the epidermis was removed. The abscissae in A,B and C are the same and represent the threshold range.

The cumulative frequency curves constructed from the data in the histograms are presented in (C). The two curves are significantly different ($P<0.001$, using the Kolmogorov-Smirnov test). The post-treatment result is effectively that of a totally high-threshold population of receptors, although it should be noted that many of the points tested post-treatment were non-responsive. B (——) represents the cumulative distribution prior to treatment; A (- - -) is the cumulative distribution after treatment.


A
100
50
Frequency (%)
Before Treatment
B
100
50
After Treatment
C
100
50
Cumulative Frequency (%)
B (Pre)
A
(Post)
61
133
205
277 >
Threshold (um/ms)

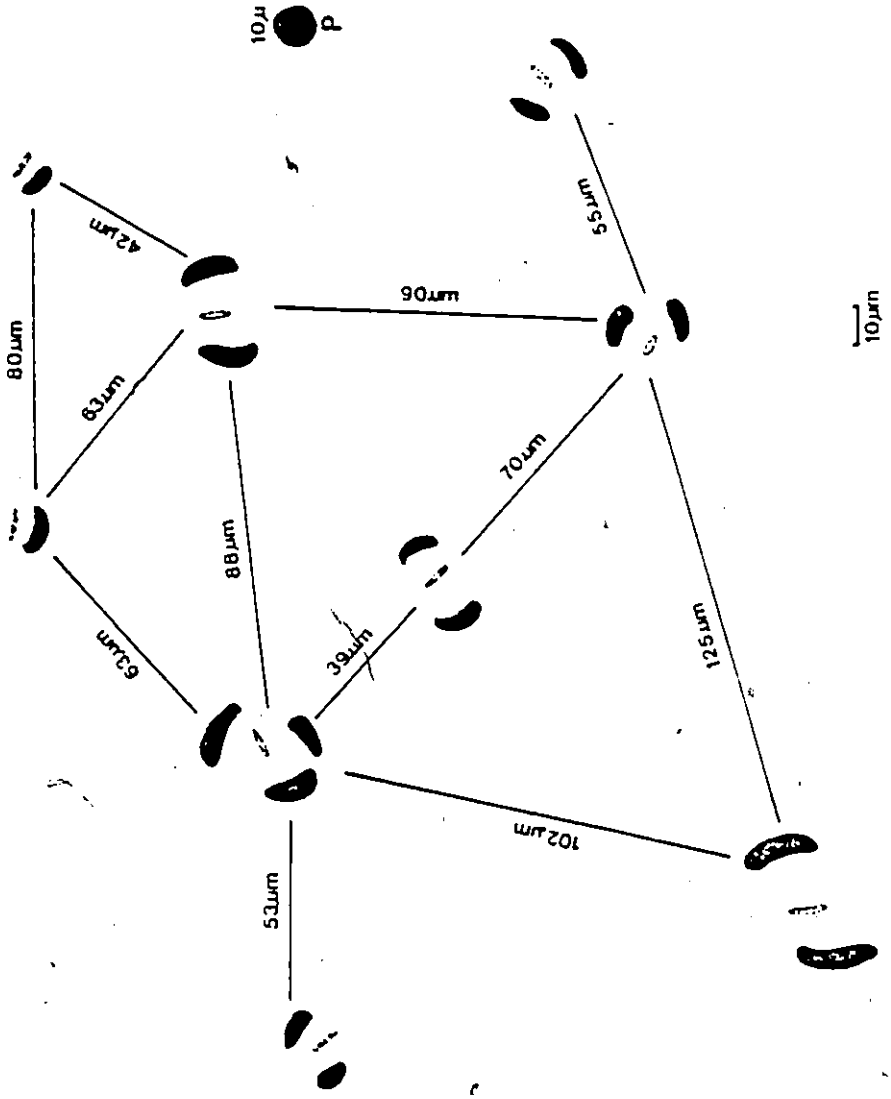
rapidly-adapting touch spots are restricted to the epidermis, and ii) that the Merkel cells are also exclusively located in the epidermis, in a ring around the gland openings. Knowing this, and the fact that the Merkel cells are associated with mechanosensitive areas in other species (e.g., Iggo and Muir, 1969; Horch et al., 1974; Parducz et al., 1977), it was of interest to determine whether the location of the touch spots and that of the Merkel cells could be correlated more quantitatively.

If the proposal that the Merkel cells are associated with the very sensitive mechanoreceptors is true, then the areas where these cells are located, the gland opening areas, should be the most sensitive to mechanical stimulation; that is, they should be coincident with the touch spots, defined as the low-threshold mechanosensitive points. To test this hypothesis, areas of skin were physiologically mapped essentially as previously described, but the systematic approach was modified; the 10 μm tip prodder was placed either directly over a gland opening, (these are 50-100 μm apart, and are visible with the dissecting microscope used on the recording set up), or slightly to one side of it. The critical stimulus required to evoke an impulse at each point was recorded. The small diameter prodder tip made it possible to stimulate between the gland opening areas without being right over the Merkel cell 'rings'. Essentially, then, the objective was to place the prodder more or less directly over the groups of Merkel cells and then in between the groups, in order to see which areas corresponded to the physiologically described touch spots (see Fig. 24 for a sketch of the

Fig. 24. The distribution of QFCs in a piece of Xenopus skin.

This is a drawing traced directly from a fluorescent micrograph of the distribution of QFCs in a sample of Xenopus skin (as in Fig. 21). The filled profiles represent the QFCs arranged around the gland openings. The measured distance between the these groupings of cells is shown; also shown to scale is the 10 μm prodder tip (P). This figure indicates that the prodder tip can be easily positioned between the gland openings, or directly on the openings.

Calibration bar: 10 μm


10µ
42µm
55µm
90µm
80µm
63µm
70µm
88µm
63µm
39µm
125µm
102µm
53µm
10µm

spatial arrangement of the glands and the relative size of the prodder tip).

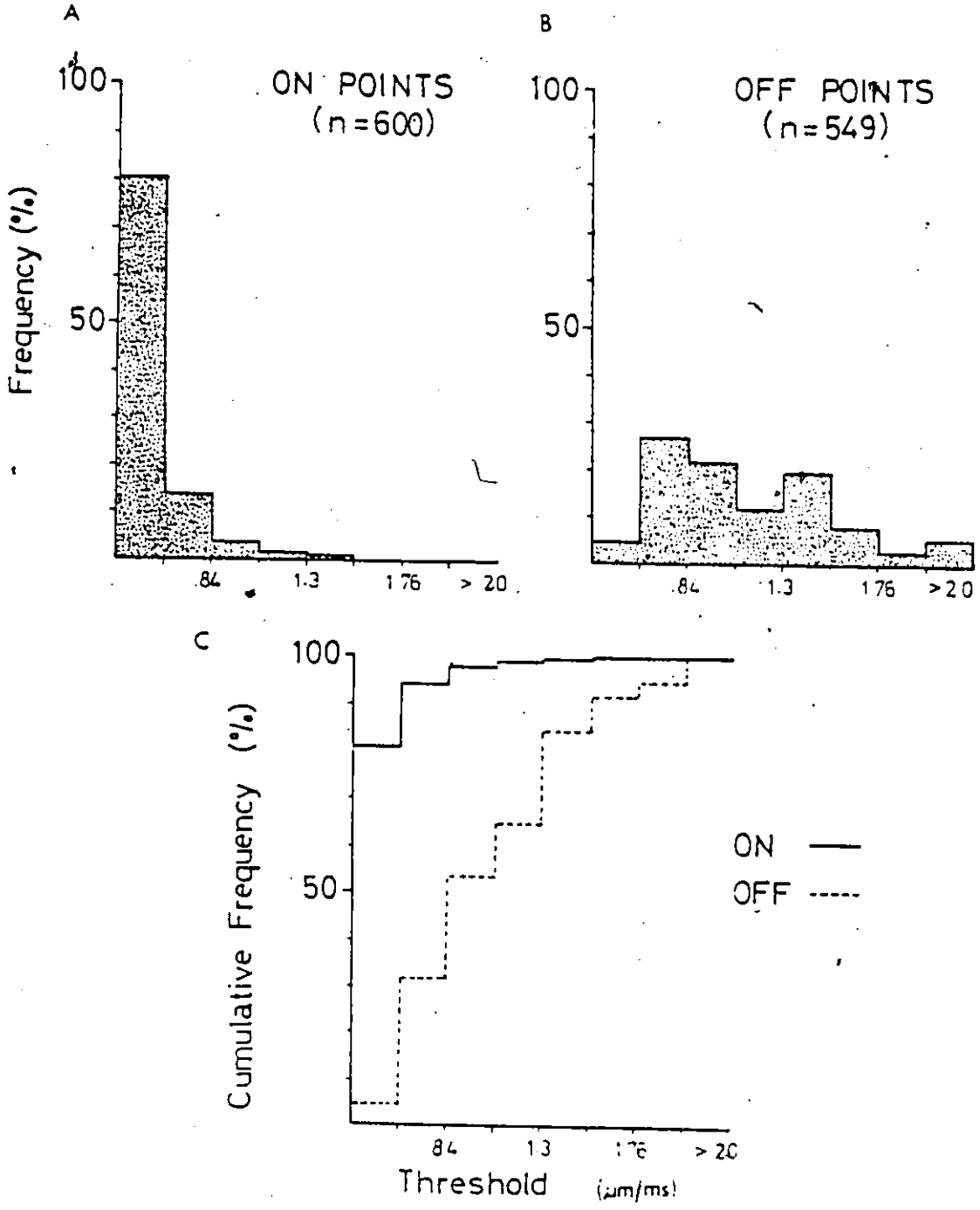
The pooled results of 12 experiments are shown in Fig. 25. It can be seen that the lowest thresholds were always obtained when the prodder was directly over the gland openings ("on" points). Higher, and somewhat more variable, values were obtained when the prodder was off to the side of, or between, the gland openings ("off" points). These results strongly support the hypothesis that the Merkel cells are associated with low-threshold mechanoreceptors in Xenopus skin. From examination of the data it was observed that moving the prodder tip 25-60 μm away from the "hot spot" increased the threshold by 1.5-4 times. These results are similar to those described in a previous section (Part I, section 8), where the prodder was moved away from a sensitive spot into non-responsive skin (adjacent to that supplied by the nerve recorded from) in 90 μm step. In that experiment, a 90 μm step away from the hot spot resulted in a 2-5 times increase in the threshold required to excite the same receptor, now 90 μm away. In the present experiment, then, the higher threshold points may well indicate the increase in threshold required to excite the adjacent low-threshold receptors. The simplest hypothesis to account for these observations is that there are no high-threshold receptors, but rather, that the results are consistent with there being a single population of low-threshold receptors, located at the gland openings, where the Merkel cells are found.

If this reasoning is correct, then maps that were done

Fig. 25. Comparison of the distribution of thresholds for "on" and "off" points.

Areas of skin were physiologically mapped by placing the prodder directly over the gland opening area ("on" points), or between the gland opening areas ("off" points), as described in the text. The pooled results of 12 experiments are presented as histograms showing the frequency of occurrence (as % of the total) of the indicated threshold ranges for the "on" points and the "off" points. The range of the low-threshold bin for the "on" distribution was 0.38-0.61 μm/ms.

The cumulative frequency curves constructed from the data in the histograms are presented in (C). The curves are significantly different ($P<0.001$, using the Kolmogorov-Smirnov test).


A
ON POINTS
(n=600)
100
50
Frequency (%)
.84
1.3
1.76
> 20
B
OFF POINTS
(n=549)
100
50
.84
1.3
1.76
>20
C
100
50
Cumulative Frequency (%)
ON
OFF
84
1.3
> 20
Threshold
(µm/ms)

systematically (90 μm steps) without determining whether the points surveyed were "on" or "off", should give a threshold distribution that is somewhere in between the "on" and "off" profiles. Fig. 26 presents the results of an experiment carried out to test this proposal. In this survey, first the "on" and "off" points were mapped, and the critical stimuli recorded. Then, in the same area, a systematic survey, in which the prodder was moved in 90 μm steps in a way that did not select for the "on" or "off" points, was done. The cumulative frequency curve constructed from this survey presents a distribution (Fig. 26) which is intermediate to that of the "on" and "off" areas.

From all these results, it can be confidently concluded (i) that there is only one population of mechanoreceptors in Xenopus epidermis, (ii) that the first bin of the threshold histograms represents the frequency with which the stimulator lands directly on the receptors, while the other bins represent the frequency with which the prodder falls off to the side or between receptors, and (iii) that the "on" positions are indeed identical with the the GO and Merkel cell locations.

Using the information from these experiments, the spacing of the receptors can be determined and the size of the receptive fields can be estimated. The spacing of the mechanoreceptors is the same as that of the gland openings, which are 50-125 μm apart. The radius of the receptive fields can now be estimated using the analysis described in Part I, section 8; using values for D taken from actual measurements of the distance between gland openings, and the range of values for x

Fig. 26. Comparison of the distribution of thresholds obtained using a systematic survey with an "on-off" survey.

An area of skin was first mapped physiologically by placing the prodder tip directly "on" the gland openings, or "off" to the sides of the gland openings. The same area of skin was then mapped systematically by moving the prodder in 90 μm steps, without regard to whether the prodder was situated "on" or "off" the gland openings ("systematic" mapping).

The histograms of threshold distribution for the "on" and "off" points are presented in (A) and(B); (C) is the histogram for the systematic survey. The cumulative frequency curves constructed from the data in the histograms are presented in (D). The abscissae in all the figures represents the threshold range. The systematic cumulative frequency curve (- - -) is significantly different from the "on" curve ($P<0.01$) and from the "off" curve ($P<0.001$). The low-threshold bin range is 0.5 to 1.0 μm/ms.

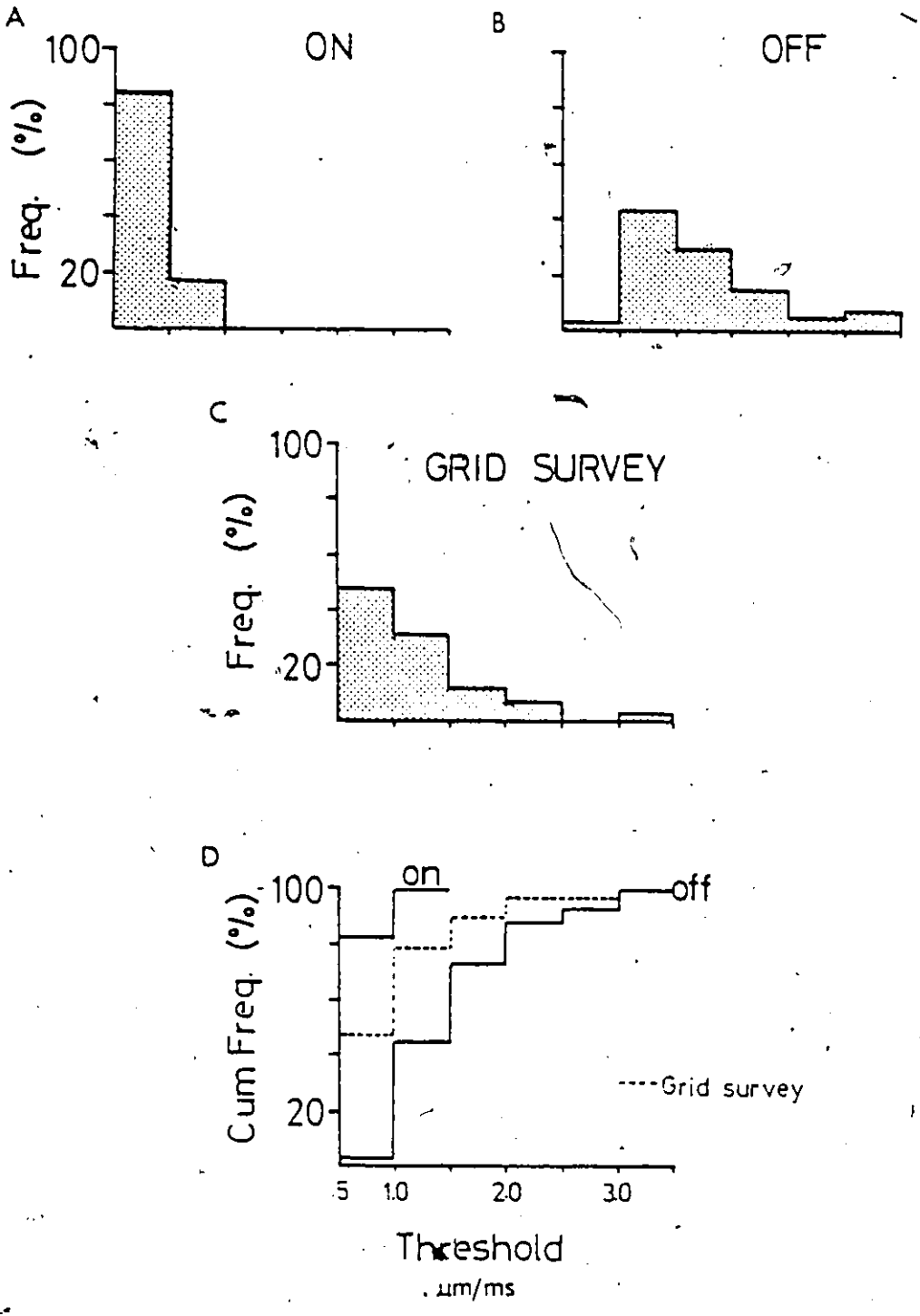

A
ON
100
Freq. (%)
20
B
OFF
C
GRID SURVEY
100
Freq. (%)
20
D
on
off
100
Cum Freq. (%)
20
Grid survey
.5
1.0
2.0
3.0
Threshold
. µm/ms

Table 2. Values of r (radius of receptive field) calculated from equation 1*, for values of x and D as determined from experimental data.

	D***			
x**	125	100	75	50
0.30	38.6	31.0	23.3	15.5
0.40	44.6	35.7	26.7	17.8
0.50	49.9	39.9	29.9	19.9
0.55	52.3	41.8	31.4	21.0
0.60	54.6	43.0	32.7	21.8
0.70	59.0	47.2	35.4	23.6
0.80	63.0	50.5	37.8	25.0

* equation 1 $xD^2 = \pi r^2$, unit of r = μm

** x = the proportion of area covered by receptive surface, determined from the range of the heights of the first bin of the frequency histograms of threshold distribution.

*** D = the distance between the centers of the receptive fields, determined from the actual spacing between the gland openings (in μm).

obtained in the present experiments (30%–80%), the radius r can be calculated by solving equation 1 (described in Part I, section 8, and Cooper and Diamond, 1977). The calculated values for r are presented in Table 2, for ranges of D and x. The radius of the receptive fields of these mechanoreceptors, ranges from 15 μm to 63 μm. An average value of r, for mid- range values of x and D, is 37 μm.

An interesting possibility is that the range of threshold values observed for the "on" points, although quite narrow compared to the "off" points, might be related to the number of Merkel cells around the gland openings, or "on" spots. For example, the lowest thresholds might be associated with touch spots that have four Merkel cells; the very low threshold points are relatively rare as are the number of gland openings with four Merkel cells around them. If all the Merkel cells at one gland opening were supplied by branches of the same axon, it might be easier to excite an impulse in an axon connected to four Merkel cells than to one Merkel cell, given that the stimulus position and strength are the same. The mid-range threshold values could be associated with touch spots that have two or three Merkel cells and the highest thresholds (of the low threshold range) might be from touch spots with only one Merkel cell. Using the quinacrine method, it should be possible in future experiments to test this possibility directly, by making a correlative map of thresholds and distribution of Merkel cells around the gland openings.

3. Summary Discussion:

The fluorescent dye, quinacrine, was used to visualize Merkel cells in whole mounts of intact skin or epidermis which had been enzymatically separated from the dermis. The QFCs can now be accepted in the vertebrate epidermis as being a genuine marker for Merkel cells (Nurse et al., 1983b); in Xenopus these were found only in the epidermis, and were characteristically arranged in a ring of 2–4 cells around the numerous cutaneous gland openings. EM examination of Xenopus skin confirmed that the Merkel cells were found in the same location as the QFCs.

Comparison of the Merkel cell distribution and density from the left and right hind limb skin indicated that the side to side variation in these parameters was negligible.

The physiological characteristics of the touch spots in Xenopus were previously presented in Part I. In Part II, it has been shown that the locations of the touch spots were coincident with those of the gland openings, and thus of the Merkel cells. Thus it is reasonable to conclude that the Merkel cells are associated with mechanoreception in Xenopus skin.

Merkel cell-neurite complexes have been shown to be associated with mechanoreceptive areas in other animals , for example, the mammalian touch domes and vibrissae (Iggo and Muir, 1969; Gottschaldt and Vahle-Hinz, 1981), and the salamander touch spots (Cooper and Diamond, 1977; Parducz et al., 1977). The role of the Merkel cells in the mechanoreceptive complex is not clear. Whether the Merkel cells act as transducers of the mechanical stimulus is unknown, although it

has been suggested that this is unlikely, at least with respect to the mammalian sinus hair follicle (Gottschaldt and Vahle-Hinz, 1981). The Merkel cells could, however, act in some way to modify the nature of the response of the nerve ending, itself the transducer, or as does the capsule of the Pacinian corpuscle, to facilitate the transfer of the mechanical stimulus to the nerve ending. Another possibility (not exclusive of those just mentioned) is that the Merkel cells are involved in trophic interaction with the nerves. Merkel cells have been shown to act as targets for growing cutaneous nerves in the salamander (Scott et al., 1981). For other animals, there is evidence, which indicates that the nerves may be necessary for the development and maintenance of the Merkel cells, suggesting that the Merkel cells may not function as targets in the same way as they do in the salamander (Brown and Iggo, 1963; English, 1977; Saxod, 1980; English et al., 1980; Nurse et al., 1983a). It is also possible that the Merkel cells may act to promote the maturation of the growing or regenerating cutaneous nerve endings to their characteristic mechanosensitive state.

The following sections (Part III) describe experiments undertaken in an attempt to clarify the role of the Merkel cell in the morphological complex it forms with the nerve endings.

Part IIIA. The role of the Merkel cell; Can it act as a target?

In addition to possible physiological interactions, it seems likely, as just discussed, that the Merkel cells and nerves are involved in trophic interactions as in the salamander (Scott et al., 1981). However, it cannot be assumed that the situation in Xenopus must be exactly like that in the salamander; it could, for example, resemble that in mammals and birds, in which the appearance of Merkel cells seems to be dependent upon the presence of nerves (English et al., 1980; Saxod, 1980). In this study, the possibility that the Merkel cell in Xenopus could act as a target during development, and particularly when it is denervated, was an attractive one. The following sections present results from experiments designed to investigate the target role of the Merkel cell in Xenopus skin.

1. Merkel cells survive denervation:

The right hind limbs of animals were denervated by sectioning and tying off all the nerves supplying the limb, as described in the Methods. These animals were then examined at various times subsequent to nerve section. At these times the animals were first examined for any reflex response to pinching or scratching of the denervated limb; in contrast to normally-innervated animals, none of the denervated animals responded to such stimuli, indicating that the limb was still denervated. The animals were injected with quinacrine (10mg per kg body wt.) 30 minutes to 1 hour prior to skin removal, and then

anaesthetized. A small piece of skin was removed from each hind limb, mounted on a microscope slide in liquid paraffin and examined with the fluorescent microscope for the presence of quinacrine fluorescent cells (QFCs), or Merkel cells. The left hindlimb served as the normally-innervated control for the denervated right limb. The skin samples were then examined for the distribution of QFCs around the gland openings by recording the number of gland openings with one, two, three or four QFCs around them; in addition, the density of QFCs was determined by counting the total number of QFCs in the sample and expressing the value as QFC per mm^2 skin area. Skin samples from animals whose right hind limb had been denervated for 4 weeks up to 30 weeks were examined.

The results of these measurements are presented in Fig. 27 and Table 3 . The distribution of QFCs around the gland openings is plotted as a histogram in Fig. 27, comparing left and right sides at various times of denervation. There is no significant difference between the control and denervated distributions at any of the sampled times after denervation, as determined using the Kolmogorov- Smirnov test to compare the distributions. Table 3 gives values for the density of QFCs in each of the samples; these values were derived from the histograms by taking the total number of QFCs counted for each histogram and dividing that number by the area of the skin sample. When the control densities are compared to the experimental densities at each time after denervation, there does not appear to be any difference between them. The possibility that following denervation

Fig. 27. Distribution of QFCs around the gland openings in control and denervated Xenopus skin.

Skin samples from denervated (right) and the contralateral (left) control hind limbs were examined for the distribution of QFCs at various times of denervation (described in the text). The skin samples from animals injected with quinacrine were examined with the fluorescent microscope and the number of gland openings (GOs) with 1, 2, 3 or 4 QFCs were recorded, and the information displayed in histograms. Each pair of histograms shows the control (on the left) and the denervated (on the right) QFC distribution for various times in weeks (the numbers above the histograms) after denervation. The abscissae and ordinates are the same for all the histograms.

The control and experimental distributions were compared (at each denervation time) using the Kolmogorov-Smirnov test, and there was no significant difference between the control and experimental distributions at any denervation time.

Distribution of Merkel cells (QFC's) around the gland openings (GO) in control (left) and denervated (x wks, right) Xenopus hind limb skin

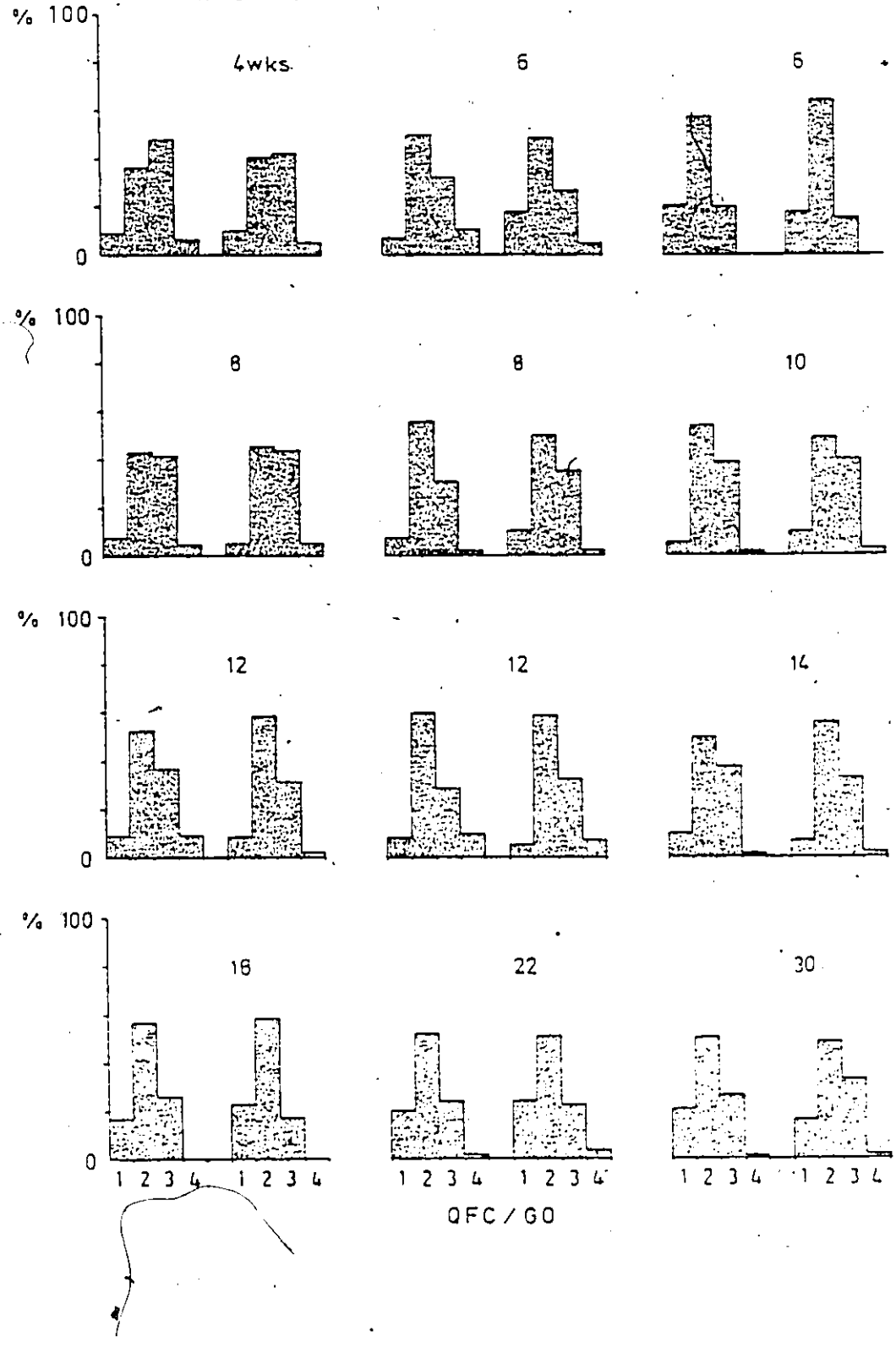


Table 3. Distribution of Merkel cells (QFCs) following denervation of the skin.

Weeks after Denervation	QFC/mm^2 * Control	Denervated	Ratio++ X_C/X_D	Diff.+ Dist.	QFC/GO** Control	Denervated
4	101.7	109.8	0.926	NS	2.5	2.4
6	117.9	111.2	1.060	NS	2.5	2.2
6	107.1	110.8	0.967	NS	1.9	1.9
8	93.2	93.6	0.996	NS	2.2	2.5
8	100.9	105.1	0.960	NS	2.2	2.5
10	102.7	102.1	1.006	NS	2.4	2.3
12	102.2	102.1	1.001	NS	2.3	2.3
12	99.7	103.0	0.968	NS	2.3	2.3
14	117.3	111.2	1.054	NS	2.3	2.3
16	60.0	62.7	0.957	NS	1.8	2.1
18	146.4	157.9	0.927	NS	2.1	1.9
22	102.3	98.2	1.041	NS	2.1	2.0
30	176.4	173.8	1.015	NS	2.3	2.2

* The values for QFC density are derived from the histograms of the distribution of QFCs/GOs, presented in Fig. 27. These values were obtained by summing the total number of QFCs observed in the skin sample and dividing the sum by the sample area. Each value represents the density of QFC/mm^2 skin area in one animal at each time period.

** GO = gland opening

\+ Diff. Dist. This is the difference in the distributions of QFCs/GOs, as presented in the histograms of Fig. 27. The Kolmogorov-Smirnov test was used to determine whether there was any significant difference in the QFC distribution in the control and denervated limbs. NS indicates that there was no significant difference ($P>0.1$) between the two distributions.

++ The ratio of the control/denervated density indicates that there is little difference between the two sides of a given animal. These ratios can be compared with those in Table 1, for normal animals.

some Merkel cells disappear from both sides, control and denervated, was examined by comparing the control values (using whole skin samples) obtained in this series of experiments with those obtained for the normal control samples presented in Table 1. It can be seen that the control values from the denervated series are within the range displayed by the normal control animals (compare Tables 1 and 3). The ratio of the control QFC density to the experimental density (Table 3) compares favorably with the ratio of the QFC density in the left side to the right side observed earlier in Table 1; this indicates that there is a small side to side variation in QFC density in any one animal, even after denervation of one limb.

The conclusion drawn from these results is that the Merkel cells in Xenopus skin survive denervation quantitatively. As a point of interest, there is evidence that Merkel cells can survive, not only in the absence of direct nervous influence, but also in a situation where possible humoral influences are excluded from having any effects on promoting their survival. In preliminary tissue culture experiments, Merkel cells were found in skin explants cultured for up to 2 weeks in the absence of nerves or any exogenous growth factors (Mearow et al., 1981).

2. Merkel cells appear in regenerated skin:

One way of testing whether the development of Xenopus Merkel cells requires the presence of the sensory nerve (in the absence of carrying out a developmental study) is to examine the appearance of

Merkel cells in skin that is regenerating in a nerve- free condition, in this case in the denervated hind limb. Before doing these experiments, it was first necessary to determine whether Xenopus skin would regenerate at all in the normally innervated limbs, and whether Merkel cells would be found in such regenerated skin.

(a) Merkel cells appear in regenerated skin in the innervated limb:

In the first series of experiments, pieces of skin ($1-2\ mm^2$) were removed from innervated hind limbs. These pieces (initial skin samples) were incubated in 10^{-5}M quinacrine and then examined with the fluorescent microscope to determine the QFC density. Over the subsequent 6 week period, the skin that regenerated in the place of that initially removed was sampled and examined histologically, to obtain some idea of the progress of skin regeneration, and with the fluorescent microscope, (following incubation in quinacrine), to look for the presence of QFCs, and to determine their density.

Light microscope examination of silver-stained paraffin sections of regenerating skin showed that at 10 days there was a well organized layer of epidermis present, along with a rather disorganized dermal layer (Fig. 28a). The dermis appeared to begin to form by migration of dermal cells from the surrounding intact skin (Fig. 28b). At this time (10 days), nerve fibres were seen in the dermis. Three weeks was the latest time examined histologically, and the dermis, although still made up of primarily fibroblast-like cells, showed some evidence of organization, increasing in complexity (i.e., in normality)

Fig. 28. Photomicrographs of sections of regenerating skin in the innervated hind limb.

Samples of regenerating skin were processed for light microscopy, as described in the Methods, and stained using a modified Ungewitter's silver stain.

(A) and (B) are micrographs are sections of 14 day regenerating skin.

(A) shows an area of regenerating skin, with both epidermis (e) and a somewhat disorganized dermis (d) present; adjacent is an intact area of normal skin (i), in which the dermis has the characteristic organized appearance.

(B) is a higher magnification of the region at the border of the regenerating and adjacent normal skin, showing appearances suggestive of migration of dermal cells (arrow) in from the normal region to the regenerating region.
e-d, epidermal-dermal boundary; d, dermis; e, epidermis; i, normal dermis.

Calibration bar: (A), 50µm; (B), 20 µm

A

B

progressively inwards from the surrounding intact area.

At each sample period, the whole regenerated skin pieces were first examined with phase microscopy and photographed; the photographs were subsequently used to measure the regenerating area at each time period after the inital skin removal. In these experiments, a distinction was made between skin that was still in the process of regeneration (regenerating) and skin that had regenerated and was very similar in overy appearance to the normal surrounding skin. The regenerating skin was generally lighter in color, thinner and had few, if any, glands present. The regenerated skin seemed almost indistinguishable in terms of outward appearance (i.e., the presence of glands and QFCs) from the surrounding skin; complete progress of skin regeneration was not, however, followed histologically. Examples of the regenerated whole skin mounts are shown in Fig. 29 A and B; also shown are drawings of skin samples removed at 1-6 weeks following the initial skin excision to illustrate the method of measurement used to compare the regenerating area with the initial area removed. Results from one experiment are presented in Fig. 30A and Table 4. Over the 6 week period the area initially removed was almost totally (94%) replaced by regenerated skin. From examination of the whole mounts (Fig. 29B), the new skin looked very similar to the adjacent skin, with the glands also reappearing (Fig. 29). The reappearance of glands in the regenerating skin was not monitored quantitatively, but it was observed that these did eventually appear.

As mentioned above, the samples that were removed for

Fig. 29. Regenerated skin in the innervated hind limb.

Photomicrographs of whole mounts of skin removed at various times after an initial excision of an area approximately 1–2 mm^2 are shown in A (removed at 1 week) and B (removed at 6 weeks after the initial excision). C–H are drawings of whole mounts of skin samples removed at 1–6 weeks after the initial excision: C, 1 week (the same sample as in A); D, 2 weeks; E, 3 weeks; F, 4 weeks; G, 5 weeks; and H, 6 weeks (the same sample as in B).

The area enclosed by the outer solid line represents the size of the sample taken at each weekly interval. The area enclosed by the inner solid line represents the area of newly-regenerating skin. The area enclosed by the dashed line is an overlay of the area of the initially-removed skin piece. The progress of skin regeneration (see Fig. 30A) was estimated by taking the ratio (initially removed area - the regenerating area / the initial area) x 100%.

Calibration bar: 0.5mm

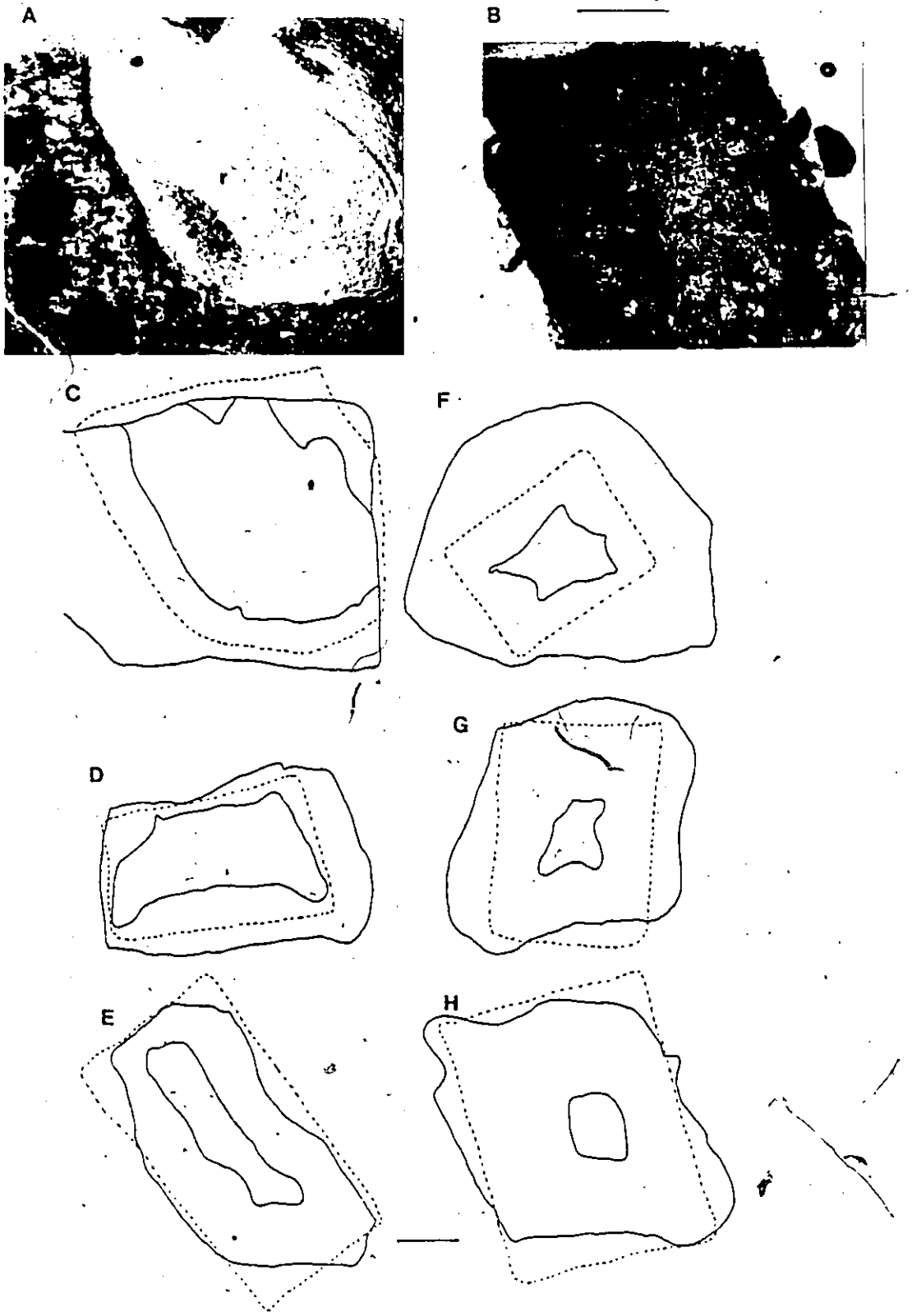

A
B
C
F
G
D
E
H

TABLE 4. Progress of skin regeneration over six weeks in the innervated hind limb

Week of Sample	Initial Area * Removed (mm^2)	Area of New Skin * at sampling time	I-R/I ** (x 100%)
1	1.83	1.12	38.8
2	0.78	0.44	43.6
3	1.41	0.23	83.7
4	0.69	0.17	75.4
5	1.01	0.09	91.1
6	1.65	0.10	94.0

* Values are calculated (in mm^2) from the drawings of the skin samples shown in Fig. 29, and represent the results from one experimental animal.

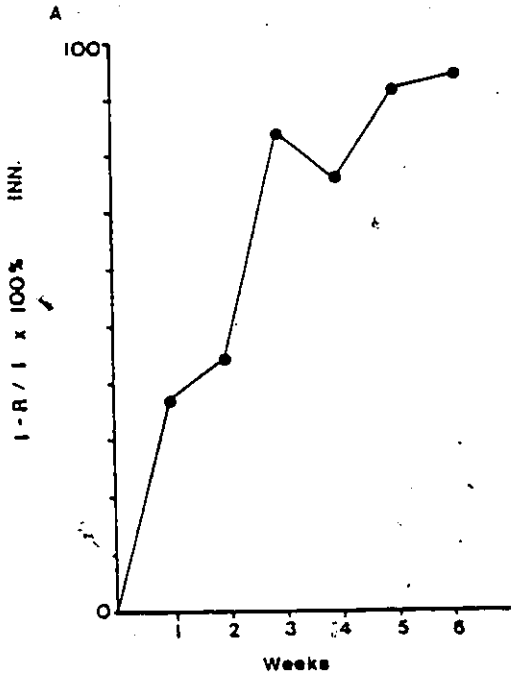
** This is the ratio of the (initial area removed - the new skin area/ the initial area), and indicates the progress of skin regeneration over the six week period.

Fig. 30. Progress of skin regeneration in innervated and nerve-free hind limbs.

The ratio of (the initial area removed - the regenerating area / the initial area) x 100% is plotted against the time after initial skin excision. (A) presents the results for the innervated hind limb (obtained from Fig 29, and Table 4).

(B) presents the same information (obtained from Fig. 32, and from Table 6)for the nerve-free limb.

The curves represent the increase in regenerated skin over the 6 week sampling period.


A
100
0
I - R / I x 100 %
INN.
1
2
3
4
5
6
Weeks

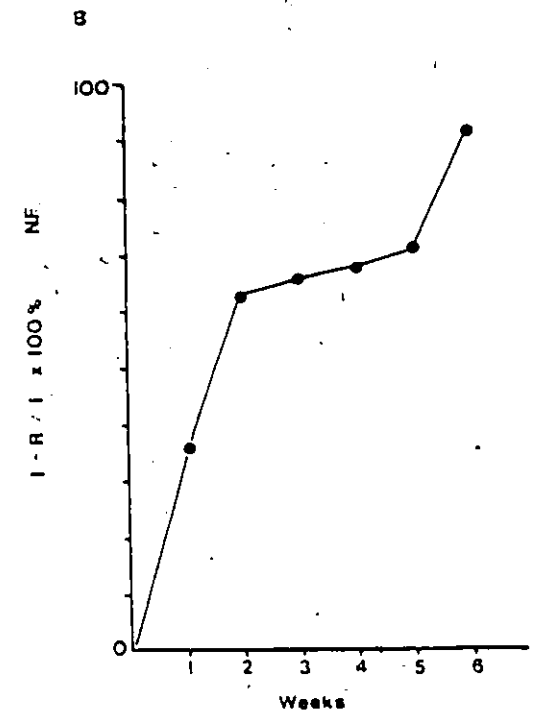

B
100
0
I - R / I x 100 %
NE
1
2
3
4
5
6
Weeks

TABLE 5. The appearance of Merkel cells in regenerated skin in the innervated hind limb.

Week of Regeneration	n	Regenerated QFC/mm^2	Initial QFC/mm^2	R/I ** (x100%)
1	3	17.1 ± 1.4	130.7 ± 10.3	13.1
2	7	27.7 ± 3.4	122.6 ± 7.2	22.6
3	7	52.5 ± 7.4	127.2 ± 5.7	41.3
4	3	77.8 ± 8.2	128.2 ± 12.8	60.7
5	7	69.2 ± 7.2	122.5 ± 4.6	56.5
6	7	114.2 ± 2.9 *	121.6 ± 6.0	93.9

* There is no significant difference between QFC density in the skin samples initially removed and the regenerated skin at six weeks ($P > 0.1$, using the Student's t test).

The QFC density measurements are given as mean ± SEM.

** This is the ratio of the regenerated QFC density / the initial QFC density.

regenerated skin area measurements were also incubated in quinacrine and then examined with the fluorescent microscope to determine QFC density. The values of QFC density in the regenerated samples (counted for an area of skin equivalent to that initially removed) are presented in Table 5, along with the initial QFC density for comparison. The number of QFCs appearing in the regenerated skin increases steadily over the six week examination period, rising to the initial values by the 6 week sample period. As mentioned above, the gland openings appeared gradually in the regenerating skin; QFCs were seen in regenerating skin in the absence of the glands, and were identified by their shape and epidermal location.

(b). The appearance of Merkel cells in regenerated skin in the nerve-free hind limb:

The same experiments described for the innervated hind limb were also performed on the denervated hind limbs.

Histological examination of silver-stained paraffin sections of denervated regenerated skin revealed that at one week there was an organized layer of epidermis present, but that dermal cells were not yet present (Fig. 31); in this Fig. the apparent migration of dermal cells from the surrounding intact area can also be seen. At 10 days of regeneration, the dermis looks very similar to that in the innervated limb, except that there were no nerve fibres observed.

Whole skin mounts were removed at each of the weekly sample times and were examined with light microscopy and photographed; the

Fig. 31. Photomicrographs of sections of regenerating skin in the nerve-free hind limb.

Samples of regenerating skin were processed for light microscopy, as described in the Methods and stained using a modified Ungewitter's silver stain.

(A) is a micrograph of a section of 7 day regenerating skin. At this stage, only the epidermis is present; dermal cells (d) appear to be migrating in (arrow) from the adjacent intact dermal region (i). g is a gland.

(B) is a micrograph of a section of 10 day regenerating skin. At this stage, there is more dermis, although rather disorganized, present (d); again, it appears that the dermal cells are moving in (arrow) from the adjacent intact skin areas (i). e, epidermis; d, dermis; i, normal dermis; g, gland.

Calibration bar: (A) 50μm; (B) 20μm

A

B

photographs were subsequently used to measure the area of the regenerating skin (Fig. 32), as described above. Estimates of the progress of skin regeneration, determined as above, are presented in Table 6 and Fig. 30B. The regenerated area in the nerve-free condition increased in area with a time course similar to that in the innervated limb. Over the 6 week period about 90% of the initial area removed was replaced by new skin. Again, the new skin appeared very similar to the surrounding area (see Fig. 32B).

The skin samples that were removed for the measurement of regenerating areas were also examined for the presence of QFCs, as described above. The results of the densitymeasurements of QFCs in the regenerated skin are presented in Table 7, and demonstrate that the QFCs, i.e., Merkel cells, do appear in the nerve-free regenerated skin. In addition, it is clear that the density of QFCs measured at 6 weeks in the new skin has recovered to the initial level determined at the time of skin removal.

In the regenerated skin samples examined both in the innervated as well as the nerve-free condition, some of the intact skin surrounding the regenerated area was also examined to determine whether there was any obvious depletion of QFCs from the surrounding intact skin. Although this was not studied quantitatively, there was no obvious depletion of QFCs in the surrounding skin. As it stands, the origin of the new Merkel cells in the regenerated skin is not known, but at least there is no evidence that they are migrating in from the surrounding area.

Fig. 32. Regenerated skin in the nerve-free hind limb.

Photomicrographs of whole mounts of skin removed at various times after an initial excision of an area approximately 1-2 mm^2 are shown in A (1 week) and B (removed at 6 weeks after the initial excision).

C-H are drawings of whole mounts of skin samples removed at 1-6 weeks after the initial skin excision: C, 1 week (and is the same sample as in A); D, 2 weeks; E, 3 weeks; F, 4 weeks; G, 5 weeks; and H, 6 weeks (the same sample as shown in B).

The area enclosed by the outer solid line represents the size of the sample taken at each weekly interval. The area enclosed by the inner solid line represents the area of newly-regenerating skin. The area enclosed by the dashed line is an overlay of the area of the initially-removed skin piece. The progress of skin regeneration (see Fig. 30B) was estimated by taking the ratio (initial area removed - the regenerating area / initial area) x 100% at each week.

Calibration bar: 0.5mm

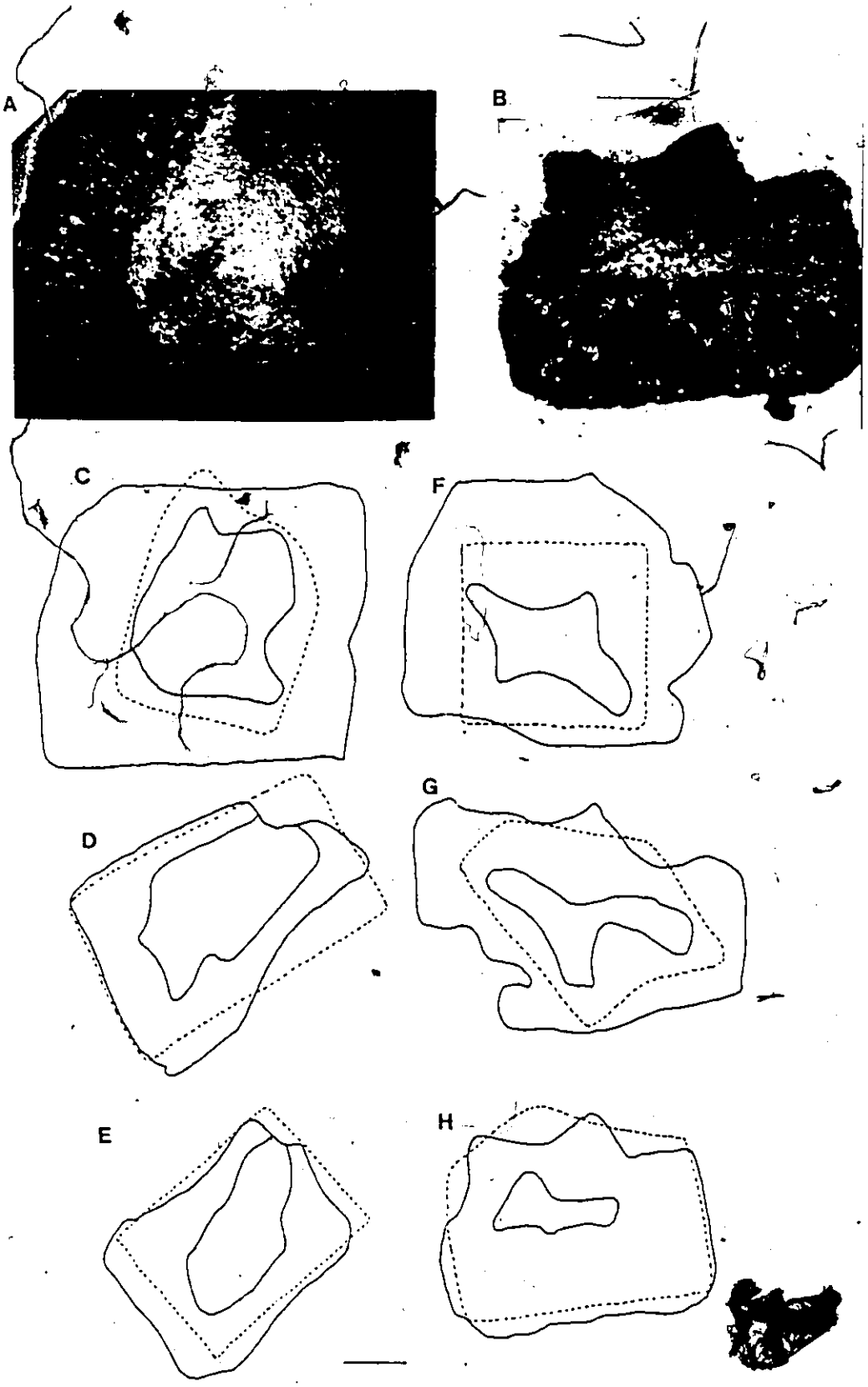

A
B
C
F
D
G
E
H

TABLE 6. Progress of skin regeneration over six weeks in the nerve-free hind limb

Week of Sample	Initial Area* Removed (mm^2)	Area of New Skin* at sampling time	I-R/I** (x 100%)
1	0.99	0.63	36.2
2	1.37	0.51	62.7
3	0.88	0.31	64.7
4	0.94	0.31	67.0
5	0.86	0.26	69.8
6	1.25	0.12	90.4

* Values are calculated (in mm^2) from the drawings of the skin samples shown in Fig. 31, and represent the results from one experimental animal.

** This is the ratio of the (initial area removed - the new skin area/ the initial area), and indicates the progress of skin regeneration over the six week period.

TABLE 7. The appearance of Merkel cells in regenerated skin in the nerve-free hind limb.

Week of skin Regeneration	n	Regenerated QFC/mm^2	Initial QFC/mm^2	R/I ** (x100%)
1	3	14.6 ± 2.1	111.5 ± 8.6	13.1
2	7	34.9 ± 6.9	123.6 ± 7.4	28.2
3	7	56.2 ± 5.2	119.7 ± 5.9	46.9
4	3	75.4 ± 9.7	118.1 ± 12.5	63.8
5	7	60.3 ± 4.9	125.4 ± 12.1	48.1
6	7	119.8 ± 7.3 *	114.1 ± 5.2	105.0

* There is no significant difference between QFC density in the skin samples initially removed and the regenerated skin at six weeks ($P > 0.1$, using Student's t-test).

The QFC density measurements are given as mean ± SEM

** This is the ratio of the regenerated QFC density / the initial QFC density.

3. Loss of mechanosensitivity following nerve section:

In the following section the recovery of mechanosensitivity following nerve regeneration and reinnervation of the Merkel cells is described. Prior to carrying out these experiments, however, a study of the time course of the loss of mechanosensitivity after nerve section was carried out, in part to discover the earliest time when the recovery of function due to regeneration of cut fibres could be determined unambiguously. It was also hoped that the physiological changes following nerve section could be ultimately correlated with morphological changes at the Merkel cell-neurite complex (see Part III, section 3).

To monitor the loss of sensitivity, all three spinal nerves serving the right hind limb were sectioned and tied off, as described in the Methods, and recordings were made from the distal stumps of the nerves still attached to the skin at various times afterwards (0-144 hrs.) to evaluate the responses to stimulation of the skin, with either the prodder or by brushing. It was found that mechanosensitivity was lost by 4-5 days after cutting the nerves; that is, by 4-5 days there was no recorded response to either prodding or brushing the skin. The sensitivity decreased in the following manner. Initially there was a change from low-threshold mechanosensitive points entirely to higher-threshold points, as though the Merkel cell-neurite complexes became high-threshold receptors during the early period following nerve section. This stage was then followed by one in which there was a

response only to brushing of the skin, and finally there was a complete loss of sensitivity to any mechanical stimulus. The results of the physiological maps that were done during this experiment are presented as histograms and cumulative frequency curves in Fig. 33. Although the nerve endings were no longer responsive to mechanical stimuli by 4-5 days after nerve section, the nerve trunks could still conduct impulses for up to 10 days; this was observed in two animals and is similar to observations made in the salamander (M. Holmes, personal communication).

It, thus, appears to take about 5-10 days for the nerves to become non-functional, and presumably to begin degenerating.

4. Return of mechanosensitivity and mechanoreceptors after nerve regeneration to:

(a) denervated skin:

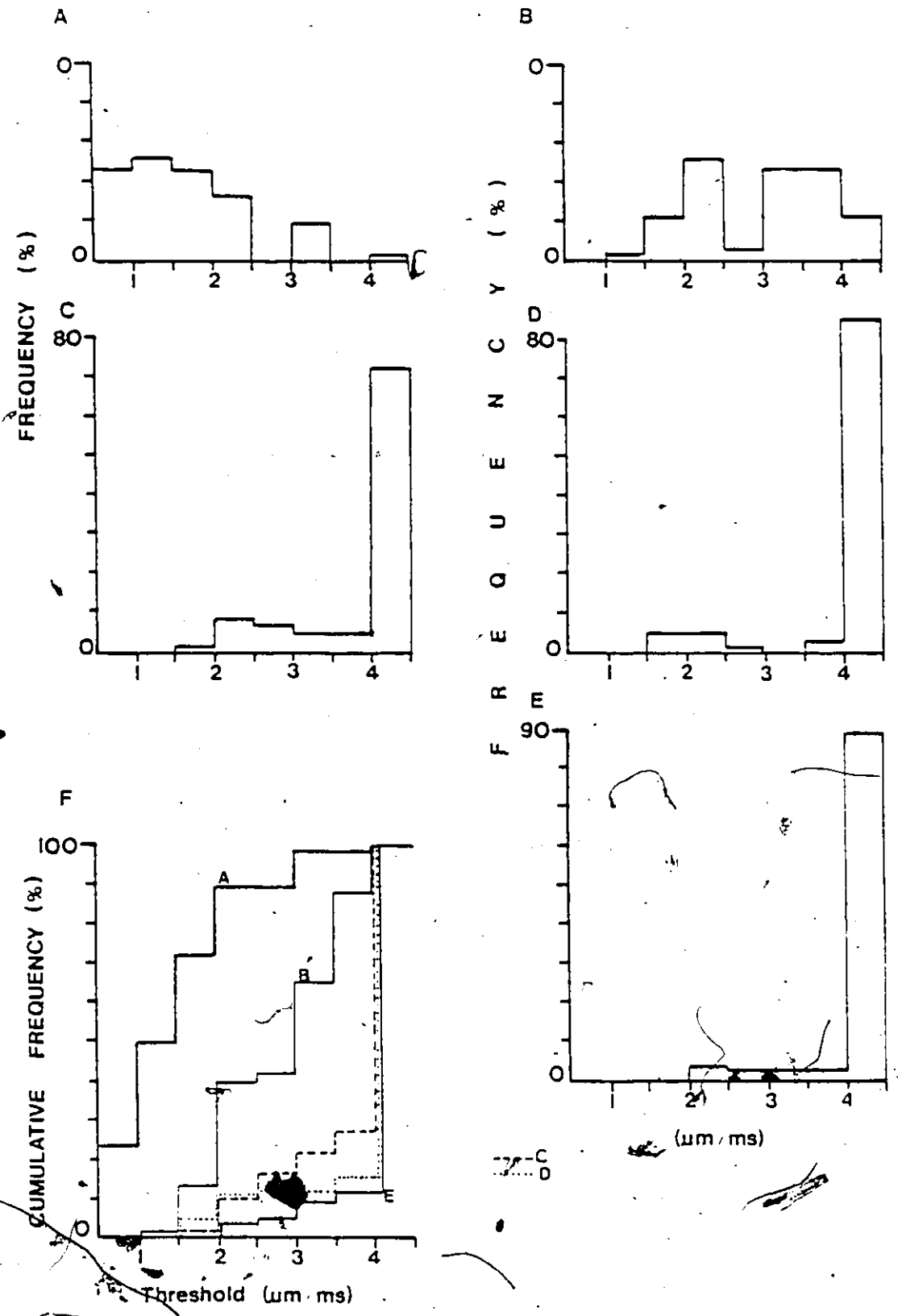
The fact that the Merkel cells of Xenopus survive denervation and also can develop (in regenerated skin) in the absence of nerves is consistent with the possibility that they could act as targets for mechanosensory nerves. The true test of their target role is whether the Merkel cells will become innervated (following a nerve lesion) by the appropriate regenerating or growing nerves. This section will present the results of experiments in which the recovery of cutaneous mechanosensitivity was monitored following a nerve crush, the questions of interest being whether the Merkel cells become innervated when the regenerating nerve fibres grow back into the skin, and whether this is

Fig. 33. Loss of mechanosensitivity following nerve section.

The loss of cutaneous mechanosensitivity was monitored by physiological mapping of the denervated skin at various times after section of the three spinal nerves serving the right hind limb.

The recordings were made from the distal portion of the selected nerve (usually nerve 8). The nerves were always electrically excitable and thus able to conduct impulses at least in the nerve trunk regions, for up to 10-12 days after mechanosensitivity of the skin disappeared (as determined by skin brushing), which was usually between 96 and 120 hours after nerve section.

The histograms show the threshold distributions at t=0 hr, (A), at t=24 hr, (B), at t=48 hr, (C), at t=72 hr, (D), and at t=96 hr (E) after nerve section. The cumulative frequency curves constucted from each of the histograms are shown in (F). The abscissae in A to F are the same and show the threshold range observed in these experiments. The curves B-E are significantly different F). The curves B-E are significantly different from curve A ($P<0.001$, using the Kolmogorov-Smirnov test).


A
B
C
D
E
F
FREQUENCY (%)
FREQUENCY (%)
CUMULATIVE FREQUENCY (%)
Threshold (µm · ms)
(µm · ms)
C
D
A
B
E

temporally, and spatially, related to the recovery of mechanosensitivity; that is, does the recovery of cutaneous mechanosensitivity require that the nerves contact the Merkel cells? As mentioned earlier, one of the ultimate aims of these studies was also to follow the time course of the physiological recovery and to determine if any stage in this recovery could be correlated with the morphological features of the reinnervation. Preliminary results concerning this last point will be presented in Part IIIB.

In this series of experiments dealing with the reinnervation of denervated skin, 2 of the 3 spinal nerves serving the right hind limb were sectioned and tied off to prevent regeneration. A 1-2 mm length of the third nerve (nerve 8) was crushed, and the nerve was allowed to regenerate. At various times after nerve crush, ranging from 3 to 14 weeks, the animals were examined. Mapping of the skin was done as previously described. While recording extracellularly from nerve 8, the skin was first stimulated by brushing; if there was response to brushing, the gross nerve fields were defined and then the response to punctate mechanical stimulation, using the prodder, was tested.

At the earliest time tested (3 weeks) there was no response to stimulating the skin in any manner. In some animals, this insensitivity persisted for up to 5 weeks; others showed responses to brushing the skin by 4 weeks. Generally, by 6 weeks all animals tested were "brush responsive"; some of these animals also displayed punctate high-threshold mechanosensitivity (see Fig. 34). Animals tested at 9-10 weeks after nerve crush displayed low- threshold

Fig. 34. Recovery of mechanosensitivity following nerve regeneration - early stages.

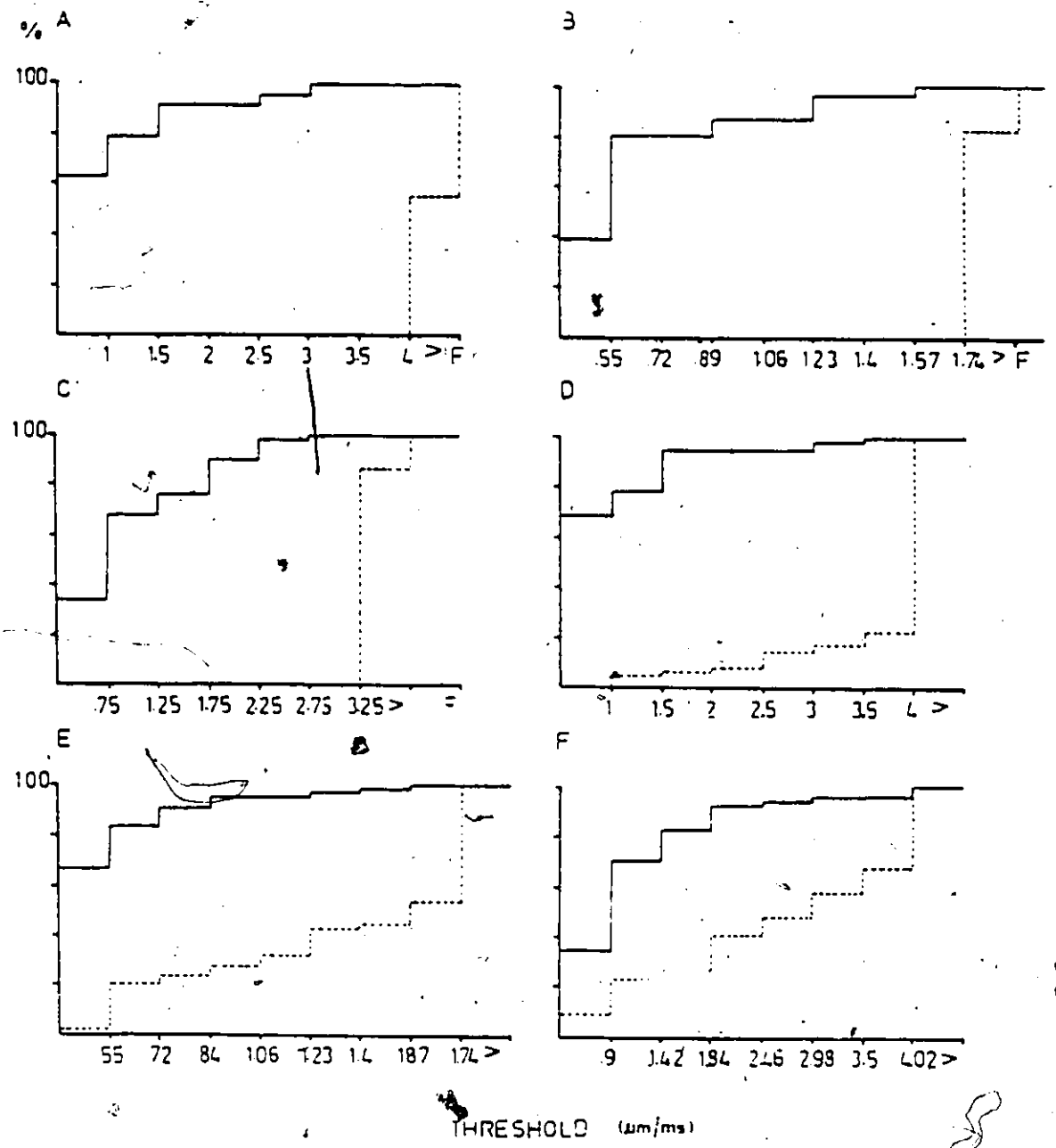
The recovery of mechanosensitivity following nerve regeneration was monitored by physiologically mapping the skin, using a brush and the mechanical stimulator, at various times after crushing the 8th nerve (as described in the text).

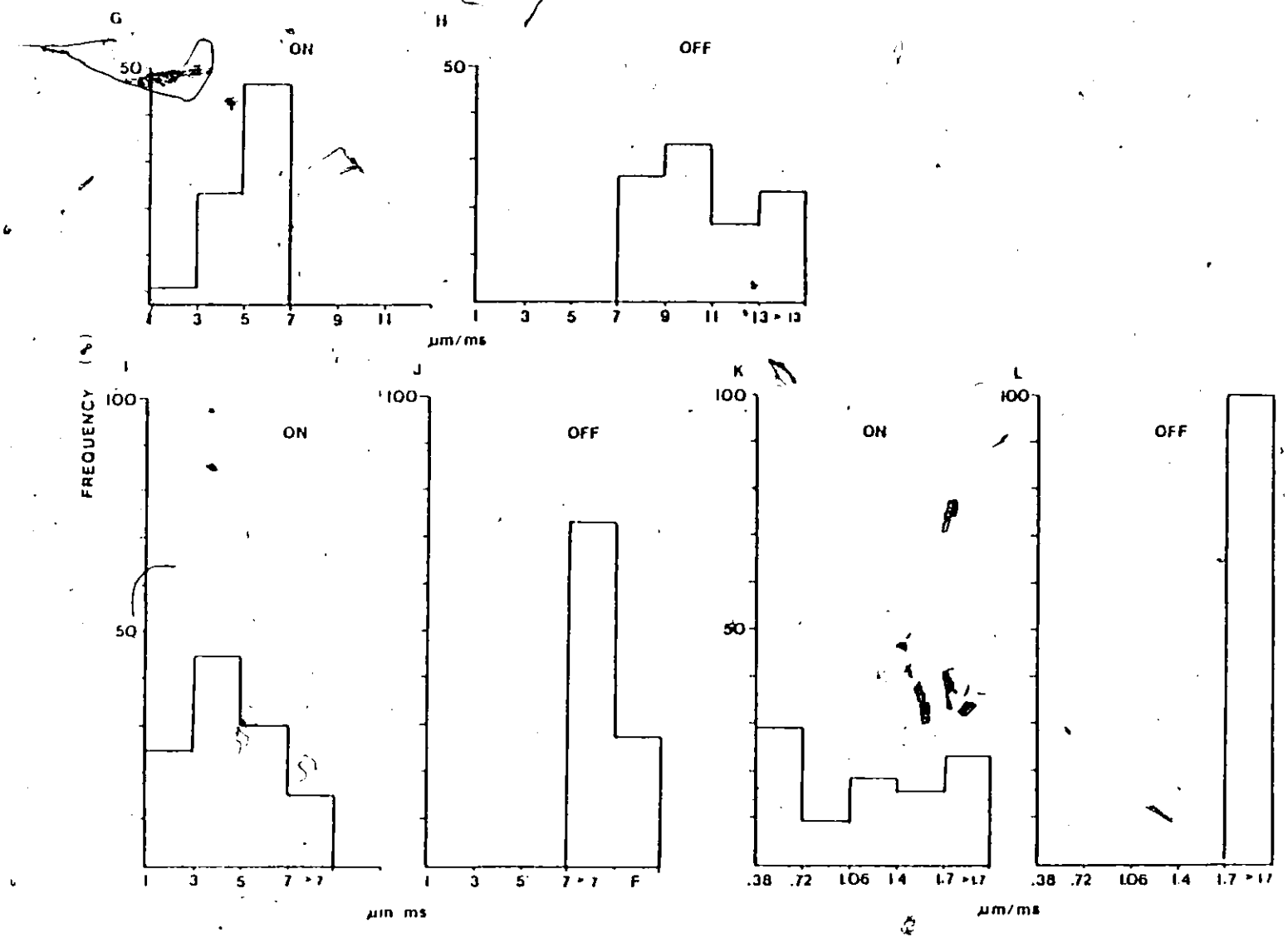
A-F present cumulative frequency curves of threshold distributions from six animals examined during the early stages of recovery of sensitivity (dotted lines). The curves for the opposite control limbs are the continuous lines. The letter "F" to the right of the abscissae in A-C represents the percentage of failures that were observed during the mapping. The ordinates in A-F are the same and represent the cumulative percentage occurrence.

A - examined at 36 days after the nerve crush
B - examined at 30 days
C - examined at 43 days
D - examined at 42 days
E - examined at 38 days
F - examined at 42 days

The experimental curves are significantly different from the corresponding control curves in each case ($P<0.001$, using the Kolmogorov-Smirnov test).

In animals B, D and E the recovery of sensitivity was also tested by comparing the responses when the prodder was located "on" the gland openings to those "off" the openings. The experimental data from B, D and E are replotted in I-J, G-H, and K-L, respectively, (see overleaf) as histograms indicating the "on" and "off" critical stimuli distributions. The "on" distributions are significantly different (i.e., lower thresholds) from the "off" distributions ($P<0.001$, using the Kolmogorov-Smirnov test).


%
A
100
1
1.5
2
2.5
3
3.5
4 > F
B
.55
.72
.89
1.06
1.23
1.4
1.57
1.74 > F
C
100
.75
1.25
1.75
2.25
2.75
3.25 >
D
100
1
1.5
2
2.5
3
3.5
4 >
E
100
.55
.72
.84
1.06
1.23
1.4
1.87
1.74 >
F
.9
1.42
1.94
2.46
2.98
3.5
4.02 >
THRESHOLD (um/ms)


G
ON
50
1 3 5 7 9 11
H
OFF
50
1 3 5 7 9 11 13 > 13
µm/ms
FREQUENCY (%)
I
ON
100
50
1 3 5 7 > 7
J
OFF
100
1 3 5 7 > 7 F
µm ms
K
ON
100
50
.38 .72 1.06 1.4 1.7 > 1.7
L
OFF
100
.38 .72 1.06 1.4 1.7 > 1.7
µm/ms

mechanosensitivity, but the range of the thresholds was still significantly different from the contralateral control values ($P<0.01$, using the Kolmogorov-Smirnov test; see Fig. 35). Recovery appeared to be physiologically complete by 11-14 weeks, since at these times there was no significant difference ($P>0.1$, using the Kolmogorov- Smirnov test) between the control and experimental cumulative frequency curves (see Fig. 36).

During the early stages (4-6 weeks after nerve crush), the recovery of mechanosensitivity progressed in a proximal-distal fashion, with the most proximal portion of the thigh becoming brush-sensitive before the more distal areas. The recovery of punctate mechanosensitivity also appeared to recover in a similar manner, with the most proximal area of the thigh displaying return of responses to the prodder in advance of the more distal areas.

In several animals examined near the end of the study, the recovery of sensitivity was tested by comparing responses when the prodder tip was "on" gland openings to those "off" them. Although recovery was not yet complete in these animals, the gland opening areas were usually the first to display any response (high-threshold, at first) to punctate mechanical stimulation; in addition, the lowest thresholds were always associated with the "on" areas (see Fig. 34 G-L).

In summary, the first sign of functional recovery is a response only to brushing of the skin, a very crude stimulus. The next stage of physiological recovery is the appearance of a high-threshold

Fig. 35. Recovery of mechanosensitivity following nerve regeneration - intermediate stages.

The recovery of mechanosensitivity following nerve regeneration was monitored by physiologically mapping the skin, using the mechanical stimulator, at various times after crushing the 8th nerve (as described in the text).

A-C present the cumulative frequency curves of the threshold distributions of three animals examined during the intermediate stages of recovery (dotted lines). The curves for the opposite control limbs are the continuous lines.

A - examined at 9 weeks after nerve crush
B - examined at 12 weeks
C - examined at 10 weeks

The experimental curves are significantly different from the control distributions (A and C, $P<0.001$; B, $P<0.01$, using the Kolmogorov-Smirnov test).

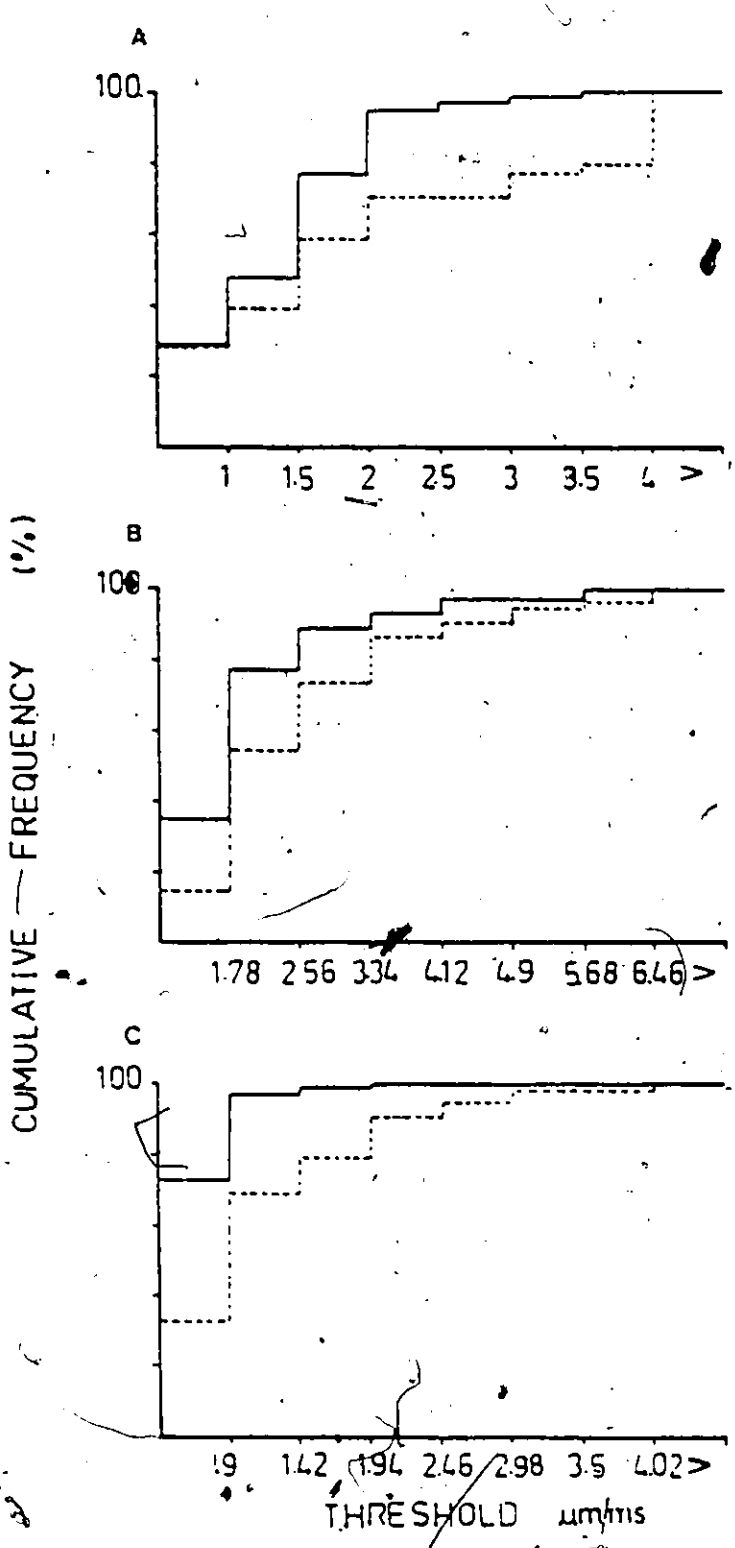

A
100
1
1.5
2
2.5
3
3.5
4 >
B
100
1.78
2.56
3.34
4.12
4.9
5.68
6.46 >
C
100
.9
1.42
1.94
2.46
2.98
3.5
4.02 >
CUMULATIVE — FREQUENCY (%)
THRESHOLD μm/ms

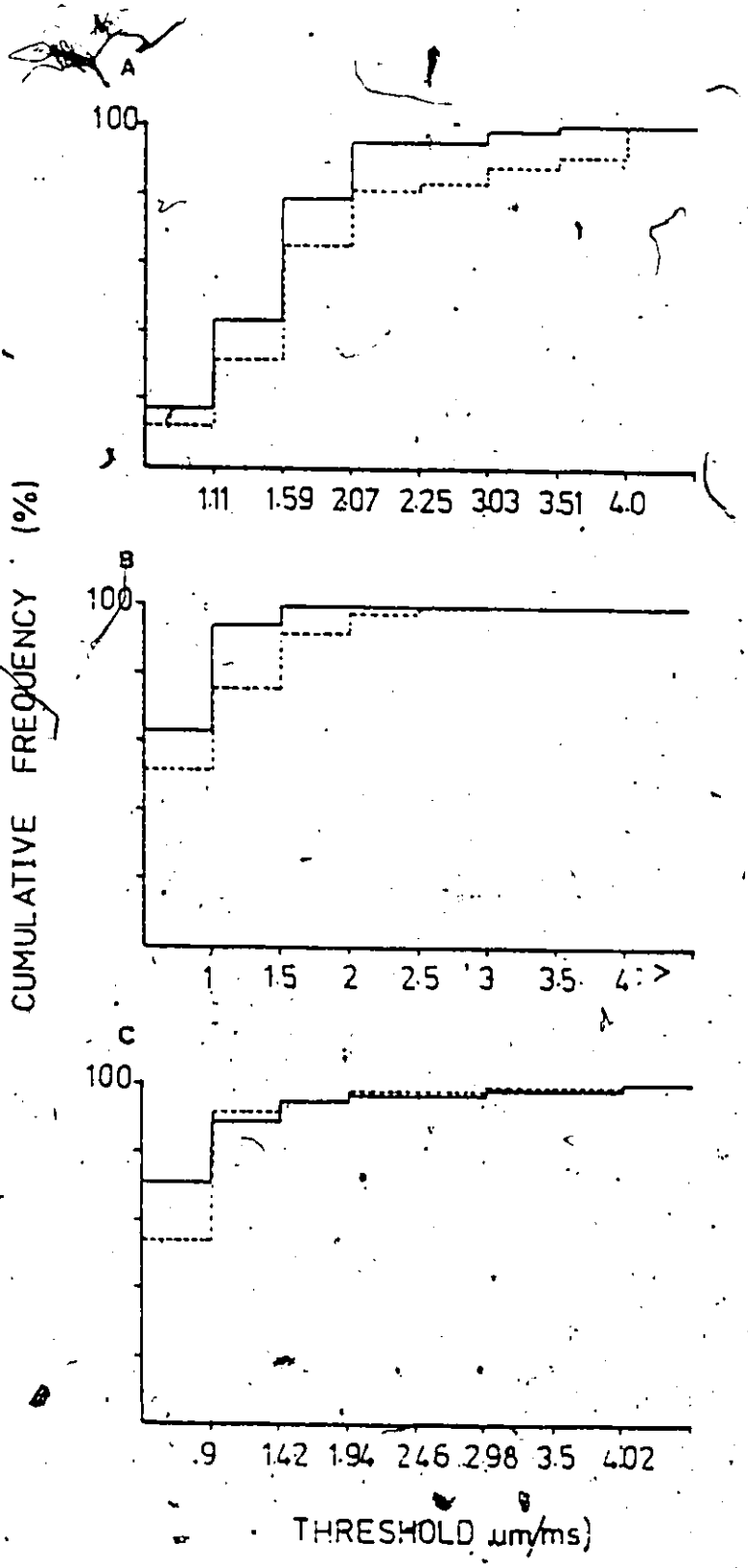
Fig. 36. Recovery of mechanosensitivity to control levels following nerve regeneration.

The recovery of mechanosensitivity following nerve regeneration was monitored by physiologically mapping the skin, using the mechanical stimulator, at various times after crushing the 8th nerve (as described in the text).

A–C present the cumulative frequency curves of the threshold distributions of three animals in which the mechanosensitivity had returned to control levels (dotted lines). The curves for the opposite control limbs are the continuous lines.

A – examined at 11 weeks after nerve crush
B – examined at 11 weeks
C – examined at 14 weeks

The experimental curves do not differ significantly from the control distributions (A and C, $P>0.1$; B, $P>0.01$, using the Kolmogorov–Smirnov test).


A
100
1.11 1.59 2.07 2.25 3.03 3.51 4.0
B
100
1 1.5 2 2.5 3 3.5 4 >
C
100
.9 1.42 1.94 2.46 2.98 3.5 4.02
CUMULATIVE FREQUENCY (%)
THRESHOLD (μm/ms)

mechanosensitivity, which is followed by a shift to low-threshold punctate mechanosensitivity.

The results (to be described below, Part IIIB) of EM examination of skin at various stages of physiological recovery show that the Merkel cells do become reinnervated.

(b) Nerve regeneration to regenerated skin:

The recovery of mechanosensitivity was also examined in regenerated skin. The animals used in the earlier experiments to examine the appearance of Merkel cells in regenerated skin were also used in this series. The recovery of mechanosensitivity in the regenerated skin was tested at 1-6 weeks after the initial excision of the skin samples (described in section 2a).

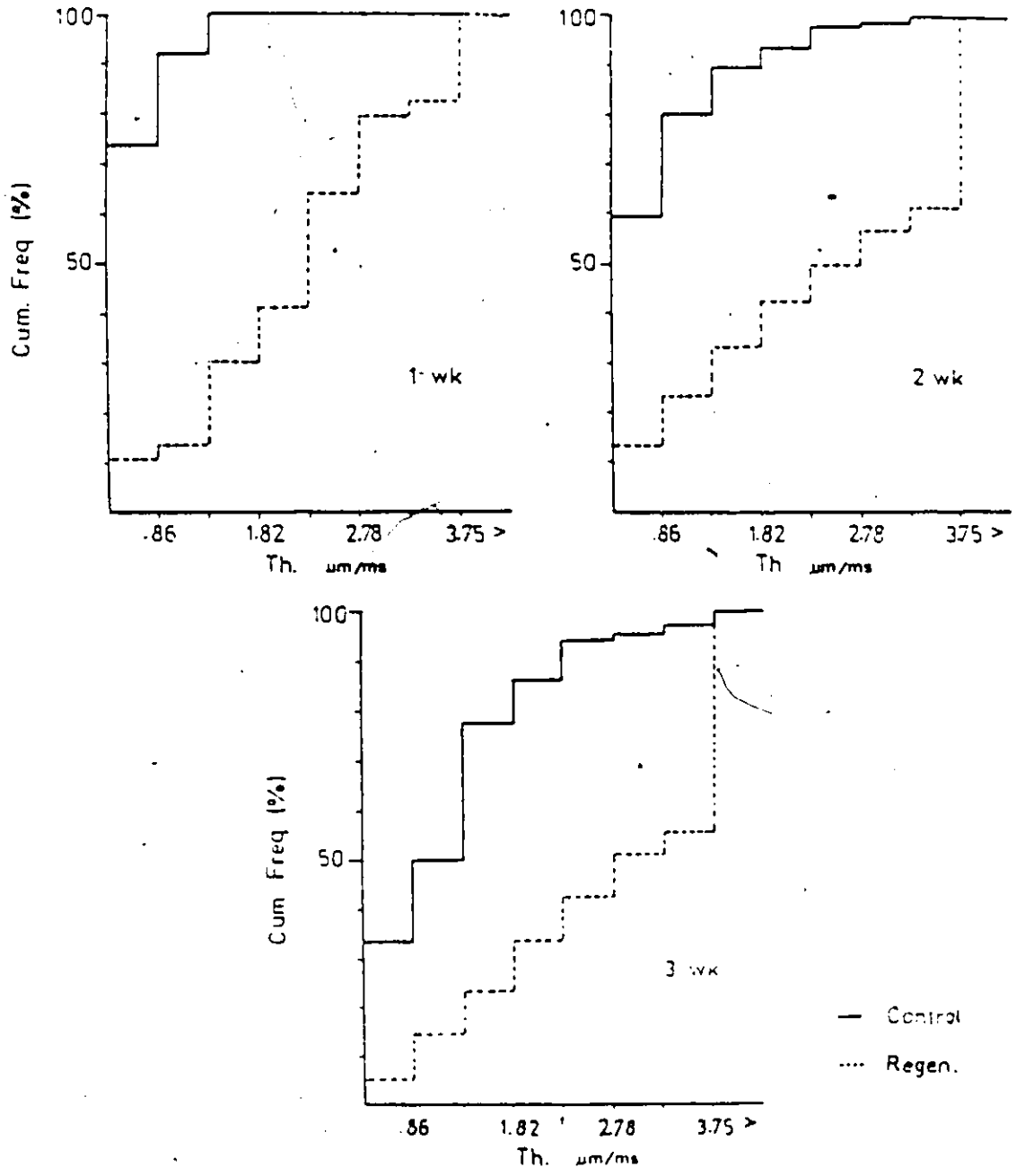
The results of the physiological mapping of the regenerated skin at 1-6 weeks are presented as cumulative frequency curves in Fig. 37. Examination of these curves indicates that, although the experimental and control curves are statistically different at all times tested ($P<0.001$, using the Kolmogorov-Smirnov test), there is a clear trend towards the appearance of normal mechanosensory function at the 5th and 6th weeks of skin regeneration.

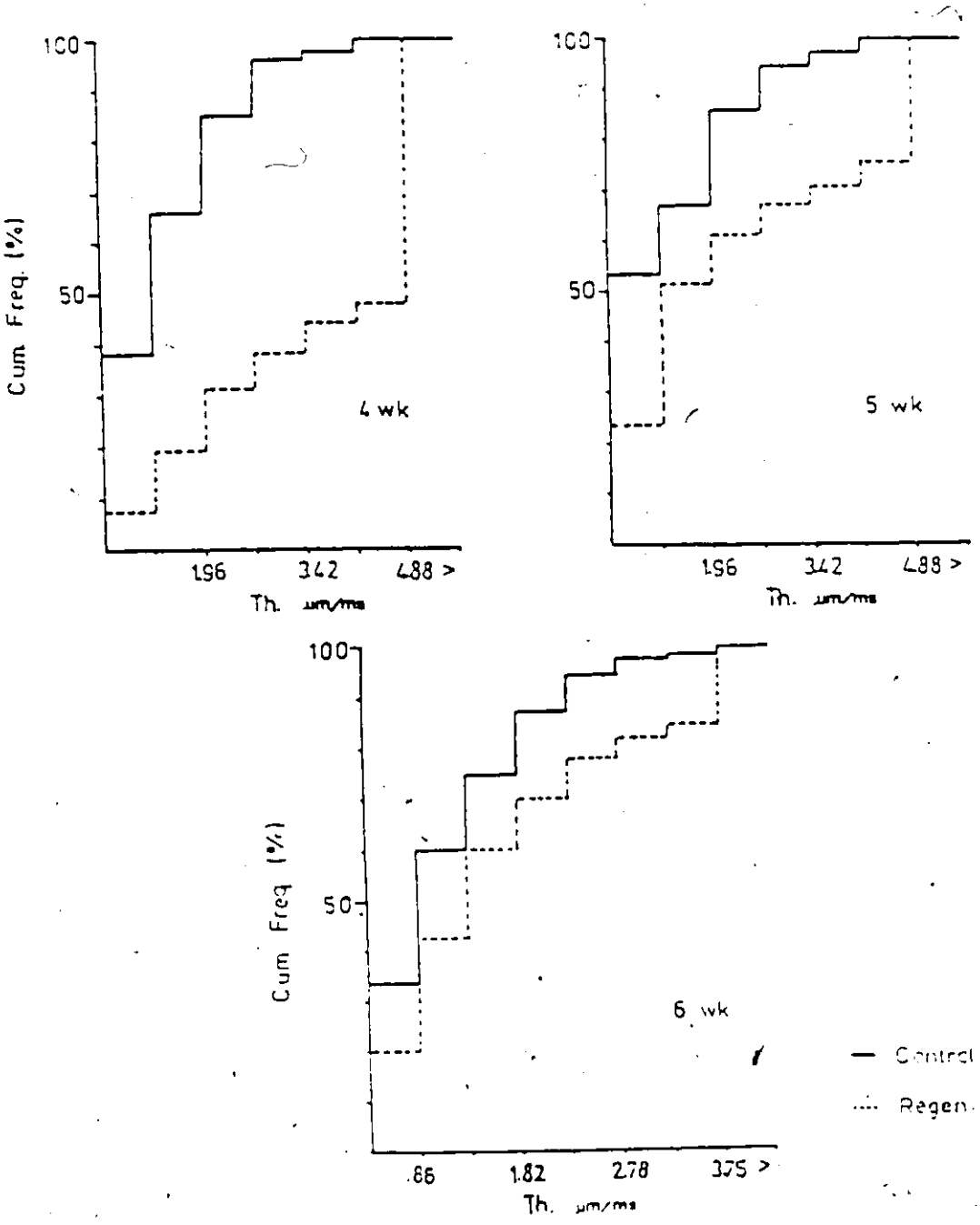
As was argued above, if it is the innervation of Merkel cells in the regenerated skin that is responsible for the development of the low-threshold mechanosensitivity, then the physiological recovery should be correlated with the appearance of Merkel cell-neurite complexes in the regenerated skin (and specifically, the touch spots

Fig. 37. Recovery of mechanosensitivity in regenerated skin.

The recovery of mechanosensitivity in regenerated skin was monitored by physiologically mapping the regenerated area, using the mechanical stimulator (as described in the text). Regenerated areas of skin were examined at 1–6 weeks after the initial removal of skin; A–F present the cumulative frequency curves of threshold distributions obtained at each weekly examination period (pooled data from 6–8 experiments for each pair of curves). A – 1 week, B – 2 weeks, C – 3 weeks, D – 4 weeks, E – 5 weeks and F – 6 weeks of skin regeneration.

In all cases, the experimental threshold distribution curves (dotted lines) are significantly different from the corresponding control (continuous lines) curves (A–E, $P<0.001$; F, $P<0.01$, using the Kolmogorov–Smirnov test).


100
50
Cum. Freq (%)
1 wk
.86
1.82
2.78
3.75 >
Th. µm/ms
100
50
2 wk
.86
1.82
2.78
3.75 >
Th µm/ms
100
50
Cum Freq (%)
3 wk
.86
1.82
2.78
3.75 >
Th. µm/ms
Control
Regen.


100
50
Cum Freq. (%)
4 wk
196
342
488 >
Th. µm/ms
100
50
5 wk
196
342
488 >
Th. µm/ms
100
50
Cum Freq. (%)
6 wk
.86
1.82
2.78
375 >
Th. µm/ms
— Control
.... Regen.

should be correlated with the location of the Merkel cells). This examination is still a future aim. However, the simpler test of examining the regenerated skin both physiologically and for the occurrence of Merkel cells (QFCs) has been done. Fig. 38 shows that the recovery of sensitivity seems to be related to the appearance of Merkel cells in the regenerated skin. In Fig. 38 (right), the difference in percentage occurrence of low-threshold mechanosensitive points (i.e., the difference in the height of the first bin of the threshold histograms) between the control and regenerated skin is presented as a function of time of skin regeneration. In Fig. 38 (left), the percentage difference in Merkel cell density between the initial skin samples and the regenerated skin as a function of time is presented. The end points of the curves indicate that when mechanosensitivity has recovered (almost to control levels), the density of Merkel cells has also returned to control values; the slopes of the curves indicate that the time course of recovery may differ slightly. Although these areas of skin were not examined with EM to see if the Merkel cells were innervated, on the basis of the previously described experiments in which regenerating nerve innervated the Merkel cells, and also on the basis of the relationship between the recovery of sensitivity and the appearance of Merkel cells in the regenerated skin, it seems probable that the Merkel cells in the regenerated skin do become innervated.

(c) Nerve regeneration to skin that had regenerated in an initially

Fig. 38. The relationship between the recovery of mechanosensitivity and the appearance of Merkel cells in regenerated skin.

The difference in percentage occurrence of low-threshold points between the control and regenerated skin at each sampling period is presented along the left ordinate (open circles). The values for the difference were obtained from the first bin of the cumulative frequency curves in Fig. 37.

The difference in Merkel cell density between the initial skin samples removed and the regenerated skin is at each sampling period is shown along the right ordinate (closed circles). The values of (N-R / N x 100%) are caluculated from the data in table 5.

Difference in % Occurrence of Low Threshold Points between Control and Regenerating Skin (C-R) %

Difference in Merkel Cell Density between Normal and Regenerating Skin $(\frac{N-R}{N} \times 100\%)$

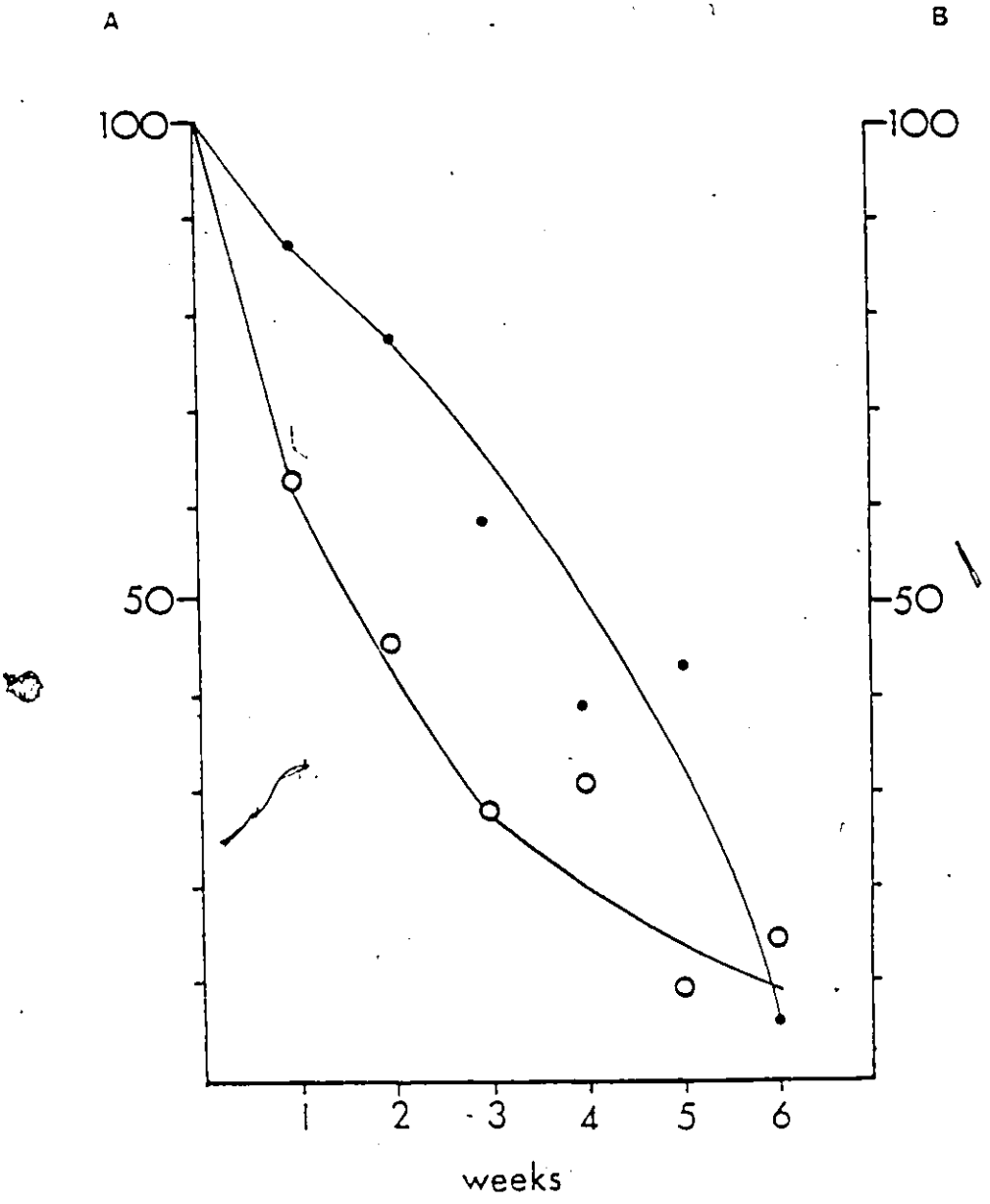
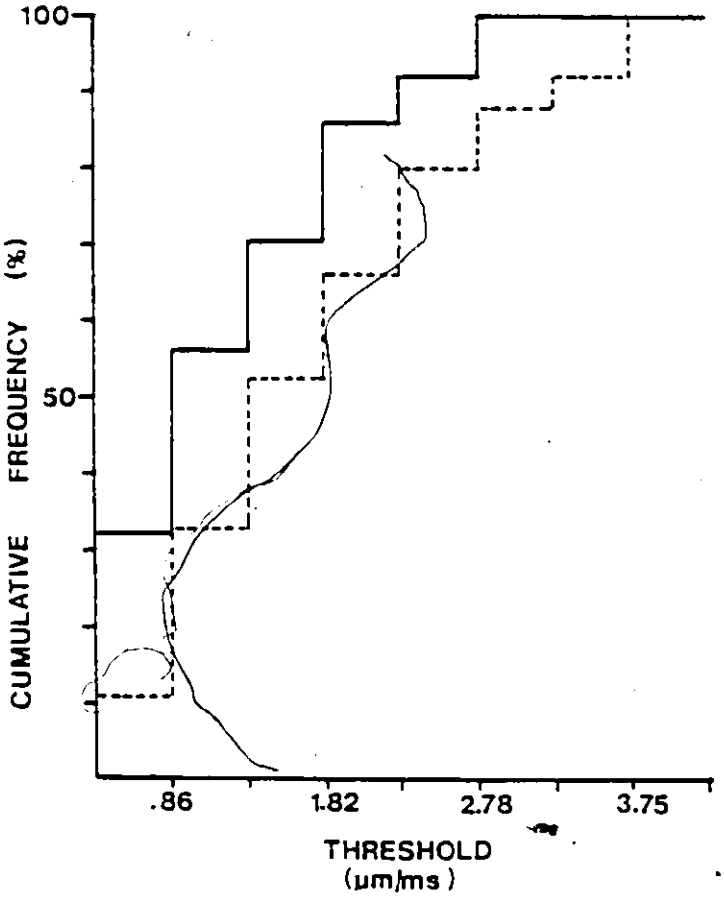


Fig. 39. Recovery of mechanosensitivity in skin regenerated in an initially nerve-free limb.

The recovery of mechanosensitivity in new skin that had regenerated initially in the absence of nerves was examined after the crushed 8th nerve was allowed to grow back into the limb.

The figure presents the cumulative frequency curves of the threshold distributions (pooled results from four experiments) from animals mapped at 7 weeks after the initial skin removal, and 9 weeks after crushing the 8th nerve (i.e., the skin was excised 2 weeks after the nerve crush).

There is no significant difference between the experimental (- - -) curve and the corresponding control (——) curve ($P>0.01$, using the Kolmogorov-Smirnov test).


100
50
CUMULATIVE FREQUENCY (%)
.86
1.82
2.78
3.75
THRESHOLD
(µm/ms)

nerve-free condition:

In this experiment, the 8th nerve was allowed to regenerate back into skin that had regenerated in an initially nerve-free condition. In the group of animals used for this experiment, the nerve was crushed 2 weeks prior to skin removal. Physiological testing was done only at 7 weeks after skin removal; the original plan was to examine these animals at 7 and 10 weeks after the initial skin removal, but unfortunately the animals did not survive past 7 weeks.

Thus at the time of physiological examination the nerve would have been undergoing regeneration for 9 weeks, and the skin would have been regenerating for 7 weeks. The regenerated skin was physiologically mapped as previously described and the results of these maps are presented in Fig. 39 as cumulative frequency curves. Examination of these curves demonstrates that there is no statistical difference between the control and experimental curves ($P>0.01$, using the Kolmogorov-Smirnov test). The number of QFCs was also counted in these samples and the density of the QFCs in the regenerating skin (116.6 ± 7.5) is not different from the initial density in these areas prior to skin excision (116.6 ± 11.2). So, the Merkel cell numbers have returned to normal, and the mechanosensitivity of the regenerated skin is also in the normal range. Again, although these samples were not examined with EM to see if the Merkel cells were innervated, given the results of the previously described experiments and the recovery of sensitivity and return of Merkel cells in this experiment, it seems likely that these Merkel cells would also become innervated.

5. Summary Discussion:

Regeneration of a crushed spinal nerve in Xenopus frogs appears to reinnervate and restore normal mechanosensory function to the skin. Physiological monitoring of the time course of reinnervation showed that the recovery of sensitivity was gradual; the first signs of functional cutaneous innervation was a response to brushing of the skin, a very crude stimulus, which occurred 5-6 weeks after the nerve lesion. Over the next 4-6 weeks, the mechanosensitivity of the skin gradually acquired the normal low-threshold characteristics; the response to localized mechanical stimulus, even in the early stages of recovery, was always rapidly- adapting.

Examination of the spatial arrangement of responsive spots indicated that the density of the touch spots also returned to control levels. There were no unresponsive gland opening areas, indicating that all the originally innervated sites had been reinnervated.

Nerve regeneration following a crush lesion, as opposed to complete transection, appears to be quite accurate and complete (see below). In fact, Sunderland (1978) states that in such lesions the regenerating axon is confined to the endoneurial tube that originally contained it, so that the pattern of reinnervation is precisely the same as the original pattern, and function is fully restored. In the present investigation, the pattern of reinnervation appears to be the same as the original pattern, and function is fully restored. In the nerve regeneration to denervated skin experiments, the regenerating

nerves could have followed the old endoneurial tubes; in the experiments where the nerve regenerated into new skin that had regenerated in place of a piece that had been excised, however, there would have been no old nerve sheaths in the new skin for the regenerating nerves to follow, and thus the Merkel cells appear to act as targets for these nerves.

Nerve regeneration, along with the reinnervation of peripheral sensory end-organs or cells, and the return of normal physiology has been observed in many other investigations. For example, Burgess et al (1974) and Horch (1979) have shown that the pattern and physiology of reinnervated cat touch domes is essentially the same as that observed prior to nerve crush. Reinnervation of primate glabrous skin was investigated by Dykes and Terzis (1979); it was demonstrated that recovery of cutaneous sensitivity following nerve crush was gradual, with more high-threshold units being observed in the early stages of nerve regeneration and a gradual recovery of the normal complement of low-threshold receptors. A similar pattern of recovery was observed in rat glabrous skin by Sanders and Zimmerman (1978).

Successful reinnervation has also been demonstrated in salamander skin, both physiologically (Johnston et al., 1975; Diamond et al., 1976; Scott et al., 1981) and morphologically (Scott et al., 1981); in the latter study, it was shown that Merkel cells that survived denervation, and Merkel cells which appeared in regenerated skin, became innervated (Scott et al., 1981). Accurate regeneration and restoration of normal function has also been observed in much

simpler systems, such as the leech (Baylor and Nicholls, 1971; Van Essen and Jansen, 1975).

In these examples, accurate functional reinnervation was attributed to mechanical guidance pathways, in the form of old Schwann tubes (e.g., Burgess et al., 1974; Johnston et al., 1975; Horch, 1979), or the peripheral portions of cut axons that had not yet degenerated (eg., Baylor and Nicholls, 1971; Van Essen and Jansen, 1975). However, in the case of the salamander skin, the Merkel cells act as targets which the nerves can 'find' even in the absence of such mechanical cues (Scott et al., 1981; see below).

Time course of nerve regeneration:

The time course of events in the present study indicates that once the regenerating fibres reach the periphery, a considerable time is required for functional recovery of the Merkel cell-neurite complexes. An estimate of the time needed for each of the stages of recovery can be arrived at as follows. Values for the rate of nerve regeneration following a crush lesion in amphibians are given by Lubinska (1964); for animals kept at 18-20°C , 0.8mm per day seems to be a reasonable extrapolation from these values. Using this rate and estimating the distance to be covered by the regenerating fibres as 1 cm (for the proximal portion of the thigh) to 3 cm (for the distal area of the thigh), the time taken for the nerves to reach the skin, once they have started to regenerate, would be about 12 (proximal) to 37 (distal) days. However, prior to the initiation of regeneration there is a latent period, during which time the neurons seem to recover

sufficiently from the retrograde effects of the lesion to begin outgrowth, and to cross the scar region. This latent period has been estimated at 4–10 days in mammals (Sunderland, 1978), and may be longer in amphibians, due to the difference in body temperature and its effect on metabolism. If this initial delay is taken as 7 days (but using the range of 4–10 days to give the bracketed range estimates) and added on to the regeneration times, it can be estimated that the cutaneous fibres should begin to reach the proximal portions of the thigh by about 20 days (range 16–22 days), and should have filled in the distal areas by about 44 (range 41–47) days.

If the rate of regeneration were faster, say 1 or 1.5 mm per day instead of 0.8 mm per day, the nerve should have reached the proximal portions of the thigh by 14 days (range 11–17 days, at 1.5 mm per day) to 17 days (range 14–20 days, at 1.0 mm per day), and should have filled in the distal areas by 27 (range 24–30 days) to 37 (range 34–40 days) days. So, by increasing the rate of regeneration from 0.8 to 1.5 mm per day, the estimated time required for nerves to reach the periphery and innervate the skin of the entire thigh drops from 2.3–6.7 weeks to 1.6–4.3 weeks.

Although the earliest signs of cutaneous sensitivity to the applied mechanical stimuli were seen at times longer than 4–5 weeks, other observations indicate that some nerves had reached the periphery by 3–4 weeks. For example, some animals exhibited escape behaviour in response to pinching of the experimental limb (in the thigh area) at 3–4 weeks; in addition, in these animals electrical stimulation of the

regenerating nerve trunk, in the area of the crush lesion, resulted in weak muscular contraction of the thigh. These observations seem to suggest that some reinnervation has taken place, and that the motor reinnervation is at least partially functional. At this time in these particular animals, there was no indication of cutaneous sensation in response to the usual test stimuli, i.e., brushing or prodding. It could be that there are regenerating fibres present in the skin and that these require more intense stimuli than that provided by the ususal test stimuli for their activation.

Responses to punctate stimulation, or prodding, appeared between 8-10 weeks, but did not reach normal low-threshold levels until 11-14 weeks after the initial nerve lesion. There is, then, a considerable delay between the earliest sign of functional cutaneous reinnervation (at 4-5 weeks) and total recovery (11-14) weeks; this time appears to range from about 6 to 10 weeks. The minimum time from the nerve crush to total recovery was 11 weeks. Fig. 40 summarizes the experimental observations.

Recovery of mechanosensitivity in regenerated skin:

The innervation of regenerated skin was monitored over a 6 week period, and although recovery was not complete, the trend was very clearly towards the normal distribution of low-threshold mechanosensitive points. In this situation, innervation of the regenerated skin is presumably achieved by regeneration of the surrounding nerve fibres that were cut when the skin patch was

Fig. 40. Summary diagram of the time course of nerve regeneration and the recovery of mechanosensitivity.

This figure summarizes the various stages in the return of mechanosensitivity, and includes estimates of the time of arrival of nerves in the skin (see text).

In the lowest portion of the figure, the estimated time (upper and lower limits) taken for nerve regeneration is presented for two different rates of nerve regeneration.

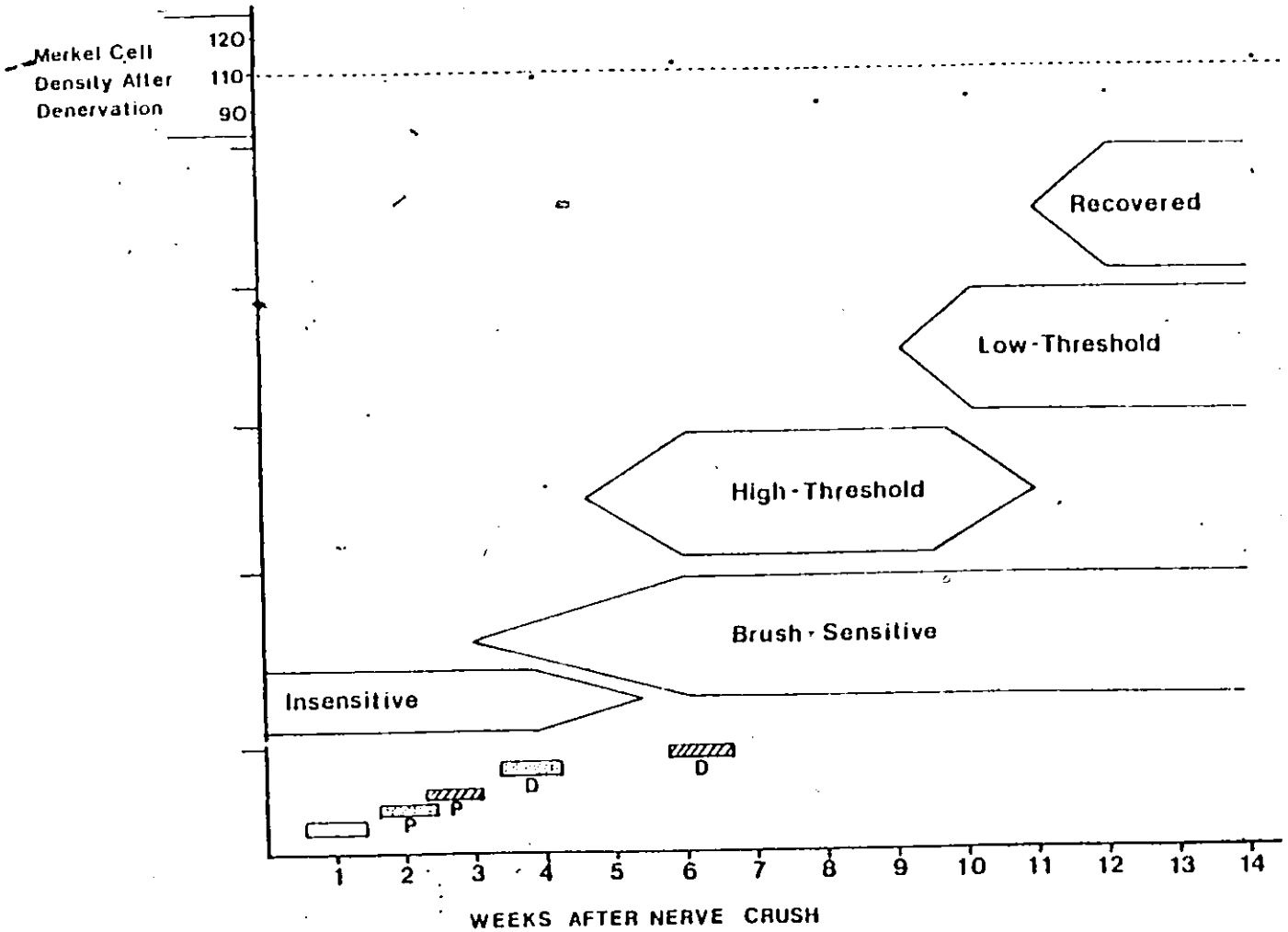
 - range of latent period before the onset of nerve regeneration.

 - estimated range of times for the nerve fibres to reach the proximal (P) and distal (D) portions of the thigh at a regeneration rate of 1.5 mm/day.

 - estimated range of times for the nerve fibres to reach the proximal (P) and distal (D) portions of the thigh at a regeneration rate of 0.8mm/day.

The upper portion of the figure shows the stages in the recovery of sensitivity, from the earliest times that each stage was seen experimentally. Note that at the "low-threshold" stage the experimental threshold distribution was still significantly different from the control distribution, but mechanosensitivity had fully returned to control values at the "recovered" stage.

The top part of the figure shows that Merkel cell density does not change following denervation of the skin.


Merkel Cell Density After Denervation
120
110
90
Recovered
Low-Threshold
High-Threshold
Brush-Sensitive
Insensitive
P
P
D
D
1
2
3
4
5
6
7
8
9
10
11
12
13
14
WEEKS AFTER NERVE CRUSH

initially excised. As previously discussed (section 4b), the recovery of mechanosensitivity follows a similar time course as the appearance of Merkel cells in the regenerated skin; it seems likely that the innervation of these new Merkel cells by the regenerating nerve fibres is responsible for the return of sensitivity.

Recovery of mechanosensitivity in skin that had regenerated in an initially nerve-free condition:

Return of sensitivity was also monitored in a situation where the nerve was allowed to regenerate into skin that had regenerated in an initially nerve-free hind limb. These samples were examined at 7 weeks of skin regeneration, at which time the nerve would have been regenerating for 9 weeks. Here, recovery seemed to be complete, since the experimental threshold profile was not significantly different from the control; in addition, the cumulative frequency curve of the 7 week regenerated skin was also similar to the previously described 6 week regenerated skin samples (from the experiments dealing with skin regeneration in the innervated hind limb). This might seem surprising, since one would expect recovery in a situation where the nerve has to regenerate over a long distance (1-2 cm, in the skin regeneration in an initially nerve-free limb) to lag behind that where the surrounding intact nerve fibres only have to regenerate over a relatively small distance (2 mm, in the skin regeneration in the innervated hind limb). However, using the estimates previously discussed, one can propose that these two different situations are at about the same stage of recovery.

For example, from the results of the previously described nerve regeneration experiments, the nerve fibres regenerating after a crush lesion would be expected to reach the skin by 3-4 weeks after the crush, and would then require another 6-10 weeks for functional recovery. So, when the samples were examined at 7 weeks of skin regeneration and 9 weeks of nerve regeneration, the regenerating nerve fibres would have had about 5-6 weeks in the skin to undergo functional recovery; this recovery period would be at the lower limit of time that seemed to be required for recovery in the previously described nerve regeneration experiments.

In the case where the skin regenerated in the presence of nerves (i.e., the innervation of the hind limb was intact), nerve fibres were observed in the regenerating skin by one week after initial skin excision. In those samples examined at 6 weeks, the nerves would have also had about 5-6 weeks in the skin for recovery; since this time is at the lower limit of the estimated time needed for functional recovery of mechanosensitivity and since there is a rather large time range involved, it is not surprising that recovery in this situation (at the 6 week sample time) is not totally complete.

Innervation of Merkel cells:

The Merkel cells in Xenopus skin survived denervation, and became innervated by nerves that regenerated back into the skin (see Part IIIB), Merkel cells which appeared in new skin that had regenerated in the presence, or absence of nerves, presumably also

became innervated when nerves grew back into the regenerated skin. The innervation of the Merkel cells in regenerated skin provides evidence for the role of the Merkel cell as a target, for in these experiments there were no old endoneurial tubes in the new skin that the regenerating nerve fibres could have followed back to the epidermis. It is possible that the regenerating nerve fibres could have been following other guidance cues, such as the location of the glands, but similar evidence from the salamander, where the Merkel cells are not specifically associated with any cutaneous structures, indicated that the nerves can still 'find' the Merkel cells in the apparent absence of any obvious guidance cues (Scott et al., 1981). The existence of a sprouting stimulus released by uninnervated Merkel cells has been proposed as a possible way in which the nerves are attracted to the Merkel cells (Cooper et al., 1977); presumably some recognition of the Merkel cells by the nerve endings (or vice versa), which might involve a similar molecular mechanism as that proposed for cell-cell recognition in other systems (e.g., Marchase, 1977), is also involved (Diamond, 1982; see also Final Discussion).

Part III B. Continuing investigation and preliminary results:

One of the ultimate aims of this study was to correlate the physiological observations during the recovery of mechanosensitivity following nerve regeneration with the fine-structural features of the Merkel cell-neurite complex, in an effort to determine the contribution of the Merkel cell to the processing of mechanosensory information . This work is part of a continuing investigation, and the results are very preliminary, since insufficient material has been examined to support a conclusive statement concerning this role.

The approach taken in this investigation was to denervate the hind limb of Xenopus frogs, and to monitor electrophysiologically the recovery of cutaneous mechanosensitivity, as discussed previously; the loss of sensitivity following nerve section was similarly monitored. Physiologically-mapped samples of skin were then excised and immediately fixed by immersion into the cold fixative. In this preliminary study, an accurate point to point correlation was not undertaken; the overall orientation of the skin sample with respect to the the physiological map was noted, but individual touch spots were not marked in such a way as to facilitate their recognition at the EM level. The objective of this study was, first, to see if the different types of physiological responses would indeed be associated with observable ultrastructural features, and second, to see how broad or narrow defined periods of physiological recovery could be, in relation to any observable morphological changes.

Ideally, one would like to be able to examine individual touch spots of known physiology; only then, could one begin to make definite conclusions regarding the role of the Merkel cell, if any, in the mechanosensory process. Such an approach will be taken in future experiments of this continuing investigation.

Skin samples examined to date have been grouped into 5 categories as follows: 1) control (i.e., normally innervated); 2) in the process of being reinnervated, but still insensitive to mechanical stimulation; 3) brush-responsive only; 4) responding to punctate stimulation (prods), but with a threshold distribution profile significantly different (higher thresholds) from the control distribution; and 5) fully recovered. Observations made on samples that have been examined so far will be discussed in the following sections.

1. Control observations:

In skin samples taken from normally innervated hind limbs, 9 Merkel cells from 3 different samples have been examined at the EM level. Each of these cells had at least one nerve ending contacting it and forming a synapse. A distinction is made between contact and synapse formation, since in a number of cases nerve endings were seen to contact the Merkel cells without forming synapses. Contact refers to close apposition of the nerve and Merkel cell membranes. Synapses were characterized by some (or all) of the following: a close apposition of the two membranes , which tend to "straighten" somewhat

over the presumed synaptic region proper, and the presence of pre- and post-synaptic cytoplasmic densities abutting the membranes of the Merkel cell and the nerve ending, and the presence of clear synaptic vesicles (in the nerve ending) or dense-cored granules (in the Merkel cell) that tended to cluster in the area of the membrane straightening and the pre-synaptic cytoplasmic densities.

Several of the cells were examined with serial thin sections to see if reciprocal synapses between the Merkel cells and nerve endings could be observed. A reciprocal synapse was characterized by evidence of two closely related synaptic junctions polarized in opposited directions, i.e., from Merkel cell to the nerve ending and from nerve ending to the Merkel cell. In each of two of the three cells examined serially, over about 5 µm, one suggestive reciprocal synapse was observed. An example of such a contact is shown in Fig. 41.

This Figure shows 3 sections through the same cell. In (A), a pre-synaptic cytoplasmic density can be seen on the nerve terminal membrane; in the same section there also appears to be a post-synaptic dense bar on the nerve membrane. In another section (B), which was 2 serial sections away from (A), the post-synaptic dense bar is seen more clearly, and a possible omega figure, which is thought to represent vesicle fusion with the membrane, is present on the nerve terminal membrane. The final micrograph (C) shows what appears to be pre-synaptic cytoplasmic densities on the Merkel cell membrane on either side of a dense-cored granule.

Synaptic areas, that were morphologically polarized in the

Fig. 41. Electron micrographs of a Merkel cell in normally-innervated control skin.

A–C are electron micrographs of an area of the same Merkel cell observed in a sample of skin (processed for EM as described in the Methods) from a control animal. A and B (but not C) are serial sections.

(A) is a view of the nerve terminal (NE) contacting the Merkel cell (MC). A possible "pre-synaptic" cytoplasmic density is visible at the nerve membrane (arrow). A possible "post-synaptic" is visible on the nearby nerve terminal membrane (the thickening indicated by the arrowhead). m, mitochondrion.

In (B), an apparent omega-figure (arrow) is visible on the nerve membrane; the post-synaptic density on the nerve membrane is still present.

In (C), there are possible pre-synaptic cytoplasmic densities at the Merkel cell membrane, flanking a dense-cored granule (arrow). There may also be a post-synaptic cytoplasmic density at the nearby nerve ending (NE) membrane.

Calibration bar: 0.5 μm

B

C

A

direction of Merkel cell to nerve, were often additionally characterized by the appearance of several dense-cored granules close to the Merkel cell membrane. Generally, there seemed to be a margin of about 200 nm around the internal perimeter of the cell which was relatively free of granules; at a synaptic site, several of the granules 'dropped' into this margin so that they were located close to the cell membrane.

Similarly, in synapses morphologically polarized from nerve ending to Merkel cell, clear vesicles tended to cluster in the area of the pre-synaptic cytoplasmic densities. In cases where the nerve was apparently post-synaptic, there was often an area under the post-synaptic dense bar that was free of vesicles.

The nerve ending appears to form several morphological contacts on the Merkel cells; examination of serial sections reveals that often one large terminal will eventually split into two or three smaller terminals; it would seem that the nerve forms numerous 'finger-like' terminals on the cells. The nerve terminals, themselves, contain numerous clear vesicles (60-80 nm diameter), several mitochondria and, occasionally, several small (60-90 nm diameter) dense-cored vesicles (Fig. 41 and 46).

In general, the cytological features of the Xenopus Merkel cell-neurite complex are the same as those already described in other species (e.g., Smith, 1967; Munger, 1971; Winkelmann and Breathnach, 1973; Parducz et al., 1977; Fox and Whitear, 1978; English et al., 1980); the one exception is that the nerve ending does not form a disc-

like termination as it does in the mammalian complex.

2. Experimental observations:

(a) Morphological observations following the loss of mechanosensitivity:

A few skin samples from animals in which the loss of mechanosensitvity was monitored have been examined. The physiological observations indicated that sensitivity decreased from low-threshold to high-threshold spots by 24-48 hours after nerve section. This was followed by response only to brushing the skin, from 72-120 hours after section, and then a complete loss of sensitivity to any mechanical stimulus.. EM examination has only been carried out on one of the sample blocks from each sample time period, and only 1-4 grids of thin sections from each of the blocks have been examined.

Fig. 42 presents 4 micrographs from samples taken at various times after nerve section. (A) and (B) are both from skin pieces taken 42 hours after nerve section; (A) shows what appears to be a degenerating terminal contacting the Merkel cell and extracellular spaces filled with nerve(?) debris. On the other hand, (B) displays several intact terminals contacting the Merkel cell, with the swollen mitochondria in one terminal being the only indication that this might not be a normally innervated cell. (C) is a Merkel cell from a sample taken 72 hours after denervation; there is a myelin figure in the one degenerating terminal. (D) is a micrograph of a portion of a Merkel cell one week after denervation, which is surrounded by a large space

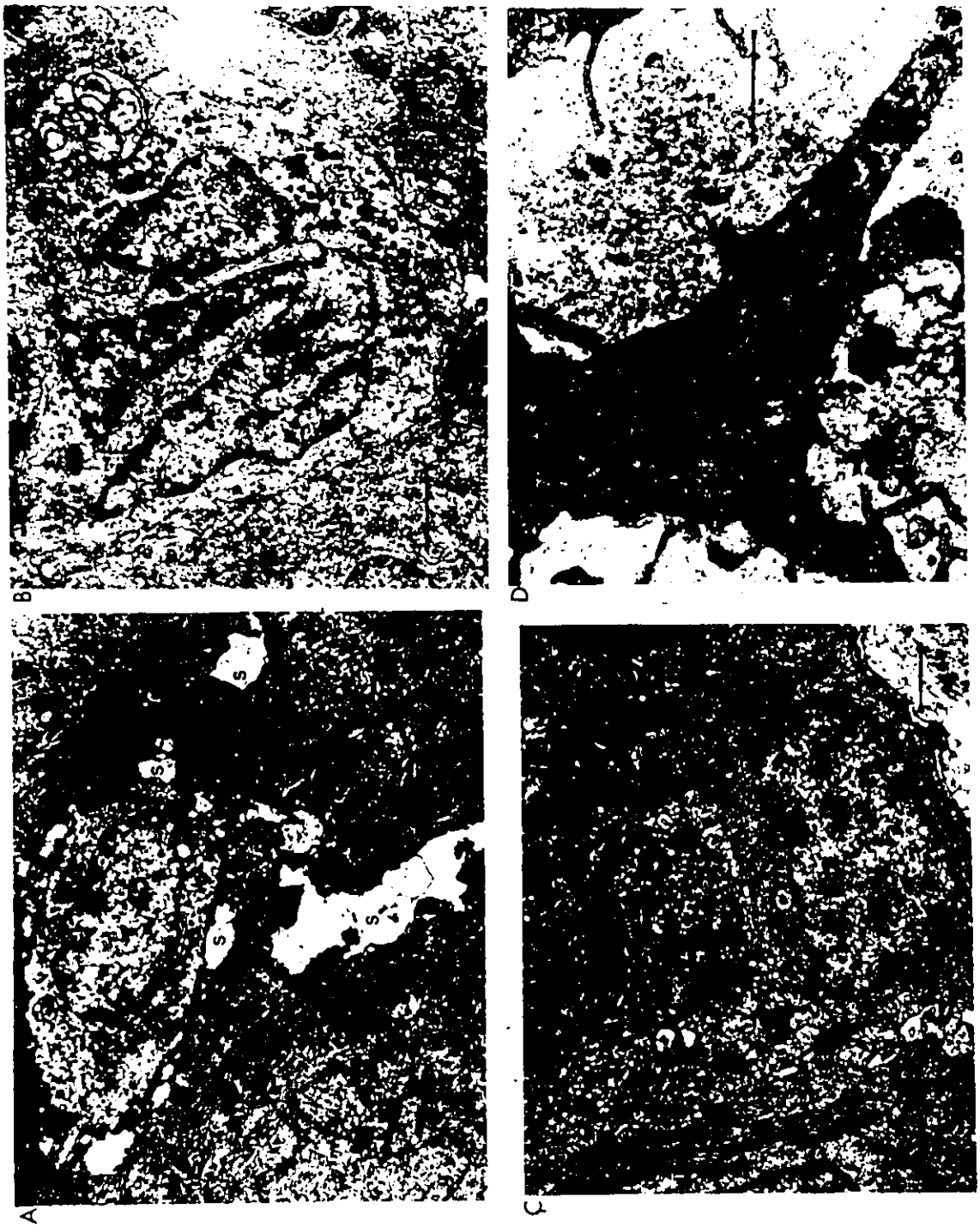
Fig. 42. Morphological observations on skin that had lost all mechanosensitivity after nerve section.

Samples of skin were taken at various times after nerve section (as described in the text) and processed for EM. (A) and (B) are electron micrographs of Merkel cells observed in 42 hr denervated skin. In (A), the extracellular space (S) contains some debris that may be from the degenerating nerve terminals. In (B), one nerve ending (at the upper right of the Merkel cell) contains swollen mitochondria, and the small space (x) may indicate a nerve ending that is "shrinking" away from the cell. The other nerve ending (at the lower right of the cell) appears normal.

(C) is an electron micrograph of a Merkel cell from a sample of skin taken 72 hrs after denervation. Notice the myelin figure (double arrowhead) in the nerve terminal.

(D) is an electron micorgraph of a portion of a Merkel cell from a sample of skin taken 1 week after denervation. Adjacent to the cell is an apparently degenerating nerve ending (NR); other amorphous material (A) also occupies the extracellular space adjacent to the Merkel cell. MC, Merkel cell; K, keratinocyte; S, extracellular space; n, nerve ending.

Calibration bar: 1 μm


B
D
S
S
S
S
A
C

that is filled with what appears to be remnants of a degenerating nerve terminal, as well as some amorphous material. In all cases, the Merkel cell itself does not show any obvious morphological effects of the nerve section, other than loss of the nerve contacts.

(b) Morphological observations during the recovery of mechanosensitivity:

As mentioned earlier, at each of the physiological stages of recovery of sensitivity following nerve regeneration subsequent to a nerve crush lesion, physiologically mapped skin samples were removed and processed for EM examination. Table 8 summarizes the observations made to date on material examined from a number of these samples.

In samples where there was no physiological response to mechanical stimulus, none of the Merkel cells examined (11) showed any nerve profiles contacting the cells; in fact, there were rarely any nerve endings observed near or around the cells. Fig. 43 shows an example of a Merkel cell from insensitive skin, taken 4 weeks after the nerve crush. There is a large space around the cell, with some debris in it, which looks like it might have been the space where the nerve had originally contacted the cell. In the insensitive samples, examination of the nerve bundles in the dermis revealed Schwann cells surrounding bundles of axons at least some of which looked normal (Fig.43b), although there were a few that appeared to be degenerating. In several cases, the 'new' bundles were very near to areas of what appeared to be degenerating nerve bundles, suggesting that maybe the

Table 8. Summary of the morphological observations made at various stages of physiological recovery of mechanosensitivity following nerve regeneration.

Physiological Stage	# Merkel Cells Examined	# Merkel Cells With Nerve Contact	# Nerve Profiles* Observed	# Nerve Endings** that Contact Merkel Cells	# Synapses*** Observed
Insensitive	11	0	0	0	0
Brush Sensitive	13	11	31	20	11
Proddable	30	15	47	22	4
Recovered	6	5	8	5	4 +
Control (Contralateral leg)	9	9	25	23	16 ++

* This is the number of nerve profiles that were observed near (not greater than 2 um away) the Merkel cells.

** This is the number of nerve profiles observed that actually contacted the Merkel cells.

*** This is the number of synaptic contacts observed between nerve endings and Merkel cells; these synapses were generally morphologically polarized from Merkel cell to nerve ending.

\+ One of the synapses observed at this stage was reciprocal, i.e., morphologically polarized from Merkel cell to nerve ending at one point, and from nerve ending to Merkel cell at an adjacent region of the same nerve terminal.

++ Four of the synapses observed in control (the contralateral leg) samples were reciprocal synapses.

Fig. 43. Electron micrographs from insensitive skin.

(A) shows a Merkel cell (MC) observed in insensitive skin from an animal examined 4 weeks after the crush lesion. A large space (S) adjacent to the Merkel cell appears to contain debris that could be the remains of the original nerve to this cell.
K, keratinocyte; bl, basal lamina; d, dermis.

(B) is a micrograph of a nerve bundle in the dermis of the same skin sample. Sc, Schwann cell; the profiles like that indicated by x could be degenerating axonal profiles.

Calibration bar: 1 μm


A
S
B

regenerating fibres do follow the old degenerating fibres or the old Schwann sheaths back to the periphery.

As mechanosensitivity begins to return to the skin, nerve endings are seen close to and contacting the Merkel cells. Table 8 indicates that 11 of 13 Merkel cells examined in the brush-responsive stage, the earliest stage of detectable sensitivity, had some form of nerve contact. Fig. 44 shows 2 different Merkel cells from the same skin sample. In (A) 3 small nerve endings are very close to the Merkel cell but do not contact the cell, at least in this section. In (B) on the other hand, not only does the nerve ending contact the Merkel cell but it also apparently forms a synaptic contact.

In the stage where punctate mechanosensitivity is recovering, only 15 of 30 Merkel cells examined had nerve contact. Fig. 45 presents 2 Merkel cells from samples that displayed low- threshold (A) and high-threshold (B) punctate mechanosensitivity. The nerve ending in (A) appears to form a synaptic contact with the Merkel cell, while in (B) the small nerve ending barely contacts the cell.

In both the brush-responsive and prod categories, it was not possible to determine if there were any particular differences in the morphological features of the Merkel cell- neurite complex that could be correlated with the physiological features. In fact, it appears that the brush-sensitive stage has more synaptic contact than the stages where punctate mechanosensitivity was returning. This observation probably results from sampling error, i.e., the amount of material examined and the EM sampling procedure. Many of the

Fig. 44. Electron micrographs of Merkel cells from reinnervated skin that was brush-sensitive.

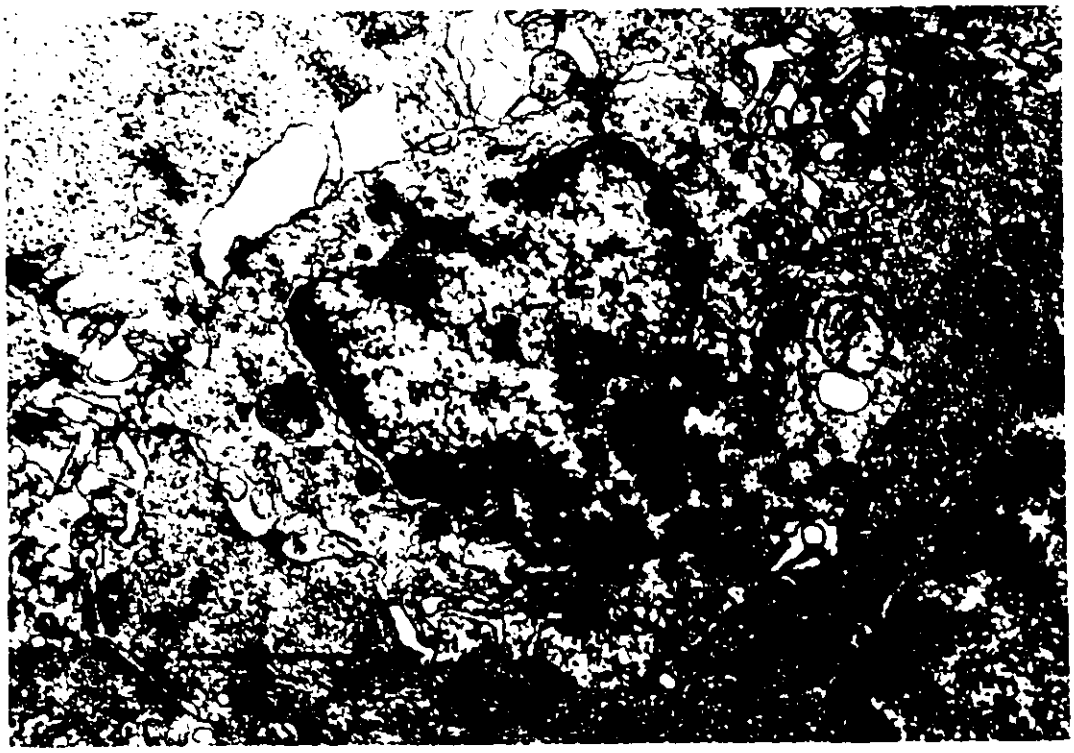
(A) and (B) are micrographs of two Merkel cells from the same sample of skin from an animal examined 6 weeks after the skin nerves were crushed. The skin was responsive only to the brushing stimulus.

In (A), there are small nerve ending profiles (n) very close to the Merkel cell (MC). K, keratinocyte.

In (B) a nerve ending (n) appears to form a synaptic junction (arrow) with the Merkel cell (MC). K, keratinocyte.

Calibration bar: 1 μm

A



B

Fig. 45. Electron micrographs of Merkel cells from reinnervated skin which was responsive to punctate mechanical stimulation.

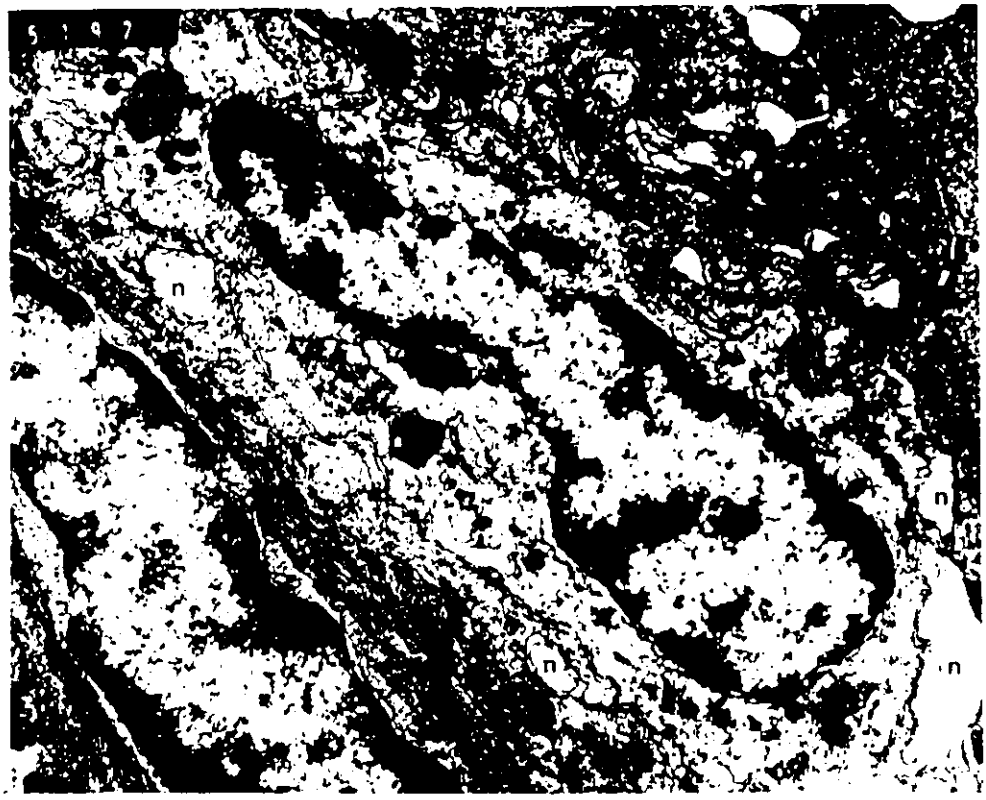
(A) is a micrograph of a Merkel cell (MC) observed in a sample of skin from an animal which was examined 9 weeks after the nerve crush; the skin displayed low-threshold mechanosensitivity, although the experimental threshold distribution curve was still significantly different from that of the control (see Fig. 35A). The nerve ending (n) contacts the Merkel cell (MC) and appears to form a synaptic junction.

(B) is a micrograph of a Merkel cell observed in a sample of skin from an animal which was examined 6 weeks after the nerve crush; the skin displayed high-threshold mechanosensitivity (Fig. 34D). There are several small nerve endings (n) present, two of which barely contact the Merkel cell in this section.
K, keratinocyte; d, dermis.

Calibration bar: 1 μm

A

B



brush-sensitive samples were examined more or less serially, i.e., several sample grids of 5-10 thin sections, separated by several semi-thin sections were looked at in the EM; usually only 5-10 sections from each block of the prod samples were examined. In these particular stages of physiological recovery, examination of individual touch spots of known physiology should provide more useful information regarding any morphological correlates of the physiological recovery, and this will be done in a future extension of the present work.

In the skin samples taken from animals that had recovered physiologically, 5 of 6 Merkel cells observed had a nerve ending contact and most of these contacts (4 of 5) were morphologically synaptic. In one particular example (Fig. 46), a possible reciprocal synapse was observed. Fig. 46 shows 4 micrographs of different sections through the same cell; (A) and (B) are serial sections, (C) is very close to these and (D) was taken about 2 μm away from the others. In (A) and (B), a post-synaptic dense bar is seen associated with the nerve membrane, and in (B) at the same area pre-synaptic cytoplasmic densities are present at the Merkel cell membrane; this is morphological evidence of a synapse polarized from Merkel cell to nerve ending. In the same terminal there is clustering of the synaptic vesicles very close to the nerve membrane, and although it is very difficult to see on this section, there appears to be some membrane densities in the region of the vesicle cluster; these observations are characteristic of a synapse morphologically polarized from nerve ending to Merkel cell. All these observations would suggest that this

Fig. 46. Electron micrographs of a Merkel cell from physiologically fully recovered skin.

A-D are micrographs of the same Merkel cell from a sample of skin from an animal examined 14 weeks after the nerve crush; the mechanosensitivity had returned to control levels.

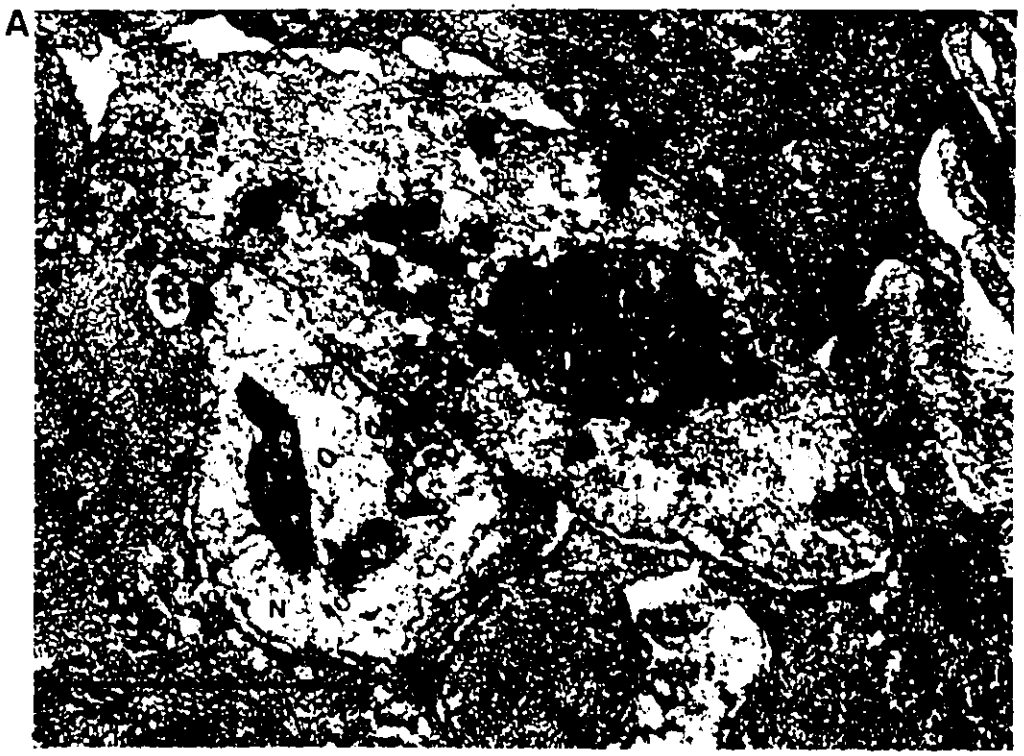
(A) and (B) are serial sections. In (A), vesicles in the nerve ending (N) cluster (filled triangle) near the Merkel cell-nerve contact area; the adjacent nerve membrane shows an apparent post-synaptic thickening (open triangle). In (B), pre-synaptic cytoplasmic densities are present at the Merkel cell membrane (single arrowheads); on the nearby nerve membrane, there is a clustering of vesicles and possible pre-synaptic cytoplasmic densities present (double arrowhead).

(C) was taken several sections away from (A) and (B), and shows another area of synaptic contact (double arrowhead) with pre-synaptic cytoplasmic densities at the Merkel cell membrane and a post-synaptic density of the nerve terminal membrane.

(D) was separated from (A) - (C) by about 2 um, and shows an area of synaptic contact with a post-synaptic density of the nerve terminal membrane (arrowhead).

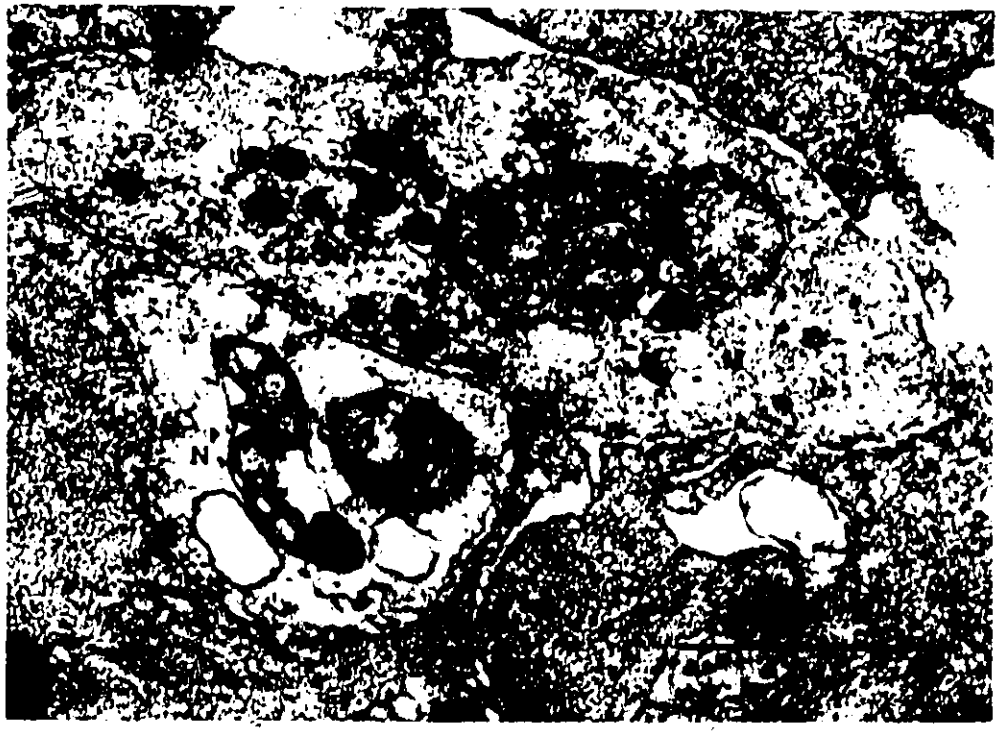
M, Merkel cell; N, nerve ending.

Calibration bar: 1 μm

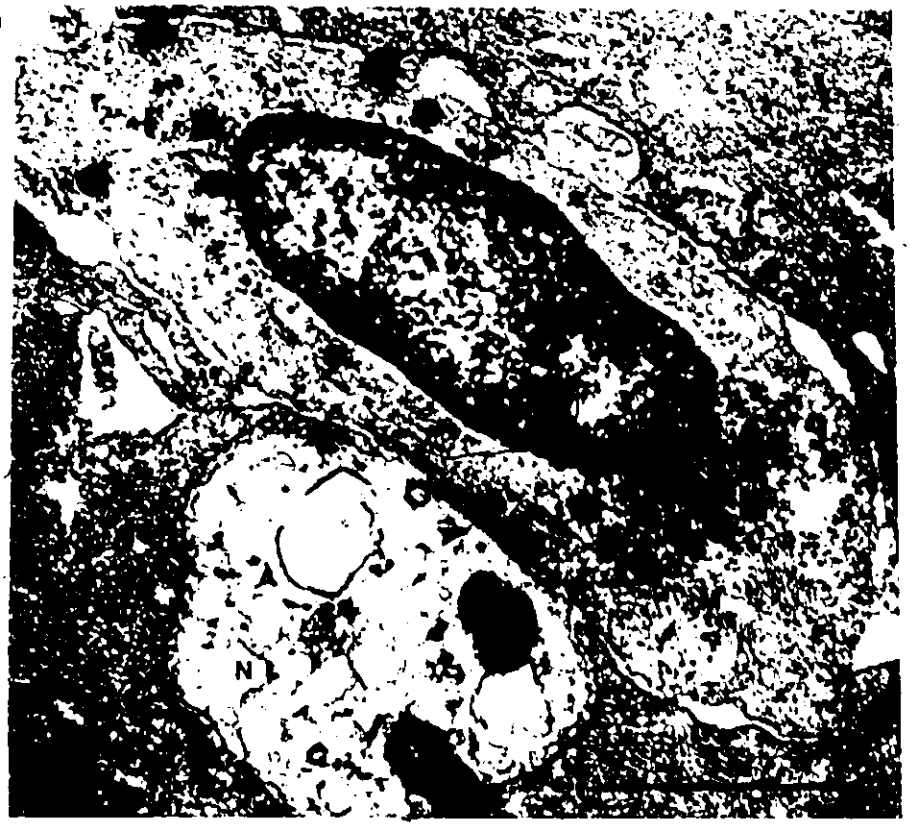

A
N


B
N

C



D



synaptic junction is indeed a reciprocal synapse. In the experimental material examined, such a reciprocal synapse was seen only in the fully physiologically recovered samples.

Clearly more material must be examined before any definitive comments can be made regarding the role of the Merkel cell in the mechanosensory process. However, to date it appears that the recovery of mechanosensitivity is correlated with the appearance of nerve-Merkel cell contacts. From preliminary results, the Merkel cell to nerve synapse develops prior to the nerve to Merkel cell synapse. However, both components of the reciprocal synapse have been seen only in totally physiologically recovered skin, suggesting that both components may be involved in normal sensory function.

Section VII

Final Discussion

The relationship between Merkel cells and mechanosensory nerves in Xenopus skin

The results of the present investigation indicate that there is a single population of rapidly-adapting, low-threshold mechanoreceptors in Xenopus skin. The location of these physiologically-defined "touch spots" is coincident with that of the gland openings, each of which is surrounded by 1-4 Merkel cells, which were visualized with the fluorescent dye quinacrine, and by EM examination. These Merkel cells were almost always found to be contacted by nerve endings. Thus it is concluded that the morphological correlate of a touch spot is one, or more, Merkel cell-neurite complex(es). If all the Merkel cells around a single gland opening are innervated by the same axon, then the innervated cluster would constitute a single sensory unit. Since in general only a single spike was obtained when the applied stimulus at any one gland opening was varied within the low-threshold range, it is likely that a single axon frequently innervated the Merkel cells around a gland opening. The stimulus required to elicit a second spike was always greater than 1.5 times the stimulus required to evoke the first spike

(Appendix Table 1), i.e., always well outside the low-threshold range of mechanosensitivity, the upper limit of which was never greater than 1.0 μm/ms. Accordingly, the individual Merkel cell-neurite complexes would be expected to have thresholds that were virtually identical, for if they were quite different, a single stimulus may give rise to multiple spikes in the same axon, though with different initiation sites.

Each grouping of Merkel cells, i.e., each touch spot, was found to be from 50 to 125 μm apart, based on the measurements of the distances between gland openings, and between OFC groupings. Using these measured values for the spacing of receptors, values for the radius of receptive fields were calculated using the equations described in Part I, section 8. The corresponding range of these calculated receptive field radii was 15 to 63 μm. It should be noted that this analysis assumes that a touch spot is a single mechanoreceptor or sensory unit in the low-threshold range without reference to the number of innervated Merkel cells. As mentioned earlier, the lowest-threshold Merkel cell-neurite complex of a gland opening must constitute this single receptor, although, as discussed above, some or all the complexes supplied by the same axon could have essentially identical thresholds. Higher-threshold complexes (if present) do not enter into the analysis, whether supplied by the same or different axons.

These results can be compared with those obtained in the salamander; in both animals, the Merkel cell-neurite complexes are

associated with discrete, low-threshold, rapidly-adapting mechanosensitive spots (Cooper and Diamond, 1977; Parducz et al., 1977). The receptor thresholds in both cases are also similar, with the range of low-threshold stimuli being 0.5-0.97 μm/ms in the salamander (Cooper and Diamond, 1977). In the salamander, however, only one Merkel cell is found at each touch spot, while in Xenopus there can be 1-4 Merkel cells at each mechanosensitive spot. The salamander touch spots were found to be about 250 μm apart, and based upon this physiological measurement the receptive field radius was calculated to be approximately 75 μm. The Xenopus mechanoreceptors are more closely arranged, as indicated by actual measurement and by the physiological mapping which showed that a higher proportion of tested points occurred within the low-threshold range (30-60% as compared to the salamander value of 20-42%). The calculated receptive field sizes of the Xenopus mechanoreceptors are thus somewhat smaller than those of the salamander; however, the salamander receptive field size must be regarded as approximate due to unavoidable errors in calculating D. The major difference between the two animals is that the Merkel cell -neurite complexes in Xenopus are arranged orderly (around the gland openings), while those in the salamander are scattered more or less randomly through the skin.

The Merkel cells as targets of mechanosensory nerves

A developmental study of the Merkel cells was not carried out so the possibility that Merkel cells act as targets during primary

development was not directly examined. The role of the Merkel cells as targets was investigated in two other situations, i.e., in denervated and newly-regenerated skin. The results bear on the question of whether Merkel cells could play such a role during development. However, in order to put the discussion of this into perspective, the origin of Merkel cells, and whether nerves are required for their development, will be addressed.

(a) Developmental origins of Merkel cells

The developmental origin of the Merkel cells is not clear. It has been proposed that mammalian and amphibian Merkel cells develop from undifferentiated basal epidermal cells, since the Merkel cells are confined to the epidermis, and have tonofilaments similar to the epidermal cells and also have desmosomal attachments with the surrounding keratinocytes (Munger, 1965; Kurosumi et al., 1969; Lyne and Hollis, 1971; Tachibana, 1979; Call and Bell, 1979; English et al., 1980; Ochiai and Suzuki, 1981); invasive cells (i.e., from the dermis) do not normally have these characteristics (English, 1977; Tachibana 1979; Ochiai and Suzuki, 1981). Other investigators suggest that the Merkel cells may be of dermal origin, since fetal human Merkel cells have been described as occurring either totally or partially within the dermis, as if they were migrating into the epidermis (Hashimoto, 1972; Winkelmann and Breathnach, 1973). In avian species the Merkel cells are normally found in the dermis, suggesting a dermal origin is possible. Saxod (1980) however suggests that they may be of a neural crest origin

and migrate into the dermis along with the early arriving nerves. On the other hand, a neural crest origin of Merkel cells is questionable in amphibians since Tweedle (1978) observed development of Merkel cells in aneurogenic salamander larvae, whose presumptive neural tissue, including the neural crest, had been removed; in these animals the Merkel cells developed normally with respect to both the morphological characteristics and number of cells present.

In the present investigation it was found that Merkel cells appeared in new skin that regenerated in place of a portion previously excised; since this occurred in a totally denervated hind limb, it appeared that nerves were not required for Merkel cell development. It is possible that some Merkel cells in the new skin had migrated in from the surrounding intact skin, but there was no evidence for a large scale (if any) depletion of Merkel cells in the surrounding skin. Thus, it seems plausible that the Merkel cells in the regenerated skin differentiated de novo. Since new Merkel cells were always seen in the epidermis (using the quinacrine fluorescent method), and never in the dermis, the results are consistent with an epithelial origin of the Merkel cells.

(b) Nerve dependence or independence of Merkel cells

The results of the experiments with newly-regenerated skin in nerve-free limbs also demonstrate that the appearance of Merkel cells is independent of nervous influence; similar conclusions were reached for Merkel cells in the salamander by Tweedle (1978) and Scott et al

(1981). In addition, it was shown that nerves are not required for the continued maintenance of Xenopus Merkel cells since these cells survived denervation for at least as long as 30 weeks.

The influence of the nerve upon the development and maintenance of the Merkel cells in other animals is not as clear cut, mainly because of the variation in the experimental results obtained from different species. For example, mammalian Merkel cells have been observed in the epidermis before nerves are seen in the epidermis; nerves are, however, observed within tens of microns away in the dermis directly below the developing Merkel cells (Lyne and Hollis, 1971; Call and Bell, 1979; English et al., 1980), and it is possible that a nerve influence on the differentiation of the Merkel cells may be exerted at a distance. In avian species, it has been demonstrated by Saxod (1978, 1980) that the differentiation of Merkel cells is clearly dependent upon the presence of sensory nerves, since in the absence of sensory innervation, no Merkel cells develop. There are also conflicting reports in the literature regarding the effect of denervation on Merkel cells. Several studies have provided evidence for the rapid degeneration and loss of mammalian Merkel cells following denervation of touch domes and vibrissae (Brown and Iggo, 1963; Palmer, 1965; Burgess et al., 1974; English, 1977; Chelysev and Vinter, 1983). There are, however, other indications that at least some mammalian Merkel cells survive denervation (Smith, 1967; Benkenstein, 1979; Hartschuh and Weihe, 1979; Nurse et al., 1983a). As discussed earlier (Background to the Investigation) a recent quantitative study suggests

that in the rat there may be two populations of Merkel cells, one which is stable and survives denervation and another labile sub-population which is sensitive to denervation (Nurse et al., 1983a).

(c) The Merkel cell is a target

The results of this investigation are consistent with the hypothesis that Merkel cells can act as targets for cutaneous mechanosensory axons. The criterion used to determine this role for the Merkel cells was that when the nerves were allowed to grow back into denervated skin, or into newly-regenerated skin, the Merkel cells already present within such skin should become innervated. The reinnervation of denervated skin by the regenerating crushed 8th nerve was monitored by following the recovery of mechanosensitivity using the physiological mapping technique. The recovery of sensitivity was gradual, but eventually acquired the normal low-threshold characteristics (discussed further below). The density of touch spots also returned to control levels and it appeared that all the originally innervated sites (i.e., the gland-opening loci) had become reinnervated. The Merkel cells were shown to survive denervation, and EM examination of samples of reinnervated skin showed that Merkel cells were indeed reinnervated.

The innervation of newly-regenerated skin was similarly monitored. Two different experiments were carried out; in one case, the innervation of regenerated skin arose from nearby nerve fibres already present in the surrounding skin. In the other situation, it

arose from a regenerating nerve that was allowed to reinnervate an otherwise nerve-free hind limb. Although in the first case (innervation by the surrounding nerves) the recovery was not complete, the trend was clearly towards the normal distribution of low-threshold points; in the second situation (regeneration of a nerve into the denervated regenerated skin) mechanosensitivity had recovered to the control level. Though the innervated regenerated skin samples have not yet been examined with EM, it is likely that the newly-acquired Merkel cells (which developed in normal numbers) do become innervated, as did their counterparts that survived denervation in skin that was subsequently reinnervated. Confirmation of this point is presently underway.

Previous studies of a similar nature in the salamander demonstrated that the eventual recovery of mechanosensitivity following nerve regeneration to denervated and to regenerated skin was associated with the innervation of Merkel cells(Scott et al., 1981); although the temporal relationship between recovery of sensitivity and innervation of Merkel cells was not examined as in the present study, it appears that Merkel cells in amphibian skin generally act as targets for cutaneous mechanosensory nerves.

There is also evidence that mammalian Merkel cells may act as targets for mechanosensory nerves. For example, during primary development the Merkel cells that appear in the mammalian epidermis at touch domes prior to nerve contact (e.g., English et al., 1980), probably act as targets by determining the termination sites of the

growing nerve fibres. This could apply even if the nerves were required to initiate the differentiation of the Merkel cells by a trophic mechanism operating at a distance. Further, the Merkel cells in the touch domes may act as targets even in the post-natal animal, since denervated domes may become successfully reinnervated by sprouting (Jackson and Diamond, 1981) or by regenerating nerve fibres (Horch, 1982; Nurse et al., 1983c). Nurse et al (1983c) have suggested that the Merkel cells which survived denervation may promote a rapid and successful reinnervation of the touch domes by acting as targets for regenerating nerves.

(d) How do the Merkel cells act as targets?

If the Merkel cells act as targets for cutaneous mechanosensory axons, one would like to know how their target influence is expressed. For example do the nerves grow randomly, and recognize the Merkel cells as appropriate ones with which to form specific associations when they contact them? Or do nerves grow selectively to the Merkel cells under some trophic influence? In the latter case, presumably "recognition" might still be required.

Nerve fibres regenerating after crush most likely follow their former (now degenerated) pathways back into the skin (e.g., Burgess et al., 1974; Sunderland, 1978), although Horch (1982) proposes that the touch dome sites exert a trophic influence on the regenerating fibres. In some of the reinnervated skin samples that were examined with EM in the present study, there were new nerve bundles running alongside old

degenerating nerves, supporting the possibility that the old paths provide guidance, probably mechanical in nature. In experiments where new skin regenerated in the place of a portion previously excised, the nerve fibres serving the adjacent skin would have to regenerate, or sprout, into the regenerated skin in the absence of any such pre-existing pathways. Similarly, in the experiments where new skin regenerated in nerve-free limbs, the regenerating nerve fibres could only have followed degenerating pathways (Schwann tubes, Sunderland, 1978) as far as the edge of the new skin patch. Therefore, in these cases, degenerating pathways could not have provided the final route for the reinnervation of the new Merkel cells. This kind of guidance could be purely mechanical, in terms of providing an easily accessible pathway for the nerves to follow, or a surface that was especially favorable for nerves to grow on (e.g., Letourneau, 1975; Bunge, 1981). Alternatively the degenerating pathway might provide some chemical attraction. For example, Skene and Shooter (1983) have shown that degenerating Schwann cells associated with the endoneurial tubes of degenerating nerves synthesize a specific protein, which accumulates in the extracellular space, at a time when nerve regeneration is occurring; they suggest that this factor might act to promote some aspect of axon growth. All of these hypotheses relating to degenerating pathways have features that involve the Schwann cells, or the various connective tissue coverings of nerves or nerve fascicles. Thus these pre-formed pathways do not provide a path directly into the epidermis, and thus directly to the Merkel cells, since the individual

myelinated nerve fibres which innervate Merkel cells normally lose their Schwann sheaths prior to their entry into the epidermis (e.g., Kruger et al., 1981). One still has to account for a means whereby the nerves successfully end up at the Merkel cells.

One possibility is that once the nerves reach the skin they grow randomly until they eventually encounter cells (the Merkel cells) which they somehow recognize as an appropriate site of termination. One might be able to distinguish between this possibility and one that involves "directed" growth to the Merkel cell if one were able to measure the average time taken for a Merkel cell population to become innervated; an entirely random growth might take somewhat longer than a directed one. In addition, with the use of histological sections, one might be able to distinguish random growth from directed growth by, perhaps, the presence in the former case of a profusion of randomly-oriented nerve fibres in the dermis and epidermis, not necessarily in the immediate area of the Merkel cells. However, since histological techniques were not used to investigate this point in the present thesis, it is not possible to distinguish between the two possibilities in this investigation.

Alternatively, the Merkel cells might release some diffusible factor (perhaps stored in the characteristic dense- cored granules) that attracts the nerve fibres to the cell; e.g., the nerves might take up the factor which could then act as a trophic substance (such a diffusible factor could probably be effective over limited distances, less than 1 mm (Crick, 1970)), or the factor might act to increase the

adhesivity of the immediate environment, thus providing a preferential pathway for nerve growth to the cell. In vitro experiments have demonstrated that growing neurites can respond to local concentrations of growth factors by orienting their processes in the direction of the source of the factor (Letourneau, 1978; Gundersen and Barrett, 1980; Campenot, 1982); other studies have shown that neurites grow preferentially along surfaces of increased adhesivity (Letourneau, 1975). A recent study dealing with the growth of chick brachial axons into wing bud mesenchyme, suggests that the target premuscle mass may act as a source of an adhesion gradient which establishes selective nerve pathways (Nurcombe and Bennett, 1983). In Xenopus Merkel cells there is no direct evidence for the storage or release of either a trophic factor or one which acts to increase local adhesivity. Earlier studies on the salamander proposed that a "sprouting stimulus" was released from denervated skin, and that this factor was responsible for inducing sprouting of the adjacent intact nerves into the denervated skin (Aguilar et al., 1973); this proposal was later extended to suggest that the Merkel cells might be releasing a factor that caused the nerves to sprout (Cooper et al., 1977; Scott et al., 1981; Diamond, 1982). Presumably such a sprouting factor could cause neurites also to grow "upstream" to the source of the factor (e.g., Letourneau, 1978; Gundersen and Barrett, 1980). It is tempting to speculate that the dense-cored granules found in the Merkel cells might contain such a factor, although the substances suggested to be present in these granules (i.e., met-enkephalin, Hartschuh et al., 1979; VIP, Hartschuh

et al., 1983; ATP, purines, Crowe and Whitear, 1978; Bock, 1980; Nurse et al., 1983b) have not yet been tested for their action as possible trophic factors.

The possibilities discussed above dealing with how the Merkel cell might exert its target influence are speculative; the present investigation does not provide any results regarding these proposals. However, preliminary experiments carried out prior to the commencement of the present study, involving the co-culture of Xenopus tadpole dorsal root ganglia (DRG) and skin explants, indicated that there was indeed preferential growth of the DRG neurites towards the skin; whether the influence of this target tissue, which was clearly exerted over distances up to 500 μm, was related to the presence of Merkel cells is unclear (Mearow et al., 1981). It could be that the skin itself exerts a gross attractive influence on the growing neurites, and then once the neurites have invaded the skin, the Merkel cells either provide a finer influence which acts to direct the growing endings, specifically towards them, or constitute recognisable targets with which the neurites form specific associations.

(e) Recognition of the Merkel cells by the nerve endings

The foregoing section discussed possible ways by which mechanosensory axons regenerating to the skin may arrive at within the general vicinity of their targets, the Merkel cells. The question that now arises is how does the final recognition occur, such that the axons stop growing and synapse with the Merkel cell. Possible mechanisms of

this recognition are suggested by other investigations of cell-cell phenomena in which various membrane macromolecules, such as glycoproteins and glycolipids, have been implicated in a "recognition" process. Numerous investigations have demonstrated the existence of specific molecules associated with different neuronal cells, and often with specific areas of these cells, which could be involved in specific cell recognition, in a manner perhaps similar, for example, to the recognition of antigens by antibodies in the immune system (e.g., Gottlieb and Glaser, 1982; Schachner, 1982; Edelman et al., 1983; Hockfield and McKay, 1983). Indeed, the sites of synaptic contact on synaptosomes from the CNS (Kelly et al., 1976) and on muscle fibres (Sanes and Cheney, 1982) are known to display specific membrane components which are different from the adjacent non-synaptic membrane. The basal lamina associated with the neuromuscular junction also displays certain molecular components which may allow it to be recognized by regenerating nerve fibres as the site of synaptic contact, even in the absence of the underlying muscle fibres (McMahan et al., 1983). Could it be that the basal lamina beneath the Merkel cells (at least in mammalian skin, in which the Merkel cells are closely associated with the basal lamina) is specialized in such a way that allows that growing nerve fibres to recognize the site as as appropriate point of entry into the epidermis, providing access to the Merkel cells close by? In amphibians, however, the Merkel cells are generally found approximately one cell layer above the basal lamina (Fox and Whitear, 1978) and thus there may still be a need for a

further influence of the Merkel cell on the nerve ending, perhaps as discussed earlier.

Although there is no information on the basal lamina as a site of specific molecules that could be recognized by mechanosensory nerves, a recent study of the lectin-binding properties of rat skin indicates that the biochemical characteristics of the Merkel cell membrane may be different from (at least) the surrounding basal cells (Rosati et al., 1983); such a difference might allow the cutaneous mechanosensory axons to recognize the Merkel cells.

Recovery of mechanosensitivity and reinnervation of Merkel cells in denervated skin

Following its denervation the recovery of mechanosensitivity in the skin overlying the thigh of Xenopus frogs was gradual. The earliest sign of functional recovery, a response in the regenerating nerves only to the brushing of the skin, was observed at 3, and sometimes not until up to 6 weeks after the initial lesion; high-threshold punctate mechanosensitivity was seen between 4.5 to 9 weeks, while recovery to the normal low-threshold state occurred between 9 and 14 weeks after the nerve crush (see Fig. 40 in Part IIIA, section 5).

The time between the earliest sign of funtional reinnervation and total recovery is, presumably, that required for the maturation of the regenerating nerve fibres, i.e., an increase in fibre diameter and myelination, perhaps the maturation of the nerve endings to the

characteristic mechanosensory state, and probably also for the physiological and morphological maturation of the Merkel cell-neurite complexes. A question that arises is whether the nerve endings acquire their sensitivity progressively as they grow along, the final low-threshold character being achieved without a requirement for any association with the Merkel cells. Another possibility is that the free nerve endings are responsive, but only to large stimuli such as brushing of the skin; perhaps also they could be responsible for the appearance of the high-threshold punctate mechanosensitive responses. The latter possibility cannot be ruled out from a consideration of the time course of the development of mechanosensitivity mentioned above. Indeed, an initial recovery of high-threshold sensitivity in the free nerve endings is supported by indications that regenerating nerve fibres are actually very sensitive to mechanical stimulation (e.g., Diamond, 1959; Sunderland, 1978; Zimmermann and Sanders, 1982).

Before further discussion of the simplest hypothesis that there is a progressive recovery of function in a single population of mechanosensory nerves, another possibility must be considered; this is that there are two normal populations of cutaneous mechanoreceptors: one, which is a high-threshold mechanoreceptor, recovers first and is responsible for the high-threshold mechanosensitivity seen at the early stages of physiological recovery; such high-threshold receptors would be normally activated (along with the low-threshold ones) by brushing the skin (perhaps the lamellated receptors described by von During and Seiler (1974) are in this high-threshold category), a stimulus which is

rather large, and involves stretching of the skin in addition to its indentation. The second population would be the low-threshold one, that recovers later than the high- threshold population, but then masks the presence of the high-threshold receptor; this could be a real possibility in the present experiments, since the mapping procedure essentially reveals only low-threshold receptors. If there are two such populations of mechanoreceptors in normal skin, the high-threshold ones would have to be in the dermis, given the results of the experiments described in Parts I and II, which showed that, while the low-threshold receptors were located exclusively in the epidermis, high-threshold responses could sometimes be recorded by stimulation of sub-epidermal structures. The presence of a high-threshold receptor population does not, however, compromise the results of these experiments in terms of determining the role of the Merkel cell.

A role for the Merkel cell in the progressive recovery of mechanosensory function

(a) The shift from high- to low-threshold mechanosensitivity

Evidence supporting the idea of a single population of mechanoreceptors that shift progressively from a high-threshold to a low-threshold form comes from observations made in several animals examined towards the end of the investigation. In these animals, the recovery of mechanosensitivity was examined by comparing responses when the prodder tip was directly "on" the gland openings to those "off" the openings. Even when recovery was not yet complete in these animals,

the gland opening areas (the "on" points) were always the first to display any detectable responses; additionally, in those skins in which all the tested points were responsive, the lowest thresholds were always associated with the "on" points. Since the "on" sites were the locations of the Merkel cells, and since these became reinnervated in the recovered skin to re-establish the morphological basis of the touch spots, then these results support the view that during reinnervation by the regenerating nerves the reconstituted Merkel cell-neurite complexes first display a high-threshold response, which eventually recovers to the characteristic low-threshold state. The results of EM examination of skin at various stages of physiological recovery (described in Part IIIB of the Results) confirm that indeed Merkel cells are reinnervated some weeks earlier than the return of low-threshold mechanosensitivity to the same skin. Assuming that the overall sequence of events in the generation of an impulse in the axon is: stimulus - receptor potential - impulse mechanism, then in the early stages of recovery, a greater stimulus must be required (than in the normal condition) to produce a receptor potential that is adequate to reach threshold and initiate an action potential. How can one account for this high-threshold state? If the nerve terminals were smaller than normal, and/or had not established a necessary connection to the Merkel cell 'tightly enough' (e.g., by desmosomes, tight junctions, or synapses), then an inadequate area of nerve ending may be available for depolarization, and thus activation of an impulse might require a larger stimulus to effect the required depolarization. This would apply whether the Merkel cell were

actively or passively involved. Suppose it were the former, i.e., that the mechanical stimulus affected the cell directly, and this led to chemical or electrical stimulation of the nerve endings. A smaller (or less tightly coupled) nerve ending would require a larger current from the Merkel cell to be excited than would a larger (or better coupled) ending (e.g., Katz and Thesleff, 1957). Alternatively, the Merkel cell might release a chemical transmitter in response to mechanical deformation, which would then depolarize the nerve ending. Although there is evidence that regenerating sensory nerve terminals may be more sensitive to certain chemical stimuli (e.g., acetylcholine, Diamond, 1959), there is also evidence that during the early stages of reinnervation of targets the amount of transmitter release from the pre-synaptic nerve ending is smaller (or even absent) (e.g., Dennis and Miledi, 1974; Carmignoto et al., 1983). If the Merkel cell were indeed a pre-synaptic element, it too might go through a similar stage, and require a larger stimulus to release an amount of transmitter necessary to depolarize the post-synaptic element (in this case the nerve ending).

These possibilities are speculative; as will be seen from the following section, the actual mechanism of, and the involvement of the Merkel cell in, the physiological response of the Merkel cell-neurite complex to mechanical stimulation is still unknown.

(b) The Merkel cell or the nerve as the mechanosensory transducer?

One aim of the correlative study of the morphology and

physiology of the Merkel cell-neurite complexes was to determine whether the recovery of mechanosensitivity following nerve regeneration to denervated skin required that the nerve endings contact the Merkel cells, and whether there were any stages of the physiological recovery that could be correlated with the fine-structural appearance of the Merkel cell-neurite complexes. The results of this particular portion of the investigation are preliminary, and represent a part of a continuing investigation. The observations made to date on skin samples taken at various stages of physiological recovery following nerve regeneration show that nerve endings are associated with the Merkel cells (although synapses are not necessarily present) from the earliest stages of recovery right through to the fully functionally recovered stage. However, apparent full morphological recovery of the complex (i.e., normal appearing synaptic junctions and the presence of a reciprocal synaptic relationship) was seen only at the stage of full physiological recovery. These preliminary results seem to indicate that contact between the Merkel cell and nerve ending is required for recovery of the characteristic low-threshold physiological response; they do not, however provide firm evidence as to whether the nerve ending or the Merkel cell is the actual transducer of the mechanical stimulation.

Several investigators have suggested that the Merkel cell acts as the transducer (Iggo and Muir, 1969; Horch et al., 1974; Iggo, 1976). This proposal is based mainly on the fact that the morphological features of the Merkel cell-neurite complex, such as the

presence of dense-cored granules and synapses, resemble features which appear in other sensory systems where a specialized epithelial cell acts as the sensory transducer, e.g., the hair cells (Wiederhold, 1976; Hudspeth, 1983) and the taste bud cells (Murray 1973; Zahm and Munger, 1983). Furthermore, the results of studies on cutaneous reinnervation in mammals, indicating that the regenerating nerve fibres are unable to generate the characteristic physiological response (slowly-adapting) until the Merkel cell-neurite complexes have been restored (Brown and Iggo, 1963; Burgess and Horch, 1973; Burgess et al., 1974; Iggo, 1976), have been interpreted as supporting the role of the Merkel cell as the sensory transducer. It has been assumed that mechanical deformation of the Merkel cell would result in depolarization of the cell membrane, which would then allow release of the dense-cored granules whose contents would depolarize the afferent nerve terminals (Iggo and Muir, 1969; Horch et al., 1974; see also Gottschaldt and Vahle-Hinz, 1981). A direct test of this hypothesis, such as recording from the Merkel cells at the time the mechanical stimulus is applied to see if a depolarization is produced in response to mechanical stimulation, has not yet been possible; nor has it been possible to abolish the supposed chemical transmission with pharmacological agents, or by manipulation of the ionic environment (e.g., Smith and Creech, 1967; Gottschaldt and Vahle-Hinz, 1982). Intracellular recording has been done in bullfrog sacculus hair cells (Hudspeth and Corey, 1977; Hudspeth, 1983), and in the taste bud cells (Akaike et al., 1976; Roper, 1983). In the hair cells, it was found that mechanical stimulus (i.e., movement of the

hair bundle in a particular direction) caused a depolarization or a hyperpolarization of the hair cells, depending on the direction of movement (Hudspeth and Corey, 1977). With regard to the taste cells, application of chemical stimuli, such as salts or acids, produced a depolarization of the taste cell; water or dilute NaCl produced a hyperpolarization of the taste cell (Akaike et al., 1976). Roper (1983) has shown that the taste cells, like the hair cells (Hudspeth and Corey, 1977) are capable of producing action potentials, in addition to the local receptor potentials.

In support of the hypothesis that the nerve ending itself is the transducer, the results of experiments carried out by Kasprzak et al (1970) are usually cited; these experiments demonstrated that it was possible to record impulses in nerves supplying newborn kitten touch domes before the Merkel cells were present. However, this requires confirmation since use of histological sections for Merkel cell identification is questionable. Furthermore, the non-sustained discharge recorded before the Merkel cells were identified was abnormal; the ability to respond to prolonged stimuli with a sustained response correlated very well with the sudden appearance of numerous Merkel cells two weeks after birth, suggesting that the Merkel cell contributes to the normal characteristics of the complex. Gottschaldt and Vahle-Hinz (1981) have also concluded that the nerve ending is the transducer, based on electrophysiological analysis of the responses of cat vibrissae Merkel cell-neurite complexes to mechanical stimuli. By measuring receptor delay in response to high frequency stimulation in

nerve fibres that innervated Merkel cells and in fibres which were from mechanoreceptors other than the Merkel cell-neurite complexes, these authors observed that the receptor delay was essentially the same in both cases, indicating that a transduction process having a similar time course was present in both types of receptor. Furthermore, they suggested that the receptor delay observed was too short (0.2-0.3 ms) for chemical transmission to have occurred between the Merkel cell and nerve ending (Gottschaldt and Vahle-Hinz, 1981). A similar receptor delay (0.2-3 ms) has been observed in the response of the Pacinian corpuscle to mechanical stimulation, where the nerve terminal is the transducer, and where no chemical mediation in the transduction process is known to occur (Gray, 1959). It should be noted, nevertheless, that there are presumed chemical synapses, e.g., of Mauthner cell axon collaterals on spinal motoneurons in fish which do have delays of fractions of a ms (Diamond, 1971).

The role of met-enkephalin as a possible transmitter in the Merkel cell-neurite complex (Hartschuh et al., 1979) was tested by Gottschaldt and Vahle-Hinz (1982) in the cat. Since the met-enkephalin antagonist, naloxone, had no effect on the responses of any type I mechanoreceptor afferent nerve fibres (those fibres which innervate Merkel cells), it was concluded that met-enkephalin was not the putative neurotransmitter released from the Merkel cell (Gottschaldt and Vahle-Hinz, 1982). However, these physiological studies need to be reevaluated in light of recent evidence. They were done in cats whose Merkel cells do not exhibit met-enkephalin-like immunoreactivity as do

those in rodents (Hartschuh et al., 1983). Mammalian species other than rodents have now been shown to exhibit vasoactive intestinal peptide (VIP)-like immunoreactivity (Hartschuh et al., 1983). Thus it is possible that such peptides in the Merkel cells may still have a neuromodulatory function, such as influencing the threshold of the sensory nerve ending.

There is still no firm evidence, either in the literature or from the present investigation, for either possibility, i.e., whether the nerve or the Merkel cell is the mechanosensory transducer.

(c) An influence of the Merkel cell on the physiological characteristics of the nerve terminal

A possible alternative role for the Merkel cell is that it influences the differentiation or maturation of the immature nerve ending into the low-threshold, rapidly-adapting mechanosensitive state. The association of the Merkel cells with the nerve terminals, which has been observed with EM during the early stages of recovery of mechanosensitivity in this study is consistent with this possibility. Of interest to this proposal is a recent report suggesting that sensory receptor cells are capable of inducing physiological characteristics in the sensory nerves that contact them (Monti-Bloch et al., 1983). These workers transplanted chemosensory organs, the carotid bodies, into the tenuissimus muscle of adult cats and allowed the muscle nerve to reinnervate the carotid bodies. In the grafts that were reinnervated, 40% of the regenerated nerve fibres responded to both stretch (a normal

stimulus for these nerves) as well as to chemosensory stimuli, such as hypoxia, NaCN, ACh, dopamine and nicotine; the responses were similar to those seen in normally-innervated control carotid bodies. Examination of these grafts revealed nerve endings in association with the glomus cells, the presumptive sensory cells of the carotid body; in one graft that did not respond to the chemosensory stimuli, examination revealed a total absence of glomus cells in the graft. In control experiments, fibres that were made to innervate a graft of adipose tissue showed no response to the chemosensory stimuli that were employed. The conclusion drawn from these experiments was that the carotid body cells, in particular the glomus cells, were capable of inducing chemosensory properties in axons that normally subserve a mechanosensory function (Monti-Bloch et al., 1983). One point which these authors do not mention, however, is the possibility that there are fibres normally present in the tenuissimus nerve which <u>can</u> respond to chemical stimuli, such as hypoxia, under normal situations. Muscle afferent nerve fibres have been reported to respond to ischemic, hypoxic conditions (Hnik and Payne, 1965; Mense and Stahnke, 1983); however, whether they are affected largely by the pressure provided by the cuff which is used to induce ischemic conditions, or by the lack of oxygen <u>per se</u>, is not clear.

<u>The reciprocal synapse</u>

One possible mechanism that might operate in the Xenopus Merkel cell-neurite complex is suggested by the observations that (a) the

Merkel cell-neurite complexes in lower vertebrates (amphibians, reptiles and birds) are associated with rapidly-adapting mechanoreceptors, while in mammals the complexes are found in slowly-adapting mechanoreceptors, and (b) that the reciprocal synapse is most commonly described in amphibians (Fox and Whitear, 1978; Mearow and Diamond, 1983), and rarely, if at all, in mammals (Munger, 1977). In other sensory systems where reciprocal synapses have been described, i.e., the olfactory system (Reese and Brightman, 1965; Rall et al., 1966), the retina (Dowling and Werblin, 1969) and the carotid body (McDonald and Mitchell, 1975), these synapses are proposed to be involved in feedback control. One could envisage a similar sort of feedback control for the Merkel cell-neurite complex in Xenopus, and in other lower vertebrates in which these complexes show reciprocal synapses. For example, mechanical stimulation of the Merkel cell-neurite complex might result in depolarization of the Merkel cell membrane and release of the contents of the dense-cored granules; the released factor could then act to depolarize the nerve terminal exciting it, and coincidentally causing release of the clear vesicles from the ending which might act to hyperpolarize the Merkel cell membrane, thus inhibiting further release of the 'excitatory transmitter'. Such a feedback control could result in a rapidly-adapting response to mechanical stimulation. In the absence of reciprocal synapses, as in the mammalian complexes, there might be only a relatively maintained response during mechanical stimulation; significantly, slow adaptation does seem to require the presence of the

Merkel cells, since several studies have shown that regenerating, and developing, cutaneous axons lack the ability to generate a sustained discharge to mechanical stimulation until the Merkel cell-neurite complexes have been restored during regeneration (Brown and Iggo, 1963; Burgess and Horch, 1973; Burgess et al., 1974; Iggo, 1976) or appear during development (Kasprzak et al., 1970).

However, it is possible that, even in the absence of reciprocal synapses, the individual Merkel cell-neurite complexes in the mammal are not slowly-adapting; conceivably, were a relatively large number of rapidly-adapting complexes, all supplied by the same axon, to fire asynchronously in response to maintained mechanical stimulation, the result would be an irregular apparently slowly-adapting response. Such a response is reported to occur in cat touch domes, for example, which are singly innervated (Horch et al., 1974; Horch, 1979). Some resetting mechanism would have to be invoked nevertheless, since the number of impulses produced during a maintained response would be greater than the number of Merkel cell-neurite complexes present. There are usually 50-100 (or more) Merkel cells associated with the touch domes (e.g., Iggo and Muir, 1969; Nurse et al., 1983a), but the number of impulses evoked in response to a maintained stimulus can excede this number, depending on the length of the stimulus (Horch, 1979).

An observation which must be considered here, however, is that the mammalian nerve endings contain large numbers of mitochondria and the amphibian terminals have relatively few mitochondria in comparison.

Large numbers of mitochondria might be expected if each Merkel cell-neurite complex was slowly-adapting, because of the requirement for large sources of metabolic energy; rapidly- adapting endings would not require such a large energy source, hence the fewer mitochondria.

Alternatively, the difference in adaptation may relate to intrinsic differences in the electrical properties of amphibian myelinated nerve fibres as compared with the mammalian myelinated fibres. For example, it is known that the duration of repetitive activity in mammalian fibres is considerably longer than that in amphibian fibres in response to a maintained constant current applied directly to the nerves (e.g., Vallbo, 1964). This could be related to reported differences in ionic currents associated with the electrical activity, since impulses in many mammalian myelinated nerve fibres do not involve appreciable potassium currents (Rogart and Stampfli, 1982) in contrast to those in frog fibres which have large potassium currents. The difference in adaptation may be a result of differences in the sodium inactivation parameter, (Hodgkin and Huxley, 1952; Gestrelius, 1983), with the rapidly-adapting receptors displaying larger values for sodium inactivation than the slowly-adapting receptors (Gestrelius, 1983).

Whether or not the reciprocal synapses are involved in the physiological function of the Merkel cell-neurite complex, they may well be involved in trophic relationships between the Merkel cell and nerve ending. For example, they may allow a two-way communication that may be necessary for the maintenance of the complex. It is not obvious

however, why such communication should occur only in the amphibian system. Possibly, it might be more important to maintain this sort of communication in a situation where loss of one or two complexes essentially results in loss of the entire receptor (i.e., in the amphibian), in contrast to the situation in mammals where loss of one or two complexes would probably have very little effect on the overall response of receptors that contain several hundreds of such complexes, such as the touch domes or vibrissae.

Summary

To summarize, the Merkel cell-neurite complex is the basis of the low-threshold, rapidly-adapting mechanosensitivity in Xenopus frog skin. Although, the physiological role (if any) of the Merkel cells is still not clear, the recovery of low-threshold mechanosensitivity following nerve regeneration seems to require that nerve endings contact the Merkel cells. If this contact is not involved in the actual processing of sensory information, it may well be involved in the maturation of the nerve ending to the normal low-threshold state.

In relation to trophic interactions between the nerves and the Merkel cells, the latter clearly are targets for the growing cutaneous nerves which terminate by contacting the Merkel cells. The mechanism by which the specific association between the nerve ending and the Merkel cell becomes established is not clear. Whether or not an attractant trophic factor, or a 'sprouting stimulus' is released by the cell to act on the nerve, it seems inevitable that the Merkel cells must be recognized by the growing fibres as their specific targets.

Finally, the role of the reciprocal synapses remains enigmatic, although preliminary observations suggest that at least some of their features may be needed for the generation of the characteristic response to mechanical stimulation.

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APPENDIX TABLES

APPENDIX TABLE 1.

Critical stimuli required to evoke:

Spike 1	xT *	Spike 2	xT	Spike 3	xT	Spike 4
1.13	6.64	7.5	7.64	8.63	12.28	13.88
1.63	3.3	5.38	5.6	9.13	7.2	11.75
2.38	1.89	4.5	2.9	7.0	4.8	11.5
1.13	2.1	2.38	7.1	8.0	8.5	9.63
0.5	3.8	1.88	10.8	5.38	14.0	7.0
0.25	8.5	2.13	13.0	3.25	21.5	5.38
0.5	2.3	1.13	3.3	1.63	6.5	3.25
0.5	3.3	1.63	11.8	5.88	21.5	10.75
2.38	1.36	3.25	1.58	3.75	2.05	4.88
1.38	1.5	2.13	5.1	7.0	7.8	10.75
0.75	5.7	4.15	12.2	9.13	13.7	10.75
0.5	13.3	6.63	15.0	7.5	22.5	11.25
0.25	17.0	4.25	18.0	4.50	—	—
1.13	3.1	3.5	6.2	7.0	7.1	8.0
1.13	1.9	2.13	7.1	8.0	—	—
0.5	7.0	3.5	10.8	5.38	16.0	8.0
0.63	12.7	8.0	—	—	—	—
0.75	5.0	3.75	9.0	6.75	17.84	13.38
0.5	8.5	4.25	10.8	5.38	15.0	7.5
0.63	6.3	4.0	11.1	7.0	12.7	8.0
0.75	7.5	5.63	9.0	6.75	10.0	7.5
0.88	1.6	1.38	3.7	3.25	—	—
0.25	13.0	3.25	27.0	6.75	32.0	8.0
0.38	12.84	4.88	24.7	9.38	42.1	16.0
0.75	9.0	6.75	17.8	13.38	21.3	16.0
0.25	17.0	4.25	19.5	4.88	—	—
0.5	16.0	8.0	21.5	10.75	—	—
$\bar{X}$ 0.83	7.11	4.08	11.24	6.75	15.06	9.65
SE 0.57	5.11	2.00	6.76	2.53	9.52	3.42
n 27	27	27	26	26	21	21

* xT - represents the multiple of the critical stimulus (threshold, required to evoke the first spike) that is necessary to evoke the second, third and fourth spikes. Critical stimulus is in units of µm/ms.

APPENDIX TABLE 2. Estimate of the accuracy of critical stimulus measurements.

Trial #	Different Test Points *											
	1	2	3	4	5	6	7	8	9	10	11	12
1	0.5	0.25	0.5	0.75	0.38	0.25	0.5	0.25	0.5	0.38	0.25	0.38
2	0.75	0.25	0.38	0.75	0.38	0.25	0.63	0.25	0.63	0.38	0.25	0.38
3	0.63	0.38	0.5	0.63	0.38	0.25	0.5	0.38	0.5	0.5	0.25	0.38
4	0.5	0.25	0.5	0.75	0.38	0.25	0.5	0.25	0.5	0.38	0.25	0.38
5	0.63	0.38	0.63	0.75	0.25	0.25	0.63	0.38	0.5	0.38	0.25	0.38
6	0.63	0.38	0.5	0.63	0.38	0.25	0.5	0.38	0.5	0.5	0.38	0.38
7	0.5	0.38	0.5	0.75	0.38	0.25	0.5	0.25	0.63	0.5	0.25	0.38
8	0.63	0.25	0.5	0.63	0.38	0.38	0.63	0.25	0.5	0.38	0.25	0.25
9	0.63	0.25	0.5	0.75	0.25	0.25	0.5	0.38	0.5	0.38	0.25	0.38
10	0.5	0.25	0.38	0.75	0.38	0.25	0.5	0.25	0.63	0.38	0.38	0.38
$\bar{X}$	0.59	0.30	0.49	0.71	0.35	0.26	0.54	0.30	0.54	0.42	0.28	0.37
SEM	0.09	0.07	0.06	0.06	0.06	0.04	0.06	0.07	0.06	0.06	0.06	0.04

* Each test point was tested ten times for the critical stimulus measurement; this was done be raising the prodder off the skin, and then lowering it back down on the same point 10 times, each time recording the critical stimulus required to evoke an action potential.

APPENDIX TABLE 3. Side to side comparison of critical stimulus distribution in control animals.

		Left Side							Right Side						
		0.29 0.43	0.44 0.57	0.58 0.71	0.72 0.85	0.86 0.99	1.00 1.13		0.29 0.43	0.44 0.57	0.58 0.71	0.72 0.85	0.86 0.99	1.00 1.13	n
A	#	27	17	1	0	4	1		31	12	3	0	4	0	50
	%	54	34	2	0	8	2		62	24	6	0	8	0	
	Cum. %	54	88	90	90	98	100	Signif.	62	86	92	92	100	100	
	*Diff.	8	2	2	2	2	0	NS							
B	#	23	15	5	0	6	1		35	10	3	0	2	0	50
	%	46	30	10	0	12	2		70	20	6	0	4	0	
	Cum. %	46	76	86	86	98	100		70	90	96	96	100	100	
	Diff.	24	14	10	10	2	0	NS							
C	#	40	9	1	0	0	0		33	14	2	0	1	0	50
	%	80	18	2	0	0	0		66	28	4	0	2	0	
	Cum. %	80	98	100	100	100	100		66	94	98	98	100	100	
	Diff.	14	4	2	2	0	0	NS							
D	#	38	10	2	0	0	0		31	9	5	0	5	0	50
	%	76	20	4	0	0	0		62	18	10	0	10	0	
	Cum. %	76	96	100	100	100	100		62	80	90	90	100	100	
	Diff.	14	16	10	10	0	0	NS							
E	#	30	12	6	0	2	0		36	9	3	0	2	0	50
	%	60	24	12	0	4	0		72	18	6	0	4	0	
	Cum. %	60	84	96	96	100	100		72	90	96	96	100	100	
	Diff.	12	6	0	0	0	0	NS							
F	#	24	11	7	11	2	1		28	10	5	10	0	3	56
	%	42.9	19.6	12.5	19.6	3.6	1.8		50	17.8	8.9	17.8	0	5.4	
	Cum. %	42.9	62.5	75.0	94.6	98.2	100.0		50	67.8	76.7	94.5	94.5	100.0	
	Diff.	7.1	5.3	1.7	0.1	3.7	0	NS							

* Diff. = $(A_{Cum.\ \%} - B_{Cum.\ \%})$. This is the actual Difference that is then compared with the calculated Difference for the Kolmogorov-Smirnov test. $D_{Calc.} = 1.95\sqrt{n_1+n_2/n_1n_2}$, at P=0.001.

APPENDIX TABLE 4. Loss of the low-threshold mechanoreceptors after trypsin treatment and removal of the epidermis.

Before Trypsin Treatment

Experiment #	Critical stimulus range; the number of observations/bin							
	0.25 0.61	0.62 0.97	0.98 1.33	1.34 1.69	1.70 2.05	2.06 2.41	2.42 2.77	2.77 *
1	13	15	10	14	1	2	1	0
2	11	13	11	15	3	3	0	0
3	27	12	13	8	3	0	5	4
4	16	18	24	5	1	0	0	0
5	8	13	11	12	1	4	0	0
6	36	12	2	0	0	0	0	0
Total # Observations (n=347)	111	83	71	54	9	9	6	4
%	31.9	23.9	20.5	15.6	2.6	2.6	1.7	1.2
Cum. %	31.9	55.8	76.3	91.9	94.5	97.1	98.8	100.0

After Trypsin Treatment

Experiment #	Critical stimulus range; the number of observations/bin							
	0.25 0.61	0.62 0.97	0.98 1.33	1.34 1.69	1.70 2.05	2.06 2.41	2.42 2.77	2.77
1	0	0	0	0	0	0	0	56
2	0	0	0	0	0	0	0	56
3	0	0	0	0	0	0	0	68
4	0	0	0	0	0	0	0	56
5	0	0	0	0	0	0	0	48
6	0	0	0	0	0	0	0	50
Total # Observations (n=334)	0	0	0	0	0	0	0	334
%	0	0	0	0	0	0	0	100
Cum. %								100

The two distributions are significantly different ($P<0.001$, using the Kolmogorov-Smirnov test).

* Critical stimuli in units of um/ms.

APPENDIX TABLE 5. Critical stimuli distributions of the "on" - "off" experiments.

"On" sets	Critical Stimuli; observations/bin (um/ms)								"Off" sets	Critical Stimuli; observations/bin						
Experiment #	0.38 0.61	0.62 0.84	0.85 1.07	1.08 1.30	1.31 1.53	1.54 1.76	1.77 1.99	2.0	0.38 0.61	0.62 0.84	0.85 1.07	1.08 1.30	1.31 1.53	1.54 1.76	1.77 1.99	2.0
1	84	14	1					1	0	4	15	8	5	7	1	10
2	9	21	9	3	1	1			0	0	7	4	14	10	9	0
3	41	1							0	6	16	7	9	2	2	0
4	42								0	16	12	4	3	4	0	2
5	50								12	27	5	3	3	0	0	0
6	42								11	21	6	0	4	0	0	0
7	40	2							0	13	12	6	9	2	0	0
8	39	3							0	10	10	7	9	5	1	0
9	50								2	23	8	3	12	2	0	0
10	50								1	25	15	3	5	1	0	0
11	25	24	5						0	3	12	13	20	3	3	0
12	10	17	8	4	3				0	0	0	7	12	7	0	16
Total # Observ. (n=600)	482	82	23	7	4	1	0	1	26 (n=549)	148	118	65	105	43	16	28
%	80.3	13.7	3.8	1.2	0.7	0.2	0	0.2	4.7	26.9	21.5	11.8	19.1	7.8	2.9	5.1
Cum. %	80.3	94.0	97.8	99.0	99.7	99.9	99.9	100.0	4.7	31.6	53.1	64.9	84.0	91.8	94.8	99.9
Diff. (on-off)	75.6	62.4	44.7	34.1	15.7	8.1	5.2	0.1								

The two sets of distributions are significantly different at $P<0.001$, using the Kolmogorov-Smirnov test. $D_{max} = 0.756$; calculated D_{max} (at $P=0.001$) = 0.115.

APPENDIX TABLE 6. The distribution of QFCs/GO following denervation.

Week after Denervation		Numbers of GOs with QFCs 1	2	3	4	Total #	Cumulative % of GOs with QFCs 1	2	3	4	
4	C*	15	61	80	10	166	9.0	45.7	93.9	100.0	
	D	10	42	43	6	101	9.9	51.5	94.1	100.0	
						Diff.	0.9	5.8	0.2	0	D_c=15.4
6	C	9	63	41	13	126	7.1	57.1	89.6	100.0	
	D	23	63	35	6	127	18.1	67.7	95.3	100.0	
						Diff.	11.0	10.6	5.7	0	D_c=15.3
6	C	73	198	70	0	341	21.4	79.5	100.0		
	D	37	133	32	0	202	18.3	84.1	100.0		
						Diff.	3.1	4.6	0		D_c=10.8
8	C	27	192	107	8	334	8.1	65.6	97.6	100.0	
	D	38	176	122	4	340	11.2	63.0	98.9	100.0	
						Diff.	3.1	2.6	1.3	0	D_c=9.3
8	C	32	168	163	19	382	8.4	52.4	95.1	100.0	
	D	13	122	117	12	264	4.9	51.1	95.4	100.0	
						Diff.	3.5	1.3	0.3	0	D_c=9.7
10	C	21	223	163	5	412	9.3	58.6	98.8	100.0	
	D	39	207	169	5	420	5.1	59.2	98.8	100.0	
						Diff.	4.2	0.6	0	0	D_c=8.4
12	C	41	222	148	4	415	9.9	63.4	99.1	100.0	
	D	21	151	81	4	257	8.2	67.0	98.5	100.0	
						Diff.	1.7	3.6	0.6	0	D_c=9.6
12	C	25	186	90	3	304	8.2	69.4	99.0	100.0	
	D	17	183	98	2	300	5.7	66.7	99.4	100.0	
						Diff.	2.5	2.7	0.4	0	D_c=9.9
14	C	38	194	146	9	382	9.9	60.7	98.9	100.0	
	D	25	183	110	6	324	7.7	64.2	98.2	100.0	
						Diff.	2.2	3.5	0.7	0	D_c=9.2
16	C	7	27	11	0	45	15.5	75.5	100.0		
	D	10	28	2	0	40	2.5	95.0	100.0		
						Diff.	9.5	19.5	0		D_c=26.5
18	C	24	84	38	0	146	16.4	73.9	100.0		
	D	36	94	28	0	158	22.8	82.3	100.0		
						Diff.	6.4	8.4	0		D_c=14.1
22	C	55	141	63	4	263	20.9	74.5	98.5	100.0	
	D	59	123	54	6	242	24.4	75.4	97.5	100.0	
						Diff.	3.5	0.1	1.0	0	D_c=10.8
30	C	45	109	57	2	213	21.1	72.3	99.1	100.0	
	D	32	98	65	2	197	16.2	65.9	98.8	100.0	
						Diff.	4.9	6.4	0.3	0	D_c=12.1

* C, control ; D, denervated skin.
Using the Kolmogorov-Smirnov test, and comparing the maximum difference between the control and denervated distributions with the calculated difference, there was no significant difference between the distributions.

APPENDIX TABLE 7. Appearance of QFCs in innervated regenerated skin.

QFC Density (QFC/mm^2) in regenerated skin sampled at weekly intervals

	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
	17.4	30.0	82.2	65.0	48.8	106.8
	19.2	45.0	64.8	75.8	50.4	108.5
	14.6	27.1	62.2	92.5	71.1	116.1
		30.6	47.2		54.3	120.2
		20.5	21.7		75.9	113.7
		21.7	50.9		86.9	[illegible]
		18.8	38.2		97.7	106.5
$\bar{X}$	17.1	27.7	52.5	77.8	69.2	114.2
SEM	1.4	3.4	7.4	8.2	7.2	2.9

QFC Density (QFC/mm^2) in initially-removed skin samples *

	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
	147.1	136.7	113.7	153.6	132.5	117.9
	133.5	142.7	137.6	118.1	123.0	111.6
	111.6	103.7	121.8	112.9	101.3	147.2
		132.0	153.6		133.9	133.5
		107.2	118.1		122.5	111.6
		138.7	112.9		112.3	129.5
		97.3	133.6		132.0	100.2
$\bar{X}$	130.7	122.6	127.2	128.2	122.5	121.6
SEM	10.3	7.2	5.7	12.8	4.8	6.0

* These samples were used as the corresponding controls to the regenerated skin samples removed at weekly intervals.

APPENDIX TABLE 8. Appearance of QFCs in nerve-free regenerated skin.

QFC Density (QFC/mm^2) in regenerated skin sampled at weekly intervals

	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
	18.7	54.5	47.6	93.0	77.0	94.6
	12.9	57.1	79.2	72.8	51.4	120.4
	12.2	24.1	51.2	60.3	57.3	92.8
		50.0	43.3		51.5	144.1
		26.7	66.7		43.6	132.8
		15.0	42.5		63.1	125.9
		16.7	62.9		78.1	128.2
$\bar{X}$	14.6	34.9	56.2	75.4	60.3	119.8
SEM	2.1	6.9	5.2	9.7	4.9	7.3

QFC Density (QFC/mm^2) in initially-removed skin samples *

	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
	127.2	133.9	104.4	108.2	81.8	128.6
	97.5	85.2	127.1	103.2	100.0	95.4
	109.9	117.2	135.3	142.8	105.3	127.6
		139.0	108.2		145.7	97.5
		127.3	103.2		129.8	109.9
		143.5	142.8		176.3	117.2
		119.3	117.0		139.0	122.7
$\bar{X}$	111.5	123.6	119.7	118.1	125.4	114.1
SEM	8.6	7.4	5.9	12.5	12.1	5.2

* These samples were used as the corresponding controls to the regenerated skin samples removed at weekly intervals.

APPENDIX TABLE 9. Recovery of mechanosensitivity following reinnervation of denervated skin; early stages.

Animal #		# points tested	Distribution of critical stimuli; cumulative percentage occurrence									
			Bin 1	Bin 2	Bin 3	Bin 4	Bin 5	Bin 6	Bin 7	Bin 8	Bin 9	Fail.
RDX26	C *	50	63.3	79.6	91.8	91.8	95.9	100.0	100.0	100.0	100.0	
	E	50	0	0	0	0	0	0	0	56.0	56.0	100.0
		Diff.+	63.3	79.6	91.8	91.8	95.9	100.0	100.0	44.0	44.0	0
RDX28	C	56	39.3	80.4	80.4	87.5	87.5	96.4	96.4	100.0		
	E	47	0	0	0	0	0	0	0	1.8	83.6	100.0
		Diff.	39.3	80.4	80.4	87.5	87.5	96.4	96.4	98.2	16.4	0
RDX13	C	50	34.0	69.0	76.0	90.0	98.0	100.0	100.0	100.0	100.0	
	E	94	0	0	0	0	0	0	87.6	100.0	100.0	
		Diff.	34.0	69.0	76.0	90.0	98.0	100.0	12.4	0	0	
RDX27	C	48	68.8	79.2	95.9	95.9	95.9	98.0	100.0	100.0	100.0	
	E	62	0	4.8	6.4	8.0	14.5	17.7	22.5	100.0	100.0	
		Diff.	68.8	74.4	89.5	87.9	81.4	80.3	77.5	0	0	
RDX29	C	66	66.7	83.4	91.0	95.5	95.5	97.0	98.5	100.0	100.0	
	E	72	1.4	20.8	23.6	27.8	32.0	43.1	44.5	54.2	100.0	
		Diff.	65.3	62.6	67.4	67.7	63.5	53.9	54.0	45.8	0	
RDX18	C	100	34.0	71.0	83.0	93.0	94.0	96.0	97.0	100.0		
	E	100	9.0	23.0	27.0	41.0	48.0	58.0	68.0	100.0		
		Diff.	23.0	48.0	56.0	52.0	46.0	38.0	29.0	0		

* C= data obtained from maps of the control (left) leg; E= data obtained from maps of the experimental (right) leg.

\+ Diff.= $(C_{cum.\ \%} - E_{cum.\ \%})$; this is the actual difference that is then compared with the calculated difference for the Kolmogorov-Smirnov test. $D_{calc.} = 1.95\ (\sqrt{n_C+n_E/n_C n_E})$ The Control and Experimental distributions are significantly different.

APPENDIX TABLE 10. Recovery of mechanosensitivity following reinnervation of denervated skin; intermediate stages.

Animal #		# points tested	Distribution of critical stimuli; cumulative percentage occurrence: Bin 1	Bin 2	Bin 3	Bin 4	Bin 5	Bin 6	Bin 7	Bin 8	
RDX24	C	89	35.9	77.5	89.9	93.3	97.8	97.8	100.0	100.0	
	E	119	15.1	54.5	73.8	87.2	91.4	94.8	96.5	100.0	
+(12 wks)		Diff.	20.8	23.0	16.1	6.1	6.4	3.8	3.5	0	$D_{calc.} = 22.8^{*}$
RDX21	C	153	72.5	97.3	99.2	100.0	100.0	100.0	100.0	100.0	
	E	150	32.7	68.7	79.4	91.4	94.7	98.0	98.0	100.0	
+(10 wks)		Diff.	39.3	28.6	19.8	8.6	5.3	2.0	2.0	0	$D_{calc.} = 22.4^{**}$
RDX25	C	104	27.8	47.9	77.7	95.0	96.9	98.8	100.0	100.0	
	E	158	28.0	39.5	59.2	71.3	71.9	77.6	80.1	100.0	
+(9 wks)		Diff.	0.2	12.4	18.5	23.7	25.0	21.2	19.9	0	$D_{calc.} = 24.6^{**}$

* Using the Kolmogorov-Smirnov test, and comparing $D_{calc.}$ with the actual difference (Diff.) between the control and experimental distributions , it was found that the two distributions were significantly different at $P<0.01$.

** Using the Kolmogorov-Smirnov test, it was found that the control and experimental distributions were significantly different at $P<0.001$.

\+ These are the times after the initial nerve lesion that the animals were examined for recovery of mechanosensitivity.

APPENDIX TABLE 11. Recovery of mechanosensitivity to control levels following reinnervation of denervated skin.

Animal #		# points tested	Distribution of critical stimuli; cumulative percentage occurrence: Bin 1	Bin 2	Bin 3	Bin 4	Bin 5	Bin 6	Bin 7	Bin 8	
RDX17	C	92	17.4	43.5	79.4	94.6	94.6	98.9	100.0	100.0	
+	E	89	12.6	32.1	65.4	81.5	83.8	88.4	90.7	100.0	
(11 wks)		Diff.	4.8	11.4	14.0	13.1	10.8	10.5	9.3	0	$D_{calc.} = 18.2^{*}$
RDX22	C	100	63.0	94.0	100.0	100.0	100.0	100.0	100.0	100.0	
+	E	100	52.0	78.0	92.0	98.0	99.0	100.0	100.0	100.0	*
(11 wks)		Diff.	11.0	16.0	8.0	2.0	1.0	0	0	0	$D_{calc.} = 17.2$
RDX14	C	108	71.3	88.9	95.1	97.0	97.0	97.9	97.9	100.0	
+	E	72	54.2	91.7	95.9	97.3	97.3	98.7	98.7	100.0	
(14 wks)		Diff.	17.1	2.8	0.8	0.3	0.3	0.8	0.8	0	$D_{calc.} = 18.5^{*}$

* Using the Kolmogorov-Smirnov test, and comparing $D_{calc.}$ with the actual absolute difference (Diff.) between the control and experimental distributions, it was found that there was no significant difference between the distributions ($P>0.1$).

\+ These are the times after the initial nerve lesion that these animals were examined for recovery of mechanosensitivity.

APPENDIX TABLE 12. Recovery of mechanosensitivity following innervation of regenerated skin.

Weeks of skin reg.		# points tested	Distribution of critical stimuli; cumulative percentage occurrence Bin 1	Bin 2	Bin 3	Bin 4	Bin 5	Bin 6	Bin 7	Bin 8	
1	C	228	73.7	92.1	100.0	100.0	100.0	100.0	100.0	100.0	
	R	193	10.9	13.5	30.1	41.0	63.8	79.3	82.9	100.0	
		Diff.	62.8	78.6	69.9	59.0	36.2	20.7	17.1	0	$D_{calc.} = 18.5^{*}$
2	C	358	59.2	80.1	89.6	93.5	97.7	98.5	100.0	100.0	
	R	471	13.4	23.2	33.1	42.2	49.8	56.2	61.3	100.0	
		Diff.	45.8	56.9	56.5	51.3	47.9	42.3	38.7	0	$D_{calc.} = 13.7^{*}$
3	C	173	33.5	50.8	77.4	86.1	94.2	95.9	97.1	100.0	
	R	477	5.5	14.3	23.1	33.6	42.8	51.0	55.4	100.0	
		Diff.	28.0	36.5	54.3	52.5	51.4	44.9	41.9	0	$D_{calc.} = 17.3^{*}$
4	C	128	38.3	66.4	85.2	96.1	97.7	100.0	100.0	100.0	
	R	180	7.2	19.4	31.6	38.3	44.4	48.3	100.0	100.0	
		Diff.	31.1	47.0	53.6	57.8	53.3	51.7	0	0	$D_{calc.} = 22.5^{*}$
5	C	282	33.3	67.0	85.8	94.3	97.1	100.0	100.0	100.0	
	R	520	23.6	51.7	61.3	67.3	70.6	75.4	100.0	100.0	
		Diff.	9.7	15.3	24.5	27.0	26.5	24.6	0	0	$D_{calc.} = 14.4^{*}$
6	C	282	34.8	60.3	75.2	87.6	94.7	97.9	98.2	100.0	
	R	405	20.0	42.7	60.2	70.6	78.3	82.0	84.7	100.0	
		Diff.	14.8	17.6	15.0	17.0	16.4	15.9	13.5	0	$D_{calc.} = 15.2^{*}$
7	C	50	32.0	56.0	70.0	86.0	92.0	100.0	100.0	100.0	
	R	200	11.0	33.0	52.5	66.0	80.0	88.0	92.0	100.0	
		Diff.	21.0	23.0	17.5	20.0	8.0	12.0	8.0	0	$D_{calc.} = 25.8^{+}$

* Using the Kolmogorov-Smirnov test, and comparing $D_{calc.}$ with the actual difference between the control (C) and regenerated skin (R) critical stimuli distributions, it was found that the distributions were significantly different at each weekly examination period, from 1 to 6 weeks ($P<0.001$)

\+ These data are for the experiment in which skin was allowed to regenerate in an initially nerve-free condition and was then innervated; the two distributions do not differ significantly ($0.1<P>0.01$).