THE NERVOUS CONTROL OF MELANOPHORES IN THE MINNOW *(Phoxinus phoxinus* (L.)) AND OTHER TELEOSTS, WITH SPECIAL REFERENCE TO THE EFFECTS OF ADRENERGIC DRUGS AND LIGHT INTENSITY

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science in the University of London

- by -

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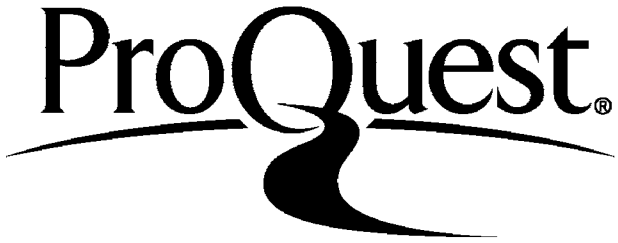

DECLARATION

The work presented in this thesis has not been accepted in substance for any other degree and is not concurrently being submitted in candidature for any other degree.

Signed .....
(Candidate)
Date .....16.6.79.....

This is to certify that the work here submitted was carried out by the candidate himself. Due acknowledgement has been made for any assistance received.

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(Supervisor)
Date .....

A B S T R A C T

The integument of the minnow *Phoxinus phoxinus* (L.) was studied using both light and electron microscopy and found to be similar in general structure to that of other teleosts. The various types of chromatophores and their location in the integument are described.

In general, melanophores have well organized microtubule systems and their possible roles in the mechanism of pigment granule movement are discussed. Fine structural and histochemical studies of melanophores suggest that their innervation is single and is probably adrenergic.

A continuous observation apparatus was employed to study in the living fish the responses to black and white background reversals of melanophores with intact innervation and of similar cells disconnected from the central nervous system by spinal nerve section. Results indicate that both pigment aggregation and pigment dispersion are active processes.

Electrical stimulation of the spinal cord, which has been shown to result in pigment aggregation, evoked pigment dispersion in paled chromatically spinal fish pretreated with bretylium, an adrenergic neuron blocking agent.

The effects of alpha- and beta-adrenoceptor agonists and antagonists on chromatically normal and chromatically spinal fish were

studied. Noradrenaline, adrenaline (alpha agonists) and, in relatively higher concentrations, isoproterenol (a beta agonist) were found to be potent pigment-aggregating agents on melanophores of chromatically normal and chromatically spinal black-adapted fish. Neither isoproterenol nor the more specific beta agonists fenoterol and isoxsuprine, injected in various concentrations were able to evoke pigment dispersion in chromatically normal white-adapted fish. However, they resulted in marked pigment dispersion (isoproterenol in relatively lower concentrations) in melanophores of chromatically spinal, prolonged white-adapted fish.

It is generally concluded that the melanophores of the minnows have only an adrenergic innervation and that the mechanisms of pigment aggregation and pigment dispersion are mediated by alpha- and beta-adrenoceptors respectively.

The effects of the incident light intensity on the rate and magnitude of the fish's response to black and white background reversals were also studied. The response of the fish was found to be constant over a wide range of incident light intensities. In the complete absence of light, or under very dim overhead illumination, melanophores in the lateral stripe showed some primary response.

The fine structure of the minnow retina was studied. The various types of photoreceptor and their responses in different conditions of illumination are described.

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CHAPTER I

INTRODUCTION AND GENERAL SURVEY OF LITERATURE

1.1. Introductory remarks

The colour changes of animals were known to the ancients, and were described for the chameleon by Aristotle, but studies concerning the physiology of the phenomenon were started in 1830 when Stark described colour changes in *Leuciscus phoxinus (Phoxinus phoxinus* L.*)*, *Gasterosteus aculeatus*, and *Cobitis barbatula*. He observed that the above fishes became dark on an illuminated black background and pale on an illuminated white background, a condition which he described as protective in nature. The discovery that chromatophores are the effectors responsible for colour changes has been attributed to Vogt, von Siebold, and Buchholz (Parker, 1948).

1.2. Structure and nomenclature of chromatophores in fishes

Chromatophores are of several types and their nomenclature is based on the pigment present. If the pigment is black or brown the chromatophores are called melanophores. Chromatophores that appear yellow are referred to as xanthophores, and the term erythrophores is used if their colour is red. Iridophore is the term given to cells which contain a guanine platelet and have iridescent properties.

1.2.1. Melanophores

Melanophores are predominently found in the dorsal skin and are described as stellate-shaped cells with cytoplasmic processes. Dermal melanophores are found at different depths under the basement lamella and are the most important cells in teleost colour changes. Epidermal melanophores, on the other hand, are fewer in number and they markedly differ from dermal melanophores in their shape. They are generally thin and elongated and are often referred to as "spindle-shaped" cells. In 1957 Falk and Rhodin studied the structure of *Lebistes reticulatus* melanophores at the ultrastructural level. They reported that melanophores, in addition to the usual cell organelles, appeared to have two cell membranes, an outer limiting membrane and an inner cytoplasmic membrane. However, electron microscopic studies of Fujii (1966a) on *Chasmichthys gulosus*, Fujii (1966b) on *Lebistes reticulatus* and Bikle *et al* (1966) on *Fundulus heteroclitus* showed that melanophores do not have the double-membrane structure as reported by Falk and Rhodin (1957) but have the single membrane usually present in cells.

The cell organelles in the melanophores include membrane-bounded melanin granules, bundles of microtubules, microfilaments, micropinocytotic vesicles, ribosomes, endoplasmic reticulum, mitochondria, centrioles, nuclei, and Golgi bodies, (Falk and Rhodin, 1957; Fujii, 1966a and b; Bikle *et al.*, 1966; Novales and Novales, 1966a; Green, 1968; Wikswo and Novales, 1972).

1.2.2. Iridophores

Iridophores are generally restricted to the dermis and are highly variable in appearance, depending on the species. Iridescent properties of these cells are generally due to the orientation of intracellular pigmentary structures (guanine platelets) which are orientated in such a way as to reflect light efficiently (Denton and Nicol, 1966; Bagnara and Hadley, 1973). Guanine, adenine and hypoxanthine have been detected in extracted skin of fish by Hitchings and Falco (1944) and Taylor (1969), and in electron microscope studies of iridophores by Kawaguti and Kamishima (1966), Setoguti (1967), Harris and Hunt (1973), Roberts *et al.* (1971). Lanzing and Wright (1974) have revealed the existence of lamellar structures with crystalline platelets of reflecting material interspersed between them. The above authors have also reported that in addition to the guanine platelets the iridophore cytoplasm consists of the usual cell organelles: oval to flat nucleus, mitochondria, endoplasmic reticulum and some microtubules.

1.2.3. Xanthophores and erythrophores

The major pigments of the xanthophores and erythrophores of fishes, amphibians and reptiles are the carotenoids and, because of the fat-soluble nature of the carotenoids, these pigment-cells were referred to in the older literature as lipophores (Fox, 1957). Also associated with yellow or red pigmentation are pteridines (Hama, 1963; Matsumoto, 1965a, b). Drosopterins, including drosopterin, isodrosopterin, and neodrosopterin, are red, while sepia-

pterins, including sepiapterin and isosepiapterin, are yellowish (Hama, 1963). Since pteridins are initially formed in these pigment granules, they are referred to as "pterinosomes". Pterinosomes usually consist of an outer limiting membrane and inner lamellae (Matsumoto, 1965).

In addition to pterinosomes, micropinocytotics, ribosomes, and Golgi apparatus have been reported in the cytoplasm of these cells (Takeuchi *et al.*, 1968; Kami-Takeuchi and Kajishima, 1971; Egner, 1971; Takeuchi and Kjaishima, 1972; Takeuchi, 1975).

1.3. Classification of chromatic responses

As early as 1909, Secerov used the terms "physiological" and "morphological" colour change with reference to the transient and quantitative changes of *Barbatula barbatula*. Although these terms are not appropriate, since both phenomena are equally physiological, they are often still retained in the literature despite many criticisms, especially by Sumner and his coworkers (Sumner, 1943). In this thesis the terms "transient" and "quantitative" will be used.

1.3.1. Transient and quantitative colour changes

Transient colour changes are rapid responses ranging in duration from seconds to hours and are based on the intracellular movements of the pigment-containing organelles. They are the main subject of this thesis.

Quantitative colour changes depend upon the formation or loss of pigment and pigment-cells and will not be considered in any

detail in the present work.

1.4. The melanophore as the chromatic effector

Melanophores are the best known of all pigment cells and have received most attention in the study of colour changes in response to the reflectivity of the background.

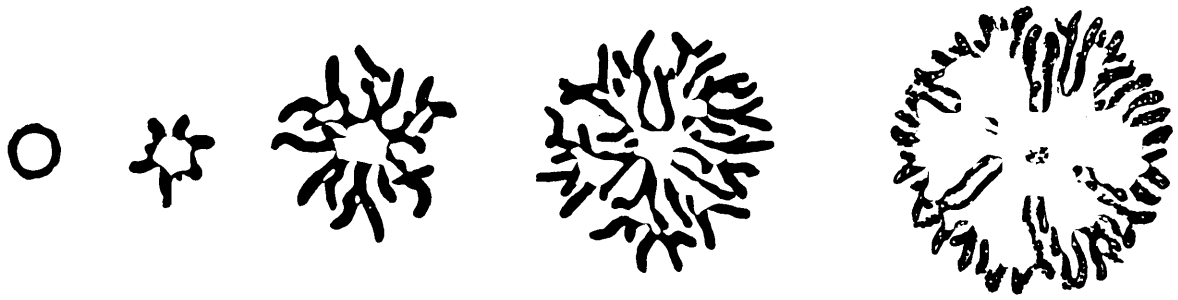
1.4.1. Assessment of melanophore response

To assess the transient responses of the melanophore a variety of methods have been developed over the years, some microscopic and some macroscopic. Hogben and Slome (1931) introduced the term *melanophore index* (MI) to assess melanophore response in amphibians. In this method the most aggregated state is designated as Stage 1 and the most dispersed as Stage 5, each increasing number from 1 to 5 representing increased pigment dispersion. This method has been modified by Healey (1951) and conveniently applied to assess melanophore response in teleosts, especially in *in vitro* experiments (Fig. I-1 p.19).

There are limitations of the MI method in *in vivo* experiments, especially in teleosts which show rapid colour changes and reactions to handling. Under such conditions only one microscopic observation of individual melanophores is generally possible even if special methods are applied to confine the fish before the observation (Hogben and Landgrebe, 1940; Neill, 1940). Repeated observations of the same fish for a long time (possibly months) is not practicable. Therefore, Healey (1967) introduced a method modified from earlier work (1940) in which the colour of the fish as seen with the naked

Fig. I-1

The Melanophore Index in the minnow *Phoxinus phoxinus* (From Healey, 1951).



The Melanophore Index From (Healey, 1951)

eye could be compared with a series of nine standard grey tints derived from the Ostwald White-Grey-Black series. As standards, Healey chose 9 points with a logarithmic relationship on the Ostwald Grey series and gave them *arbitrary numbers*; 0 (very light grey) to 8 (very dark grey). For convenience, Healey referred to these numbers as the derived Ostwald index (D.O.I.). Both the MI method and the DOI method have received some criticism because of the fact that they are basically subjective. Several workers have used photoelectric devices to estimate melanophore responses in fishes by measuring either reflected or transmitted light from a unit area of pigmented surface. These methods have the advantage of being objective and rapid (Hill *et al.*, 1935; Smith, 1936; Novales and Novales, 1966b; Fujii and Novales, 1968, 1969a; Finnin and Reed, 1970; Fujii and Miyashita, 1975). The three major arguments against the photoelectric measurement of melanophore responses are, firstly, the extreme difficulty of measuring melanophore responses in living fishes; secondly, the other kinds of chromatophore that might interfere with the recordings; thirdly, the different sizes and density of distribution of melanophores.

1.4.2. The mechanism of intracellular pigment movement

Early workers used the terms expansion and contraction of melanophores to describe the spreading of melanin over a larger or smaller surface of the integument. They believed that melanophores performed amoeboid movement and extension and withdrawal of pseudopodia-like branches were responsible for the dark or pale appearance of the animal respectively. This concept was first

challenged by Spaeth (1913) when he studied the responses of a particular melanophore to black and white background reversals. By means of photographs Spaeth demonstrated that the branches of individual melanophores always displayed the same profile. However, the terms 'expanded' and 'contracted' remained in use until Matthews (1931) clearly showed that they are inappropriate. Matthews (1931), as a result of his work on *Fundulus* melanophores using tissue culture techniques, reported that during centrifugal and centripetal movement of pigment the cell-outline remained unaltered. Matthews' observations that melanophores have fixed boundaries in which pigment migration takes place have been confirmed in recent studies using phase contrast and electron microscopy. Therefore the terms 'expanded' and 'contracted' have been replaced by 'dispersed' and 'aggregated' (or 'concentrated') states of pigment granules (Bikle, *et al.*, 1966; Fujii, 1966a, b; Green, 1968). A great deal of attention has been given by some researchers to the investigation of the nature of the mechanism by which centrifugal and centripetal movement of pigment granules takes place. Marsland (1944), working on *Fundulus* melanophores, suggested that the aggregation of pigment granules is correlated with gelation of the protoplasm containing them, whereas melanin dispersal involves cytoplasmic solation. Therefore, Marsland suggested that the sol-gel states of protoplasm, however they might have arisen, result in dispersed and aggregated states of pigment granules within the cell.

Kinosita (1953, 1963) proposed an electrophoretic mechanism for melanin granule movements. By means of microelectrodes he

measured the membrane potential of the centrosphere and peripheral branches of *Oryzias latipes* melanophores. He showed that when the melanophore was under the influence of aggregating agents such as adrenaline or K^+ the centrosphere became less negatively charged than the peripheral branches. On the other hand, when the melanophore was under the influence of dispersing agents such as atropine or physiological saline the centrosphere became more negatively charged than the peripheral branches which, consequently, resulted in the distal migration of pigment granules. On the basis of the above results Kinosita suggested that the negatively charged pigment granules migrated electrophoretically through the cytoplasm in a direction away from the more negatively charged region as a result of the potential gradient.

On the ultrastructural level Falk and Rhodin (1957), in an attempt to explain the nature of the mechanism involved in pigment granule migration, claimed that their electron micrographs of *Lebistes* melanophores showed that the melanophore has two cell membranes, an outer limiting membrane and an inner cytoplasmic membrane. The latter invests the pigment granules like a sack. They believed that the contraction and relaxation of the fibrils present in the space between the inner and outer membrane of the cell are responsible for aggregation and dispersion of pigment granules in the cytoplasmic sack. However, Fujii (1966b) working on the same species, was unable to confirm Falk and Rhodin's observations, and reported that the melanophores were enclosed by the usual single thin membrane only. Fujii considered that the mechanism proposed by Falk and Rhodin was based on misinterpretation

of their electron micrographs and thus should be discounted.

Bikle *et al.* (1966) studied the kinetics of pigment migration within the melanophores of *Fundulus heteroclitus*. They reported that their observations by light microscopy indicated that pigment granules move along relatively fixed channels arranged parallel to the long axis of the cell processes. At the fine structure level, the above authors observed bundles of microtubules in the cytoplasmic processes of the cell regardless of whether the granules were being aggregated or dispersed. Therefore, Bikle *et al.* concluded that microtubules, in addition to defining the channels in which the granules move, also function as cytoskeletal elements which help to maintain the extended form of the melanophore processes. Green (1968), working on the same species as the above (*Fundulus heteroclitus*), confirmed the results of Bikle *et al.* (1966) regarding the participatory role of the microtubules in mediating pigment granule migration. Moreover, the difference in the centripetal and centrifugal movement of pigment granules led Green to propose that granules behave as though they are embedded in a cytoplasmic continuum that 'expands' during dispersion and 'contracts' elastically during aggregation. However, the composition of this gel-like continuum and its relationship with microtubules or any other cytoplasmic element was not clarified. Microtubule depolymerising agents such as colchicine and vinblastine, which are known to disrupt microtubules, were also found to inhibit pigment migration (Wright, 1955; Junqueira and Porter, 1969; Wikswo and Novales, 1969; Malawista, 1965, 1971). Furthermore, Wikswo and Novales (1972) showed that treatment with colchicine always was accompanied

by a reduction in the number of microtubules.

Porter (1973) suggested that pigment granule movement is associated with the depolymerization and repolymerization properties of microtubules during pigment aggregation and dispersion respectively. This suggestion was challenged by the recent work of Murphy and Tilney (1974). Working on *Fundulus heteroclitus* melanophores, they showed that the density and distribution of microtubules remains unchanged in both the dispersed and aggregated states of pigment granules and thus concluded that the microtubules in *Fundulus* melanophores are relatively stable organelles and do not depolymerize upon aggregation of pigment granules. However, Murphy and Tilney supported the idea of microtubule-dependent granule movement in the melanophores but suggested an alternative mechanism based on active association of pigment granules and microtubules. They proposed that "cross-bridges" of ATPase nature might exist on the microtubules functioning as an active site in sliding pigment granules along a fixed array of microtubules.

On the other hand, Schliwa and Bereiter-Hahn (1975) reported that, though the microtubules clearly participate in the process of pigment migration, they are not the sole cytoplasmic agent controlling pigment migration. These authors suggest that in addition to the microtubules, a microtubule-independent contractile system might be involved, regulating pigment migration. Junqueira and Farias (1976) investigated erythrophores and xanthophores of fourteen species of teleosts. They observed large numbers of filaments in the cytoplasm of these cells and, based on this

observation, they suggested the possibility of microfilament participation in pigment migration. Moreover, along the same line that microtubules are not the main agent responsible for pigment migration, Junqueira *et al.* (1977), based on previous observations of the presence of microfilaments in adrenaline-treated melanophores of *Fundulus* but not in the control melanophores (Junqueira *et al.*, 1974), studied the effect of sympathomimetic compounds (adrenaline, 5 HDA, 6 HDA) on the ultrastructure of the melanophores of these species of teleosts. The aggregation effect of the sympathomimetic agents used was always found to coincide with the appearance of abundant filaments in the cytoplasm at the ultrastructural level. The above results were taken as further support for the possible participation of these filaments in pigment migration processes. However, Byers and Porter (1976), working on *Holocentrus ascensionis*, found that the erythrophores of this fish had no microfilaments, and their observations, based on a study of whole-cell preparations in Stereo high Voltage Electron Microscopy (HVEM), suggested that the microtubules guide linear motion and that the granules are suspended in a dynamic microtrabecular system that withdraws during pigment aggregation and is restructured during pigment dispersion. A very recent report by Schliwa and Euteneuer (1978), provided further support for Byers and Porter's (1976) suggested mechanism for pigment migration. Schliwa and Euteneuer (1978) studied the melanophores of *Pterophyllum scalare* and *Gymnocorymbus ternetzii* *in situ* (within an isolated scale) or *in vitro* (after isolation from the surrounding tissue). Schliwa and Euteneuer reported that, apart from microtubules, they observed a trabecular system and

suggested a similar mechanism for pigment granule migration to that suggested by Byers and Porter (1976) for the erythrophores in *Holocentrus ascensionis*.

1.4.3. Responses of melanophores to light

Responses of melanophores to light may be divided into two categories, primary and secondary responses.

1.4.3.1. Primary responses

Primary responses of melanophores to light are responses which occur through routes other than the eyes.

1- a dermal response which is independent of the central nervous system and the pituitary, the melanophores behaving as independent effectors.

2- a coordinated non-visual response which is independent of the eyes but involves either nervous or endocrine coordination between a stimulus received by a receptor other than the eye and the melanophore.

1.4.3.2. Secondary responses

These are coordinated visual responses of melanophores to light and are dependent upon the nature of the background; the intensity of pigment dispersal is related to the ratio of direct incident light to the reflected light from the background. Thus, on a black background where light is almost entirely absorbed the melanophores are dispersed, whereas on a white background where light is almost fully reflected the melanophores are aggregated.

Hogben (1942) and Waring and Landgrebe (1950) used "secondary ocular" and "tertiary ocular" terms for white and black background responses respectively. The above terminology was criticized by Fingerman (1959) on the basis that it could cause confusion.

1.5. The eye and chromatic responses

As early as 1858 Lister reported that the eye is the main organ concerned with the chromatic responses in vertebrates. Pouchet (1872, 1876), working on the trout *Salmo trutta*, found that removal of the eyes abolished the ability of the fish to adapt to the background.

1.5.1. Differentiation of the retina

Von Frisch (1911), working on the trout, suggested the possibility of retinal differentiation into a dorsal and ventral region as far as the chromatic responses are concerned, the dorsal part being responsible for white adaptation and the ventral part being responsible for black adaptation of the animal. Sumner (1933) concluded from the results he obtained by covering the eye of *Fundulus* with celloidin caps painted with Indian ink, that "the shade which a fish assumes upon a given background is determined by the relative luminosity of the upper and lower portions of the visual field." However, Sumner himself criticized his results on the basis that the fishes' eyes were badly damaged and also that the responses of fishes with transparent corneas were not the same as the responses of normal fishes on a white background. Butcher and Adelman (1937) performed experiments on *Fundulus heteroclitus* similar to those of

Sumner using thick papers as blinders which could be fixed in place by slipping them under a series of stitches made in a rectangular area around the eye. They also concluded that when the dorsal portion of the retina was illuminated the fish became pale and that when the ventral portion of the retina, but not the dorsal, was illuminated the fish darkened. In a further series of experiments they severed the eyes of 22 fishes from their connections, except those of the optic nerve, the eyes were then rotated 180^{o} and were then stitched in their new position. They observed that the fish treated in this manner became slightly pale to intermediate if they were illuminated from above, regardless of the white or black vessels in which they were placed. If the fish were illuminated from below and kept in dark vessels, they found that 13 out of the 22 showed a distinct darkening. They concluded that the dorsal and ventral portions of the retina are physiologically different, and that stimulation of the upper part of the retina results in lightening and that of the lower part of the retina in darkening.

Butcher (1938) also demonstrated, by destroying the retina surgically, that if the dorsal portion of the retina was removed the fish became dark regardless of the shade of background and assumed intermediate shades when kept in total darkness. Destroying the ventral portion of the retina, the fish became pale on a white and intermediate on a black background. Butcher (1938) also presented some evidence that not only different regions of the retina are physiologically different, but that they also differ anatomically. He found two distinct regions in the retina of

Fundulus. The dorsal region, comprising about 70% of the total retinal area, was found to contain rods and single and double cones. The lower region had only rods and double cones. He also reported the existence of a specialised crescentic ridge found in the ventral region containing more double cones and rods than any other part of the eye.

1.5.2. The ratio hypothesis

The ratio hypothesis was originally proposed by Keeble and Gamble (1904) to explain colour changes in some crustacea. According to this hypothesis, the ratio of direct to reflected light which reaches the eye of the animal determines the degree of integumentary pigment dispersal. On a black background the value of this ratio is high because a very small proportion of incident light will be reflected which therefore results in maximal pigment dispersion. On the other hand, on a white background, the ratio of incident to reflected light will be very small so that there will be maximal pigment concentration. On any background of intermediate shade the intensity of pigment dispersion is determined by the value of the ratio on that specific background. In other words, the greater the value of the ratio, the greater the dispersion of pigment. However, conflicting results were obtained by some investigators. Sumner (1911), experimenting on the turbot, set up experimental conditions and tried to obtain a very low ratio of light above to light below by covering the vessels containing the turbot and admitting light from below only. Instead of the expected paling, the fish darkened to some extent. Sumner believed that the darkening

was due to unwanted reflections. Mast (1916), from work with flounders, concluded that "the interaction of the light received from the different immediate sources is probably not so simple as is demanded by the ratio hypothesis of Keeble and Gamble."

Sumner and Keys (1929) tested the responses of *Hypsopsetta guttulata* with two backgrounds on which the illumination was such that the ratio in one case was greater, in the other less than that which would ordinarily exist. They concluded that their results were consistent with the ratio hypothesis but that the ratio can not be quantatitively interpreted. Pearson (1930), working on *Ameiurus*, adopted Sumner and Keys' (1929) technique and obtained similar results, but by altering the experimental conditions (i.e. fairly intense illumination from above and below at the same time) he found responses which were not consistent with the ratio hypothesis. Brown (1936) measured the ratio existing on a series of black, grey and white backgrounds and found that at intensities of 1.75 foot-candles or more the diameter of the melanin masses of *Ericymba buccata* varied directly with the ratio.

More recently, Gentle (1968), working on the minnow *Phoxinus*, studied the structure of the retina and the effects of retinal lesions and concluded that surgical removal or destruction of the dorsal retina by high intensity light resulted in the fish being fully dark on a black and intermediate on a white background. Similar destruction or removal of the ventral retina resulted in the fish being intermediate on the black and pale on a white background. From the above results Gentle concluded that the whole of the retina

is important in chromatic adaptation and considered that the ideas of retinal differentiation and the ratio of direct to reflected light are far too simple to be applicable.

1.6. Neural control of chromatophores

As early as 1852, Brücke provided data suggesting that colour change in the chameleon was controlled by nerves. Pouchet (1872-76) demonstrated that the rapid aggregation of pigment in the melanophores of the turbot (*Scophthalmus maximus*) is controlled by the sympathetic nervous system and that the eyes are the principal receptor organs involved. Removal of the eyes abolished the colour change reflex. In tracing the pathways of the chromatic nerve fibres Pouchet found that section of the autonomic chain in the tail region caused a darkening of the skin posterior to the point of section. The chromatic fibres emerge from the autonomic chain to innervate the integument by way of the spinal nerves, section of a spinal nerve below the point of union with the ramus communicans causing a darkening of the skin innervated by that spinal nerve. Section of the spinal nerve, immediately on leaving the spinal cord and before its union with the ramus communicans, on the other hand, gave no effect. The head region was found to be innervated by chromatic fibres of the trigeminal nerve.

Von Frisch (1910, 1911), on the basis of the conclusions reached by Pouchet, described the pathways of chromatic fibres from the brain to the melanophores. Working on the minnow (*Phoxinus*), he made transections in the spinal cord, the autonomic chain, spinal

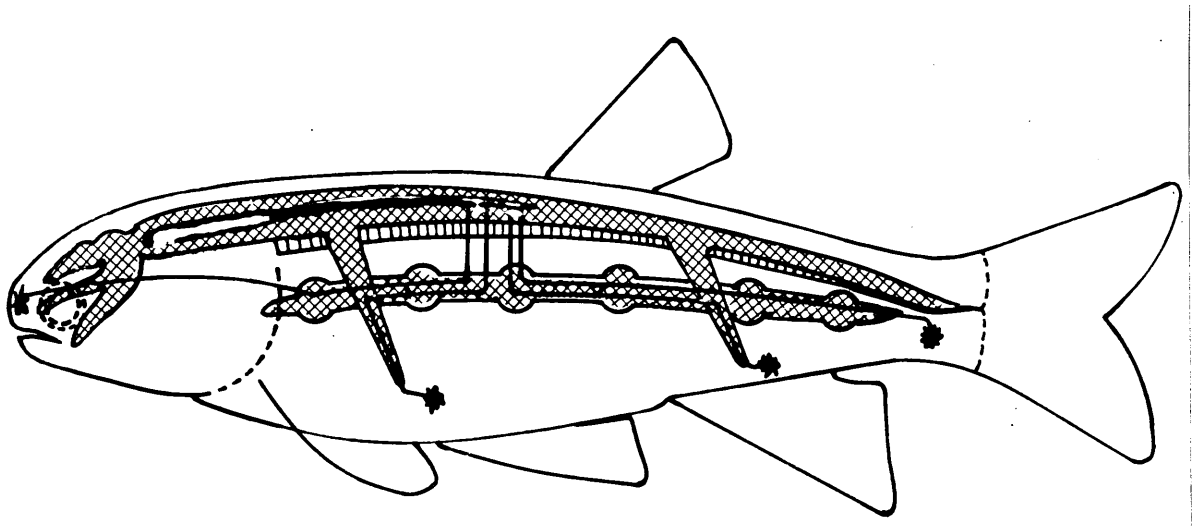
nerves and the trigeminal nerve at different levels and showed that the chromatic fibres leave the spinal cord in the region of the 15th vertebra to enter the sympathetic chain. There they or their post-ganglionic associates run forwards and backwards, passing out with the spinal nerve to supply the skin melanophores of the body and tail. The melanophores of the head are innervated by the trigeminal nerve (Fig. I-2 p. 33).

Von Frisch's experiments also involved electrical stimulation of various parts of the nervous system. He demonstrated that if the autonomic chain was stimulated behind the region of the emerging point of the pigment fibres from the spinal cord, only paling of the body posterior to the point of stimulation was observed. Stimulation of the spinal cord posterior to the 15th vertebra did not cause any paling; stimulation of the spinal cord at any point anterior to the 15th vertebra resulted in paling of the whole body. Von Frisch also reported that stimulation of the medulla oblongata also brought about paling of the whole body. On the other hand, he reported that if electrical stimulation was applied to the diencephalon or if there was illumination of the pineal region in the absence of any other light stimuli, this resulted in overall darkening of the body.

According to the above results, von Frisch concluded that (1) a paling centre in the medulla oblongata exercises a tonic influence on the melanophores through the chromatic pathway and keeps them in a state of active aggregation and (2) a centre in the brain exists which can inhibit the medullary paling centre

Fig. I-2

The pathway of chromatic fibres in the minnow *Phoxinus phoxinus* (after von Frisch, 1911).



either when stimulated electrically or through the retina as a result of an illuminated black background. Inhibition of the tonic influence of the medulla results in a passive pigment dispersal of the melanophores. On a white background the stimulation of the retina terminates the inhibition effect of the brain on the tonic influence of the medulla oblongata and so keeps the melanophores in an active state of aggregation. Von Frisch's publications received subsequent support by many other workers as far as the autonomic outflow from the spinal cord is concerned.

1.6.1. The nature of the neural control

There is general agreement that teleost melanophores are innervated by at least pigment-concentrating axons. Evidence for such axons has been obtained by electrical stimulation of nerve tracks and denervation experiments. The concentration of pigment in response to electrical stimulation of the appropriate nerve in innervated tissues, but not in denervated ones, has been reported in fishes by numerous workers (von Frisch, 1911; Spaeth, 1913; Schaefer, 1921; Wyman, 1924; Parker, 1935a; Abramowitz, 1936a; Osborn, 1938; Wykes, 1938; Parker and Rosenblueth, 1941; Gray, 1956; Pye, 1964a and many others). Moreover, it is established that pigment concentration in teleost melanophores is controlled by an adrenergic mechanism (Spaeth, 1916; Bray, 1918; Abolin, 1925). Following the discovery by von Euler (1946) that noradrenaline is the transmitter generally involved at the neuro-effector junction in mammals, its effect on teleost melanophores was investigated and was reported to be strongly aggregating on the pigment (Umrath, 1957;

Fujii, 1961; Fänge, 1962; Scheline, 1963; Pye, 1964a;Scott, 1965). Furthermore, pharmacological studies by Healey and Ross (1966) and Grove (1969a, b) on *Phoxinus* indicated that the melanophores in this fish are more sensitive to noradrenaline than to adrenaline. This is consistent with the results with mammals and they concluded that the transmitter involved is probably noradrenaline. Regarding the presence of pigment-dispersing fibres, there has been considerable indirect evidence supporting their existence but their nature has been the subject of controversy for many years. Many attempts to clarify the situation have been made and the earliest and most publicised argument for the presence of melanin-dispersing axons was presented by Parker and his coworkers.

The undermentioned headings are areas of investigation which throw light on the nature of the pigment-dispersing mechanism.

1. Responses of denervated melanophores (caudal band experiments).
2. Experiments on chromatically spinal fish and on fish after section of the sympathetic chain.
3. Experiments on regeneration of chromatic fibres.
4. Studies on structure and ultrastructure of melanophore innervation.
5. Experiments involving administration of drugs affecting the autonomic nervous system and/or electrical stimulation.

1.6.1.1. Responses of denervated melanophores (caudal band experiments).

Studies concerning the responses of caudal band melanophores started when Wyman (1924) produced such bands in *Fundulus* by fine

transverse incisions across the base of the tail fin, separating a group of melanophores in the fin from their innervation, and producing a well-defined dark band of dispersed melanophores running from the cut to the margin of the fin. Wyman found that electrical stimulation of the spinal cord failed to produce aggregation in the melanophores of the band. However, he found that the band melanophores of a fish kept on a white background faded partially in a few hours after the operation. Wyman's conclusions were that sudden interruptions of central nervous influences caused the dispersion of melanophores within the band.

Mills (1932a, b), working in Parker's laboratory, induced caudal bands in *Fundulus* as had Wyman. She not only found that the band had faded on a white background but that some melanophores on the edge of the band dispersed readily on a black background but failed to aggregate readily on a white background. To explain the above results Mills suggested that neighbouring dispersing and concentrating axons do not always innervate the same melanophores.

Parker (1933, 1934a) extended the work of Mills on caudal band melanophores. He observed that the rate of dispersion of pigment in caudal band melanophores on a black background and the rate of pigment aggregation on a white background is slower than in innervated melanophores outside the band region. He claimed that the above results provided evidence for double innervation, that the slow response of the melanophores within the band is due to diffusion of neurohormone substances into the caudal band from adjoining regions where the innervation is intact. In further experiments Parker (1935b)

showed that in a previously faded band a second cut made posterior to the first one induced a second dark band distal to this second cut. Parker explained the revival of dispersion in the band melanophores produced by a second cut as being due to persistent injury discharges in the dispersing fibres only. Parker (1934b) considered the results of experiments in which he claimed that the presence of a cold block prevents the development of the band posterior to the block as further evidence in support of the hypothesis of persistent injury discharges. Sand (1935), Young (1950), and Waring (1942, 1963) have criticized Parker's hypothesis and found his evidence to be very inconclusive.

However, as far as Parker's observations are concerned, Osborn (1938a, b) and Wykes (1938), both working on *Ameiurus*, reported similar observations, except for the revival of the caudal band by a second cut which they failed to obtain.

More recent investigations on caudal band melanophore response were performed by Umrath and Walcher (1951), Gray (1956), and Fujii and Novales (1969b). Although they agreed that there might be a set of fibres regulating active pigment dispersion, they did not accept Parker's sustained injury discharge hypothesis as an interpretation of the results and offered alternative explanations.

Umrath and Walcher (1951) interpreted the formation of a caudal band after nerve section in *Macropodus* as being the result both of separation from the tonic influences of the medullary paling centre, as suggested by von Frisch (1911), and stimulation of the

dispersing fibres due to cutting, as suggested by Parker (1935b). They also suggested that entry of water into the wound stimulated nerve fibres.

Gray (1956), working on *Phoxinus*, performed caudal band experiments on normal as well as hypophysectomized fish. He found that the band of affected melanophores regained its ability to respond to background reversal after some time in both normal and hypophysectomized fish. He therefore discounted the possibility that pigment dispersion after nerve section could be due to a dispersing pituitary hormone circulating in the blood. Moreover, Gray suggested that the marginal fading of the affected melanophores on a white background and their marginal dispersion when a fish with a faded band was subjected to chromatic spinal section indicated the activities of aggregating and dispersing neurohormones diffusing marginally into the affected area. From the above observation, Gray confirmed Parker's observations on other teleosts. However, Gray did not accept Parker's interpretation that dispersion is caused by continued injury discharges in the dispersing (but not the aggregating) fibres and gave the alternative interpretation that melanophores possessed some kind of inherent dispersing quality within themselves which comes into play when the central nervous control is removed by nerve section. Such melanophores are refractory to aggregating neurohormones in the beginning, but later lose their refractoriness and become sensitive to it. The above argument was based on observations by Cannon and Rosenblueth (1937) on mammals that supersensitivity in denervated tissue develops, not immediately,

but some time after nerve section.

Fujii and Novales (1969b) followed Parker's procedure on the caudal fin of the goby *Chasmichthys*. They failed to produce the revival of dispersion in the faded band melanophores by a second cut and found that a cold block did not prevent the development of the band posterior to the point of application, as was claimed by Parker. Fujii and Novales, on the basis of their results and the results recorded by Fujii (1959b), concluded that the formation of a caudal band distal to incision is a result of separation of the melanophores within the band from nervous control and that the initial dispersion of the band melanophores is due to spontaneous release of the dispersing neurotransmitter from the presynaptic structures involved. On the other hand, Fujii (1959a), working on the caudal band of the goby *Chasmichthys gulosus*, reported that the store of the dispersing transmitter in the remnant axons seems to disappear 3-5 hours after the incision at 26^{o}C. Therefore, to account for the relatively longer lasting dispersed state of the affected melanophores after incision (days or weeks), they suggested that after the disappearance of the neurotransmitter from the remnant axons, the pigment granules within the melanophores of the band distribute passively by Brownian movement without being affected by the neurotransmitter. They discount the effects of dispersing or aggregating neurotransmitter released from the innervated area, and suggest their possible inactivation by enzymes, probably acetylcholinesterase and catecholamine-o-methyltransferase.

1.6.1.2. Experiments on chromatically spinal fish and on fish after section of the sympathetic chain.

Healey (1940, 1948, 1951), working on the minnow *Phoxinus*, examined in detail the response of melanophores to background reversal after various operations, i.e. the chromatic response of chromatically spinal fish (i.e. spinal section anterior to the level of outflow of the chromatic fibres); chromatic response in fish sectioned in the sympathetic chain; and the chromatic response of fish after hypophysectomy alone or in combination with spinal sectioning. The results of the above experiments not only suggest the presence of dispersing fibres controlling an active pigment dispersion but, if the dispersion is to be maintained, the presence of the pituitary is essential. Therefore, Healey suggested that the initial dispersion in response to an illuminated black background is evoked by nervous action and supplemented thereafter by a secretion from the pituitary.

1.6.1.3. Experiments on regeneration of nerve fibres.

Parker and Porter (1933) and Abramowitz (1935, 1936a) published, as a result of their work on *Fundulus*, data concerning the rates of regeneration in the severed chromatic fibres of the caudal band. They reported that the portion of the nerve distal to the cut degenerates in about 2 weeks, after which time regeneration begins. They assessed the regeneration rate of dispersing and aggregating fibres by transferring a white adapted fish with a faded caudal band to a black background. The darkened area of the caudal

band due to the background reversal indicated the extent of regenerated dispersing fibres. By transferring black adapted fish with a dark band to a white background an indication of the extent of regenerated aggregating fibres could be obtained. They also reported that when the chromatic fibres were in the process of regeneration they could find some melanophores which could disperse and aggregate their pigment completely, some which could disperse but not aggregate their pigment completely and some which could aggregate but not disperse completely, and some which could neither disperse nor aggregate at all. Abramowitz interpreted the above as a result of irregularities in the relative speed of aggregating and dispersing fibre regeneration.

Healey (1967) provided experimental evidence for the regeneration of nerve fibres controlling colour changes after spinal cord section anterior to their outflow. He observed some reconstitution of rapid colour changes nine months after the operation. The above observation was further supported when Healey subjected a fish in which rapid colour change was reestablished to spinal section anterior to the level of the first. This was followed by darkening of the whole animal, suggesting functional chromatic regeneration in the cord. Moreover, Healey observed situations where a rapid pigment-aggregating mechanism and a rapid pigment-dispersing mechanism were not equally reconstituted. Such a condition is difficult to explain without assuming an active pigment-dispersing nervous mechanism.

1.6.1.4. Studies on structure and ultrastructure of melanophore innervation

As early as 1893 Ballowitz (1893a, b) and Eberth and Bunge (1895) demonstrated by means of a silver impregnation technique a network of nerve fibres associated with teleost melanophores. A similar innervation was reported by Wyman (1924) in *Fundulus*. However, Whitear (1952) was unable to identify with certainty melanophore motor fibres in the minnow. Gray (1956) reported that minnow nerves are highly refractory to histological technique. Ahmad (1970) suggested that this refractoriness could have been the reason for Whitear's inability to show an innervation similar to the one reported by earlier workers. More recently, Jacobowitz and Laties (1968) and Falck *et al.* (1969), using a histochemical fluorescent technique for catecholamine-containing structures, were able to show nerve fibres in the vicinity of melanophores, probably loaded with catecholamine.

As far as ultrastructural evidence for the concept of double innervation of teleost melanophores is concerned, Bikle *et al.* (1966) on *Fundulus*, Fujii (1966a), Fujii and Fujii (1966) on the goby *Chasmichthys*, Fujii (1966b), Fujii and Novales (1969b) and Fujii and Taguchi (1970) on the guppy *Lebistes*, claimed that their electron micrographs have shown some evidence in favour of double innervation of fish melanophores. Generally, their argument was based on a correlation between the fine structure of mammalian nerve-terminals (adrenergic and cholinergic nerve-terminals) and nerve endings found in the vicinity of the melanophores. They found agranular vesicles

of about 50 nm (500 Å) and granular vesicles of about 100 nm (1000 Å) in diameter which were considered to be the sites for a melanin-dispersing transmitter and a melanin-aggregating transmitter respectively, because of their similarity to the mammalian neuro-vesicles.

Quite recently Schliwa (1976) investigated the fine structure of melanophore innervation in the angelfish *Pterophyllum scalare*. He made the suggestion based on his electron micrographs of thin serial sections, that the pre-synaptic elements are not nerve terminals but are varicosities of the peripheral nerve fibre. Moreover, he found a single fibre which formed two *en passant* synapses with a single melanophore. He therefore concluded that since a terminal synapse has never actually been reported, it is very likely that the majority of nerve-melanophore contacts are, in fact, *en passant* synapses.

1.6.1.5. Experiments involving administration of drugs affecting the autonomic nervous system and/or electrical stimulation.

A. General Review

Giersberg (1930) and von Gelei (1942), both working on the minnow *(Phoxinus)*, found that by treating the fish with ergotamine and acetylcholine followed by electrical stimulation a darkening of the animal resulted. The darkening was attributed by these authors to be the result of melanophore-dispersing fibre excitation. Moreover, von Gelei further concluded that his experiments demonstrated the pathways of the dispersing fibres. According to him, dispersing

fibres do not have the same path as von Frisch's aggregating fibres. He claimed to show that they emerge from the spinal cord at the level of the 1st or 2nd spinal nerve to enter the autonomic chain where they run posteriorly to supply the melanophores.

Healey (1954) studied the effects of spinal section between vertebrae 1 and 15 and of anterior autonomic section on the melanophores of *Phoxinus*. A statistical treatment of the results of the combined operations of section of the spinal cord and sympathetic chain at various levels did not indicate that von Gelei's fibres, if they existed at all, play any part in colour change. Gray (1956) criticized Giersberg and von Gelei's claims regarding melanophore-dispersing fibres after ergotamine and electrical stimulation in that the darkening could be due to a reversal action of adrenaline on the melanophore after treatment with ergotamine. Gray's suggestion was consistent with the general view held with regard to mammals (Goodman and Gilman, 1955). A further invalidity of von Gelei's dispersing-fibres was put forward by Pye (1964a). He demonstrated that a darkening response similar to that of von Gelei's in an ergotamine-treated fish could be produced by electrical stimulation of the spinal cord at any level and even in small isolated sectors of the cord.

Many attempts have been made to prove the existence of melanophore-dispersing fibres by stimulating the pigment-aggregating and pigment-dispersing fibres independently. Parker and Rosenblueth (1941) working on the catfish *Ameiurus*, claimed that they had stimulated the pigment-dispersing fibres independently by altering

the frequency, duration and intensity of repetitive pulses. Pye (1961, 1964a) criticized Parker and Rosenblueth's conclusions on the basis that the electrodes that they used were not of a non-polarisable type, that the response time was extremely long when compared with that normally encountered during chromatic stimulation, and that stimulation with the stated variables might have blocked conduction in aggregating fibres. Fujii and Novales (1969a), as a result of their work on a split tail-fin preparation of *Fundulus*, reported that no pigment dispersion was obtained in response to electrical stimulation, although various parameters of the stimulating pulses were changed.

More recently, Kinosita and Ueda (1970), working on an isolated scale of *Oryzias latipes*, claimed that they had stimulated the pigment-aggregating fibres and pigment dispersing fibres independently by a careful selection of the frequency and the strength of the repetitive stimulation. According to them, at low frequency stimulation the somewhat dispersed pigment in the melanophore in Ringer's solution dispersed further whereas an increase in frequency was followed by concentration. Also, if the preparation was stimulated by sine-wave alternating currents (Frequency: 50 HZ), the state of the melanophore was found to depend on the strength of the stimulus, a relatively strong stimulus resulting in pigment aggregation while a weaker stimulus resulted in dispersal followed by aggregation. With a still weaker stimulus only pigment dispersion was observed.

On the other hand, as described on page 34, pharmacological studies have revealed that the pigment aggregation is controlled

by an adrenergic mechanism. However, no clear picture has emerged from such studies about the nature of the transmitter associated with the mechanism of pigment dispersion, in spite of the fact that it has often been stated to be cholinergic (Parker, 1948). Results obtained from the administration of acetylcholine and related substances have always been associated with conflicting observations. Healey and Ross (1966), Grove (1967) on *Phoxinus*, Reed and Finnin (1972) on *Pterophyllum eimekei* and Miyashita and Fujii (1975) on *Lebistes reticulatus* have reported that their investigations on the effects of acetylcholine and other parasympathomimetic substances have provided no support for a cholinergic mechanism being concerned in the dispersion of pigment in the melanophores.

Gray (1955) suggested that the darkening which follows injection of adrenergic blocking agents could be due to "reversal" of adrenaline effects. In mammals, for such "reversals" to take place, the presence of antagonistic alpha and beta adrenoceptors is required, (Furness and Costa, 1974; Furness and Burnstock, 1975). Fujii (1961) and Watanabe *et al.* (1962) suggested the possibility of adrenergic mechanisms for both pigment aggregation and pigment dispersion in teleost melanophores. Before discussing the effects of adrenergic drugs reported recently on teleost chromatic systems a brief outline of the organisation, physiology and pharmacology of the adrenergic neurons will be given.

1.7. Organisation of the autonomic nervous system

Langley (1921) in his monograph introduced the term "autonomic

nervous system" and stated, furthermore, that the system is composed of two main subsystems, the sympathetic and parasympathetic. Dale (1937) reported that the sympathetic and parasympathetic efferent pathways are composed of a chain of at least two neurons, a preganglionic neuron, and a postganglionic neuron. Preganglionic neurons of the sympathetic nervous system pass *via* white *rami communicantes* and make synaptic connections with postganglionic neurons in the paravertebral or prevertebral ganglia. Preganglionic neurons of the parasympatheic system make synaptic connections with postganglionic neurons in ganglia lying in or near the innervated organs. Dale also reported that the preganglionic neurons of both sympathetic and parasympathetic systems are cholinergic. The postganglionic parasympathetic neurons are known to be solely cholinergic, while the postganglionic sympathetic neurons are mainly adrenergic (see Bacq, 1933; von Euler, 1956; Campbell, 1970).

As regards the anatomy of the autonomic system in teleost fish it is essentially similar to that seen in mammals (Burnstock, 1969). However, a sacral parasympathetic outflow is not present in teleost fish (Young, 1931).

1.7.1. The adrenergic fibres

The adrenergic fibres usually are unmyelinated and leave the sympathetic ganglia as postganglionic fibres. They receive an excitatory cholinergic input from the preganglionic sympathetic fibres and probably an inhibitory input from intraneuronal release

of catecholamine. A diagram to show the hypothetical types of receptors involved in adrenergic transmission was shown by Burnstock and Costa (1975) in their monograph on adrenergic neurons (Fig. I-3 p.49).

1.7.2. Adrenergic nerve-endings

The adrenergic varicose endings are found close to an effector, usually a smooth muscle cell. They run parallel to the effector cell and each axon may have several contacts with the same cell. As far as the structure of adrenergic endings is concerned, de Robertis and Pellegrino de Iraldi (1961) were the first to describe them. Adrenergic nerve varicosities contain synaptic vesicles and mitochondria. Three types of vesicles have been reported:

1 - small granular vesicles which are predominant in number, characteristically 30-60 nm in diameter and contain an electron-dense granule;

2 - large granular vesicles whose proportion is very small when compared with the small granular vesicles, 60-120 nm in diameter;

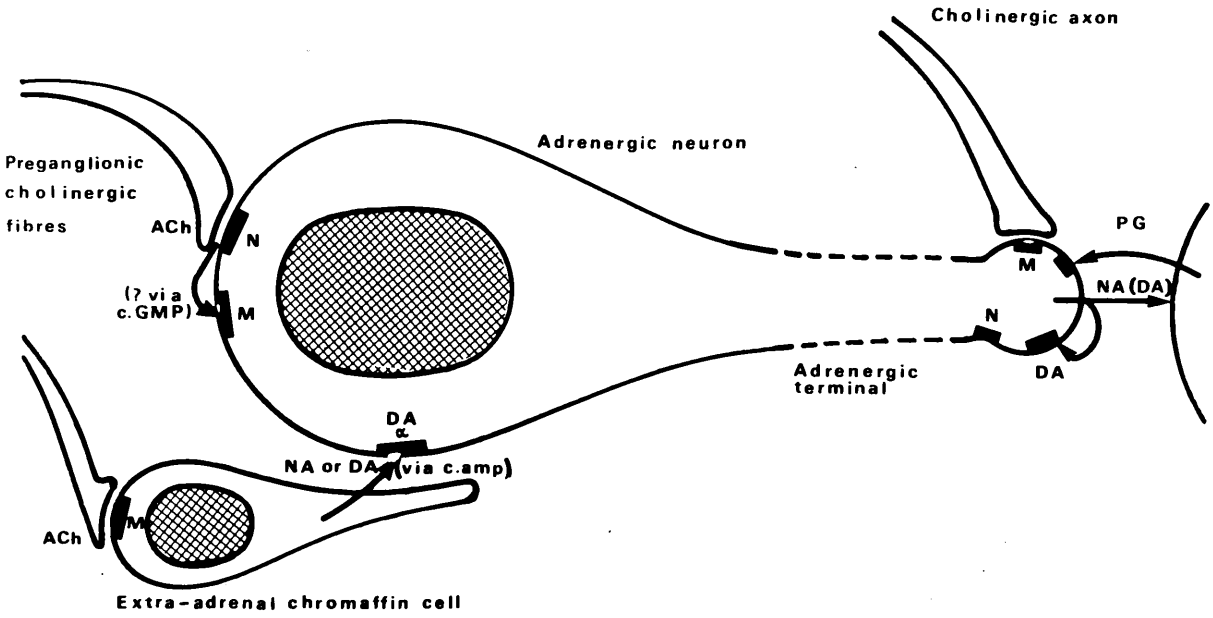
3 - agranular vesicles which are electron-lucent, and their numbers vary from one nerve ending to another, and also on the tissue preparation procedure, Hökfelt (1968, 1969, 1973), Burnstock (1970) and Tranzer (1973).

Electronmicroscopic visualization of the granules (dense cores) of the small granular vesicles is only possible if an appropriate fixation procedure is applied. It is suggested that

Fig. I-3

"The hypothetical types of receptors involved in adrenergic transmission and located on adrenergic cell bodies, adrenergic terminals, axons and extra-adrenal chromaffin cells in sympathetic ganglia. Preganglionic cholinergic fibres excite adrenergic neurons through nicotinic receptors (N). Acetylcholine diffuses away from the nicotinic synapse and activates muscarinic receptors (M), their physiological role being unclear. Adrenergic neurons may receive inhibitory inputs of intraganglionic origin from extra-adrenal chromaffin cells. These cells appear to receive an excitatory preganglionic input mediated through muscarinic receptors (M) and inhibit adrenergic neurons through α-adrenoceptors which are highly sensitive to dopamine (DA). Both nicotinic and muscarinic receptors are present on adrenergic terminal axons, but acetylcholine released from neighbouring cholinergic fibres acts mostly through muscarinic receptors to inhibit noradrenaline release. Noradrenaline and dopamine released from adrenergic axons act on α-adrenoceptors located on the adrenergic terminals, inhibiting further release of transmitter. Prostaglandin (PG) released from the effector tissue may affect transmitter release from adrenergic terminals."

(From Burnstock and Costa, 1975).


Cholinergic axon
Adrenergic neuron
Preganglionic
cholinergic
fibres
ACh
N
(? via
c.GMP)
M
PG
M
NA(DA)
N
Adrenergic
terminal
DA
DA
α
NA or DA
(via c.amp)
ACh
M
Extra-adrenal chromaffin cell

the granule (dense core) is best preserved in permanganate fixation followed by glutaraldehyde-trichromate-osmium tetroxide, glutaraldehyde-osmium tetroxide, and formaldehyde (see Bloom 1972 and Hökfelt and Ljungdahl, 1972).

Tranzer and Thoenen (1967) reported that treatment with 5-hydroxydopamine (5-OHDA), a 'false' transmitter, formed dense osmiophilic material in all the empty and dense core vesicles in adrenergic nerve-endings specifically while the vesicles of cholinergic nerve endings remained empty. Moreover, they reported that 5-hydroxydopamine, in addition to being a specific marker for adrenergic vesicles, provided strong evidence that the empty and dense core vesicles in adrenergic nerve terminals represent a potentially uniform population of cell organelles differing only in their degree of amine filling. So far as the origin of the small and large vesicles in adrenergic nerve-endings is concerned, the matter is not clear. Geffen and Ostberg (1969) and Smith (1971) suggested that the large granular vesicles are probably formed in the perikaryon and are transported to the nerve-endings where they are transformed into the small granular vesicles after the release of their contents, namely the neurotransmitter and possible associated enzymes.

1.7.3. Physiology of adrenergic transmission

1.7.3.1. Release of noradrenaline

While the association of noradrenaline with the vesicles and the fact that nerve impulses release noradrenaline stored in

vesicles and not extravesicular noradrenaline is well established (Smith and Winkler, 1972), the significance of such structures (storage vesicles) for transmitter release phenomena is not yet certain. Results obtained from studies involving electron microscopy, chemical, immunological and enzymatic analysis have suggested the possibility that noradrenaline is released from adrenergic nerves by a process of exocytosis (Smith and Winkler, 1972; de Potter, 1973). Smith (1973) illustrated some hypothetical mechanisms involved in the release of noradrenaline from sympathetic nerve fibres (Fig. I-4 p.52).

1.7.3.2. Peripheral control of noradrenaline release: Presence of pre-synaptic alpha and beta adrenoceptors regulating release of the transmitter

Iversen (1967) reported that the firing rate of the action potentials which invade the terminals mainly determine the amount of released noradrenaline.

Brown and Gillespie (1957) reported that in the presence of phenoxybenzamine, an alpha-adrenoceptor blocking agent, nerve stimulation in the perfused cat spleen resulted in an increase in the outflow of noradrenaline. These authors postulated that post-synaptic alpha-adrenoceptors are important utilization sites for the transmitter released and their blocking by an alpha blocking agent results in the overflow of the transmitter. However, the discovery that phenoxybenzamine was able to inhibit neuronal uptake by Iversen (1965) and extraneural uptake (Iversen, 1967; Iversen and

Fig. I-4

Hypothetical mechanisms for the release of noradrenaline (NA). From Smith (1973).
Mechanisms of type I involve the diffusion of NA out of the vesicle, into the cytosol and across the cell membrane.
In mechanism type II, the vesicle itself moves and releases NA directly into the extracellular space.
(ADP: adenosine 5'-pyrophosphate; ATP: adenosine 5'-triphosphate).

I(a). The stimulus for release acts on the cell membrane, making it more permeable to NA, so that NA in the cytosol can rapidly pass out of the cell. This lowers the concentration of NA in the cytosol, displacing the equilibrium between NA in the vesicles and NA in the cytosol, causing the NA in the vesicles to enter the cytosol.

I(b). The storage complex of NA and adenosine triphosphate inside the vesicles is dissociated or made less stable.

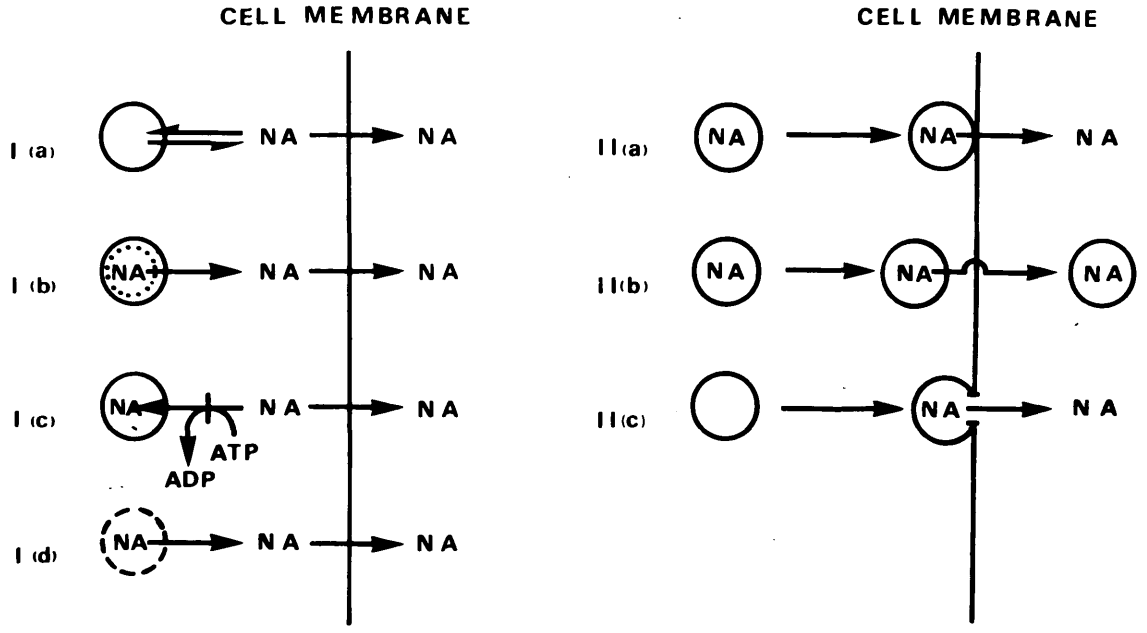
I(c). The energy-dependent pump in the vesicle membrane, which maintains a high concentration of NA inside the vesicle, is inhibited, and any NA that diffuses out of the vesicle cannot be pumped back in again. This is the most likely mode of action of reserpine.

I(d). The vesicle membrane is partly dissolved, allowing NA to pass out unhindered.

II(a). Vesicle and plasma membranes come into close contact (a "tight" or "gap" junction) and NA passes across the two membranes into the extracellular space.

II(b). The intact vesicle is ejected from the cell.

II(c). The vesicle and plasma membranes fuse, and then rearrange to form an opening through which the contents of the vesicle can pass into the extracellular space; this process is called exocytosis.


CELL MEMBRANE
I (a)
NA
NA
I (b)
NA
NA
NA
I (c)
NA
NA
NA
ATP
ADP
I (d)
NA
NA
NA
CELL MEMBRANE
II(a)
NA
NA
NA
II(b)
NA
NA
NA
II(c)
NA
NA

Langer. 1969) led to the hypothesis that alpha-adrenoceptors located on the membrane of adrenergic terminals are involved in regulating release of noradrenaline through negative feed-back mechanisms mediated by the neurotransmitter itself. In support of the above, it has been reported that administration of alpha-adrenoceptor agonists exogenously inhibits transmitter release during nerve stimulation, (Starke 1972). Further support for the hypothesis was obtained when it was reported that the overflow in the presence of an alpha-adrenoceptor blocking agent was obtained regardless of whether the postsynaptic receptor was of the alpha or beta type (Langer *et al.*, 1977). Moreover, Langer (1977) has recently postulated the presence of pre-synaptic beta-adrenoceptors in adrenergic nerve-endings acting as a positive feed-back mechanism. Evidence in support of the assumption is based on evidence that the administration of low concentrations of isoprenaline, a beta agonist, increases the amount of noradrenaline released during nerve stimulation at low frequencies in several adrenergically innervated organs (Langer *et al.*, 1976a; Stjarne and Brundin, 1976a). Fig. I-5 page 54 , is a schematic drawing for the autoregulation of noradrenaline release during nerve stimulation with a description of a working hypothesis for the participation of the pre-synaptic alpha- and beta-adrenoceptors in the regulation of transmitter released as postulated by Langer (1977).

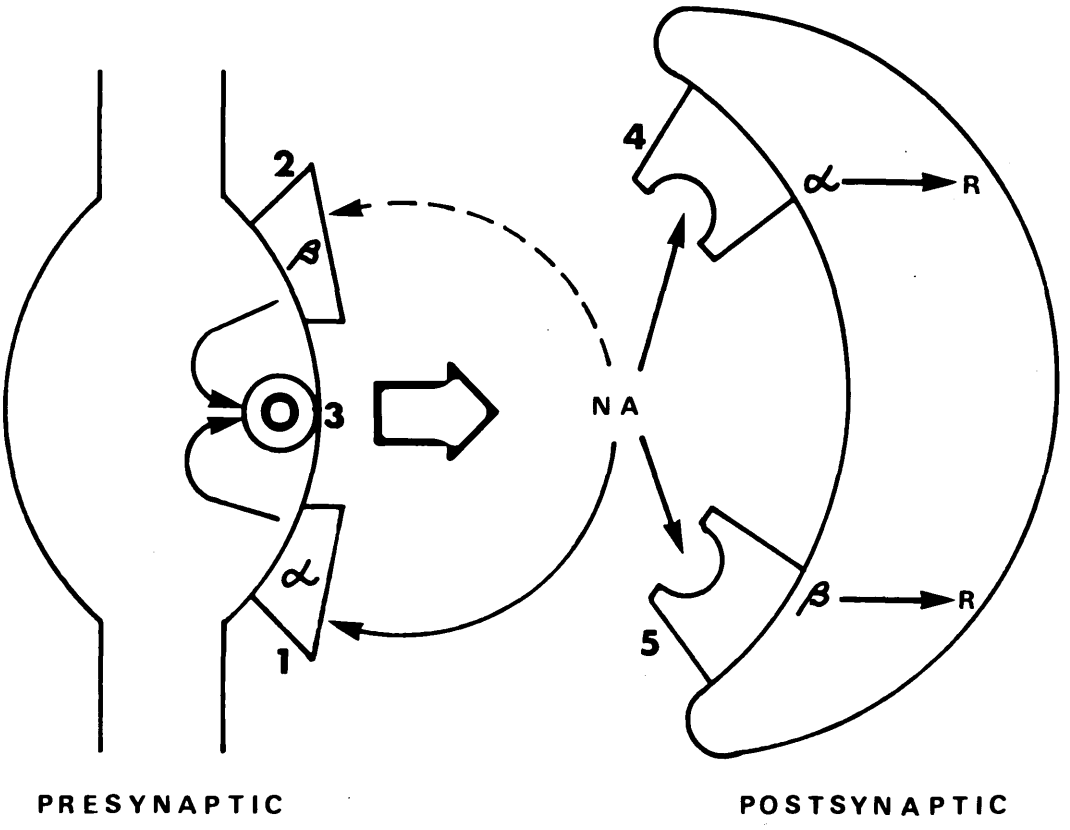
1.7.3.3. Inactivation of released noradrenaline

Several mechanisms are involved to facilitate the inactivation of released noradrenaline:

Fig. I-5

"Role of the presynaptic α- and β-adrenoceptors in the regulation of noradrenaline release during nerve stimulation. During noradrenaline (NA) release at low frequencies of nerve stimulation (when the concentration of the released transmitter in the synaptic cleft is rather low) the positive feed-back mechanism mediated by presynaptic β-adrenoceptors is activated leading to an increase in transmitter release. As the concentration of released noradrenaline increases, a threshold is reached at which negative feed-back mechanism mediated by presynaptic α-adrenoceptors is triggered, leading to inhibition of transmitter release. Both presynaptic feed-back mechanisms are present in nerves, irrespective of the α or β nature of the receptors that mediate the response (R) of the effector organ."

(From Langer, 1977).


2
β
3
α
1
NA
4
α
R
β
R
5
PRESYNAPTIC
POSTSYNAPTIC

(a) Neuronal uptake : this is re-uptake of the released transmitter by nerve-endings. This mechanism plays an important role in prompt termination of the effect of noradrenaline and can be inhibited by drugs such as cocaine (Iversen, 1971, 1973).

(b) Extraneural uptake : this mechanism was first reported by Iversen (1967) when he observed the accumulation of noradrenaline in smooth and cardiac muscle if they were exposed to high concentrations of noradrenaline.

(c) Metabolization by enzymes : noradrenaline taken up by a process of neuronal or non-neuronal uptake may be catabolized by the enzymes monoamine oxidase or catechol-o-methyltransferase (Langer *et al.*, 1972).

(d) Diffusion into the blood stream : some of the released noradrenaline is inactivated through diffusion into the blood stream (Rosenblueth, 1950; Su and Bevan, 1970).

Possibly other mechanisms remain to be discovered.

Figure I-6 page 56 is a schematic illustration of the basic mechanisms involved in the synthesis, storage, uptake and release of noradrenaline from a varicosity of the adrenergic terminal axon innervating smooth muscle (after Burnstock and Costa, 1975).

1.7.3.4. Pharmacological properties of adrenergic receptors

The contradictory responses of various organ systems to the same sympathomimetic amine, e.g. relaxation in one end organ and stimulation in another, led Ahlquist (1948) to propose the

Fig. I-6

The basic mechanism involved in the synthesis, storage, uptake and release of NA from a varicosity of the adrenergic terminal axon innervating smooth muscle.

(lNA) - large storage pool of vesicular NA

(sNA) - small storage pool of vesicular NA

(cNA) - cytoplasmic NA

(MAO) - monoamine oxidase

(COMT) - catechol-o-methyl transferase

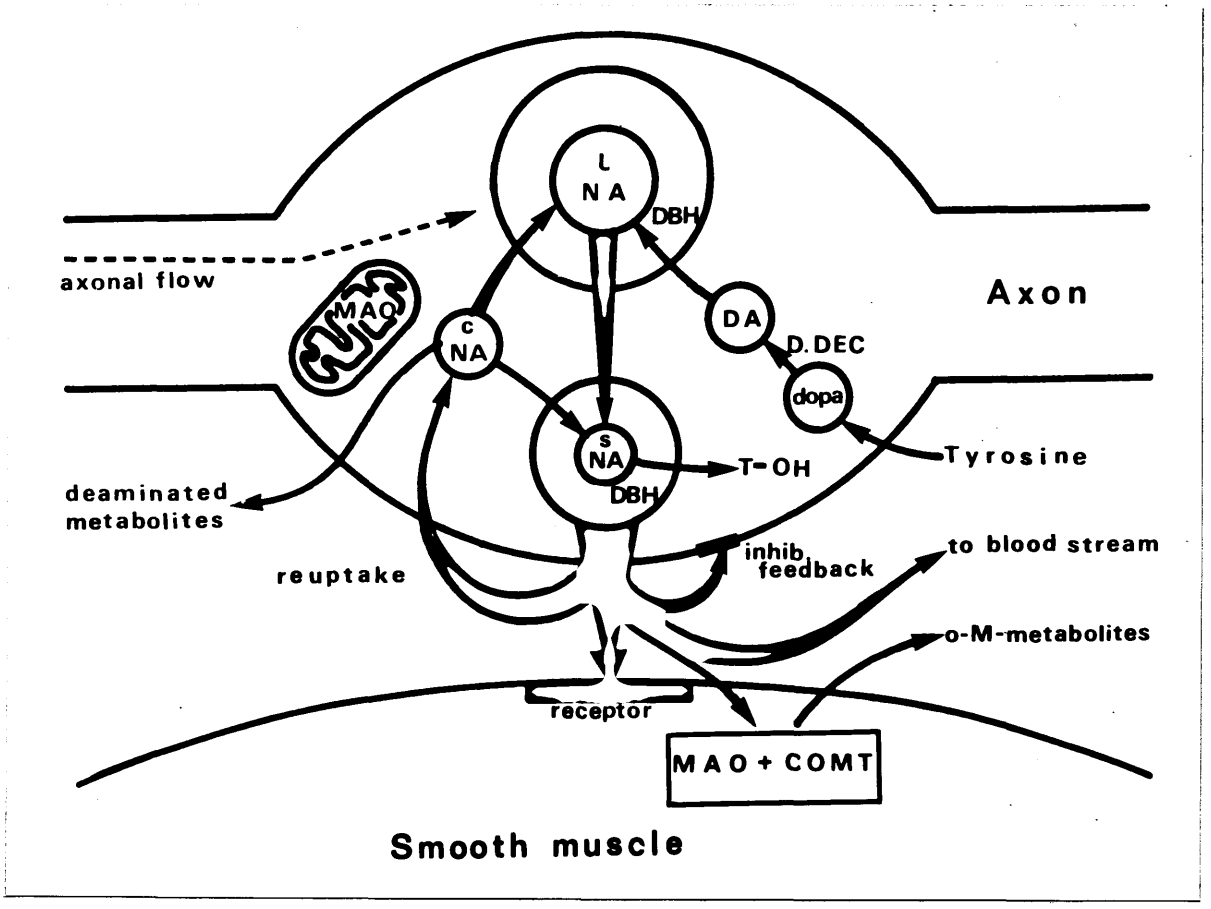
(DA) - dopamine

(T-OH) - tyrosine hydroxylase

(D.DEC) - dopa decarboxylase

(DBH) - dopamine-beta-hydroxylase

(o-M-metabolites) - o-methylated-metabolites


L
NA
DBH
axonal flow
Axon
MAO
c
NA
DA
D.DEC
dopa
Tyrosine
s
NA
T-OH
DBH
deaminated
metabolites
reuptake
inhib
feedback
to blood stream
o-M-metabolites
receptor
MAO + COMT
Smooth muscle

presence of two different types of adrenergic receptor sites called alpha and beta. Ahlquist's proposal was based on the relative effectiveness of five sympathomimetic amines - noradrenaline, adrenaline, isoprenaline and the alpha-methyl derivatives of noradrenaline and adrenaline - on a variety of isolated tissues and intact animal systems. Ahlquist found two orders of activity. For one adrenaline was the most effective and isoprenaline the least, whereas for the other isoprenaline was the most and noradrenaline the least effective. The above concept was not fully accepted until Powell and Slater (1958) introduced dichloroisoprotenal (DCI) as a specific blocking agent for the beta receptors.

The concept of dual adrenergic receptors has recently been reviewed by Furchgott (1972), Jenkinson (1973) and Ahlquist (1976). Burnstock and Costa (1975) defined the alpha-adrenoceptor as the one which mediates a response pharmacologically characterized by: (a) - a relative motor potency series in which adrenaline is more or equal to noradrenaline which is more than phenylephrine which is more than isoprenaline; and (b) - the susceptibility to specific blockade by phentolamine, dibenamine or phenoxybenzamine at relatively low concentrations. The beta-adrenoceptor is one which mediates a response pharmacologically characterized by: (a) a relative molar potency series (in the presence of a blocker of nerve amine uptake) which may be isoprenaline greater than noradrenaline greater than adrenaline greater than phenylephrine, the relative potencies of adrenaline and noradrenaline being reversed in certain tissues; and (b) a susceptibility to specific blockade by either propranolol or

pronethalol at relatively low concentrations.

1.7.3.5. Beta-adrenergic subtypes

Lands *et al.* (1967), Furchgott (1972) and Jenkinson (1973) indicated that the beta-adrenergic receptors might be subdivided into beta 1 receptors (cardiac and lipolytic) and beta 2 receptors (bronchial, vascular and intestinal). The argument for β_1 and β_2 subclassification is based on the fact that a number of beta-agonists exhibit a considerable selectivity for β_2-receptors (bronchial, vascular and intestinal) (Brittain *et al.*, 1970). It was also found that butoxamine, a beta antagonist, can block vascular beta-responses (β_2) to a greater extent than cardiac beta-responses (β_1), (Levy and Wilkenfeld, 1969), and that practolol (another beta antagonist) was found to block cardiac beta-responses (β_1) to a greater extent than vascular beta-responses (β_2) (Bristow *et al.*, 1970).

1.7.3.6. Adrenergic receptors and cyclic nucleotides

Rall *et al.* (1957) and Robinson *et al.* (1970) reported the association of catecholamine and adenylate cyclase when they found that c-AMP was the mediator for adrenaline-induced hepatic glycogenolysis. Regarding the association of adrenergic receptors and adenylate cyclase, Furchgott (1972) and Triggle (1972) reported that in tissues classified to possess beta receptors, application of beta agonists resulted in activation of adenylate cyclase and thus an increase in the level of cyclic AMP. Moreover, Burges and Blackburn (1972) and Lefkowitz (1975) reported that the specific β_2

receptor agonists were found to be more effective activators for adenylate cyclase in tissues classified under β_2 subdivision. Consistent results with the above were obtained when inhibiting effects of β_1 and β_2 antagonists on adenylate cyclase were tested. Therefore the above authors concluded that these receptors (beta types), if not an integral component, are closely associated with the adenyl cyclase system. As far as the relationship between alpha-adrenergic receptors and adenylate cyclase is concerned, investigations in a number of systems where stimulation of alpha and beta receptors has opposing physiological events have suggested that stimulation of alpha receptors might cause inhibition of adenylate cyclase and a decrease in intracellular c-AMP levels (Abe *et al.*, 1969; Volicer and Hynie, 1971; Andersson, 1973).

1.8. Involvement of alpha and beta postsynaptic adrenoceptors in melanophore control

On the mammalian pharmacological criteria, the presence of postsynaptic alpha-adrenoceptors mediating pigment aggregation in some teleosts has been reported (Iga, 1968 in *Oryzias latipes*; Fujii and Novales, 1969 in *Fundulus*; Grove, 1969 in *Phoxinus*; Reed and Finnin, 1972 in *Pterophyllum eimekei*; Fernando and Grove, 1974a, b in *Pleuronectes plastessa*; Fujii and Miyashita, 1975 in *Lebistes reticulatus*). As far as the possible involvement of beta-adrenoceptors in melanophore control is concerned, recent studies on some amphibians and reptiles have revealed their presence in melanophores, in which they mediate pigment dispersion (Graham, 1961; Goldman and Hadley, 1969a, b; Bagnara and Hadley, 1973; Taylor and

Teague, 1976).

Goldman and Hadley (1969a), working on *Anolis carolinensis*, found that norepinephrine (noradrenaline), which normally results in pigment aggregation, in the presence of alpha-adrenergic blocking agents causes pigment dispersion. Because the pigment dispersing effect of noradrenaline was blocked by the beta-adrenoceptor blocking agents, the above authors concluded that the pigment dispersion mechanism in *Anolis* is mediated through stimulation of the beta receptor. The above conclusion was further supported by the fact that isoproterenol, a synthetic beta agonist, enhanced the darkening of *Anolis* skin after alpha blockade by a greater degree than noradrenaline. The relative effectiveness of adrenergic agonists in darkening after treatment with alpha-adrenoceptor blocking agents was found by the same authors to be in the following order: isoproterenol is greater than adrenaline which is greater than noradrenaline which is greater than phenylephrine. In an attempt to demonstrate the existence of adrenergic beta receptors mediating pigment dispersion in teleosts, Reed and Finnin (1972), working on spinal angelfish, *Pterophyllum eimekei*, provided evidence that the melanophores possessed, in addition to the alpha-adrenoceptors which mediate pigment aggregation, beta-adrenoceptors mediating pigment dispersion. They kept the melanophores in a constant state of partial pigment aggregation by a selective low pulse rate of electrical stimulation. Intraperitoneal injection of isoproterenol resulted in a transient pigment dispersion. The above authors concluded that this transient pigment dispersion suggested the presence

of beta-adrenoceptors mediating pigment dispersion.

More recently Miyashita and Fujii (1975) and Fujii and Miyashita (1975) in *in vitro* experiments with split fin preparations of guppies, *Lebistes reticulatus*, reported that postsynaptic beta-adrenoceptors appeared to take part in the pigment dispersion response, and suggested that the endogenous beta stimulating amine responsible for the darkening reaction of living fish may be adrenaline. This suggestion is based on the fact that when adrenaline was applied in concentrations lower than that required to produce pigment aggregation, pigment dispersion resulted. Various synthetic beta agonists - isoproterenol, protokylol, metaproterenol, methoxyphenamine and isoxsuprine - have all proved to be potent dispersing agents and were antagonised by beta-adrenoceptor blocking agents like propranolol and dichloriso-proterenol.

1.9. Hormonal control of chromatophores

Early investigations by Smith (1916a, b) on hypophysectomized frog tadpoles and Spaeth (1918) on the isolated scale of *Fundulus* clearly demonstrated that the pituitary gland is in some way involved in the control of integumental colouration in amphibians and teleosts. Hogben and Winton (1922) were able to conclude that colour changes in amphibians are the result of an agent released from the posterior lobe of the pituitary. Swingle (1921) provided strong evidence for the *pars intermedia* of the posterior lobe being the precise site responsible for the release of the darkening agent (melanophore-dispersing hormone = melanophore stimulating hormone MSH). In further

publications, Hogben and Winton (1923) and Hogben (1924) postulated a dual hormone mechanism acting antagonistically, B-substance mediating pigment dispersion and W-substance mediating pigment aggregation, and that the *pars tuberalis* might be either the site for the aggregating hormone secretion or might stimulate some other organ to secrete an agent which is inhibitative and antagonistic to the pigment dispersing hormone of the posterior lobe. However, Jørgensen and Larsen (1960) and Jørgensen (1962), working on *Bufo* and *Xenopus* reported that their results did not indicate the presence of a hormone controlling pigment aggregation in the above animals. As far as the effect of pituitary extracts on teleost melanophores are concerned, there is good evidence to believe the existence of a melanophore dispersing hormone in the pituitary of the catfish *Ameiurus*. Abramowitz (1936b) and Osborn (1938a) reported the failure of hypophysectomized *Ameiurus* to adapt to an illuminated black background to the same extent as an unoperated fish. A further line of evidence in support of the presence of melanophore dispersing-hormone in *Ameiurus* was obtained as a result of experiments involving injection of pituitary preparations.

Darkening of *Ameiurus* in response to injected mammalian posterior-lobe pituitary preparations was reported by Odiorne (1933.), Parker (1934c, 1941), Kleinholz (1935), Abramowitz (1936b), Osborn (1939a) and in response to its own pituitary extracts by Parker (1934c), Osborn (1938a, b), Healey (1940, 1948). Also the following observations of Abramowitz (1936b), that a faded denervated caudal band (p. 35) darkened if the fish was injected with mammalian

pituitary extracts; Veil (1938), the darkening of paled hypophysectomized fish when injected with intermedin and Osborn (1938b) on the lesser extent of darkening of a caudal band produced in paled hypophysectomized fish when compared with darkening which would normally be observed if an unoperated fish were subjected to the same operation, were all considered to provide further support for the presence of a darkening hormone of pituitary origin in this fish.

On the other hand, Matthews (1933) reported that the removal of the pituitary in *Fundulus heteroclitus* did not in any way interfere with its normal background adaptation. This was considered to indicate that the pituitary is not involved in colour change response in this fish.

In *Phoxinus phoxinus* Healey (1940, 1948) has demonstrated that chromatically spinal minnows lose their ability for rapid colour change but still change colour slowly in response to a black and white background in a manner similar to that of the hormonally controlled amphibians. However, this slow adaptation of chromatically spinal fish to illuminated black and white backgrounds is abolished if the fish is subjected to hypophysectomy, the fish remaining dark. In agreement with this, injections of extracts and implants of their own pituitary have a paling effect on spinal as well as intact fish. Therefore Healey concluded that the pituitary of *Phoxinus* contained a paling hormone. Healey observed that hypophysectomized but otherwise intact *Phoxinus* failed to adapt fully for more than a short time to black and white backgrounds, which suggested that though initial dispersion and aggregation of pigment is evoked by nervous mechanisms,

the presence of dispersing and aggregating agents of pituitary origin are essential to maintain a consistent dark and pale tint of fish on illuminated black and white backgrounds respectively. Furthermore, Healey's results obtained from partial removal of the pituitary suggest that the anterior lobe and the posterior lobes of the pituitary in *Phoxinus* might be the sites controlling hormonal paling and darkening of the fish respectively.

Based on Hogben and Slome's (1931) bihormonal hypothesis, Neill(1940) and Healey (1951) studied the time relations of hormonal colour change in *Anguilla* and spinal *Phoxinus* and they both concluded that their results indicated the presence of antagonistic aggregating and dispersing hormones. However, Kent (1959) criticized the logic on which Hogben and Slome (1931, 1936) had based their bihormonal hypothesis. Kent's criticism is based on the fact that the time relation studies do not necessarily indicate the nature of the involved coordination. Moreover, Kent (1960), based on results obtained by subjecting the pituitary extract of *Phoxinus* which causes pigment aggregation in *Phoxinus* and pigment dispersion in *Ameiurus* to paper-electrophoresis, concluded that the fraction always contained a *Phoxinus* aggregating factor and an *Ameiurus* dispersing factor, which suggests that they are one and the same thing. On the other hand, Enami (1955)working on *Parasilurus asotus*, reported that aqueous extracts of this fishes hypothalamus and pituitary produced aggregation at the site of injection and darkening over the rest of the body. He also occasionally observed that if a high dose of extract was injected generalised paling all over the

body resulted. Based on the above results Enami suggested that both aggregating and dispersing hormones were present, and partial separation of the two was possible. Enami considered the melanophore-concentrating hormone (MCH) to be neurosecretory, originating in the hypothalamus and stored in the anterior lobe of the pituitary. Imai (1958) working on the same fish as Enami and on the carp, *Cyprinus carpio*, confirmed Enami's results.

However, histological studies on the pituitary of several teleosts by Baker (1963) indicated that in some black-adapted species a cell type of the *pars intermedia* showed hyperactive cytological structures but she failed to detect any activated cells in the pituitary of the white-adapted ones, suggesting that melanin-dispersing hormone (MSH) is the sole pituitary agent controlling fish melanophores. Therefore, the hypothesis suggested originally by Hogben and his co-workers that there are two antagonistic principles, e.g. MSH or a related melanin-dispersing peptide and MCH, has not been universally accepted.

Another endocrine organ which has been observed to participate in the chromatic physiology of vertebrates is the *pineal body*, first reported by McCord and Allen (1917). Hoar (1955), working on *Oncorhynchus nerka*, reported that removal of the pineal gland and the eyes resulted in more intense darkening of the fish than if the eyes only were removed. Lerner and Case (1960) were able to isolate the active substance from bovine pineal gland and they called it melatonin and found it to be a potent paling agent for

frog skin. However, the role of the pineal gland in teleost colour changes is uncertain and requires further investigation.

CHAPTER II

GENERAL MATERIAL AND METHODS

2.1. Source and general treatment of the animals

The European minnows, *Phoxinus phoxinus* (L.), were obtained from the River Lea in Hertfordshire. The fish were given a bath in 'Parasan', followed by a bath in 'Myxosan' (Piscisan Ltd., 34/38 Church Street, Enfield, Middx. EN2, England.) to prevent ecto-parasites and myxobacteria in the fish population. The fish were then transferred to disinfected sinks (Bactosan disinfectant, Piscisan Ltd.) in the laboratory and supplied with running water and air. The fish were fed on alternate days with minced ox heart, and once a fortnight with 'Bemax' (wheat germ) to provide them with the necessary vitamins. All uneaten food and faeces were regularly removed from the sinks and the sinks were disinfected by 'Bactosan' from time to time as a matter of precaution.

2.2. Operative technique on the minnow *Phoxinus phoxinus* (L.)

The same operative procedure used by Healey (1940, 1967) was followed. During the operation the fish were anaesthetized in 0.5% urethane in tap water. After deep anaesthesia the fish were placed on the operating support, which was made of a zinc tray measuring 10 x 15 cm and filled with paraffin wax. Two rows of pins were embedded at the side of a groove in the wax. By the use

of two rolls of moistened filter paper at the side of the groove the fish was protected from dehydration and supported with the help of an elastic band which was attached to the pins and drawn over the fish. The anaesthesia was maintained during the operation by siphoning 0.25% urethane through a funnel and then by a tube down to the mouth from a Winchester quart bottle placed on a shelf above the operating table. If the respiratory movements of the animal were found to be weakening, the anaesthetic solution could be replaced by respiratory water contained in a second Winchester bottle, also directed into the funnel. A third Winchester bottle was placed on the same shelf to siphon a jet of fresh water teleost Ringer (Young, 1933) through a fine glass tube with a fine point. The jet of Ringer helped visibility and washed away the blood during the operation. All operations were carried out under a binocular microscope.

2.2.1. Spinal sectioning posterior to the 15th vertebra

This operation was carried out on fish which were going to be used in the continuous observation tank, in experiments requiring chromatically normal fish. A section through the spinal cord posterior to the 15th vertebra has no effect on the chromatic behaviour of the fish (von Frisch, 1910, 1911, p.32), and at the same time it reduces the tendency of the fish to struggle when confined.

The operation procedure was as follows:-

a clean longitudinal cut, approximately 1 cm long, was made with a sharp knife through the underlying muscles down to the neural arch.

The bones of the neural arch were removed by means of a fine dental drill burr and a small part of the spinal cord was exposed. Using a very fine knife manufactured by J. Weiss and Company, 11 Wigmore Street, London, W.1., the exposed spinal cord was subjected to two fine sections 0.5 mm apart and the piece of the cord in between was removed to ensure a complete separation of the anterior and posterior portions of the spinal cord. The posterior portion was then further destroyed by the fine dental burr and the wound was sutured using a finest curved braided silk eyeless needle manufactured by Ethicon Limited, Scotland. After the operation the fish was transferred to the post-operative aquarium with running water and after full recovery from the anaesthetic the fish was subjected to background reversal to confirm normal colour changes.

2.2.2. Post-operative care

Post-operative treatment was given to the operated fish by means of salt water baths (3% NaCl in tap water) for 3 minutes, twice a day for the first week and thereafter once a week. Application of Povisan (Pisisan Limited, 34/38 Church Street, Enfield, Middx. EN2, England) to the wound was found to be very helpful in the early stages of healing.

2.2.3. Spinal sectioning anterior to the 15th vertebra

The operative procedures were the same as above excepting that after the removal of a very small piece of the cord at the level of the sectioning (but large enough to ensure that re-establishment of nervous connection did not take place during the time of

the experiment) both the anterior and the posterior portions of the cord from the site of sections were left intact. The spinal cords of fish in this group were usually sectioned at the level of vertebra 10 in order to disconnect the integumentary melanophores from the central nervous control and at the same time to ensure that the fish maintained its normal balance after recovery in the aquarium. However, in another group of fish which were required in experiments concerning electrical stimulation of the pigmentary fibres, the operation was carried out more anteriorly at a level anterior to that of vertebra 5, in order to provide a reasonable length of these fibres to be electrically stimulated. Since these fish were unable to maintain their balance they were suspended over an illuminated black or white background (Healey, 1940). These operations abolished the rapid colour changes of the fish in response to background reversal through the separation of the integumentary melanophores from the medullary paling centre.

2.2.4. Spinal nerve section

This operation provided animals in which a group of melanophores were separated from the spinal cord as well as from the medullary paling centre. A cut of approximately 2 cm in length to one side of the dorsal line through the underlying muscles was made with a sharp knife and the vertebral column was exposed. The spinal nerves on one side were severed over 3-5 segments. A dark strip of dispersed melanophores which resulted immediately after the operation confirmed the disruption of the chromatic fibres to the affected melanophores. The above operation was always combined with spinal

cord section posterior to vertebra 15 to allow the observation of these separated melanophores in the continuous observation apparatus.

2.3. The continuous observation apparatus used in studying the responses of the minnow and the recording of the results

The continuous observation apparatus used in the experimental work of the present thesis was designed by Healey (unpublished). His original apparatus was used by Gray (1956). The apparatus in its later form, modified by Healey (Fig. II-1 p.72) consisted of a rectangular tank measuring 15 x 20 x 12 cm^3 made of black perspex except for the observation face which was made of very thin transparent perspex (a). A platform (b) on which the experimental animal rested was made of black perspex and could be removed from the observation tank in a vertical direction by an attached arm (c). The fish was exposed to background reversal by means of open-top square boxes (d) painted black or white and some with different shades of standard greys (Healey, 1967) on the inside with an arch-shaped inlet through which the experimental fish could be exposed to the background reversal. These boxes were built on a rectangular piece of perspex which, at the same time, served as a base for an attached arm used to facilitate background reversal without disturbing the fish.

The observation tank was based on a wooden experimental housing in which an electronic photographic flash was fixed (e). Experimental fish, either chromatically spinal or chromatically normal were confined on the platform by means of a fine cotton loop through the

Fig. II-1

The continuous observation apparatus used in studying the responses of the minnow *Phoxinus phoxinus*.

a - The observation tank

b - The platform with the experimental animal shown confined

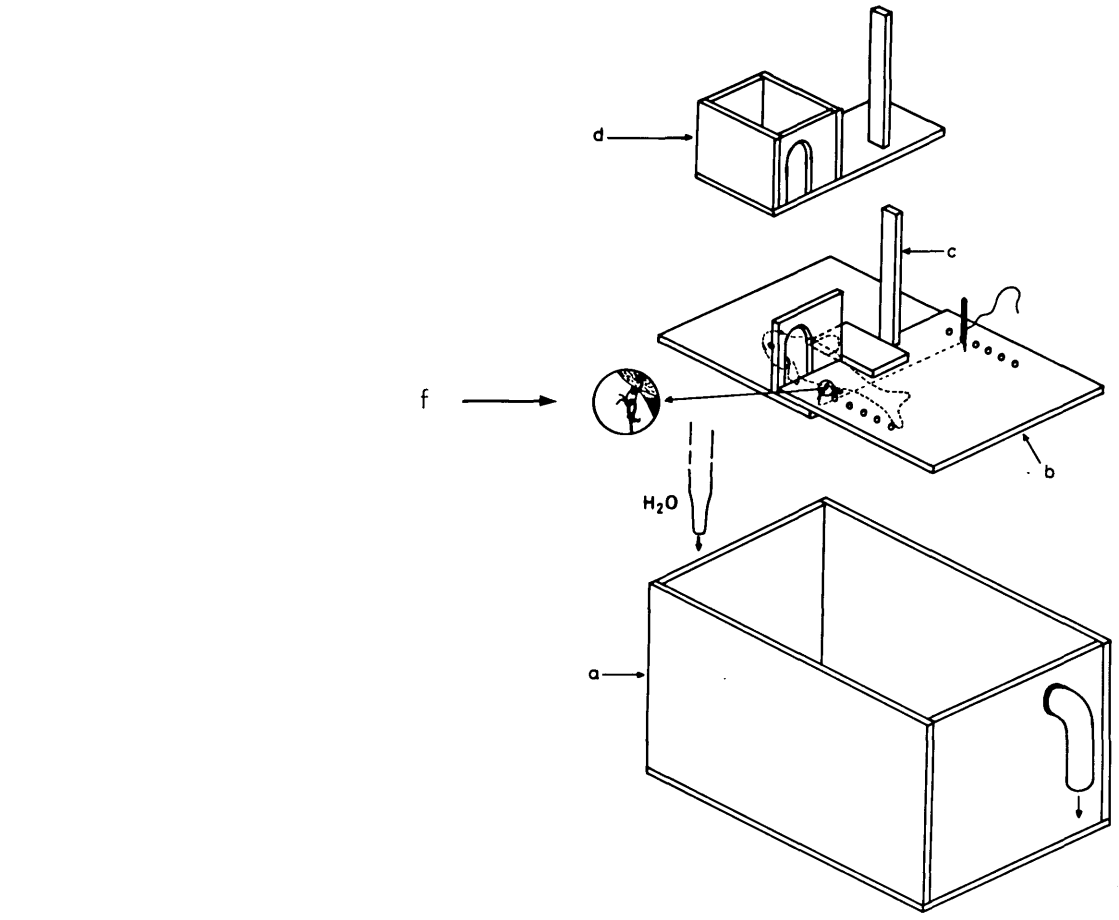
c - The attached arm of the platform for convenient placing of it in the observation tank.

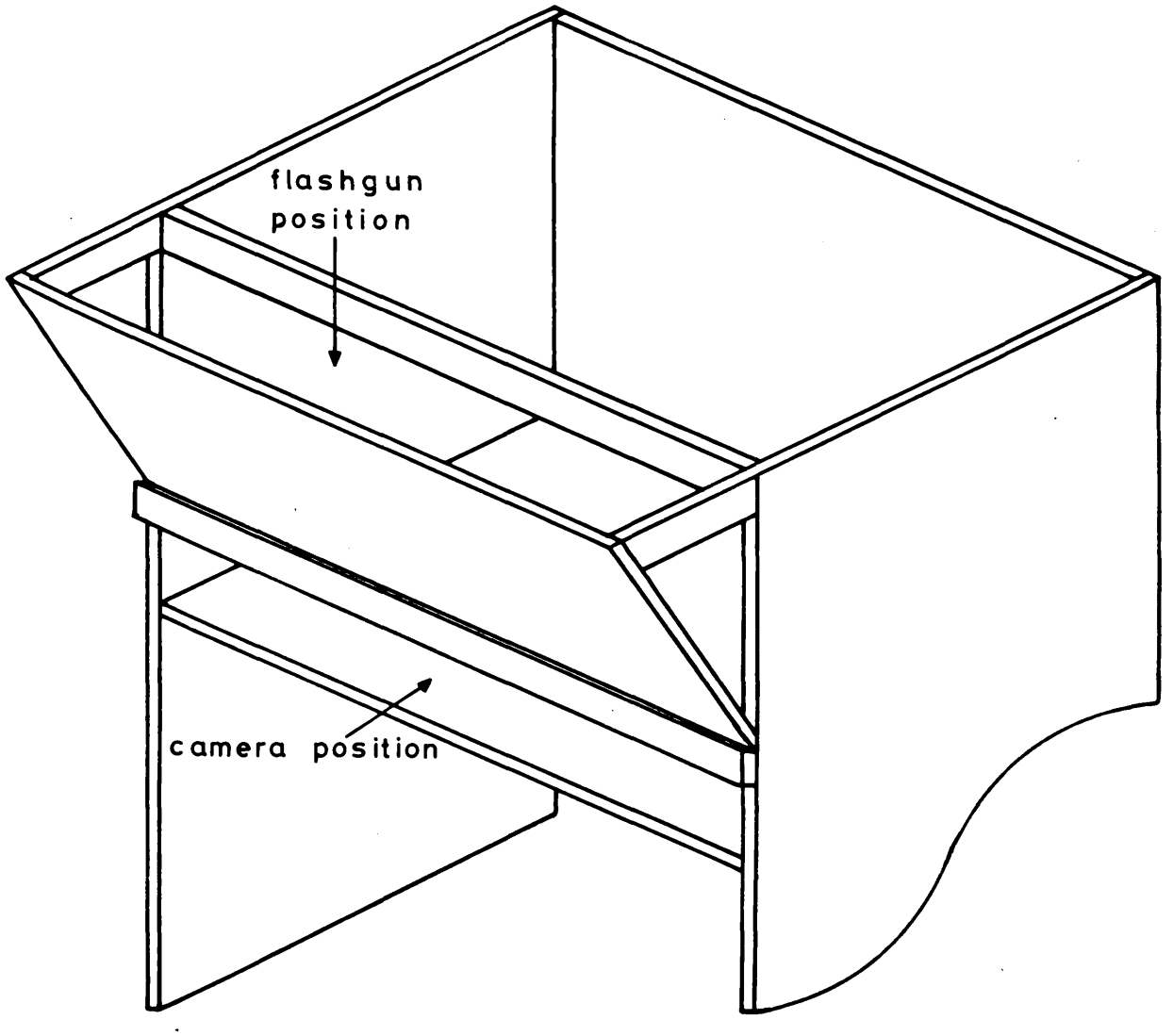
d - Open-top square boxes used for exposing the fish to different shades of grey.

e - Wooden experimental housing on which the observation tank was based. The positions of the flashgun and camera are shown.

f - Different arrangements of loops used in confining the fish.

(For further details see the text).


d
c
f
b
H_2O
a


flashgun
position
camera position

integument at the anterior base of the anal fin. A second loop was made at the end of a fine nylon thread and was connected to the cotton loop attached to the fish by means of a third fine cotton loop (f).

The continuous observation tank was supplied by a slow but constant streams of water and an overhead illumination by means of a 60 watt domestic lamp, 50 cm above the water surface.

In the chromatically spinal and chromatically normal experimental fish, the site of the operation (spinal section) was well anterior of the site of confinement. Therefore, any pain stimulus which might be set up by the loop of thread pulling on the integument would not reach the brain. Furthermore, the operative technique greatly reduced the movement of the fish.

For recording the results the responses of the melanophores under study were recorded using the Melanophore Index by means of a Nikon microscope mounted on a base which was adjustable both horizontally and vertically. Taking the caudal fin as a marker, the 3rd lateral patch of melanophores from the caudal fin was taken as the site for microscopic observations. The Melanophore Index (M.I.) of the melanophores was not individually recorded but a general estimate of their values was recorded in the area of observation in different fish. The plots were drawn through means of these estimates. Standard deviations of the means are shown by vertical lines where they exceeded the limit of the plotting symbols. Also photomicrographs were taken using either a camera attached to the

microscope, or using a Nikon camera with 55m f/3.5 micro auto lens attached to a Nikon bellows focusing P.B-5. The photomicrographs were then studied to determine the responses of the animals.

C H A P T E R III

FINE AND ULTRASTRUCTURE STUDIES OF THE CHROMATOPHORES OF THE MINNOW *Phoxinus phoxinus* (L.) WITH SPECIAL REFERENCE TO MELANOPHORES AND THE NATURE OF THEIR INNERVATION

3.1. Introduction - purpose of the investigation

While a great deal of information has been obtained about the physiology of colour changes in the minnow *Phoxinus phoxinus* (L.), the fine structure of the chromatophores in this fish has not yet been studied at the electron microscopic level. Therefore, this chapter of the present thesis is designed to cover the following topics:

(a) To study the fine structure of the chromatophores in this fish and to compare it with the studies available on the fine structure of chromatophores in other teleosts.

(b) Since the initial description of microtubules in the melanophores of *Fundulus* and their probable role in mediating the intracellular movement of the pigment granules by Bikle *et al.* (1966), a great deal of information has been reported on the same lines (Chapter I, p. 23). However, no work of this nature has been reported on the animal under present investigation. Therefore, it was thought necessary to study the relationship between the microtubules, melanin granules and other structures in the melanophores

of *Phoxinus* and to compare the results with those available from similar studies on other teleosts.

(c) The histochemical nature of the melanophore innervation in teleosts has not yet been investigated at the electron microscopic level and because minnows are known to have an active nervous control of their melanophores, they were thought to be a suitable species for such a study. 5-hydroxydopamine (5-HDA), a 'false' sympathetic transmitter, has been reported by Tranzer and Thoenen (1967) to be a specific marker for adrenergic nerve terminals in mammals. It was hoped that by adopting the same techniques in the present investigation some light could be thrown on the histochemical nature of the melanophore innervation.

3.2. Method and materials

3.2.1. Electron microscopy

The difficulties encountered in ultrastructural studies of skin are generally due to the different sectioning requirements of the dermal and epidermal layers with their difference in hardness and texture within one block. The scales in teleost skin make the situation far more difficult. Furthermore the epithelial barrier and collagen-rich matrix surrounding the chromatophores might well retard the rate of fixation of the chromatophores. However, the following procedures were found to give satisfactory results.

3.2.1.1. Pre-treatment

(a) In experiments designed to study the relationship

between the microtubule apparatus of the melanophores, the pigment granules, and the changes in cell morphology, one group of fish received a dose of noradrenaline (20 mg/kg) in order to obtain a state of pigment aggregation and another received a dose of tolazoline (10 mg/kg) in order to obtain a state of pigment dispersion, 30 minutes before decapitation.

(b) In experiments designed to study the nature of melanophore innervation, a similar technique to that reported by Tranzer and Thoenen (1967) was adopted. Each member of a group of experimental fish was given four doses of 5-HDA (Sigma, P.O. Box 14508, St. Louis, Mo. 63178 U.S.A.) (40 mg/kg) intraperitoneally over a period of 48 hours. Another group of fish received the same number of injections over the same period of time, but these contained only Young's fresh water Ringer and served as controls. Four hours after the last injection pieces of skin from a decapitated fish from each group were processed separately for electron microscopy.

3.2.1.2. Fixation

Specimens were dissected out immediately after decapitation of the fish and were transferred directly to the fixative. The primary fixative used was a mixture of paraformaldehyde-glutaraldehyde (Karnovsky, 1965) buffered in phosphate buffer at pH 7.4 for 2 - 4 hours at $4^{o}C$. The specimens were then washed very thoroughly through 3 - 5 rinses of phosphate buffer with a minimum of four rinses per hour and then were left for an extra hour in the last rinse at $4^{o}C$. The specimens were next post-fixed for one hour in 1% osmium tetroxide buffered to pH 7.4 in phosphate buffer at room temperature. 1% osmium

tetroxide was prepared from 2% solution supplied by Messrs, B,D,H, Chemicals Ltd., Poole, England in 10 ml vials.

3.2.1.3. Dehydration

After treatment with osmic acid the specimens were rinsed thoroughly in distilled water with a minimum of two rinses, the first one rapid and the second for 15 minutes. Then the specimens were passed through a graded series of acetone followed by propylene oxide as follows:

30% acetone	15 minutes
50% acetone	15 minutes
70% acetone	15 minutes
90% acetone	15 minutes
100% acetone, two changes	30 minutes each
50:50 propylene oxide/acetone	10 minutes
100% propylene oxide, two changes	15 minutes each

Propylene oxide was added gradually to the specimens in 100% acetone, and brought to a concentration of about 50% propylene oxide over a period of about 10 - 15 minutes.

3.2.1.4. Infiltration and embedding

Resin mixture - The embedding resin introduced by TAAB (TAAB Laboratories, 52 Kidmore End Road, Emmer Green, Reading, England) was selected for all experiments. This is a developed epoxy resin and has the following valuable characteristics:

1 - relatively low viscosity

2 - good cutting characteristics

3 - can be used with uncoated grids

4 - thermostability with little or no shrinkage upon polymerization

A wide range of hardness could be obtained by using different proportions of the hardeners, DDSA and MNA. BDMA was used as the accelerator. For most of the work in the present investigation resin mixes were made up according to the following schedule:

TAAB resin	20 g.
DDSA	10 g.
MNA	10 g.
BDMA	0.8 g.

Process of infiltration - After two changes of propylene oxide, the resin mixture was added to the vials containing the specimens according to the following schedule:

10:1 propylene oxide/resin	overnight (on rotator)
10:5 propylene oxide/resin	4 hours "
10:10 propylene oxide/resin	4 hours "
100% resin	overnight "
100% resin changed	overnight "

Embedding moulds were filled with fresh resin and specimens were placed in the centre of the resin with fine tweezers with the desired orientation.

Polymerisation - The moulds filled with resin containing the

specimens were placed in an oven at 60°C for 48 hours. The specimen blocks were released by flexing the moulds and were later cut out and stuck to resin blanks with araldite adhesive for mounting in the microtome.

3.2.1.5. Sectioning

Sections were cut with a glass knife on a Huxley Ultramicrotome. The glass knives used were made on an LKB Knife Maker. Sections were floated on distilled water. Sections which showed silver/gold interference colours were picked up on copper grids (200 m) and coated with formvar film. Positioning the block in the microtome in such a way as to cut the dermal layer first, followed by the epidermis, using a slow cutting speed, usually gave satisfactory results.

3.2.1.6. Staining

The sections were stained on the grids with lead citrate (Reynolds, 1963) for 5 - 10 minutes.

All staining was carried out by floating grids face downwards on drops of the stain placed on a dental wax slab kept in a covered Petri dish whose floor was covered with filter paper soaked in a saturated solution of sodium hydroxide. The staining with lead citrate was carried out in the presence of sodium hydroxide in order to achieve an atmosphere of low CO_2 tension and so to avoid contamination with lead carbonate. This appears as black insoluble deposits in sections.

After the staining the section-containing grids were rinsed thoroughly with distilled water and then touched on their edge with a filter paper disc to drain them.

Sections were examined in a Corinth 275 microscope.

3.2.2. Light microscopy

Thick sections for general skin topography were cut from specimens embedded in TAAB resin at 1 - 2 μ thick with glass knives on a Huxley Ultramicrotome. These sections were mounted on glass slides and stained with a mixture of 1% Toluidine Blue in 1% borax solution (O'Brien *et al.*, 1964), which stains well the material embedded in epoxy resins. Whole-mount preparations of skin were made by fixing pieces of skin in 10% formal-saline. After a rapid dehydration in acetone, the specimens were mounted on glass slides in D.P.X.

Light microscope observations were made by means of a Zeiss Universal photomicroscope.

3.3. Observations

The general structure of the integument of *Phoxinus phoxinus* (L.) was found to be very typical of teleost fish in general and can be divided, according to Herikson and Matoltsy (1968), into epidermis, dermis and hypodermis. Only the epidermis and, in particular, the dermis will be considered here.

3.3.1. The epidermis

The epidermis consists of a few to several epidermal cell

layers. The distal epidermal cells are flat and are characterized by the presence of the so-called epidermal surface pattern (e.s.p.) Lanzing and Wright (1974) plate III-1 page 83.

In line with the previous observation of Herikson and Matoltsy (1968) and Roberts *et al.* (1972) on other teleosts, minnow epidermis was found mainly to consist of the so-called filament-containing cells. These cells extend from the basal lamina, which separates the dermis from the epidermis, to the epidermal surface.

The filament-containing cells are characterized by the following:

a - Their relatively smaller size when compared with other epidermal cells;

b - Their cytoplasm is packed with fine filaments;

c - They show numerous desmosomes;

d - Their plasma membrane is usually highly infolded;

e - Their nucleus is centrally located and surrounded by cytoplasmic elements: endoplasmic reticulum which, particularly towards the periphery, can be distinguished as granular, mitochondria and Golgi bodies (Plate III-1, 2, 3 pp. 83, 84).

As is shown in Plate III-3 page 84 a number of other cell types are present in the epidermis. These cells are unicellular glands such as mucus cells and club cells.

Plate III-1

Surface filament-containing cell showing epidermal surface pattern (arrows)

Cytoplasmic organelles present are:

(GERT) granular endoplasmic reticulum; (MIT) mitochondria; and (G) golgi.

Note: desmosomes between adjacent plasma membrane (arrows).

Mag. X 2,600.

Plate III-2

Illustrating the highly infolded nature of the plasma membrane usually observed in the filament-containing cells.

Mag. X 45,000.


MIT
GERT

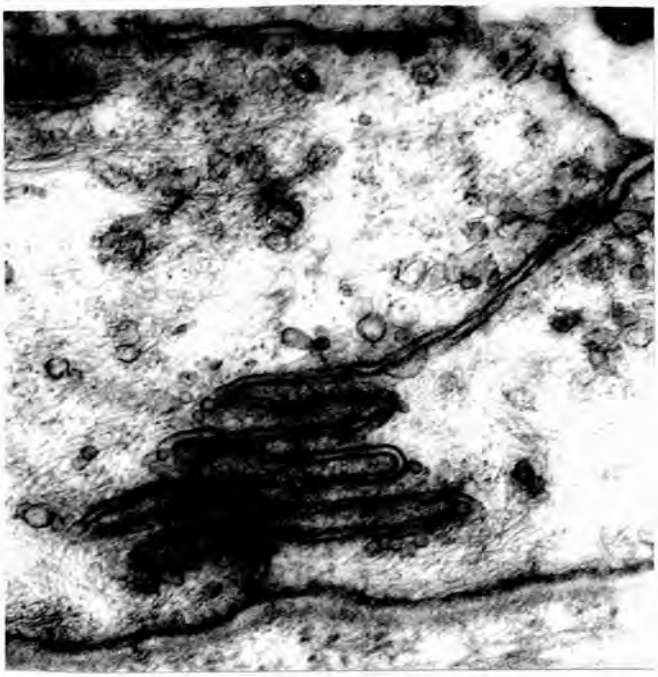


Plate III-3

A mucus cell completely filled with secretory granules (SG), surrounded by filament-containing cells (FCC). Peripheral parts of two club cells (CC) can be observed.

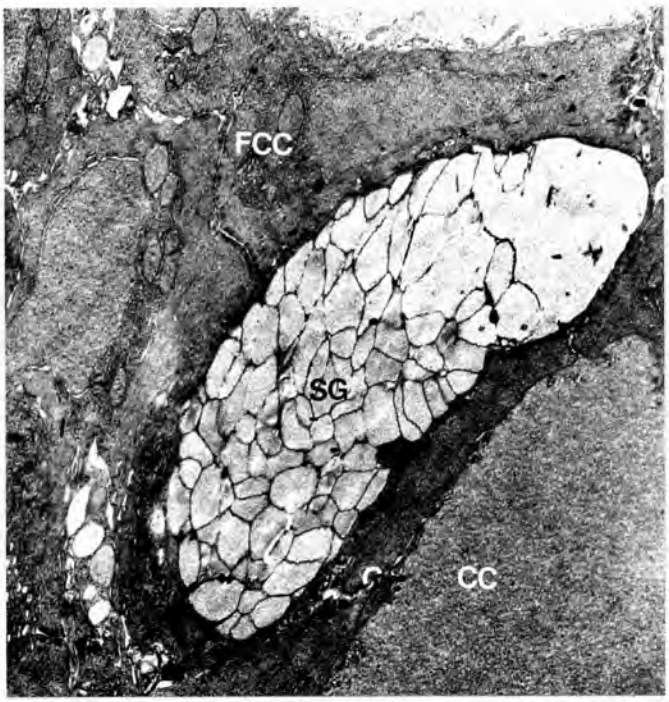
Note: the size of the filament-containing cells compared with the surrounding cells and their centrally located nucleus surrounded by cytoplasmic organelles.

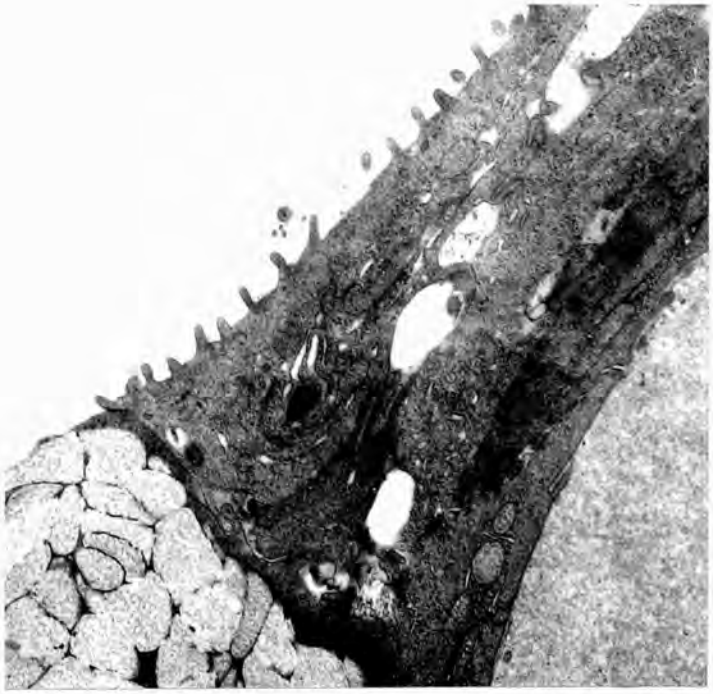
Mag. X 7,200.

Plate III-4

Illustrating a mucus cell which has reached the epidermal surface to release its contents.

Mag. X 10,400.


FCC
SG
CC



Mucus cells are filled with secretory granules of low electron density which appear to be filled with a fine textured filamentous material (Plate III-3 p. 84).

These cells provide mucosity to the epidermis and were often seen releasing their contents over the skin (Plate III-4 p. 84).

Club cells are characterized by their relatively large size, their centrally located nucleus and perinuclear organelles. The cytoplasm of these cells was found to be filled with fine fibrillar material. The chemical nature of their contents is not yet known. Von Frisch (1941) suggested their involvement in the fright reaction (Plate III-5 p. 86).

3.3.2. The dermis

The dermis was found to consist of masses of collagen bundles, different types of chromatophores and bundles of myelinated and non-myelinated axons (Plate III-6 p. 88).

3.3.2.1. Light microscopy

3.3.2.1.1. Melanophores

Light microscopic observations of longitudinal sections of the skin showed melanophores which appeared to be elongated cells with long processes and were found at varying depths beneath the basal lamella surrounded by dermal collagenous fibres.

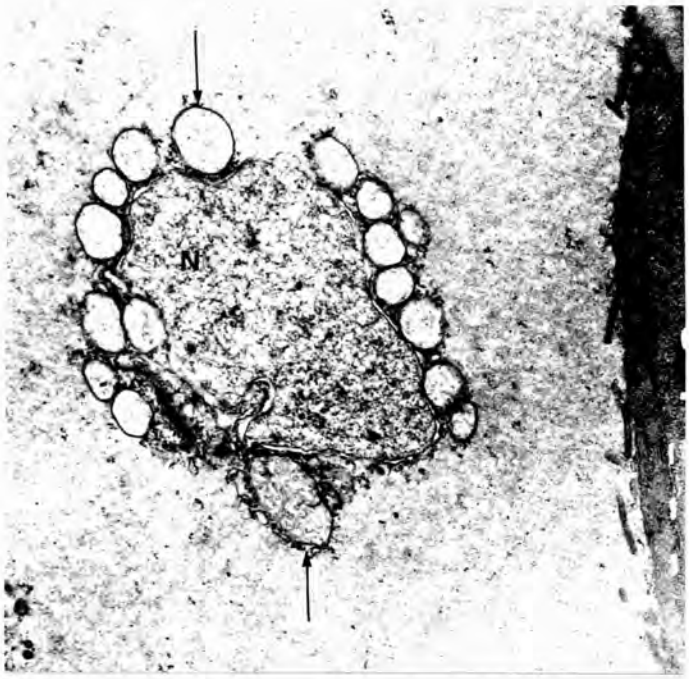
The epidermis is generally free from melanophores. Masses

Plate III-5

Central part of a club cell.

Note: the centrally located nucleus (N) having irregular shape, surrounded with perinuclear organelles (arrows) and the cytoplasm filled with fine fibrillar material.

Mag. X 10,800.


N

of melanin granules which occasionally appear in the epidermis may be clumps of pigment in the process of extrusion from the integument (Plate III-7 p. 89).

3.3.2.1.2. Xanthophores and erythrophores

Fixed preparations of xanthophores and erythrophores for light microscopy were not possible, probably because of the carotenoid solubility in xylene during the clearing procedure. However, these cells were observed microscopically in living, chromatically normal fish confined in the continuous observation tank (p. 71).

Xanthophores were found to be present throughout the skin. The erythrophores in the minnow are only present at the base of the fins and ventral skin, their observation being generally only possible when their pigment is dispersed by injecting the fish with an extract of whole plaice (*Pleuronectes*) or minnow (*Phoxinus*) pituitary. Plate III-8 page 90 is a colour micrograph of the base of the caudal fin to show different types of chromatophores.

3.3.2.1.3. Iridophores

Iridophores in whole-mount preparations using transmitted light were not easily distinguished. However, by using dark field microscopy, reflecting platelets were seen to be interspersed between melanophores (Plate III-9, 10 p. 91).

3.3.2.2. Electron microscopy

Plate III-6

Showing the dermis consists largely of masses of collagen bundles (CB). This electronmicrograph shows two types of chromatophores and bundles of myelinated and non-myelinated axons present in the dermis.

Note: the dermis is separated from the epidermis by a basal lamella (BL)

Mag. X 10,800.

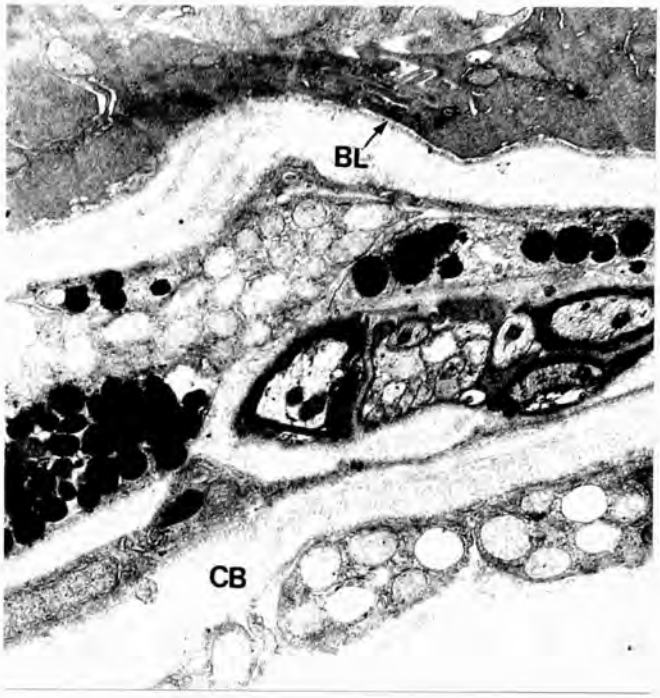

BL
CB

Plate III-7

A photomicrograph of a longitudinal section through the skin of *Phoxinus phoxinus* showing the general structure of the integument.

(MC) - mucus cell

(MEL) - melanophore

(SC) - scale

(BL) - basal lamella

Mag. X 1200

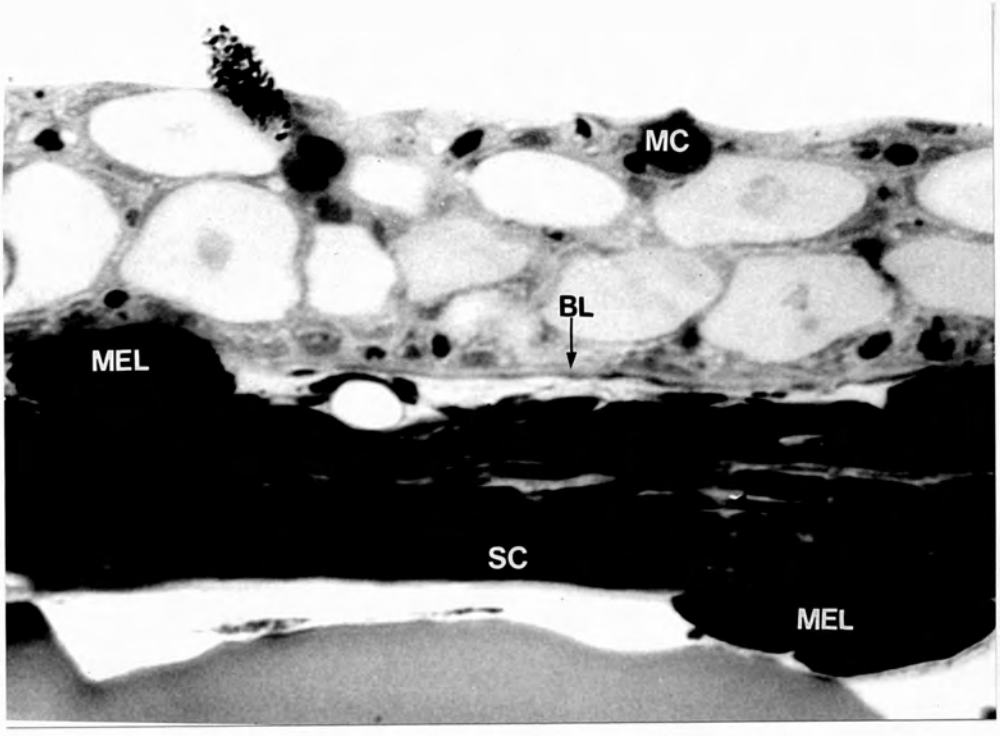

MC
BL
MEL
SC
MEL

Plate III-8

This colour photomicrograph is of a living fish to show different types of chromatophores of the minnow *Phoxinus phoxinus*.

(MEL) - melanophores; (XAN) - xanthophores; (ERY) - erythrophores.

Mag. X 20.

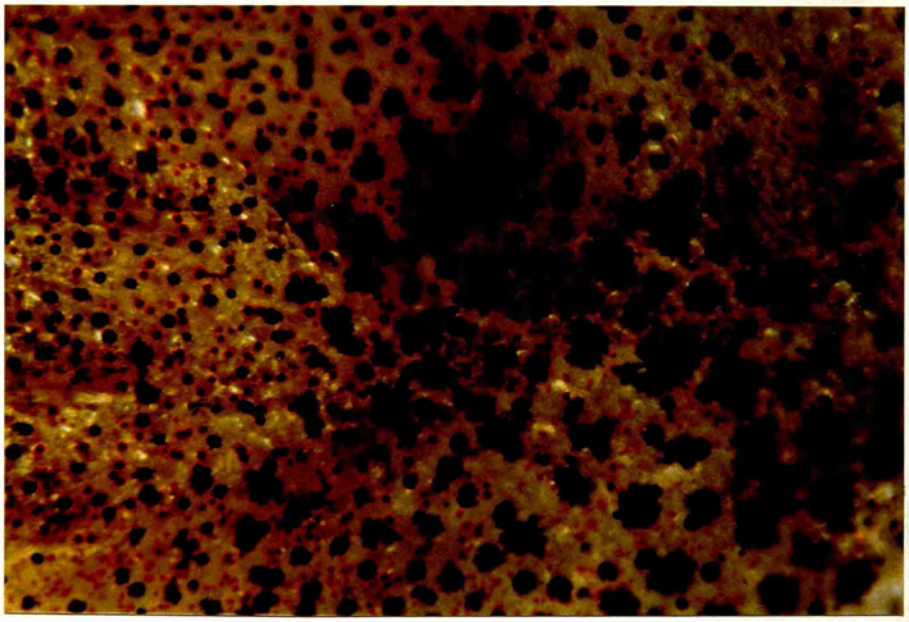


Plate III-9

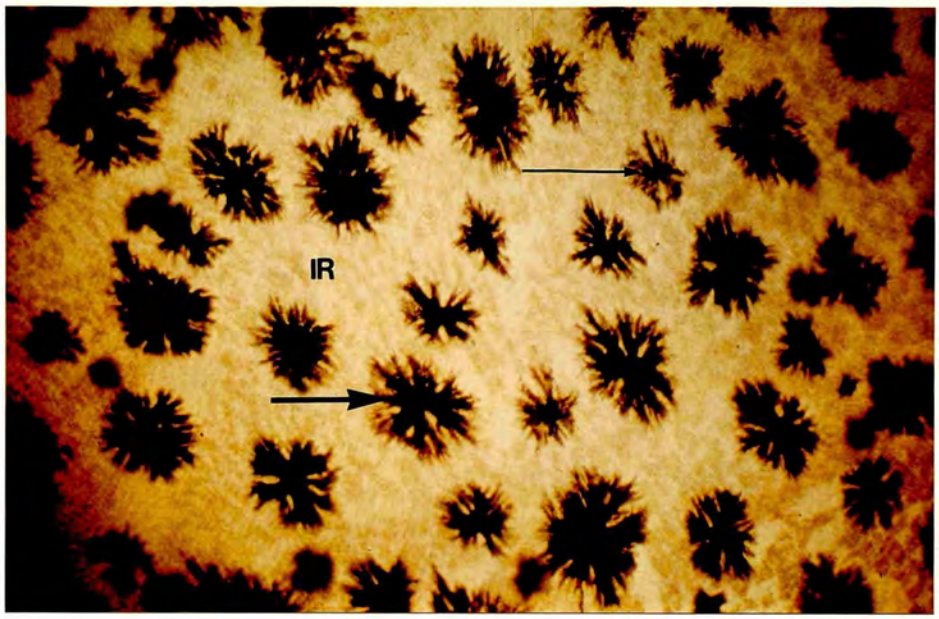
A colour photomicrograph of a fixed whole-mount preparation. (IR) - iridophores are seen interspaced between melanophores.

Mag. X 120.

Plate III-10

This is another photomicrograph from the same area as above, but taken under dark-field microscopy. Guanine platelets (GP) are well demonstrated. The same sized arrows show the corresponding melanophores in the above figure.

Mag. X 120.


IR


GP

3.3.2.2.1. Melanophores

Electron microscope studies confirmed the light microscope observation, that the epidermis is free from melanophores. Melanophores were found to occupy an area from just beneath the basal lamina of the epidermis to deep in the dermis. They have a single plasma membrane, the cytoplasm inside being filled with numerous spherical highly electron-dense melanin granules. These granules are surrounded by a membrane and measure about 0.45 μ in diameter. Besides the melanin granules, the cytoplasm contains smooth endoplasmic reticulum, numerous mitochondria of various shapes, micropinocytotic vesicles some of which seem to be fused with the plasma membrane, a granular fibrous nucleus with prominent nucleoli, bundles of microtubules and some microfilaments, the latter being more numerous in the cell processes (Plates III-11, 12, 13 pp. 93, 94).

3.3.2.2.1.1. Organization of microtubules in relation to the cell structure and the pigment granules

Microtubules in the melanophores of *Phoxinus phoxinus* were found to be 22 nm (220 Å) in diameter and were observed to run parallel to each other in the cell centrosphere and the cell processes. These microtubules were found to have a very close association with the pigment granules and seemed to provide reasonably well defined channels for the pigment granules during their centripetal and centrifugal movements (Plate III-14, 15 p. 95).

In the aggregated state of the pigment granules the cytoplasm of the cell centrosphere as well as of the processes was found to

Plate III-11

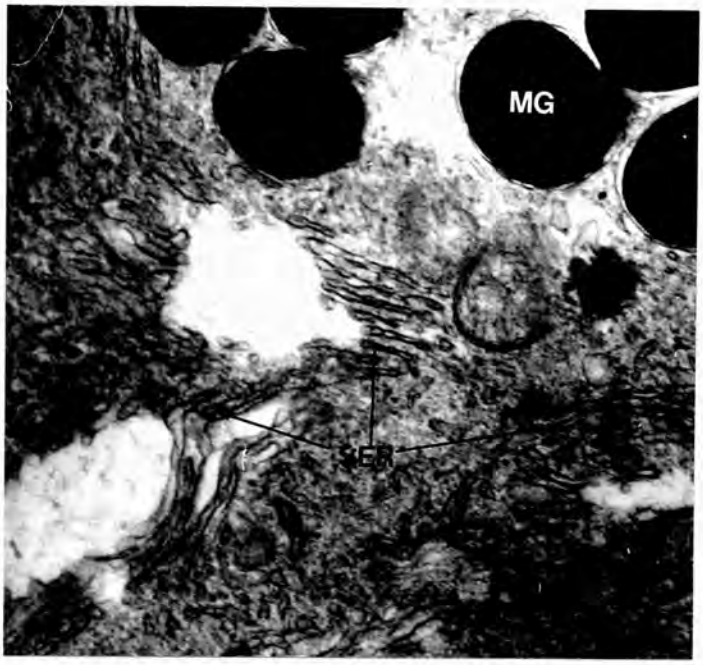
Electronmicrograph of a melanophore with aggregated melanin granules showing the presence of smooth endoplasmic reticulum (SER) and membrane-bounded melanin granules (MG).

Mag. X 45,000.

Plate III-12

Electronmicrograph of a process in a melanophore free of melanin granules showing mitochondria (MIT) cut in the plane of their long axis.

Mag. X 45,000.


MG
SER

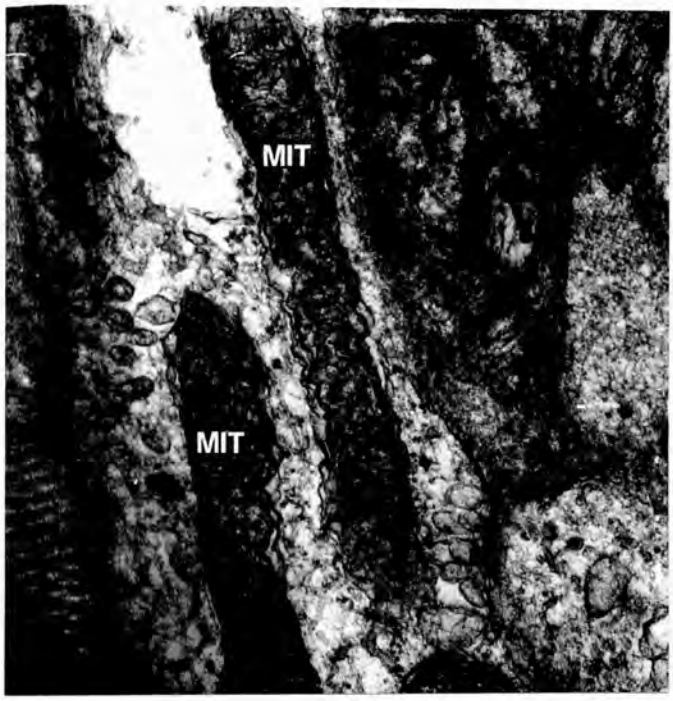

MIT
MIT

Plate III-13

Electronmicrograph of a process in a melanophore cut longitudinally and free of melanin granules to show microtubules (MT) and microfilaments (MF).

Note: micropinocytotic vesicles fused with the plasma membrane (MV).

Mag. X 72,000.


MV
MF
MT

Plate III-14

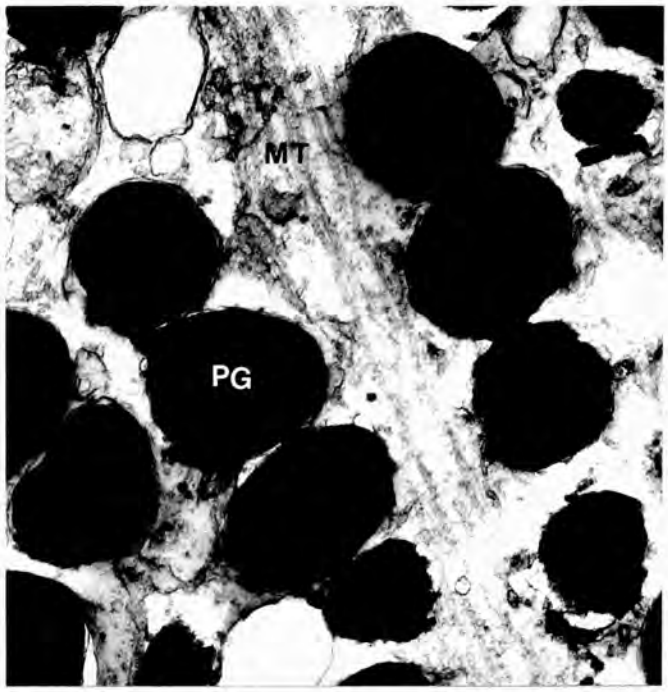
Longitudinal section through the centre of a melanophore showing pigment granules (PG) in linear columns, having a close association with bundles of microtubules (MT).

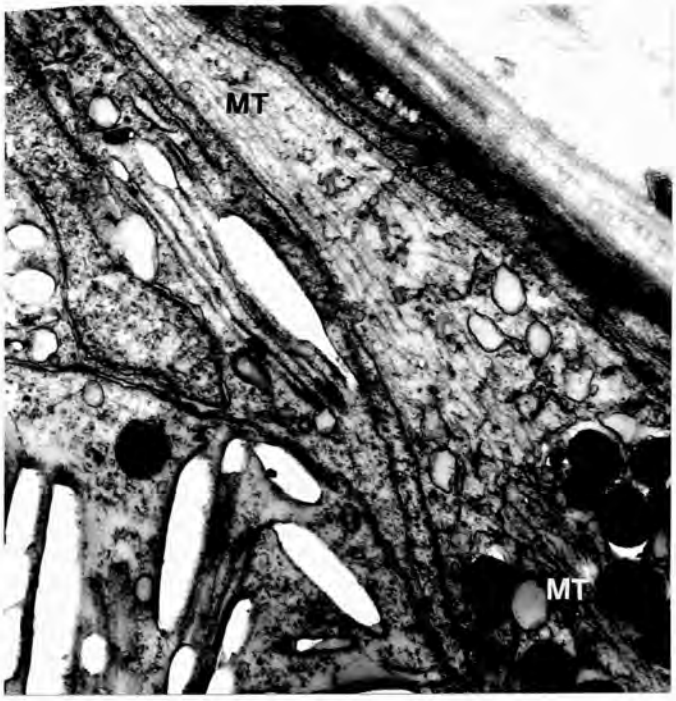
Mag. X 45,000.

Plate III-15

This is a lower magnification of a cell process belonging to the same melanophore as that above. This shows microtubules (MT) running along the entire area from the cell centre to the cell process.

Mag. X 18,000.


MT
PG


MT
MT

be densely packed with microtubules and showed no sign of depolymerisation.

3.3.2.2.1.2. Change in cell shape during pigment migration

Although the size and shape of the melanophores are variable, generally speaking big differences were observed between the shape of the melanophores in which the pigment granules were completely dispersed and the shape of the melanophores in which pigment granules were completely aggregated. In melanophores with their pigment granules in a state of complete dispersion the centrosphere appeared flat and the nucleus was found to be central and elongated. The processes and the cell body showed fairly uniform diameters (Plate III-16, 17 p. 97). Melanophores with their pigment granules fully aggregated were found to have large hemispherical centrospheres and a nucleus usually located at the cell periphery at the base of a process (Plate III-18 p. 99). The processes of melanophores in which the pigment granules were fully aggregated were found to have collapsed as a result of the withdrawal of the granules from them. However, they showed a fairly uniform diameter throughout their length.

3.3.2.2.1.3. General observations on melanophore innervation and associated vesicles

Observations with the electron microscope revealed the presence of unmyelinated nerve axons in the vicinity of the melanophores. The axons were found to contain microtubules, microfilaments and a few mitochondria. These axons were enveloped by another

Plate III-16

Electronmicrograph of a melanophore with dispersed pigment granules, nucleus (N).

Note: the flat appearance of the centrosphere.

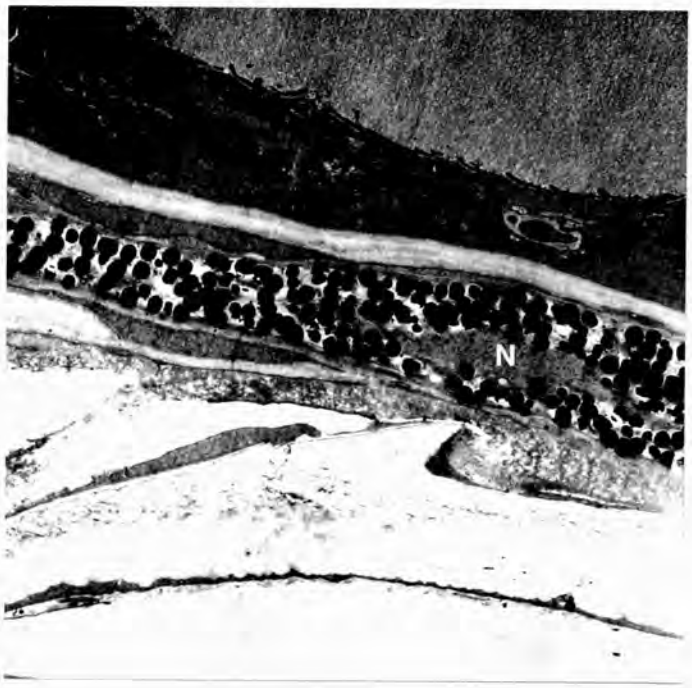
Mag. X 5,250.

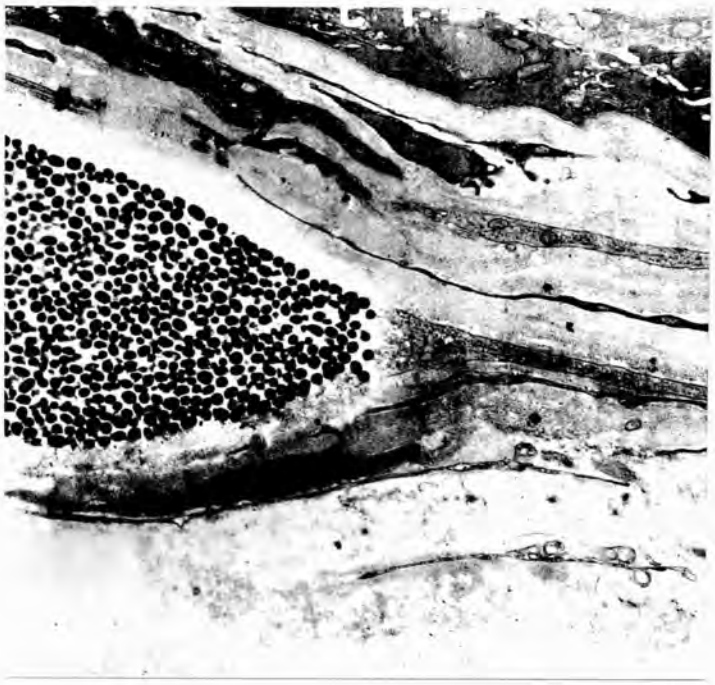
Plate III-17

Electronmicrograph of a melanophore with aggregated pigment granules.

Note: the hemispherical appearance of the centrosphere and fairly uniform diameter of the process.

Mag. X 5,250.


N



connective tissue sheet which appeared to be composed of collagen fibres of endoneurium. The unmyelinated axons observed are probably derived from nerve trunks composed of myelinated and unmyelinated nerve fibres usually found in the dermis in the proximity of the melanophores (Plate III-19 p. 100).

The nerve endings, which are probably varicosities (Schliwa, 1976), measured about 0.6 μ in diameter and were found usually invaginating the melanophores. Several of these varicosities were found to supply a single melanophore both at the process and at the cell body. These nerve endings were identified by their presynaptic specialization, that is, the presence of synaptic vesicles and mitochondria. Some tubular structures were also found among the presynaptic elements (Plate III-20 p. 100). Two populations of vesicles were observed as far as size is concerned, small vesicles measuring 33 nm (330 Å) in diameter and large vesicles measuring about 100 nm (1000 Å) in diameter. Based on their proportions, the nerve endings can be divided into two types:

a - Nerve endings in which the majority of the vesicles are of small size with few larger ones;

b - Nerve endings in which the majority of the vesicles are of larger size with few small ones (Plates III-20, 21 pp. 100, 102).

Despite the fact that no appropriate fixative was used to preserve the dense cores usually present in the vesicles of adrenergic nerve endings (Richardson, 1966; Geffen and Livett, 1971), it was interesting to note that some of the vesicles of both populations

Plate III-18

Longitudinal section through a basal part of a process showing that, through the aggregation of granules to the centrosphere, the nucleus has been pushed to the periphery and takes the shape of the peripheral part of the cell body and the process. (N) - nucleus; (NO) - nucleolus; (CP) - cell process; (PG) - pigment granules.

Mag. X 10,800.

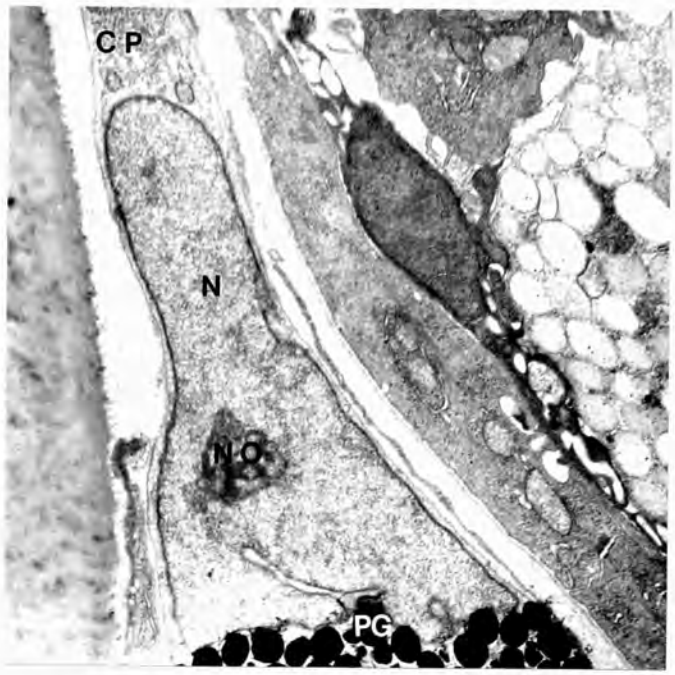

CP
N
NO
PG

Plate III-19

Nerve axons, surrounded by collagen fibres of the endoneurium (EN) and perineural lamella (PL). (SH) - Schwann cell process; (A) - axons.

Note: their proximity to a collapsed process of a melanophore, showing fairly uniform diameter with parallel bundles of microtubules. The second process with membrane bound granules belongs to a xanthophore (XAN).

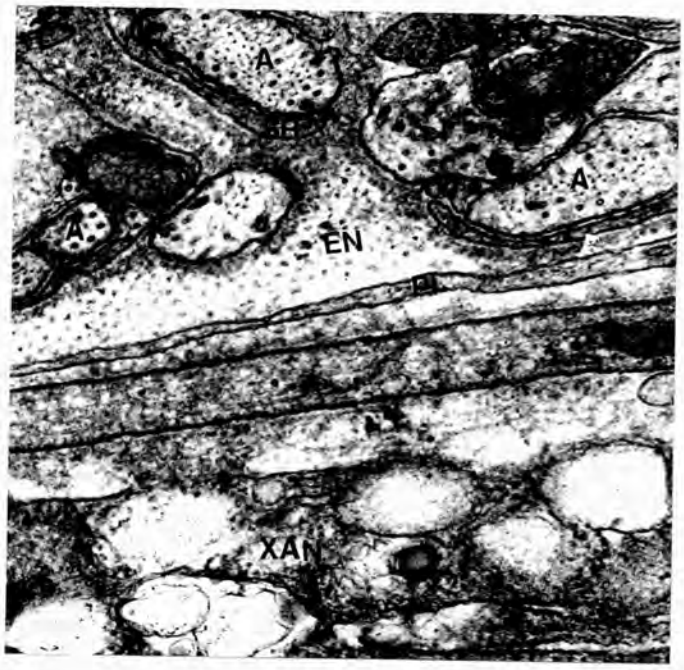
Mag. X 45,000.

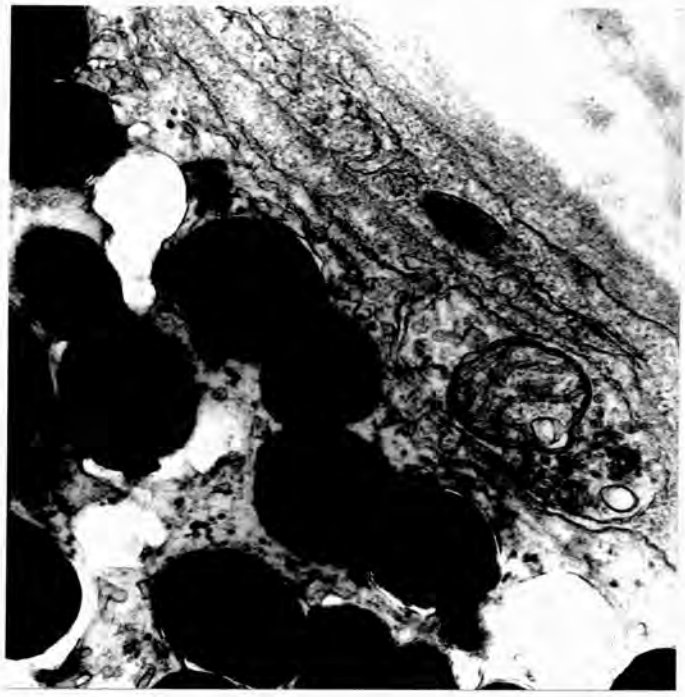
Plate III-20

Showing a nerve-ending invaginating the cell body of a melanophore containing electron-dense vesicles the majority of which are of the small type.

Note: some large granular and agranular vesicles can also be seen.

Mag. X 52,500.


A
A
A
EN
XAN



appeared to be granular. Occasionally axon profiles were observed in the proximity of the melanophores totally containing micro-tubules (Plate III-22 p. 103). Similar observations have been made in mammals and considered to be an area between two varicosities (Gabella, 1976). The distance between the nerve-endings and the melanophores in the areas of intimate contact ranged from 15 - 20 nm (150 - 200 Å).

No definite membrane specialisation (electron-dense material) at the pre- and postsynaptic membranes was observed at the contact points of the nerve-endings of the melanophores.

3.3.2.2.1.4. The histochemical nature of the vesicles

5-Hydroxydopamine, a 'false' sympathetic transmitter is known to be a specific marker for adrenergic nerve-endings in mammals (see pp. 76, 77).

Plate III-23 page 104 shows three nerve-endings making intimate contact with a process of a melanophore. One of these endings is of the type in which the large vesicles constitute the majority of the vesicles present. This plate clearly demonstrates that both populations of the vesicles have interacted with 5-hydroxydopamine equally and appear highly osmiophilic, indicating that the vesicles in both types of nerve endings probably have the same chemical nature.

The tubular structures in the nerve-endings which were described earlier on page 98 also showed a dense precipitate

Plate III-21

A nerve-ending containing larger electron-dense vesicles.

Mag. X 45,000.

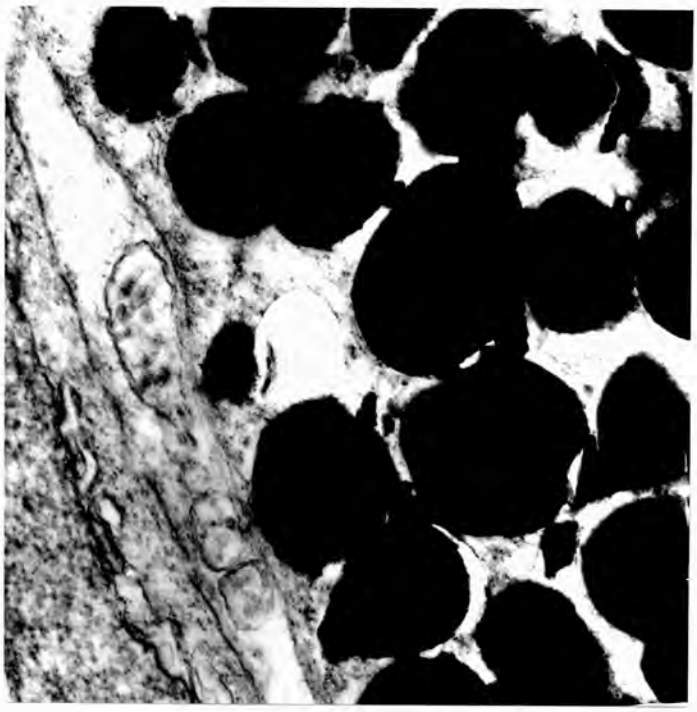


Plate III-22

A nerve profile in the proximity of a melanophore containing numerous microtubules.

Mag. X 72,000.

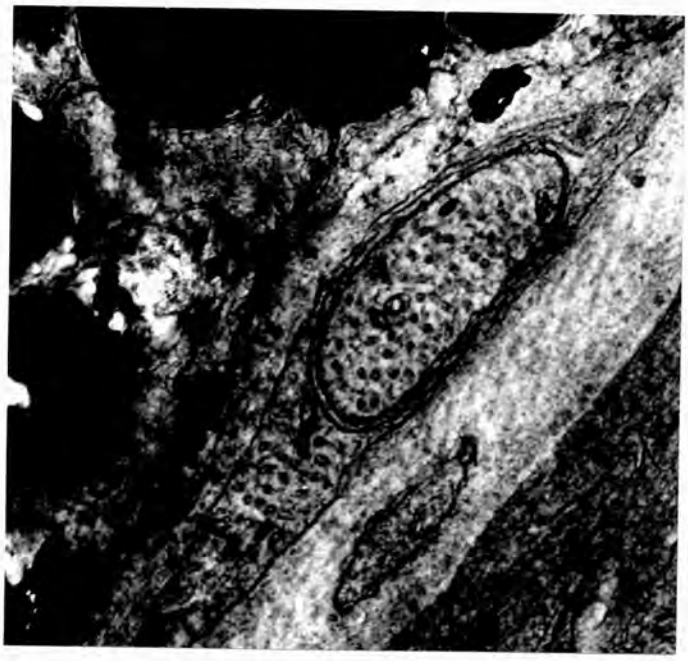


Plate III-23

This shows three nerve endings in a fish treated with 5-HDA. The endings are in close contact with a process of a melanophore in which the pigment granules are aggregated to the centrosphere of the cell.

Note: the synaptic vesicles (V) are highly filled with osmiophilic material. (MP) - melanophore process; (MIT) - mitochondria; (MT) - microtubules.

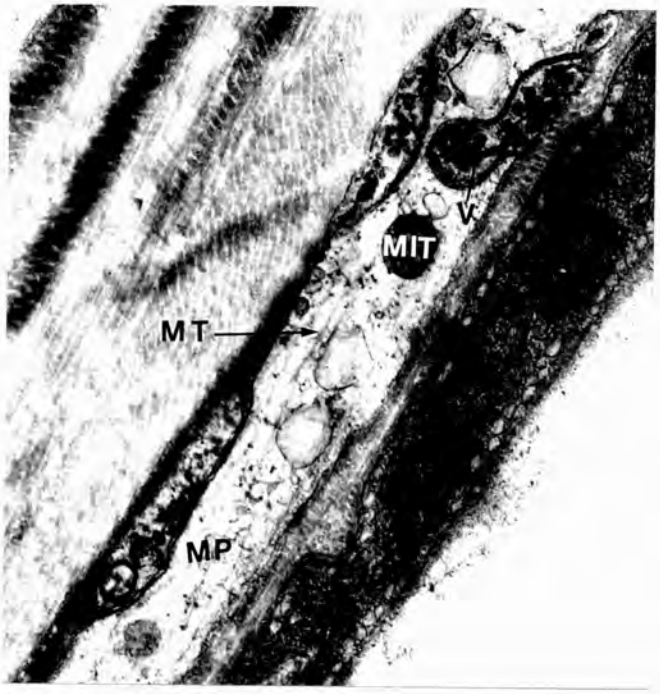
Mag. X 27,000.

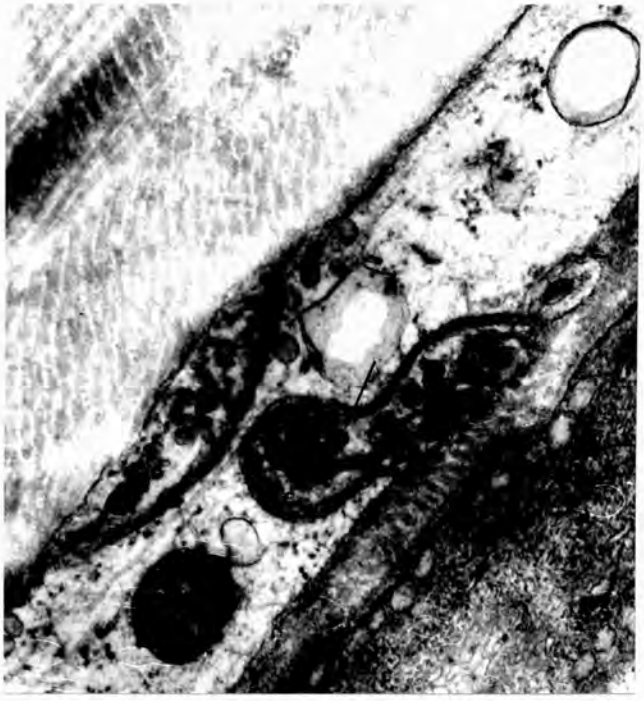
Plate III-24

A higher magnification of two of the above nerve terminals.

Note : the synaptic vesicles' limiting membrane (arrow) and the tubular structure which appeared highly osmiophilic as well.

Mag. X 45,000.


V
MIT
MT
MP



(Plate III-24 p. 104). Similar observations were made by Tranzer (1972) on mammalian adrenergic nerve endings and were thought by him to be extra-vesicular compartments for catecholamine.

3.3.2.2.2. Iridophores

Iridophores are restricted to the dermis and they lie between dermal melanophores and xanthophores. Their cytoplasm is totally occupied by groups of parallel lacunae measuring 2500 - 3000 nm in length and approximately 90 nm thick. These groups of parallel lacunae usually are seen to have varying angles to each other (Plate III-25 p. 106). The lacunae are surrounded by a double limiting membrane and are filled with electron-lucent material, which usually shows empty spaces (Plate III-25 p. 106). Examination of thick sections reveals the presence of dense inclusions within the lacunae. These dense inclusions are probably guanine platelets which are not retained in thin sections (Plate III-26 p. 106).

Processes of iridophores are often seen in very close contact with neighbouring melanophores and iridophores but no desmosomes have been found between them (Plate III-27 p. 107). The nuclei of these cells are elongated in shape with a prominent nucleolus (Plate III-28 p. 107).

Other elements present in the cytoplasm of these cells are numerous mitochondria, granular endoplasmic reticulum and free ribosomes (Plate III-25 p. 106).

3.3.2.2.3. Xanthophores

Plate III-25

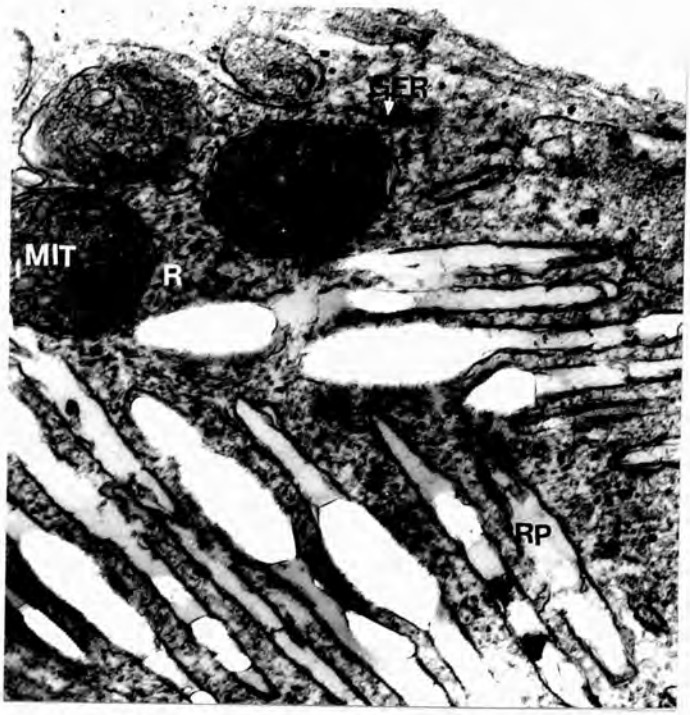
Electronmicrograph of part of an iridophore showing two groups of reflecting platelets (RP). The platelets are regularly arranged in groups, but the groups are not at the same angle. (MIT) - mitochondria; (GER) - granular endoplasmic reticulum; (R) - ribosomes.

Mag. X 45,000.

Plate III-26

Electronmicrograph of a thick section to show the dense inclusions within the lacunae of iridophores.

Mag. X 7,200.


SER
MIT
R
RP

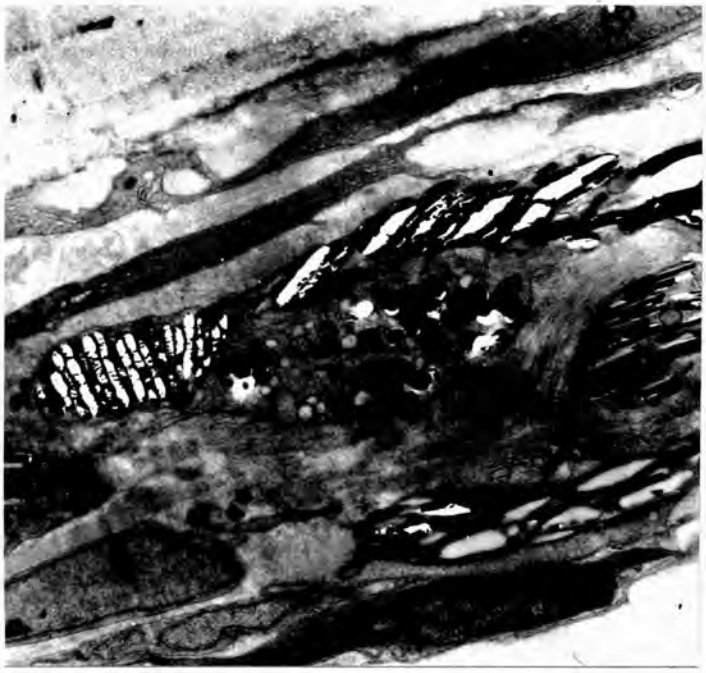


Plate III-27

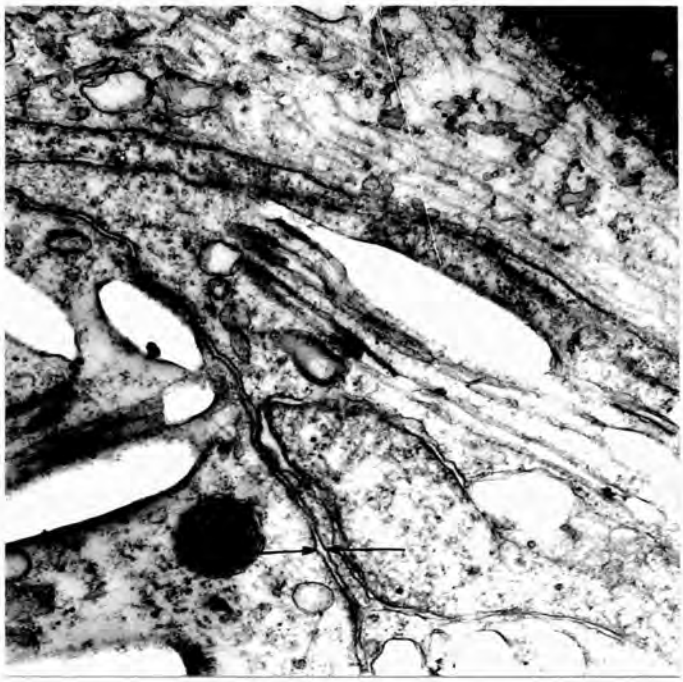
This shows adjacent iridophores, only separated by a very narrow intracellular space (arrow).

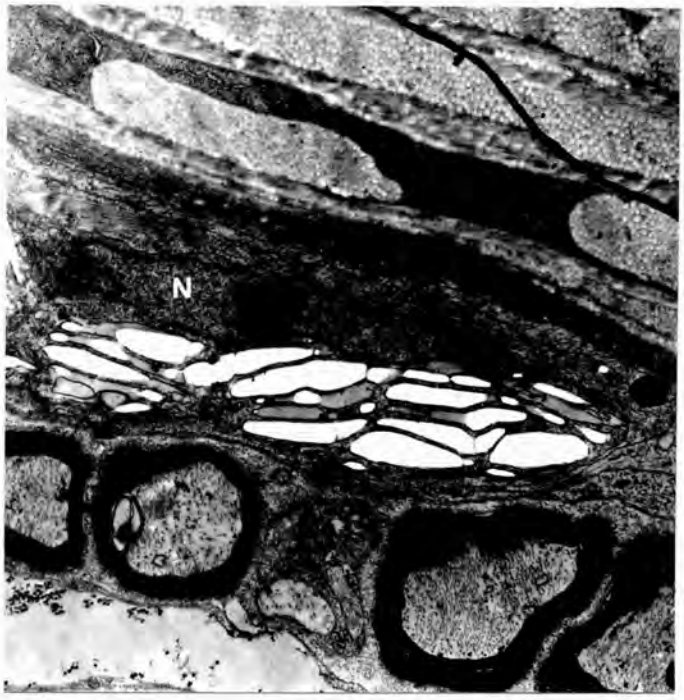
Mag. X 27,000.

Plate III-28

Showing elongated shaped nucleus (N) with a prominent nucleolus in an iridophore.

Mag. X 10,800.




N

Xanthophores are present in both the dermis and the epidermis. These cells were found to show very fine granulations which occupied the central region of the cell. The nuclei have an irregular shape with a very well defined double membrane (plate III-29 p. 109). Most of the cell cytoplasm is occupied by round-shaped membrane bound vesicles of low electron density measuring about .5 μ in diameter. These vesicles are considered to be pterinosomes and probably contain sepiapterin (Yasutomi and Hama, 1971).

3.3.2.2.4. Erythrophores

Erythrophores in the minnow *Phoxinus*, as was mentioned earlier (p. 87), are only present at the base of the fins and ventral side of the fish. To facilitate their visualisation their pigment was dispersed by pre-treating the fish with a dose of plaice (*Pleuronectes*) pituitary extract.

The dermis from such a specimen as seen under the electron microscope was found to possess two groups of cells both containing two different electron-dense pigment droplets as far as the size is concerned. The term pigment droplets and not pigment granules is employed because they were found to lack a limiting membrane. In one group of these cells only, membrane-bounded electron-lucent granules similar to those present in xanthophores were observed. (Plate III-30 p. 109). These membrane-bounded granules are probably pterinosomes and the cell containing them is likely to be an erythrophore. The nature of the cell containing solely pigment droplets is not clear.

Plate III-29

Low magnification, electron micrograph showing the presence of xanthophore (XAN) in the epidermis. (PT) - pterinosomes; (FG) - fine granules at the centre; (N) - nucleus; (NO) - nucleolus.

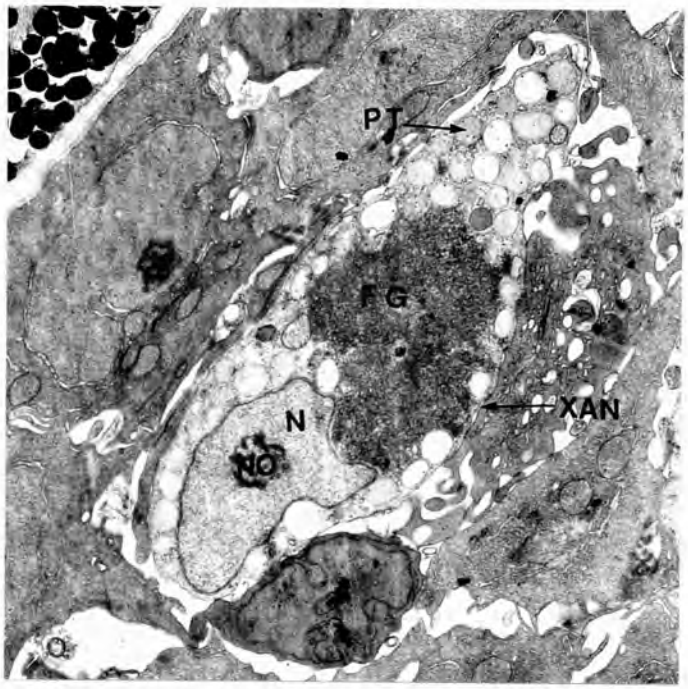
Note: The well-defined double membrane of the nucleus.

MAG. X 7,200.

Plate III-30

This is an electron micrograph of the ventral skin showing adjacent branches of probably an erythrophore (ERY) and of an unidentified cell. (PT) - pterinosome; (PD) - pigment droplet; (MT) - microtubules; (MF) - microfilaments.

MAG X 45,000.


PT
FG
XAN
N
NO

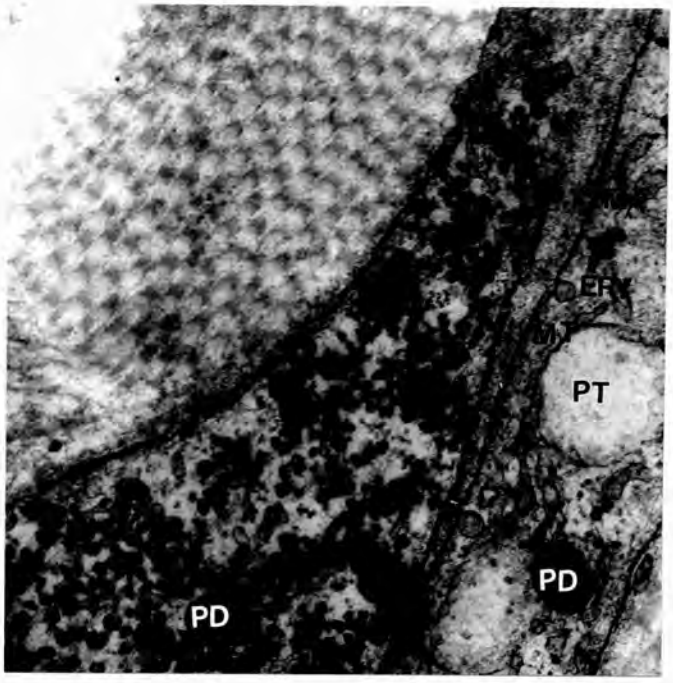
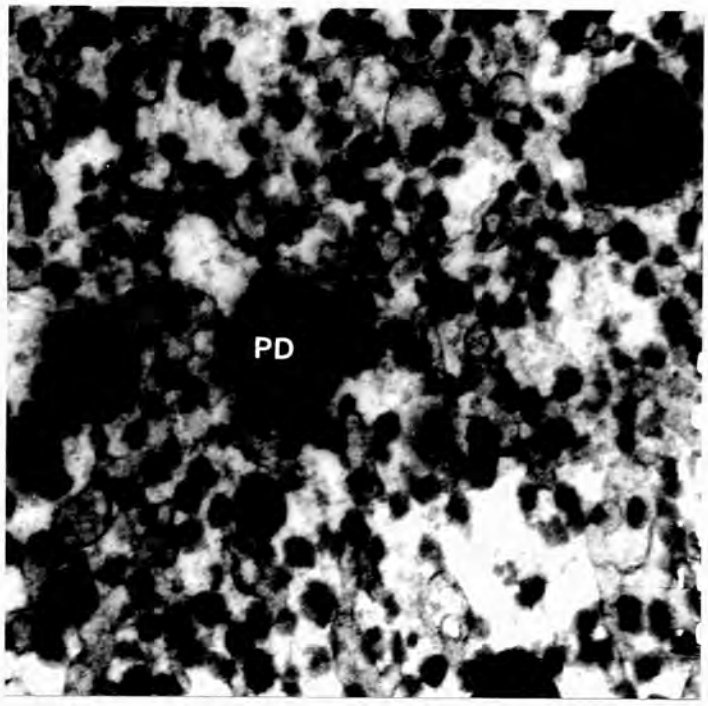

ER
PT
PD
PD

Plate III-31a, b

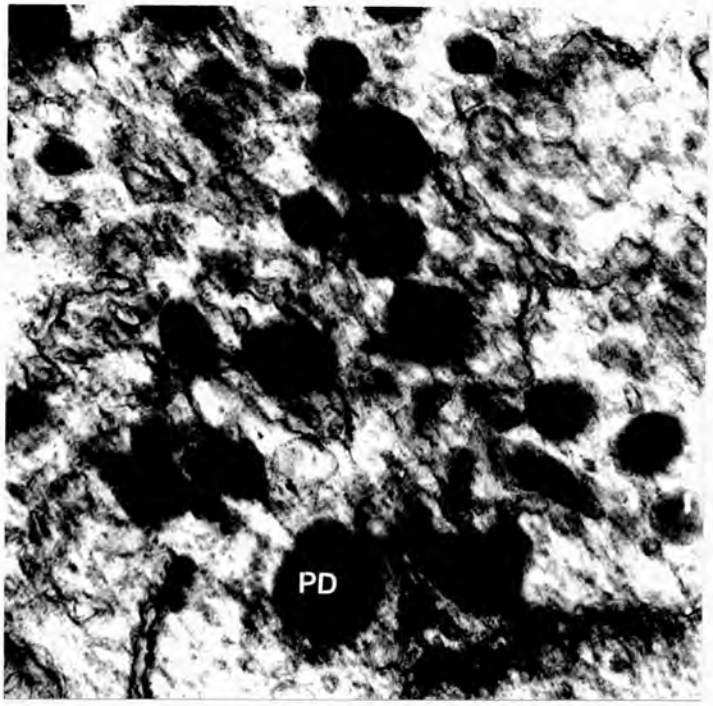
Showing the higher electron-density of the larger pigment droplets (PD) in fish treated with pituitary extract (a) and their lower electron-density in untreated control fish (b).

Note : the absence of the smaller droplets in the cell of the untreated fish.

Mag. X 72,000.



a



b

Specimens from the same area of untreated fish revealed the same basic structure as in the treated ones, excepting that the larger pigment droplets were less electron-dense and the smaller ones were absent (Plate III-31a, b p. 110). Similar droplet structures have been reported by Hama and Hasegawa (1967) and were considered to be the possible compartments for xanthophylls.

3.4. Discussion

The epidermis in vertebrates is a stratified epithelium and its main function is to protect the organism from its environment and to preserve the osmotic constancy. To fulfil the above functions two developmental pathways are observed, depending on the environmental adaptation. Animals such as mammals and, to a lesser extent, amphibians are invested with protective keratinizing epithelium in relation to their relatively dry environment. Teleosts, on the other hand are invested with a non-keratinizing epithelium which contains a variety of unicellular glands.

Results obtained in the present investigation confirm the general topography of the epidermis reported by Whitear (1970) in the minnow *Phoxinus* and in other teleosts by Herikson and Matoltsy (1968), Whitear (1970), Roberts *et al.* (1972), Lanzing and Wright (1974) and Whitear (1977). The functional interpretation of the fine structure of the epidermal cells is beyond the scope of the present thesis and will not be discussed.

3.4.1. The melanophores

The epidermis in the minnow *Phoxinus* seems to be free of melanophores and the different focusing requirements during the whole-mount observations are probably due to the depth of their position in the dermis. Falk and Rhodin (1957) reported that the melanophores of *Lebistes reticulatus* have two cell membranes, dividing the cell into two regions. The inner region contains the nucleus, melanin granules and other cytoplasmic organelles and the outer region contains filaments. These observations were first challenged by Fujii (1966b) working on the same fish. Fujii reported that the melanophores had the usual single plasma membrane. The chromatophores, including the melanophores in the minnow *Phoxinus*, are invested by a single plasma membrane and this condition seems to be universal, applying to melanophores generally. Therefore, one may justifiably discount the report by Falk and Rhodin (1957) and assume, as suggested by Fujii (1966b), that these authors misinterpreted their electron micrographs.

3.4.2. Microtubules and the mechanism of pigment movement in melanophores

Microtubules are well known organelles existing in almost all types of cell. Their function ranges from maintenance of cellular structure to the mediation of intracellular movement of many particles and the motility of cilia and flagella (Bardele, 1973; Roberts, 1974).

The present investigation shows that the microtubules in the minnow, *Phoxinus*, run parellel to the longitudinal axis of the cell processes. The alignment of melanin granules and their very close

parallel arrangement to the microtubules indicates that microtubules in the melanophores, in some way or other, are involved in the translocation of pigment granules. Since the observations by Bikle *et al.* (1966) of microtubules in the melanophores of *Fundulus heteroclitus*, the same relations have been reported in the chromatophores of different species of fish (Green, 1968; Fujii and Novales, 1969; Egner, 1971; Wikswo and Novales, 1972; Schliwa and Bereiter-Hahn, 1973). On the basis of the similar observations made in the present study, the suggestion made by Porter (1973) that pigment granule movement is associated with the depolymerization and repolymerization of microtubules during pigment aggregation and dispersion respectively, seems unlikely to be correct. In the present work the microtubules were found not to depolymerize and remained extended from the cell centre to the processes in melanophores in which the pigment granules were completely aggregated by adrenaline. The above observation is consistent with the observations reported by Murphy and Tilney (1974) working on *Fundulus heteroclitus*.

The change in the cell shape from discoidal (melanin granules dispersed) to hemispherical (melanin granules aggregated) confirms the previous observations made by others on different fishes (Obika, 1976; Porter, 1973; Schliwa and Bereiter-Hahn, 1973). The above change might have a great significance in the mechanism of pigment granule movements if it were not merely due to the passage of the granules from and to the cell processes.

Finally, based on the morphological evidence obtained as a result of the present investigation and similar investigations by

other workers together with the results of experiments with colchicine, vinblastine, low temperature and high hydrostatic pressure, (Malivista, 1965, 1971; Junqueira and Porter, 1969; Murphy and Tilney, 1974), the participation of microtubules in the mechanism of pigment movement is confirmed. Whether the microtubules merely provide a defined, physical channel, or whether they are actively involved in the above mechanism, is not clear. The close association of the linearly arranged pigment granules and the microtubules indicates a possible affinity between the granules and the microtubules. This led Murphy and Tilney (1974) to speculate on the existence of 'cross-bridges', probably ATPase involved in the sliding of the pigment granules along a fixed array of microtubules. However, neither their electron micrographs nor the electron micrographs from the present study show any 'arms' or'bridges' extending between granules and microtubules. On the other hand, Byers and Porter (1976) working on the erythrophores of *Holocentrus ascensionis* and Schliwa and Enteneur (1978) working on *Pterophyllum scalare* melanophores, studied the three-dimensional organisation of the plasmic matrix and concluded that it consists of a system of fine fibrillar strands (microtrabeculae) connecting microtubules, pigment granules and the plasma membrane of chromatophores in the state of pigment dispersion. During pigment aggregation these microtrabeculae withdraw, allowing the granules to aggregate to the cell centre along the fixed arrays of microtubules.

In the present thesis the three-dimensional organisation of the melanophores has not been studied and the subject remains to

be investigated.

3.4.3. Melanophore innervation

As early as 1893 Ballowitz, using conventional light microscopy with silver staining techniques, was able to demonstrate a network of nerve fibres associated with teleost melanophores. This observation was confirmed by Wyman (1924). However, Whitear (1952) reported that she was unable to identify with certainty melanophore motor innervation in the minnow *Phoxinus*. Ahmad (1970) explained Whitear's inability as being probably due to the highly refractory nature of the nerve fibres in the minnow to histological procedures. From the results obtained in the present investigation, the direct innervation of melanophores in the minnow *Phoxinus* is confirmed at the ultrastructural level.

In the present work, the relationship between nerve profiles and melanophores was not studied in serial sections. Therefore, the term "nerve-endings" is employed to define the portion of the nerve in the area of the intimate contact with melanophores showing presynaptic elements. The term "nerve-endings" does not necessarily mean the actual nerve termination. According to Jacobowitz and Laties (1968) and Falck *et al.* (1969) the fluorescent specimens of the nerve fibres associated with melanophores showed a series of bright varicosities, indicating that contacts with melanophores are by means of varicose nerve fibres. Fujii and Taguchi (1970) were not able to confirm the above relationship at the ultrastructural level in the teleost *Lebistes reticulatus*. However, Schliwa (1976)

clearly demonstrated by means of serial ultra thin sections that the innervation of melanophores in the angelfish *Pterophyllum scalare* is by means of nerve fibre varicosities.

The only criterion so far available for distinguishing adrenergic endings from other types of endings in electron microscopy, is the presence in the former of granular synaptic vesicles of about 30 - 60 nm in diameter, constituting the majority of the vesicle population at nerve-endings (Geffen and Livett, 1971). The results obtained in the present investigation are strongly indicative of adrenergic innervation of melanophores in *Phoxinus*. Similar innervation has been suggested in other teleosts (Jacobowitz and Laties, 1968 in *Tautogalabrus adspersus*; Falck *et al.*, 1969 in *Salmo gairbneri)* by experiments using catecholamine histochemical fluorescent methods and by ultrastructural studies (Schliwa, 1976 in *Pterophyllum scalare*).

The conclusion of Bikle *et al.* (1966) that their electron micrographs demonstrated dual innervation of the melanophores in *Fundulus* is not justified since they were merely based on observations that several nerve-endings were found to be associated with a single melanophore. The association of several nerve-endings with a single effector does not necessarily indicate a functional difference. Fujii (1969) and Fujii and Novales (1969b) claimed to have provided ultrastructural evidence in favour of cholinergic and adrenergic innervation of melanophores in the guppy *Lebistes*. They based their argument on the electron-lucent and electron-dense properties and the size of the synaptic vesicles within the same nerve-endings.

They associated the small electron-lucent vesicles (50 nm diameter) with the storage sites for acetylcholine and the large electron-dense (100 nm diameter) vesicles with those for catecholamine. On the basis of the well established result in mammals the above argument cannot be considered to be valid. In mammals it has been proved by means of histochemical and radioautographic methods that cholinergic nerve endings exclusively contain electron-lucent vesicles while adrenergic endings contain granular and agranular vesicles. The appearance of granular and agranular vesicles in adrenergic nerve endings depends on the method of fixation and the level of NA stored (Geffen and Livett, 1971). Moreover, the arguments of Fujii (1969) and of Fujii and Novales (1969b) are challenged by the results obtained in the present investigation. The positive interaction of all the vesicles, regardless of their size and their electron appearance, with 5-hydroxydopamine strongly indicates that they have the same chemical nature. Furthermore, positive responses of vesicles in the different nerve endings found to be associated with the same melanophore indicate that these nerve endings are the same and strongly suggest that they are adrenergic. One may therefore conclude that there is a direct adrenergic innervation of melanophores (if not the only innervation) in the minnow *Phoxinus*. Further consideration of the results obtained will be discussed in Chapter V.

3.4.4. Other chromatophores

Carotenoids and pteridines in xanthophores and erythrophores are responsible for the yellow and red appearance of these cells

respectively. Hama *et al.* (1963) introduced the term petrinosomes to designate the granules which contain pteridine.

Pterinosomes in xanthophores and erythrophores in *Phoxinus* were found to have very low electron density and either no inner structure or few particulate inclusions. This type of pterinosome has been considered to be the onset of pterinosome development (Kamei-Takeuchi and Hama, 1971; Takeuchi, 1975).

The significance of the pigment droplets in the erythrophores and their abundant appearance in the cells usually found to be adjacent to erythrophores is not clear and remains to be investigated.

It appears from the electron micrographs that the first stage of development of electron-dense inclusions in iridophores is the formation of a narrow membrane bounded elongated bodies (lacunae). Then, these bodies seem to develop an inner membrane bounded by electron-lucent substance in which, gradually, the electron-dense inclusion will be deposited. A similar explanation to the above has been suggested by Lanzing and Wright (1974) working on *Tilapia massambica*. The inclusions are assumed to be crystals of guanine (Kawaquti and Kamishima, 1966a; Setoguti, 1967; Harris and Hunt, 1973). Since guanine has been detected from extracted skin of fish (Hitchings and Falco, 1944; Lee *et al.*, 1969; Taylor, 1969), the above assumption is justified.

The absence of the electron-dense inclusions from most of the ultra thin sections is probably due to their loss during the sectioning procedure, as was suggested by Setoguti (1967).

Although occasional myelinated and unmyelinated nerve fibres were found in the proximity of iridophores, xanthophores and erythrophores, no actual nerve-endings were found to be associated with these pigment cells.

CHAPTER IV

RESPONSES TO ILLUMINATED BACKGROUNDS OF MELANOPHORES AFFECTED BY SPINAL NERVE SECTION

4.1. Introduction

In the general introduction and survey of literature (pp. 35-39) it was pointed out that the results of experiments on caudal band melanophores of several teleosts have been interpreted as indicating an active pigment dispersion mechanism.

In the present study experiments were designed to compare the responses to illuminated backgrounds of melanophores separated from the central nervous system by spinal nerve section with those of caudal band melanophores reported by earlier workers.

Before commencing the experiments the validity of the continuous observation apparatus (p. 71) as a tool to investigate the chromatic responses to an illuminated background was examined.

4.2. Methods

For the method of fish confinement, a description of the apparatus, recording the results and operating procedure see Chapter II, pages 73-74 . The temperature of the water was not controlled and ranged between 18 - 20°C.

4.3. Results

4.3.1. Evaluation of the continuous observation apparatus

Gray (1955) reported that confinement of minnows causes abnormal chromatic responses to both black background and white background adaptation (beyond one hour) when the animals tend to aggregate pigment.

In the present study measures were undertaken to ensure that the apparatus used would minimize the effect of confinement and would provide the fish with the most normal environment possible.

To test the adequacy of the apparatus, the responses of the fish to a black background and a white background and their subsequent reversal and also to the standard grey series of the D.O.I. (Healey, 1967) of values 2, 4, and 6 were studied.

4.3.2. Responses of chromatically normal fish confined in the continuous observation tank to an illuminated black background and an illuminated white background

A group of minnows with a previous history of an intermediate background (white sink mottled in black) were subjected to spinal cord section posterior to the 15th vertebra. After recovery from the operation and the macroscopic test on their colour change (p. 69) some of the operated fish were transferred to a white painted glass aquarium and others to a black painted glass aquarium.

These aquaria were supplied with running water and the level

of water was maintained by glass siphons.

Twenty-four hours after the operation macroscopic examination of the fish showed that they had fully adapted to their respective backgrounds. Following the procedure already described (Chapter II, p. 71), fish from both the above aquaria (one at a time) were transferred to the continuous observation tank and were exposed to their respective backgrounds.

Continuous microscopic observation for two hours of the confined fish exposed to an illuminated black background showed a constant state of maximum pigment dispersion throughout the period of the observation. Confined fish exposed to an illuminated white background had melanophores which were in a state of maximum pigment aggregation except for a very few melanophores which did not show full pigment aggregation (Plate IV-1a, b p. 123).

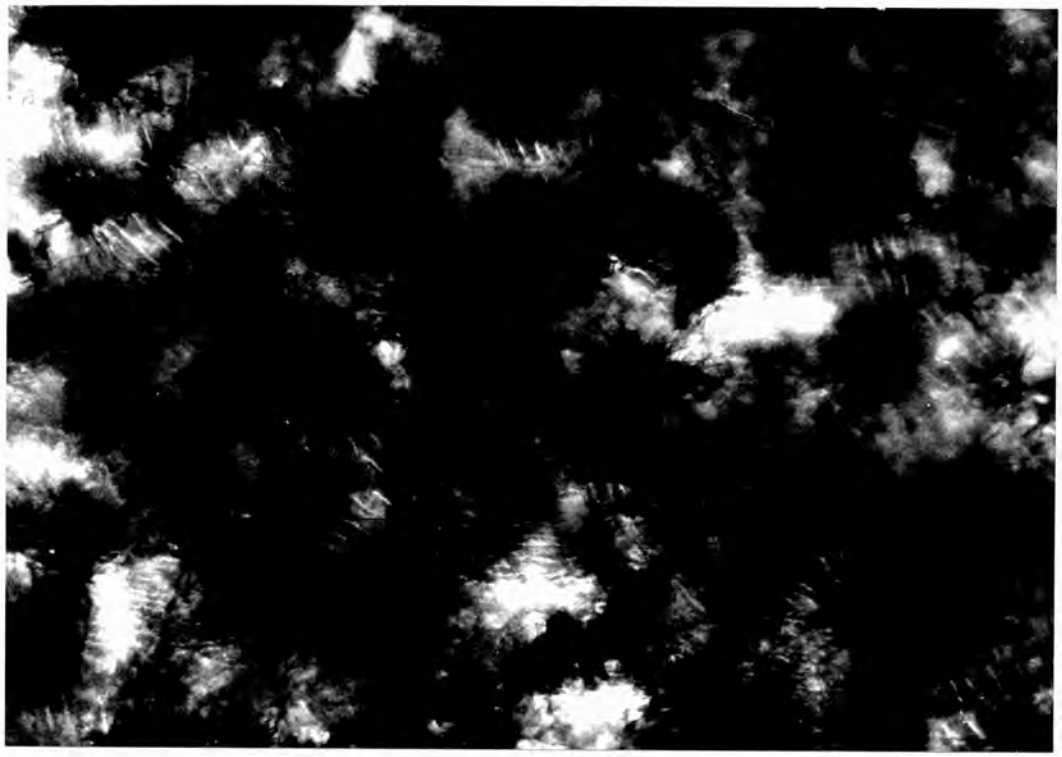
However, the apparatus did show some limitations. Some of the confined fish struggled and this resulted in incomplete dispersion of their pigment when exposed to an illuminated black background. This paling which often follows disturbance of the fish (von Frisch, 1911) has been attributed by later workers to the sudden release of adrenaline into the blood stream.

Furthermore, in some cases confinement beyond two hours was found to result in a slight aggregation in fully black adapted fish. Therefore, continuous microscopic observation of melanophores was limited to a period of two hours and fish which struggled were

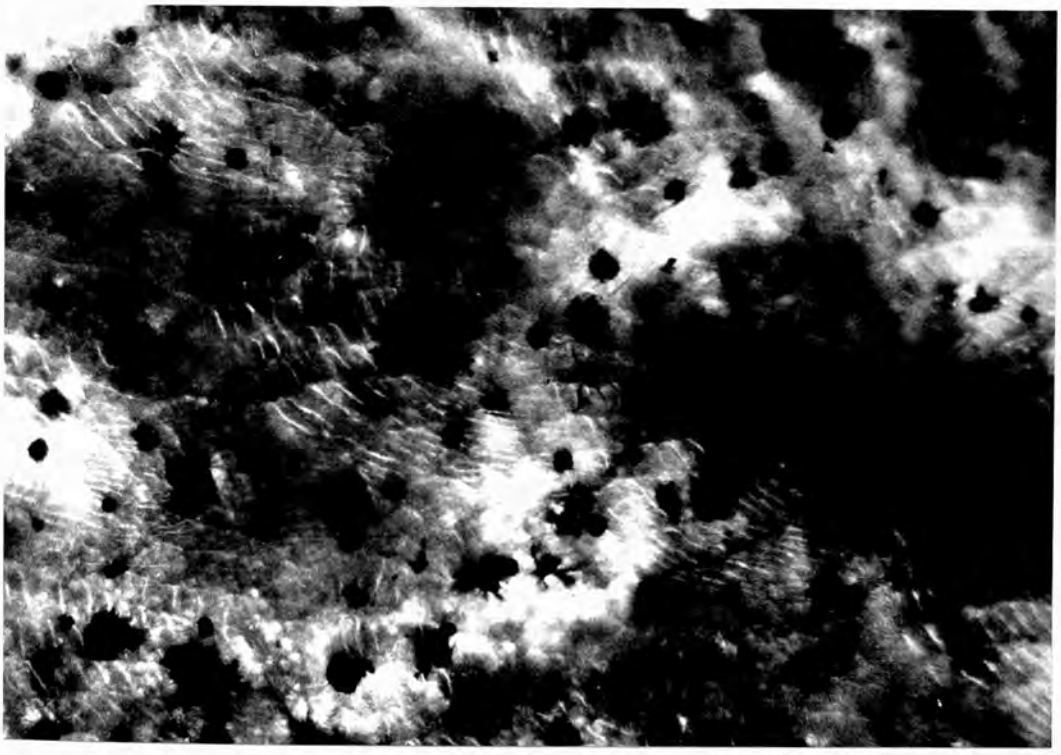
Plate IV-1 a,b

Photographs a and b show the responses of a chromatically normal *Phoxinus phoxinus*, confined in the continuous observation tank to an illuminated black background (60 minutes) and an illuminated white background (60 minutes) respectively.

Mag. X 110.



a



b

discarded.

4.3.3. Responses to background reversals of chromatically intact fish confined in the continuous observation tank

Figure IV-1 page 125 shows the colour changes of chromatically intact spinal fish confined in the continuous observation tank when subjected to background reversals (mean values for 6 fish). Curve A shows responses of previously white adapted fish (7 days) to a black background. Curve A' shows the responses of the above fish when the background was reversed to a white background.

Curve B shows responses of previously black adapted fish (7 days) to a white background. Curve B' shows the responses of the above fish when the background was reversed to black.

The melanophores used for the recordings have been described in Chapter II, page 73.

The above curves are well in agreement with the results reported in studies on chromatic responses and time relations by Healey (1951, 1967) and Grove (1967), working on the minnow *Phoxinus*, and by Hogben and Landgrebe (1940) and Neill(1940) working on other teleosts.

The curves indicate that the initial responses are fast and are followed by much slower responses. Healey (1951, 1967) explained the above results along the lines of similar explanations given by Hogben and Landgrebe (1940) and Neill(1940) that the initial and relatively fast colour changes in response to illuminated backgrounds

Fig. IV-1

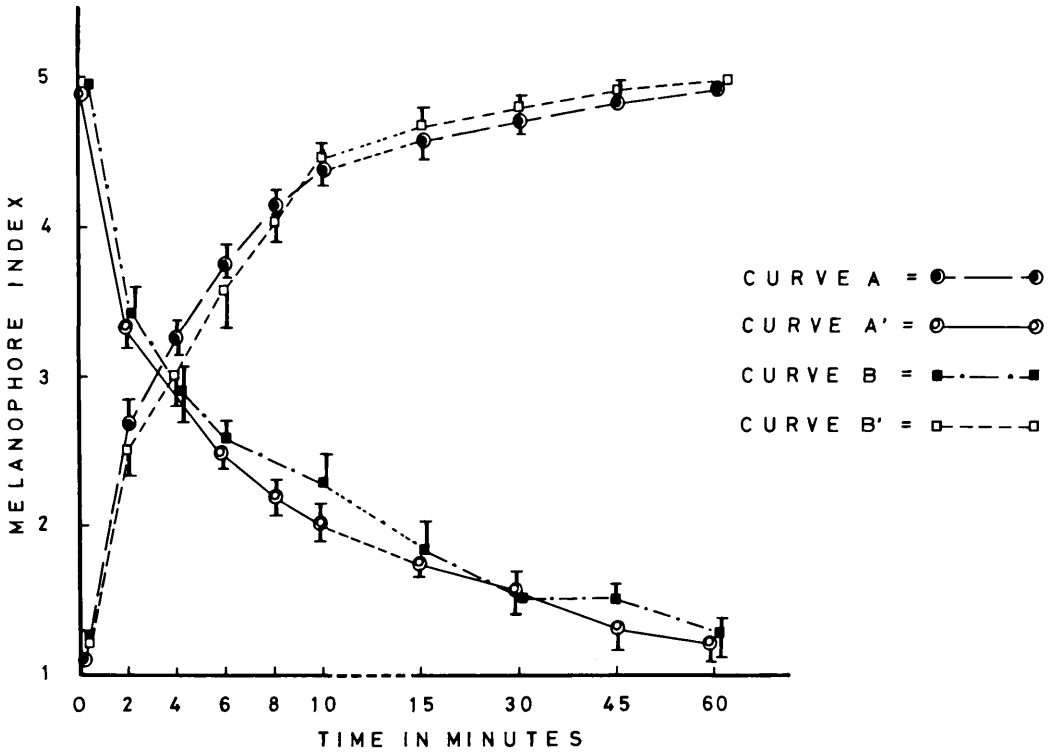
Responses of chromatically normal *Phoxinus phoxinus* to black and white reversal.

Curve A : previously white-adapted (7 days) to black backgrounds (6 animals.

Curve A': reversing the background of the above fish after 60 minutes on black, to white.

Curve B : previously black-adapted (7 days) to white background (6 animals).

Curve B': reversing the background of the above fish after 60 minutes on white, to black.


MELANOPHORE INDEX
5
4
3
2
1
0
2
4
6
8
10
15
30
45
60
TIME IN MINUTES
CURVE A =
CURVE A' =
CURVE B =
CURVE B' =

are controlled by the nervous system which is thereafter reinforced and followed by a slower hormonal change.

The full reaction of fish confined in the present apparatus to illuminated backgrounds and the constancy in the state of their reaction for as long as two hours indicate the reliability of the apparatus used in the studies of chromatic responses. Moreover, the close agreement of colour change curves obtained compared with those previously reported reinforces the above reliability.

However, to gain more confidence in the results which will be based on the use of this apparatus, it was subjected to further tests.

Melanophores of minnows are known to have different thresholds to stimuli. Melanophores in barred regions of the skin are known to have a higher threshold in response to aggregating stimuli and a lower threshold in response to dispersing stimuli, the reverse being true for melanophores outside the barred region of the skin (Healey, 1954, 1965; Grove, 1967).

According to the above, melanophores inside the barred region of the skin are first to disperse in response to an illuminated black background and last to aggregate in response to an illuminated white background.

Therefore, to see whether the fish confined in the present apparatus will respond similarly, a larger area of the skin was observed using a Nikon camera with micro-auto lens attached to a Nikon bellows (see page 74). Magnification obtained by the

above method facilitated the observation of more than one of the lateral patches of melanophores and, at the same time, individual responses of melanophores could be seen.

A similar sequence of responses was observed: melanophores in the barred region were found to be the first to disperse and the last to aggregate while the reverse was true for the melanophores outside the barred region. Plate IV-2a, b, c page 128 shows the responses of a white adapted fish (2 weeks in an illuminated white background and 15 minutes exposure to a white background in the observation tank) to a black background. Furthermore, with the similar previous adaptation fish were exposed to different shades of greys, D.O.I. 2, 4, and 6 in sequence, 30 minutes exposure on each. It was observed that on progressively darker backgrounds the melanophore patches became darker and larger and the state of the melanophore response remained constant as long as the fish was exposed to the same background (Plate IV-3a, b, c p. 129).

All the results obtained from the above experiments strongly indicate normal chromatic responses of the fish while confined to the continuous observation tank. Therefore it is justifiable to conclude that while the limitations mentioned above should be considered, the apparatus used in the present investigation is nevertheless a valuable tool in experiments requiring continuous microscopic observation of melanophores.

4.4. Pigment aggregation in melanophores separated from central control by spinal nerve section in response to prolonged white adaptation

Plate IV-2a, b, c

Differential responses of melanophores in different regions of the skin to illuminated backgrounds.

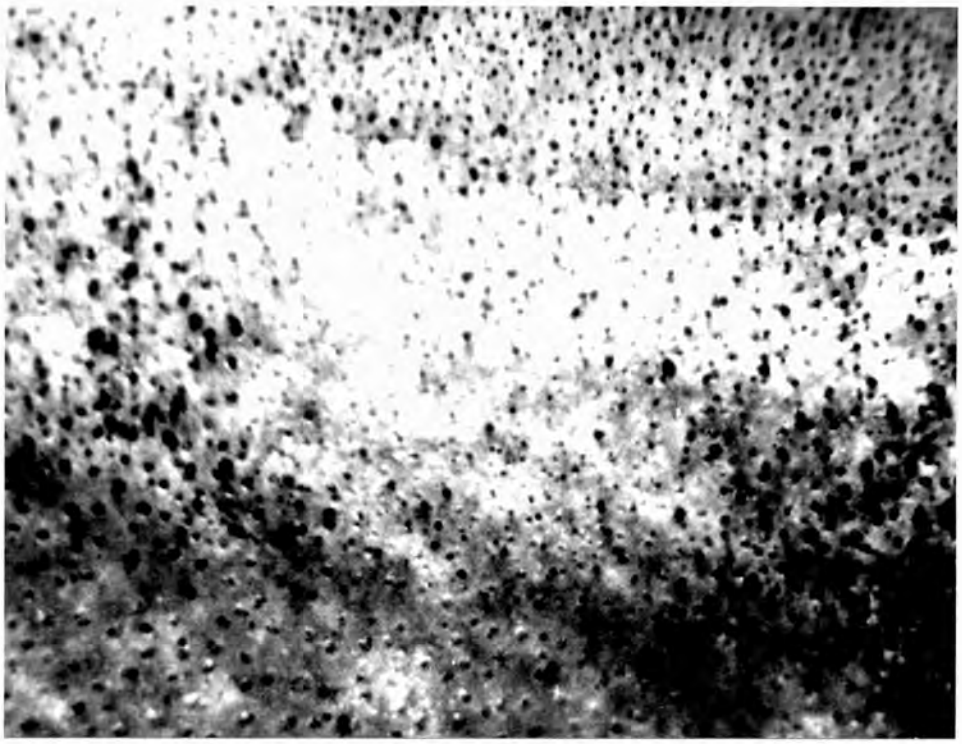
a - 30 minutes exposed to a white background

b - 2 minutes after reversal to a black background

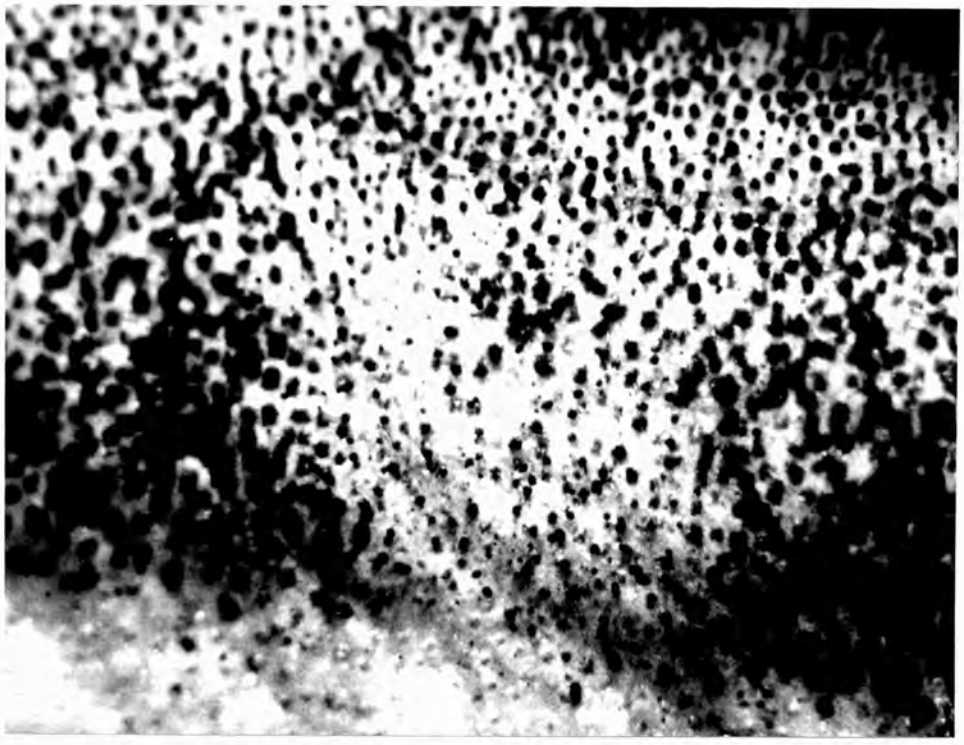
c - 10 minutes after reversal to a black background

Note : melanophores in the barred region of the skin are the first to disperse in response to a black background.

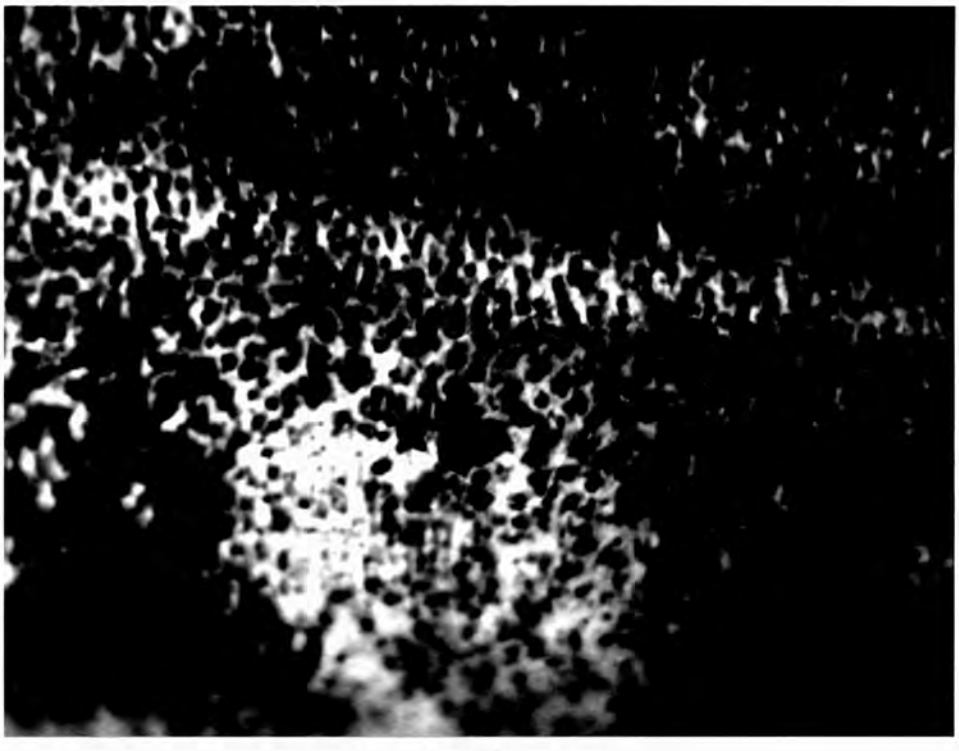
Mag. X 18.



a



b



C

Plate IV-3a, b, c

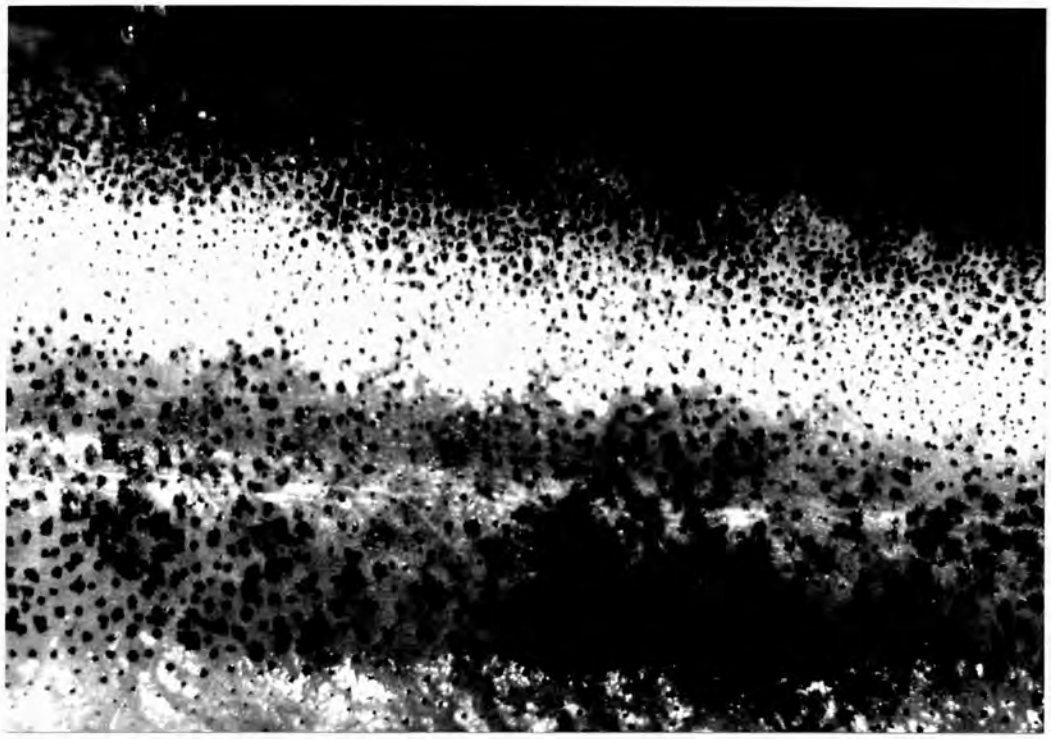
Photographs a, b, and c show a gradual increase in the degree of pigment dispersion on progressively darker shades of grey.

a - 30 minutes exposed to the D.O.I. 2

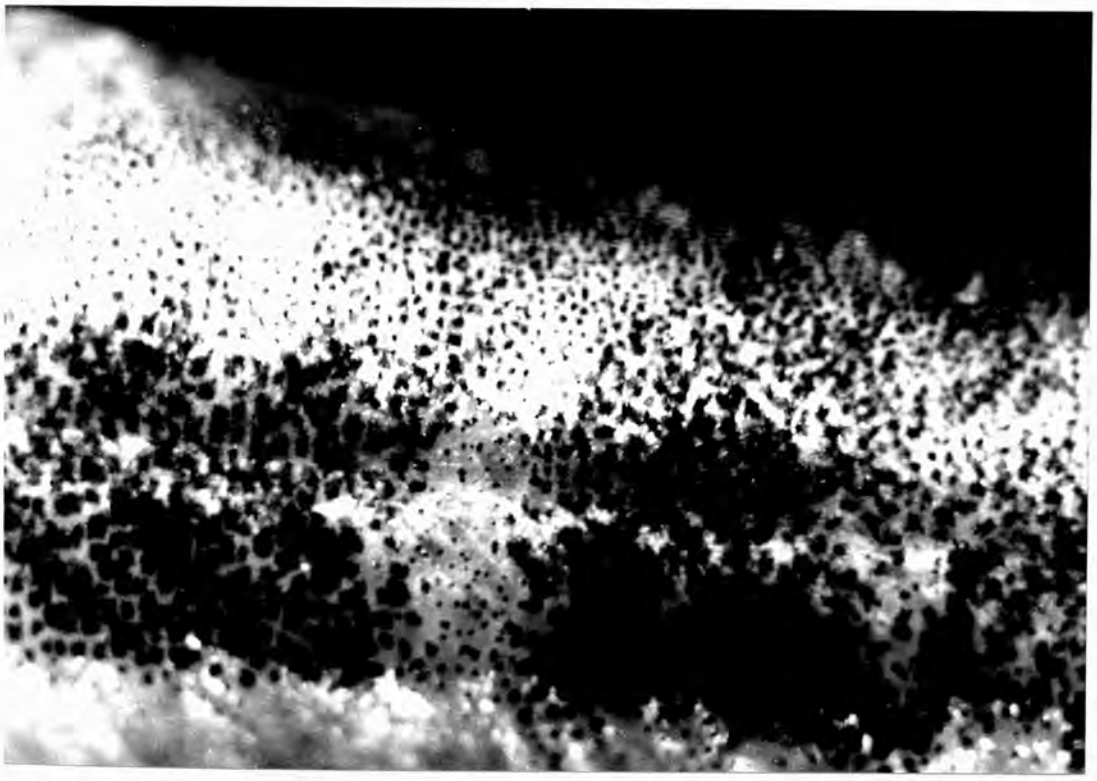
b - 30 minutes exposed to the D.O.I. 4

c - 30 minutes exposed to the D.O.I. 6

Mag. X 15.



a



b

c

A fish adapted to an intermediate background (white sink mottled with black) was subjected to the combined operations of spinal nerve section (3 - 5 spinal nerves) and spinal cord section posterior to the 15th vertebra.

4.4.1. The first paling on a white background after the operation

After the operation the fish was transferred to an illuminated white background. As a result of the operation a dark strip of melanophores with dispersed pigment granules was observed, indicating the area affected by the operation (Plate IV-4a p. 132). These melanophores affected by the peripheral nerve transection are known to remain dispersed in spite of the fish being on a white background. However, the dispersed pigment granules gradually aggregate on a white background. The time required for this aggregation to be accomplished varies from several hours to several days (Mills, 1932a, b; Parker, 1933, 1934a; Gray, 1956; Fujii and Novales, 1969b). Probably, this variation in the time required for the affected melanophores to aggregate on a white background depends on the size of the area affected by the operation. The larger the size the longer the time required on a white background to bring about the pigment aggregation in all the affected melanophores.

In the present work, in order to study the gradual pigment aggregation on a white background in melanophores affected by the operation, the fish were transferred to an illuminated white background in the continuous observation tank. Thirty minutes after transferring the fish to the continuous observation tank, a photo-

graph was taken covering an area with melanophores affected by the operation and surrounded by unaffected, neighbouring melanophores. After exposing the photograph, the fish was immediately released from the continuous observation tank and was transferred to an illuminated white background. Plate IV-4a, b, c, d page 132 show the gradual pigment aggregation in the affected area in response to an illuminated white background, 4 hours after the spinal nerve section and on the 1st, 2nd and 3rd postoperative days respectively. By comparing photographs a and b page 132 it is evident that pigment aggregation occurs at the margin of the affected area. This observation is consistent with the marginal fading of the caudal band melanophores reported by Mills (1932a, b) working on *Fundulus* and Gray (1956) working on *Phoxinus*. However, as is also evident from the photographs, the aggregation of the pigment granules in the melanophores at the periphery of the affected area is not in that strict sense. Some melanophores right at the centre of the affected area are seen to have aggregated pigment, while some of the peripheral ones have their pigment granules quite dispersed (Plate IV-4b p. 132).

The above experiment was repeated on another two fish with the same background history and two other fish, one with prolonged white background history (6 months) and the other with prolonged black background history. All the results were consistent.

4.4.2. Responses of the separated melanophores to background reversals

4.4.2.1. Response to background reversal on the 1st postoperative day

Plate IV-4a, b, c, d

Pigment aggregation in melanophores affected by spinal nerve section on an illuminated white background.

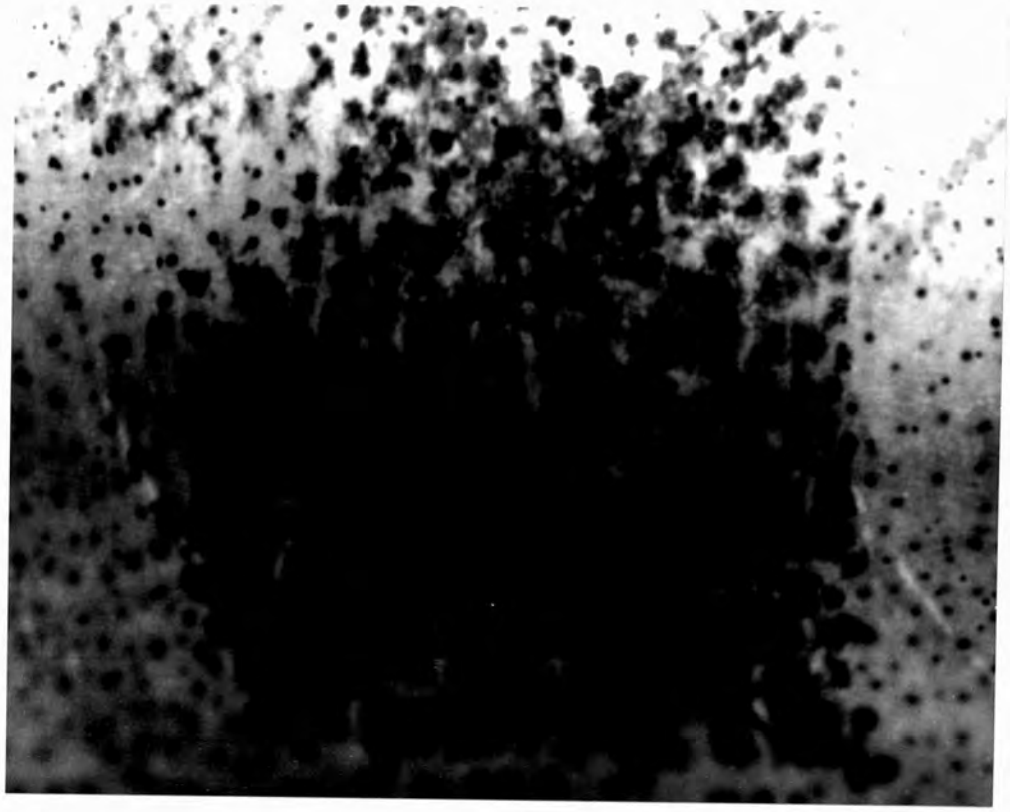
a - state of the melanophores 4 hours after the operation

b - state of the melanophores on first post-operative day

c - state of the melanophores on second post-operative day

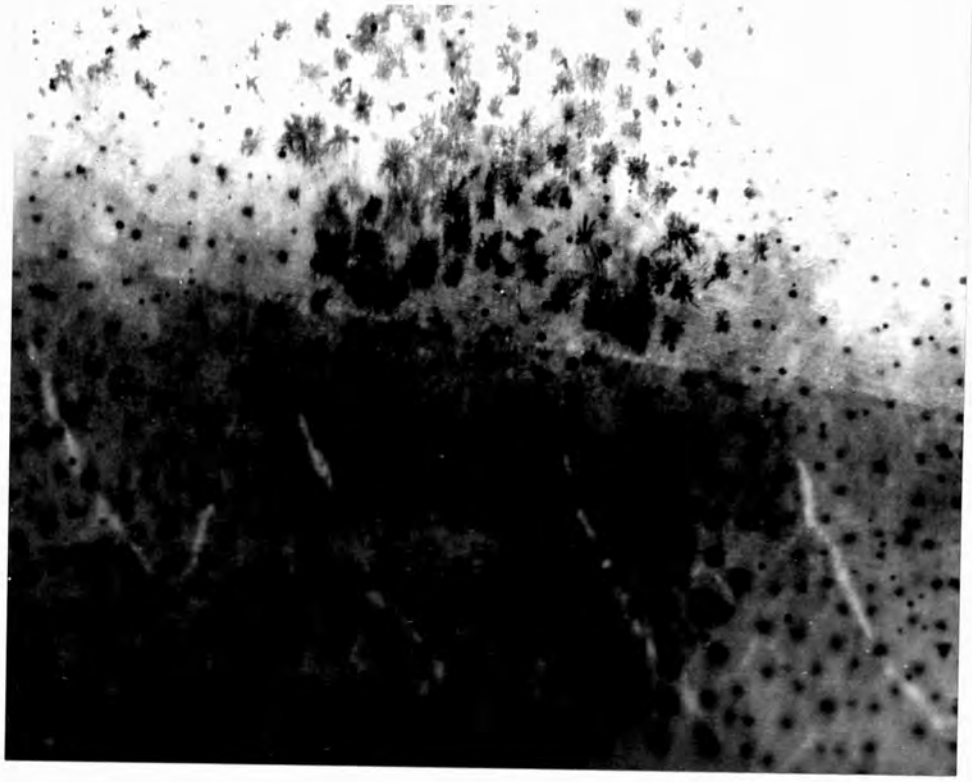
d - state of the melanophores on third post-operative day

Mag. X 20.

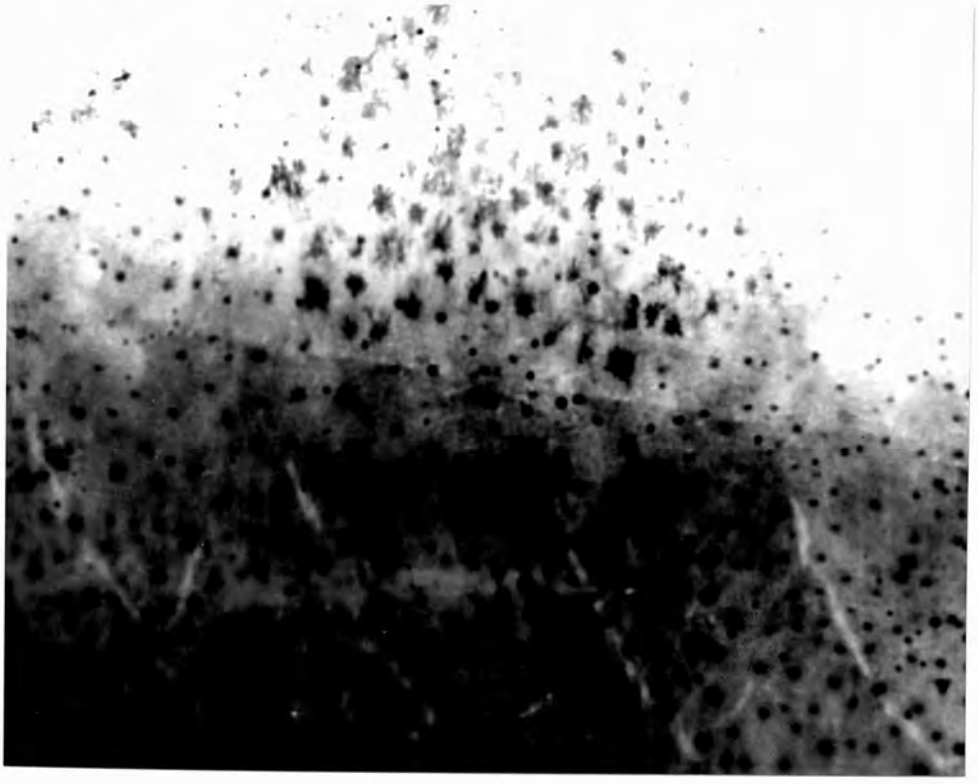


a

b



c



d

A prolonged white adapted fish (6 months) was operated on in a similar manner to the fish in the previous experiment. After the operation the fish was kept on an illuminated white background. Approximately 24 hours after the operation the fish was transferred to the continuous observation tank and exposed to an illuminated white background. Plate IV-5a page 135 shows the state of pigment granules in the melanophores affected by the operation and the neighbouring melanophores in response to the illuminated white background for more than 24 hours. The photograph was exposed 30 minutes after the confinement of the fish in the continuous observation tank. Upon the reversal of the background from an illuminated white to an illuminated black background melanophores with their innervation intact readily responded to the background by dispersing their pigment Plate IV-5b page 135 (after 10 minutes on black). Some melanophores in the area affected by the spinal nerve section operation did disperse their pigment when the background was changed to illuminated black. Some of these melanophores were at the periphery of the affected area and some at the centre. No distinct difference in the rate of the pigment dispersion could be observed between the melanophores in the area affected by the spinal nerve section and those with intact innervation. After the fish was exposed to the illuminated black background for 15 minutes, the background was reversed to white. As a result, melanophores with intact innervation responded readily by aggregating their pigment granules. However, it is interesting that those melanophores in the area affected by the spinal nerve section which dispersed their pigment rapidly in response to the illuminated black background

(15 minutes) failed to aggregate their pigment upon reversal of the black to white again for as long as 60 minutes (Plate IV-5c p. 135). This observation resembles that reported by Mills (1932a) where she stated *"It frequently happened that melanophores on the edge of the completely denervated area expanded readily on a black background, but failed to contract over white"*. Mills explained the above observation by assuming a double innervation of melanophores, that is, that the antagonistic nerve fibres mediating pigment aggregation and pigment dispersion have different distributions. However, before commenting on the above, results obtained from further experiments on the responses of the separated melanophores will be presented.

4.4.2.2. Responses to background reversal on the 2nd postoperative day

A fish which had been adapted to an illuminated white background after the combined operations, spinal nerve section and spinal cord section posterior to the 15th vertebra, was transferred to the continuous observation tank. Plate IV-6a page 137 was exposed 30 minutes after the fish was confined in the continuous observation tank and exposed to an illuminated white background. Upon reversal to an illuminated black background, some of the melanophores in the area affected by the spinal nerve section showed some aggregation of their pigment granules, while melanophores with intact innervation were in the process of pigment dispersal in response to the black background. This contrast of the responses of the two

Plate IV-5a, b, c

Responses of melanophores affected by spinal nerve section to background reversal on 1st post-operative day. After the operation the fish was transferred to an illuminated white background.

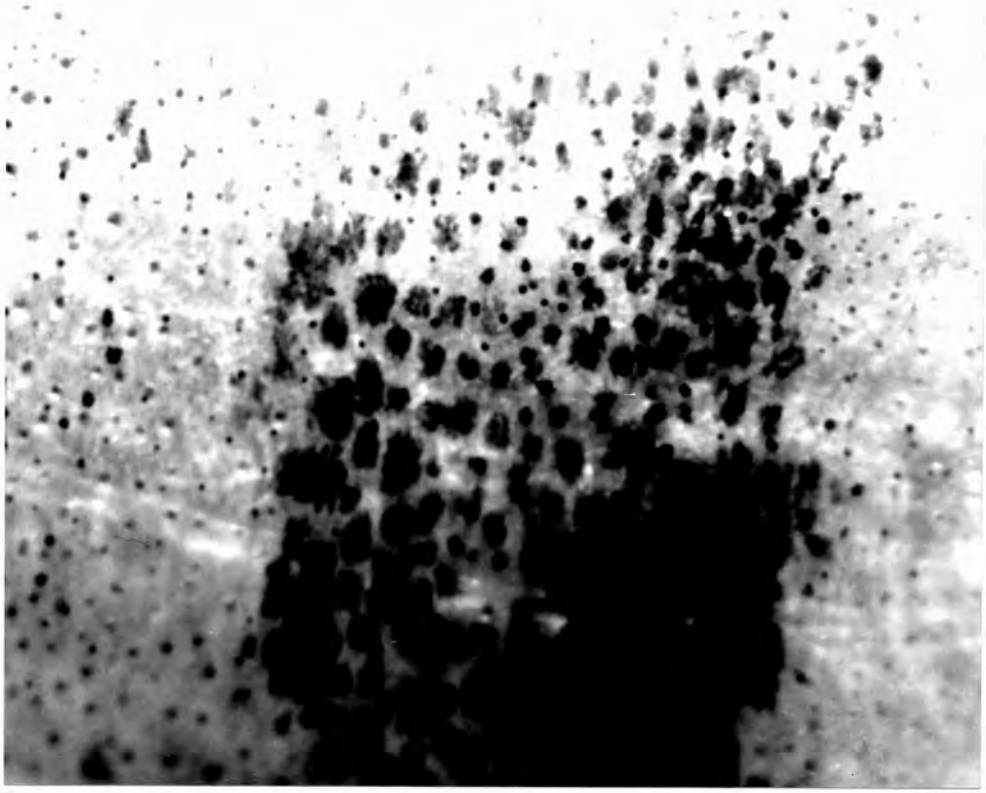
a - state of melanophores affected by the operation and neighbouring innervated melanophores exposed to an illuminated white background in the continuous observation tank for 30 minutes

b - responses of the above melanophores on the reversal of the background to black (10 minutes)

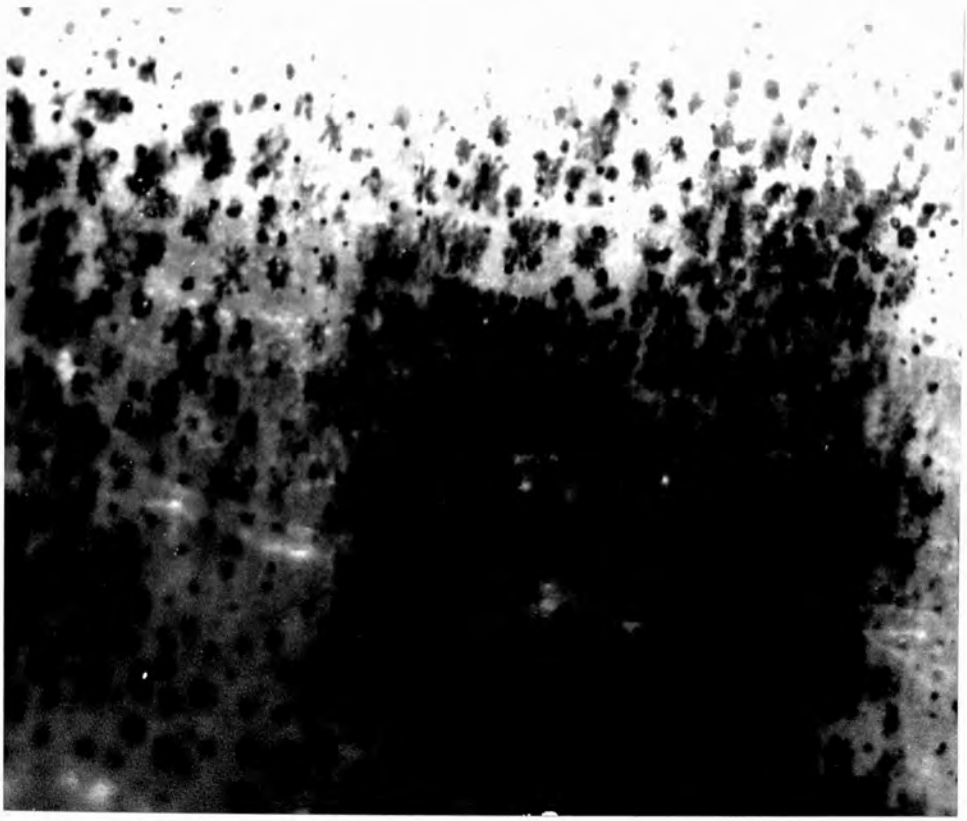
c - responses of the above melanophores on re-exposing the fish to the white background

Note : melanophores in the affected area dispersed readily on exposing the fish to black background (10 minutes), but have failed to aggregate on re-exposing the fish to white background.

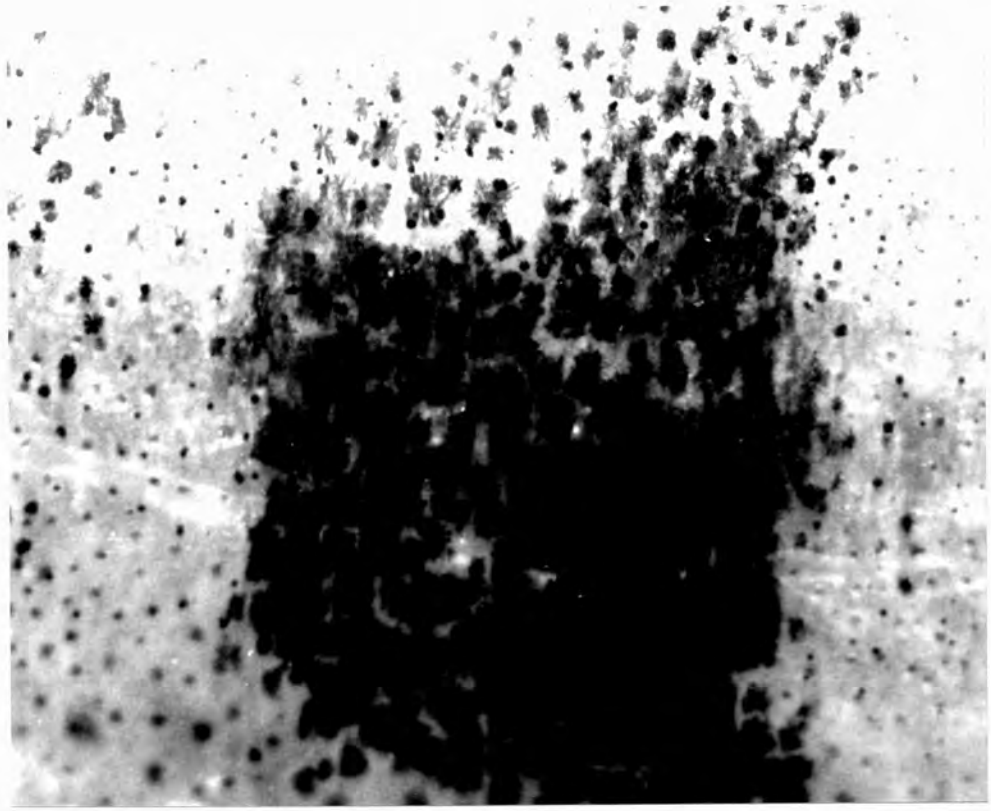
Mag. X 20.



a



b



c

groups of melanophores lasted for about 10 minutes. Thereafter, melanophores in the affected area started to respond positively to the illuminated black background by dispersing their pigment granules (Plate IV-6b, c, d p. 137). Reversing the background from black to white resulted readily in pigment aggregation in the melanophores with intact innervation. However, melanophores in the area affected by the spinal nerve section remained dispersed (Plate IV-6e p. 137). The latter observation is consistent with the observation in the previous experiment, (responses on the 1st postoperative day), so far as the failure of those melanophores in the affected area which dispersed their pigment granules in response to the relatively short exposure of the fish to a black background, to aggregate at the same rate, is concerned. The additional observation in this experiment, was the pigment aggregation in melanophores in the area affected by the spinal nerve section upon the reversal of the background from an illuminated white background to an illuminated black background. This pigment aggregation might result from the sudden release of adrenaline into the blood stream caused by a fright response of the fish (Smith, 1931; Gray, 1956). The released adrenaline would affect melanophores in the separated area to a greater extent than those with intact innervation for the following possible reasons:

(a) - Melanophores with intact innervation are directly interacting with the illuminated black background, therefore, the effect of the released adrenaline would be less noticeable;

(b) - Melanophores in the affected area are more sensitive to the released noradrenaline as a result of possible functional denervation

Plate IV-6a, b, c, d, e

Responses of melanophores affected by spinal nerve section to background reversal on the 2nd post-operative day. After the operation the fish was transferred to an illuminated white background.

a - 30 minutes exposed to an illuminated white background in the continuous observation tank

b - 6 minutes after the background was reversed to black

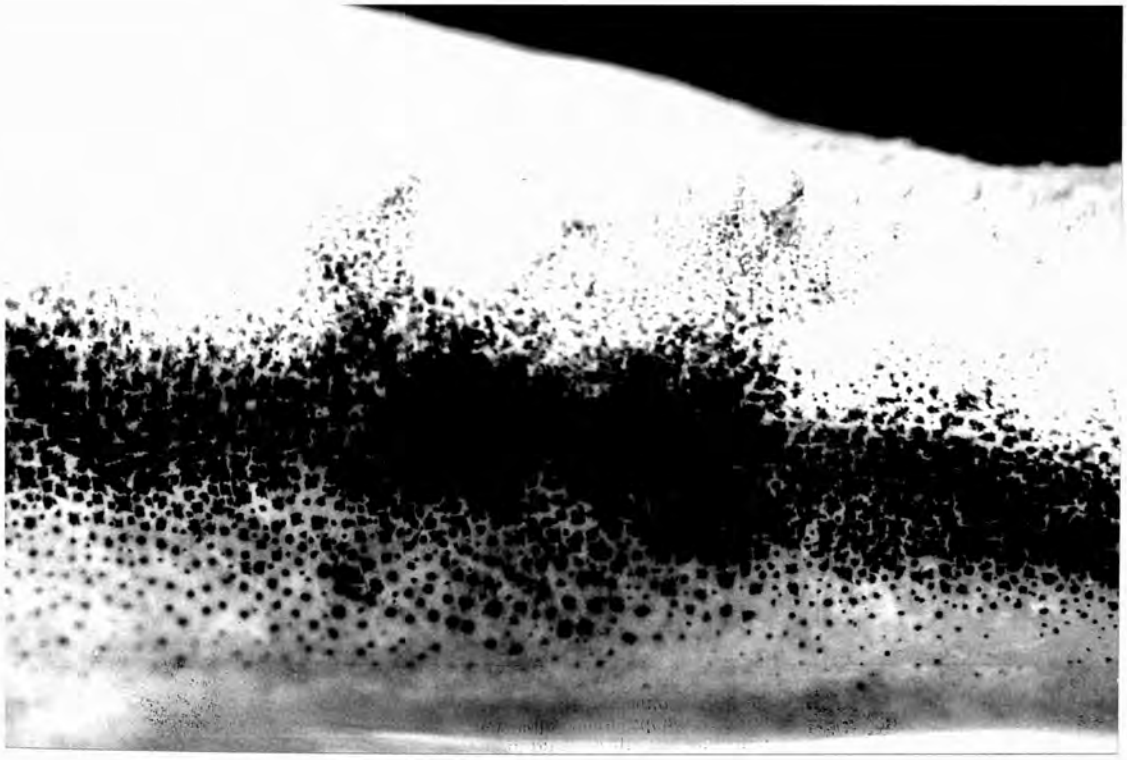
c - 10 minutes after the background was reversed to black

d - 15 minutes after the background was reversed to black

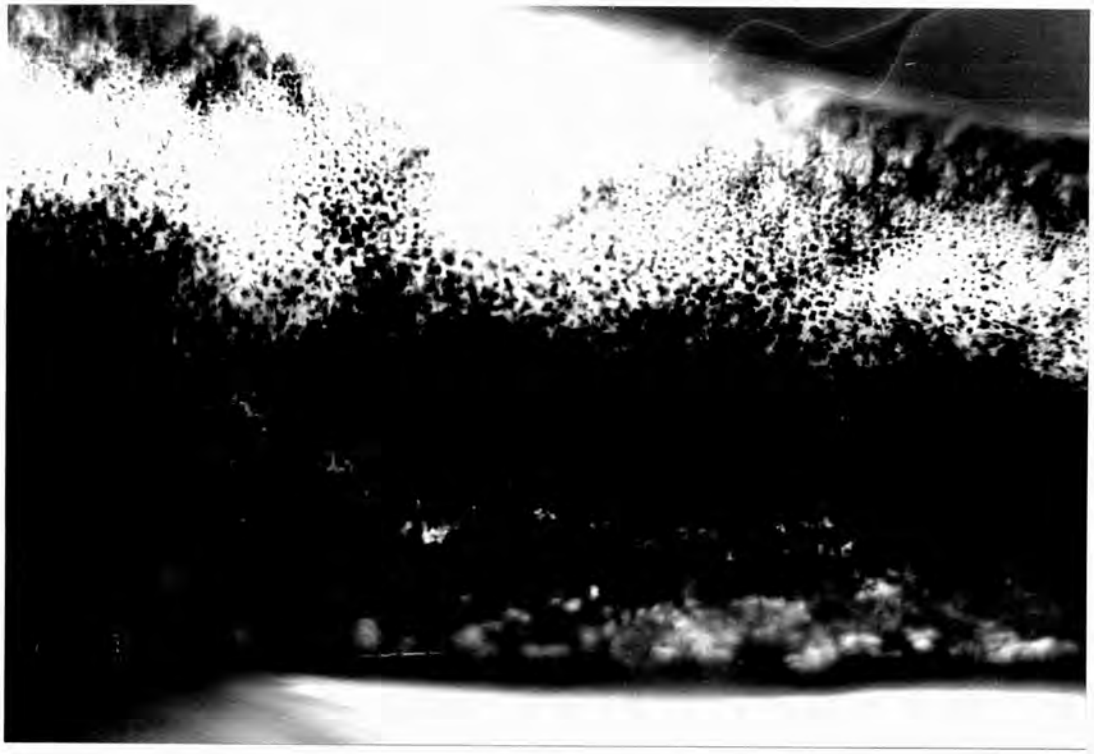
e - 30 minutes after re-exposing the fish to white

Mag. X 14.

a



b



c

d

e

(Trendelenburg, 1963);

(c) - A combination of both factors a and b.

4.4.2.3. Response to background reversal on the 7th postoperative day

The melanophores in the area affected by the spinal nerve section were found to aggregate their pigment almost completely if the fish was kept on an illuminated white background without any interruption. A fish under such experimental conditions was transferred to the continuous observation tank, again over an illuminated white background. Plate IV-7a page 139 shows the almost fully aggregated pigment granules in melanophores affected by the spinal nerve section, after the fish had been kept for a week on an illuminated white background and for 30 minutes over an illuminated white background in the continuous observation tank. Upon the reversal of the background to black in the continuous observation tank, melanophores with intact innervation responded readily to the black background by dispersing their pigment. Plate IV-7b page 139 shows the response of the fish to the black background, 30 minutes after the background reversal. As is evident from the photograph, melanophores in the area affected by the spinal nerve section have only slight pigment dispersion. By changing the background back to white, pigment granules in melanophores with intact innervation readily aggregated. However, melanophores in the area affected by the spinal nerve section not only did not show any sign of pigment aggregation, but continued to disperse their pigment granules despite

Plate IV-7a, b, c

Responses of melanophores affected by spinal nerve section to background reversal on the 7th post-operative day. After the operation the fish was transferred to an illumunated white background.

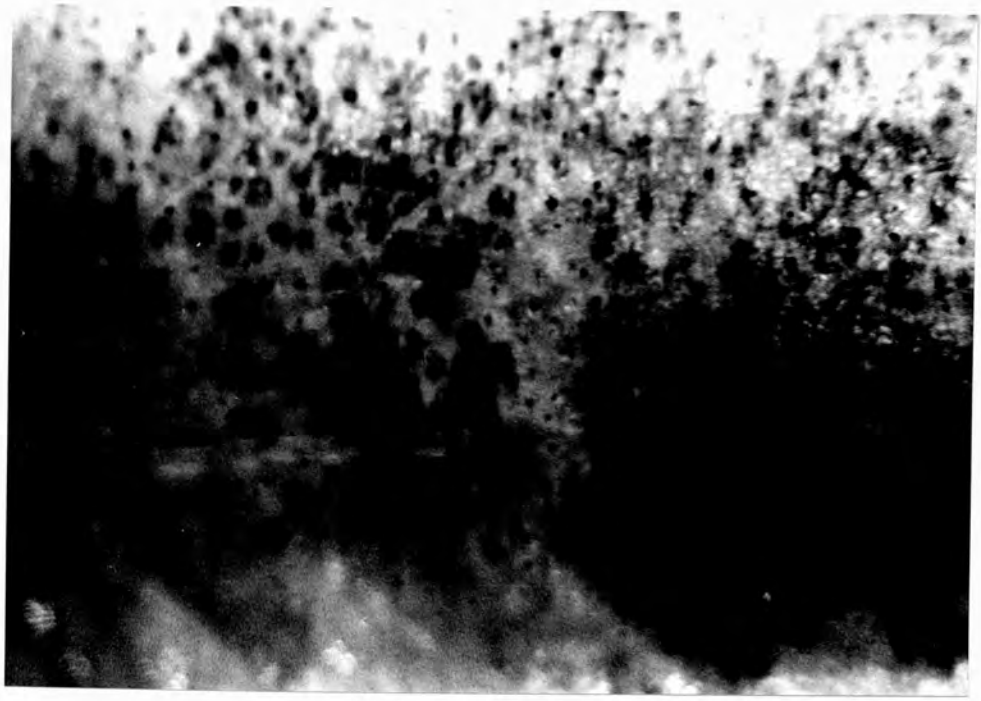
a - 30 minutes exposed to illuminated white background of the continuous observation tank

b - 30 minutes after the background was reversed to black

c - 30 minutes after re-exposing the fish to white

Mag. X 20.

a



b

c

the fish being exposed to an illuminated white background, Plate IV-7c page 139 shows the dispersion of the pigment granules in the area affected by the spinal nerve section, after reversal of the background to white (30 minutes).

The above results confirm Mills' (1932b) observation that, if a fish with a denervated area was transferred from a black background to a white background, melanophores in the denervated area which were in the process of dispersing their pigment as a result of being exposed to a black background, continued to disperse their pigment granules even after transferring the fish to a white background.

All the experiments described in the above sub-sections were repeated at least five times and consistent results were obtained.

4.5. Discussion

4.5.1. The continuous observation apparatus

Healey's modified form of the continuous observation apparatus used in the present study is a simplified version of his previous apparatus used by Gray (1956). Although the apparatus is simple and easy to construct, the results obtained on minnow colour changes in response to illuminated black background, white background and their reversal are in full agreement with the responses of free swimming fish to the same (Healey, 1967; Grove, 1967). The consistency in the degree of the response of confined fish to a shade of grey and the gradual dispersion or aggregation of pigment

granules in response to a darker or lighter background respectively strongly suggest that the confinement has no effect on the normal chromatic responses of the fish to illuminated backgrounds. However, the slight aggregation of pigment granules after long exposure (beyond two hours) of the confined fish to black background restricted the period of the continuous microscopic observation to two hours only.

4.5.2. Responses of melanophores affected by spinal nerve section

In describing the experiments and the results the term "denervated melanophores" was not employed to designate the melanophores separated from the central nervous control by the spinal nerve operation. This term is improper because the distal part of the severed nerves, although they eventually might degenerate, remain for some time physiologically functional. In mammals transection of post-ganglionic sympathetic fibres results in their permanent distal degeneration (Thoenon, 1972). Taking the disappearance of the stored noradrenaline at the nerve terminal as a measure, Thoenon reported that the time varies from 18 - 48 hours. It is very likely that the time for the severed fibres in poikilotherms to degenerate would be longer, since tissue temperature and, consequently, metabolic rate is lower than in mammals. However, the main purpose of the present experiments was only to compare the effect of illuminated background reversals on melanophores affected by the operation with that on the neighbouring melanophores with intact innervation.

The dispersion of pigment granules which follows the transection of the appropriate nerve fibres has been given several explanations (Chapter I, page 34). In brief they can be summarised as the following :-

(a) Dispersion is passive and results as soon as impulses in the sympathetic aggregating fibres cease either due to nerve section or black background presentation (von Frisch, 1911).

(b) Dispersion is active and is due to sustained injury discharges in dispersing fibres but not in aggregating fibres (Parker, 1948).

(c) Dispersion is active and is probably due to some dispersing mechanism of the melanophores which comes into play after the cessation of the aggregating stimuli in the sympathetic fibres (Gray, 1956).

(d) Dispersion is active; however, the nature of the mechanism mediating this dispersion is obscure (Healey, 1948, 1951, 1954, 1967; Healey and Ross, 1966; Grove, 1969a, b).

The results obtained in the present study confirm that the pigment dispersion mechanism is an active mechanism. This was clearly demonstrated in the experiments concerning the effect of black background presentation on the affected melanophores. The fast pigment dispersion (minutes) in those of the affected melanophores which had aggregated as a result of prolonged white adaptation (hours) can only be explained if an active mechanism of pigment dispersion is assumed. This dispersion is almost certainly elicited by the same agent mediating active pigment dispersion in

melanophores with intact innervation in response to a black background. However, the nature of this agent is not yet known. Mills (1932a, b), Parker (1948) and Gray (1956) have suggested that pigment dispersion in the caudal band melanophores in response to a black background is the result of the invasion of dispersing neurotransmitter released from neighbouring intact melanophores. Similarly, the above authors explain the aggregation of pigment in caudal band melanophores on a white background as being the result of invading aggregating neurotransmitter from adjacent intact nerve endings. The above explanation was challenged by Fujii and Novales (1969b) on the basis that the released neurotransmitter would be rapidly degraded by enzyme action and other inactivating mechanisms (Chapter I, pp. 39, 53). However, since the dispersion of pigment granules in the affected area takes place in a reasonably short time (minutes) and since the affected melanophores are densely surrounded by melanophores with intact innervation, then at least a small proportion of a released dispersing neurotransmitter might be expected to diffuse into the affected area before being totally inactivated. Therefore, based on the interpretation of the responses of caudal band melanophores which almost applies to the responses of the melanophores affected by the spinal nerve section, it might appear to be justifiable to assume a dual melanophore innervation. However, the main shortcomings of the above assumptions are :-

(1) How can the dispersion which follows the transection in the chromatic fibres be accounted for? Parker's sustained injury discharge theory in the dispersing fibres, but not in the aggregating

fibres has been strongly criticised by many workers (Chapter I, p. 37, Healey, personal communication).

(2) Despite many attempts to identify the chemical nature of the proposed dispersing fibres, the results reported were inconsistent and no clear picture has emerged (Chapter I, p. 46). Therefore, the double innervation of fish melanophores remains for the moment in question.

A further discussion of the results presented in this chapter will be dealt with later (Chapter V).

CHAPTER V

THE EFFECTS OF ADRENERGIC DRUGS ON THE CHROMATIC SYSTEM IN THE MINNOW *Phoxinus phoxinus* (L.)

5.1. Introduction and purpose of the investigation

Pharmacological studies on the chromatic system of *Phoxinus phoxinus* using various autonomic drugs have clearly demonstrated that the fibres mediating melanin granule aggregation within melanophores are adrenergic (Chapter I, p. 34). It is fairly well established that the pigment dispersion is mediated by an active mechanism but the nature of such a mechanism is in question. Parker (1948) and his school held the view that pigment dispersion is mediated by cholinergic fibres but this has not received any general support from recent pharmacological studies involving the administration of acetylcholine and associated drugs (Chapter I, p. 46). On the other hand, results indicating that both pigment aggregation and pigment dispersion are probably mediated by adrenergic mechanisms have also been reported (Chapter I, pp. 46, 60, 61). Such reports can be interpreted by the dual antagonistic adrenergic receptor concept of Ahlquist (Chapter I, p. 55).

Adrenaline "reversal" effect on melanophores was first observed by Barbour and Spaeth (1917) working on isolated scales of *Fundulus*. They not only showed that adrenaline was a potent aggregating agent

but also demonstrated that exposure of the isolated scale to ergot followed by adrenaline resulted in pigment dispersion within the melanophores instead of the usual aggregating effect. As was described in Chapter 1, page 46 , Gray also suggested adrenaline "reversal" to explain the dispersion of pigment following electrical stimulation of chromatic fibres in fish treated with ergotamine. However Pye (1964b) working on *Phoxinus phoxinus in vivo* and *in vitro* was not able to demonstrate adrenaline "reversal" in preparations pre-treated with ergotamine. Grove (1967) made an extensive survey of the effects of mammalian autonomic drugs. He found that bretylium (an adrenergic neuron blocking agent) injected into the body cavity of chromatically normal fish (*Phoxinus phoxinus*), darkened the animals. However, similar injections into chromatically spinal white adapted fish caused considerably less darkening. Furthermore, he found that chronic treatment of the fish with bretylium results in their inability to adapt fully to black or white backgrounds. Based on the above observations, Grove suggested that bretylium, which in mammals is known to accumulate in adrenergic neurons, should have a similar action on minnow chromatic fibres, then it is possible that the postulated dispersing fibres are adrenergic as well. However, results from his experiments with alpha and beta adrenergic agonists and antagonists did not support the suggested dual adrenergic receptors on melanophores involved in both pigment aggregation and dispersion mechanisms.

On the other hand, Healey and Ross (1966), working on *Phoxinus phoxinus*, did show some indication of adrenaline "reversal" in

their *in vivo* experiments using preparations pre-treated with ergotamine. Therefore, in view of the above results, experiments described in this chapter were designed in an attempt to throw further light on this problem.

Table 1 page 148 gives a list of drugs used in the present investigation and their chemical structure.

5.2. Methods

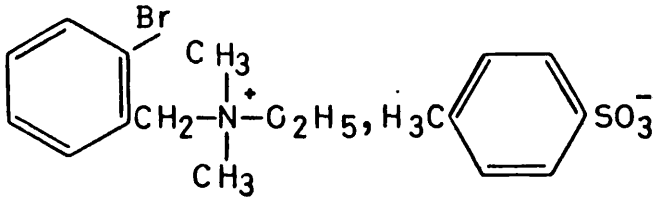
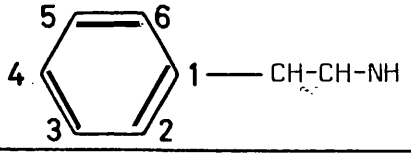
5.2.1. Recording of results

For graphical analysis of the results, the effects of drugs on the chromatic responses of the fish to illuminated backgrounds were recorded microscopically according to the method already described (Chapter II, p. 73).

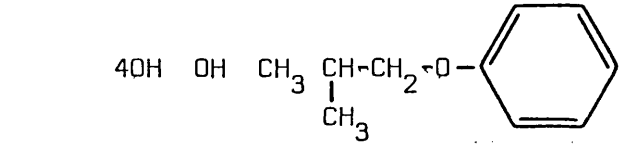
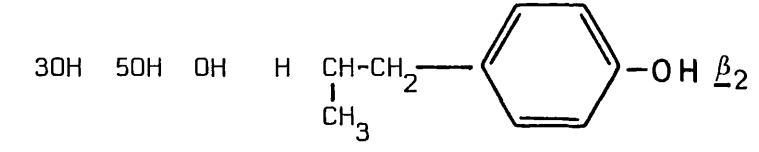
5.2.2. Drug administration

To overcome the difficulties and inaccuracy involved in weighing small amounts, stronger concentrations than required were made up in Young's (1933) fresh water teleost Ringer. The solutions were then brought to the appropriate concentration by dilution. The drug to be used was always prepared freshly before the commencement of the experiment. Drugs were administered intraperitoneally into the body cavity using a fine hypodermic needle. Care was taken to avoid damaging any internal organ by keeping the needle parallel to the body wall. Fish confined to the continuous observation tank were injected while they were confined on the platform by gentle removal of the platform from the tank.

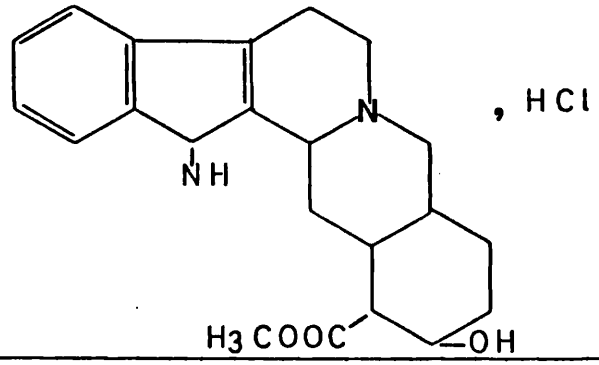
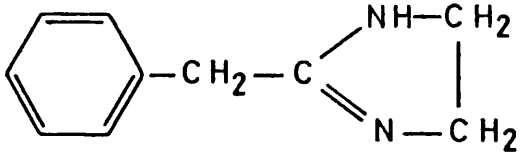
Table V-1

DRUG	CHEMICAL STRUCTURE	SITE OF ACTION	SUPPLIED BY
Adrenergic Neuron Blocking Agent BRETYLIUM TOSYLATE		Adrenergic Neurons	BURROUGHS WELLCOME & CO. The Wellcome Foundation Ltd London England
Adrenergic Agonists			
NORADRENALINE ACID TARTRATE	3OH 4OH OH H H	α-Adrenooeptors	WINTHROP LABORATORIES Sterling-Winthrop House Surbiton Surrey
ADRENALINE ACID TARTRATE	3OH 4OH OH H CH_3	α&β "	JOHN BELL & CROYDEN 50 Wigmore Street London, W.1.

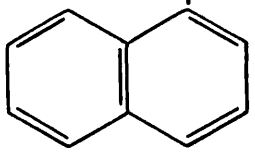
Continued .....

DRUG	CHEMICAL STRUCTURE	SITE OF ACTION	SUPPLIED BY
Adrenergic Agonists			
ISOPROTERENOL SULFATE		$\beta_{1,2}$ (α) Adrenoceptors	SIGMA CHEMICAL COMPANY P.O. Box 14508 St. Louis MO.63178 U.S.A.
FENOTEROL HYDROBROMIDE		$\beta_2(\beta_1)$ "	PHILIPS-DUPHAR B.V. P.O. Box 2 WEESP - The Netherlands
ISOXSUPRINE HYDROCHLORIDE		β_2 "	PHILIPS-DUPHAR B.V. P.O. Box 2 WEESP - The Netherlands

Continued .....

DRUG	CHEMICAL STRUCTURE	SITE OF ACTION	SUPPLIED BY
Adrenoceptor Antagonists			
YOHIMBINE HYDROCHLORIDE	 , HCl	α-Adrenoceptors	BDH Poole England
TOLAZOLINE HYDROCHLORIDE	 , HCl	α - Adrenoceptors	CIBA Ciba Laboratories Ltd Horsham Sussex

Continued .....

DRUG	CHEMICAL STRUCTURE	SITE OF ACTION	SUPPLIED BY
Adrenoceptor Antagonists			
PROPRANOLOL HYDROCHLORIDE	 $OCH_2\text{-}CH(OH)\text{-}CH_2\text{-}NH\text{-}CH(CH_3)_2$, HCl	β-Adrenoceptors	ICI Alderley Park Cheshire England

Free swimming fish were injected by scooping the fish to be injected gently from the water and holding them securely in a fine net. The site of the injection was an area just behind the pelvic fins and to the side of the mid line. The doses were calculated as moles of active substance injected per body weight of the fish. The injected volume was limited to 0.1 ml to avoid any substantial leakage. Operated fish are known to lose weight (Grove, 1969a), therefore, in calculating the injected dose, the method used by Grove was employed.

Mean weight of a group of normal fish	3.5 g	S.D. $\pm$ 0.2
Mean weight of a group of spinal fish	3.2 g	S.D. $\pm$ 0.05
Mean weight of a group of spinal nerve fish	3.3 g	S.D. $\pm$ 0.1

5.2.3. Control injections

Control injections to find out the effect of Young's Ringer and the fright response of the fish (von Frisch, 1911) on melanophores were given to both the operated and unoperated fish. Consistent with Grove (1967) the injection was found not to have any significant effect on the chromatic responses of the fish. Black adapted fish were found to show slight paling which only lasted for about one minute. Freshly operated fish, chromatically spinal and spinal nerve sectioned, did not show any paling. However, black adapted chromatically spinal fish (2 weeks after the operation) showed a greater paling response which also lasted longer, about 2 - 3 minutes (Fig. V-1 p. 150).

Fig. V-1

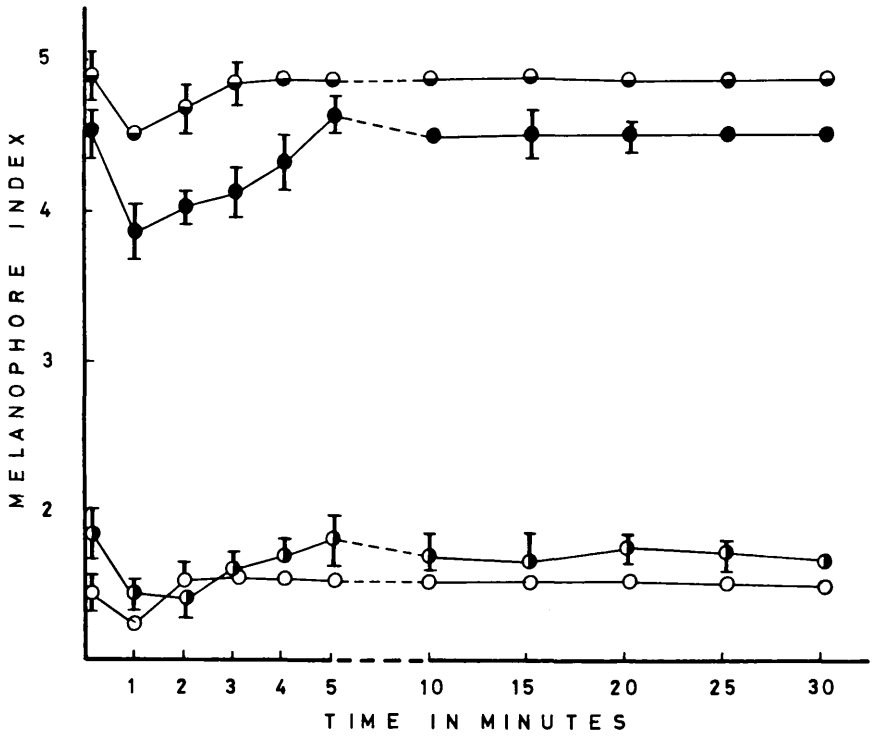
Effects of control injections (Young's ringer) on chromatically normal and chromatically spinal black-adapted and white-adapted *Phoxinus phoxinus*.

○ - chromatically normal white-adapted (3 animals)

◑ - chromatically spinal white-adapted (3 animals)

◒ - chromatically normal black-adapted (3 animals)

● - chromatically spinal black-adapted (3 animals)


MELANOPHORE INDEX
5
4
3
2
1
2
3
4
5
10
15
20
25
30
TIME IN MINUTES

5.2.4. Temperature

Unless otherwise mentioned, the temperature was not controlled and ranged during the course of the experiments between 12 - 18°C.

5.2.5. Electrical stimulation

5.2.5.1. Holding and preparation of the fish

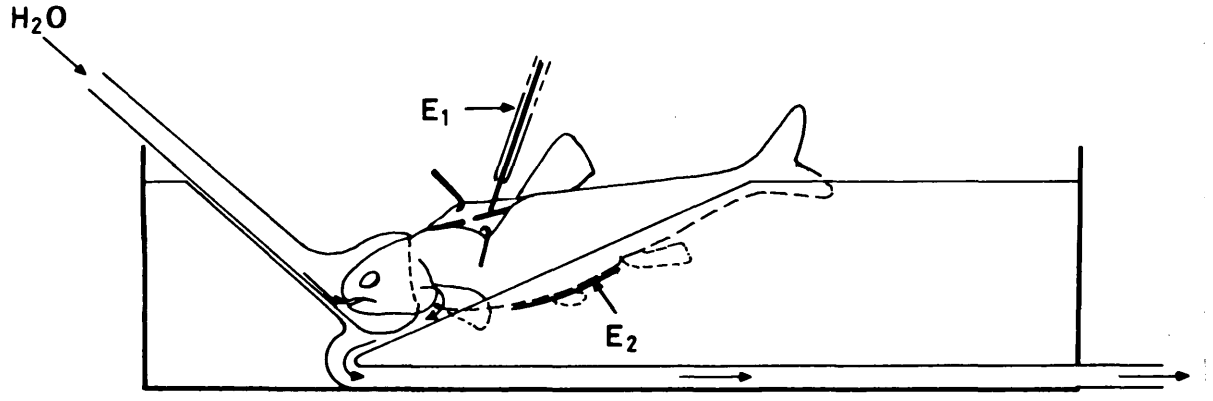
Fish were prepared for electrical stimulation according to the methods described (Chapter II, p. 70). The stimulating apparatus consisted of an operating tray built of Perspex measuring about 18 cm x 10 cm. A cell in which the fish to be stimulated could rest was built in the tray by means of paraffin wax and plasticene. The cell was designed so that the animal could be orientated with the body sloping down from the tail. This was done to facilitate drainage of the respiratory water from the site of stimulation thus avoiding any possible short circuits during the experiment. The respiratory water was maintained through a glass tubing drawn to accommodate the head comfortably and, at the same time, serve as a support to keep the fish in its upright position. The fish being spinal, settled well and did not struggle. The water was drained from the cell through an outlet by means of a groove at the side of the tray (Fig. V-2 p. 152). A small incision just posterior to the site of the first operation was made and the spinal cord was exposed. The wound was held apart by means of hooks. The stimulating electrode was placed on the spinal cord posterior to the point of the first section by means of a manipulator.

Fig. V-1

The apparatus used to study the effect of electrical stimulation on the spinal cord in living fishes.

E_1 - Active electrode

E_2 - Indifferent electrode


H_2O
E_1
E_2

5.2.5.2. Preparation of the stimulating electrodes (Ag/AgCl non-polarizable electrodes)

Fine silver wire ca. 3 cm in length and 22 S.W.G. was sealed with Araldite into clean soda-glass tubing ca. 5 - 6 mm o.d. and of a suitable length ca 15 cm. Care was taken to provide a length of wire ca 1.5 cm projecting from the end of the tube. The wire was kept free of Araldite as much as possible. Four of these electrodes were conveniently coated with silver chloride at a time. The outer projection of the wire was dipped in concentrated nitric acid (2 seconds), washed in distilled water and then in acetone. A ring of thick-walled rubber tubing was placed over each glass tube and the latter was then suspended through a hole in a piece of Perspex of such size that the rubber collar would not pass through. Into each tube mercury was poured to a depth of ca. 3 cm. By means of copper wires coated with mercury these tubes were connected to the anode (+) of a cell of approximately 1.5 V. The cathode of the cell led through a mercury-coated copper wire and mercury to a platinum plate sealed into a glass tube. The circuit also included a variable resistance and a milliameter. The silver electrode and the platinum plate were suspended in 1N HCl in a litre beaker. A current of ca. 0.2 mA for each 1.5 cm of 22 S.W.G. silver wire was passed until there was a clear coating of chloride. Then the current was increased to 1 mA. The total current passed per 1.5 cm of 22 S.W.G. silver was ca. 1 mA for 1 hour.

In order to avoid any damage which might be caused by

inserting a silver electrode into the spinal cord, the electrode was inserted in a soda-glass tube containing a Ringer-agar wick.

The indifferent electrode was a heavier silver wire (40 S.W.G.) placed beneath the body of the fish.

5.2.5.3. Stimulation parameters

The stimulation was by means of a mains operated square wave generator with a low output impedance manufactured by Scientific and Research Instruments Ltd. (335 Whitehorse Road, Croydon CRO 2HS, England) (mode 6020) giving a pulse of 2 ms width, with variable frequency and voltage.

5.3. Results

5.3.1. The effects of bretylium (an adrenergic neuron blocking agent) on various minnow preparations

5.3.2. Chromatically normal fish

Bretylium tosylate (Burroughs Welcome), is known to block specifically adrenergic neurons and results in a decrease in the output of the neurotransmitter (noradrenaline) to a very low level (Boura and Green, 1959). It does not block the effect of end organs to circulating catecholamines. In relatively high doses it exerts some sympathomimetic actions (Burn, 1963). Consistent with the results reported by previous workers (Healey and Ross, 1966; Grove, 1969a, b) injections of 2.85×10^{-5} moles/kg bretylium resulted in the darkening of the animal. This dose caused considerable

pigment dispersion in 15 minutes and this lasted for approximately one hour. Thereafter, the fish started to respond to an illuminated white background by aggregating the pigment granules and so becoming paler. Plate V-1 page 156 shows the response of chromatically normal white adapted fish to the above mentioned dose of bretylium. However, full adaptation of the treated fish to white background was not accomplished during the microscopic observations. Macroscopic observation of free swimming fish treated with bretylium revealed that, for these fish to achieve full white adaptation, 3 - 4 days were required.

5.3.3. Chromatically spinal fish (decentralized melanophores)

White adapted fish were subjected to spinal cord section anterior to the 15th vertebra. As a result of this operation the fish darkened. Consistent with Healey's (1965) observations, it was observed that sectioning of the spinal cord progressively more anterior to the 15th vertebra was followed by a greater extent of darkening. However, the above relation was limited to an area between just anterior to the level of the 15th vertebra and the level of the 10th vertebra. The site of section of the spinal cord in fish used in the experiments to be described was always anterior to the level of the 10th vertebra. After the operation the fish were adapted to a white background to obtain a condition in which the melanophores were disconnected from their central nervous control and at the same time the pigment granules within them are aggregated. To obtain the above condition, it was found

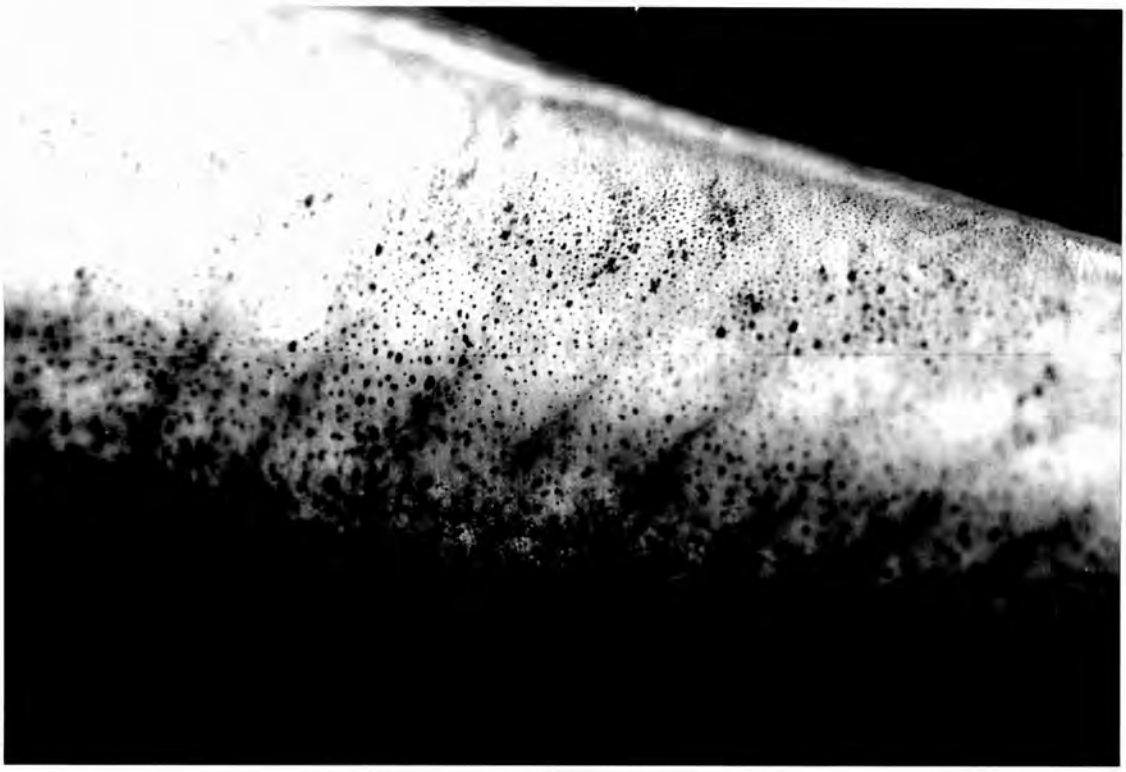
Plate V-1

Effects of bretylium on chromatically normal white-adapted *Phoxinus phoxinus*.

a - state of melanophores before the injection

b - state of melanophores 30 minutes after the injection (2.85×10^{-5} moles/kg).

Mag. X 15.



a

b

that the operated fish should spend at least 2 weeks on a white background. After this period the fish appeared to be fairly pale, but not as pale as chromatically intact fish on a white background.

Plate V-2 page 158 shows the response of a chromatically spinal fish paled on a white background to a dose of 3.13×10^{-5} moles/kg bretylium. As is evident from these photographs, no significant dispersion of the pigment can be observed. The failure of the above dose which caused very considerable pigment dispersion in chromatically normal fish, to bring about a clear pigment dispersion in chromatically spinal white adapted fish is well in line with the previous observations of Healey and Ross (1966) and Grove (1969a, b).

5.3.4. Melanophores separated from the spinal cord by spinal nerve section

Fish with spinal nerve section were adapted to an illuminated white background for a week to allow the separated melanophores to aggregate. This provided a condition in which a group of melanophores was at least, if not truly denervated, separated from central nervous control as well as the spinal cord and was surrounded by melanophores with intact innervation. To compare the effect of bretylium on melanophores with intact innervation and separated melanophores within the same fish, a dose of 3×10^{-5} moles/kg of bretylium was injected into the above mentioned fish. It was found that, as in chromatically intact fish, the above dose caused almost complete pigment dispersion in melanophores with intact innervation. Melanophores separated by section of the spinal nerves were not affected

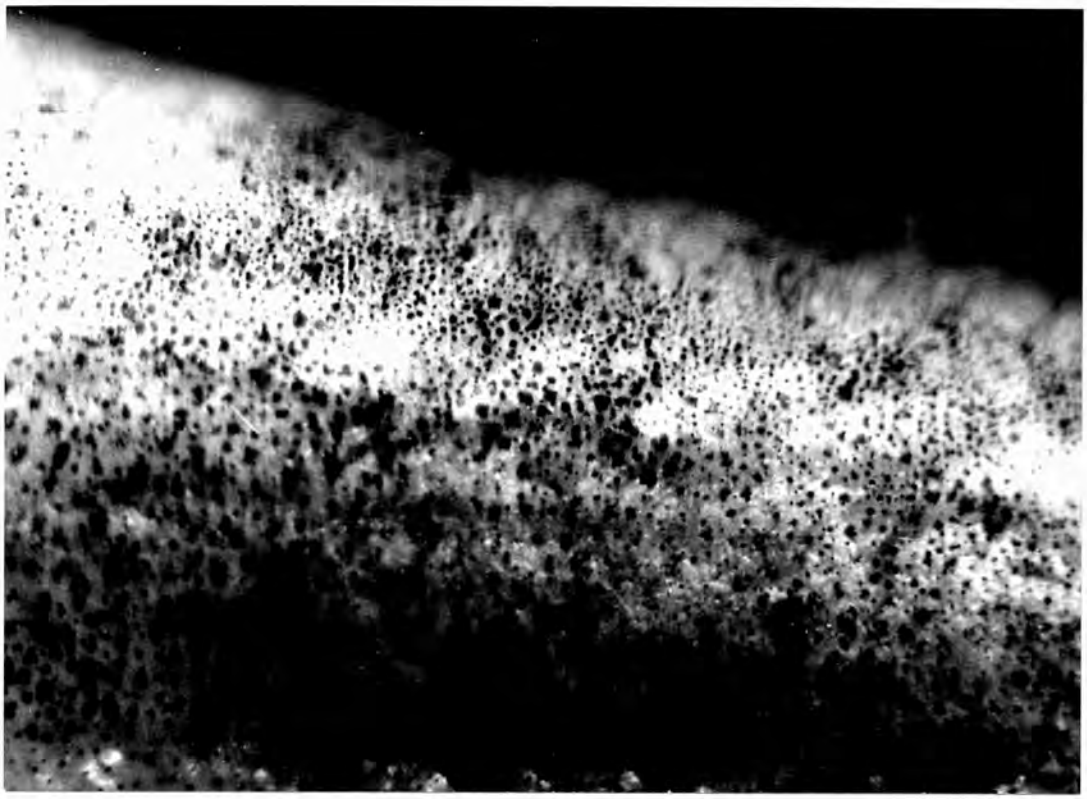
Plate V-2a, b

Effects of bretylium on chromatically spinal prolonged white-adapted *Phoxinus phoxinus*.

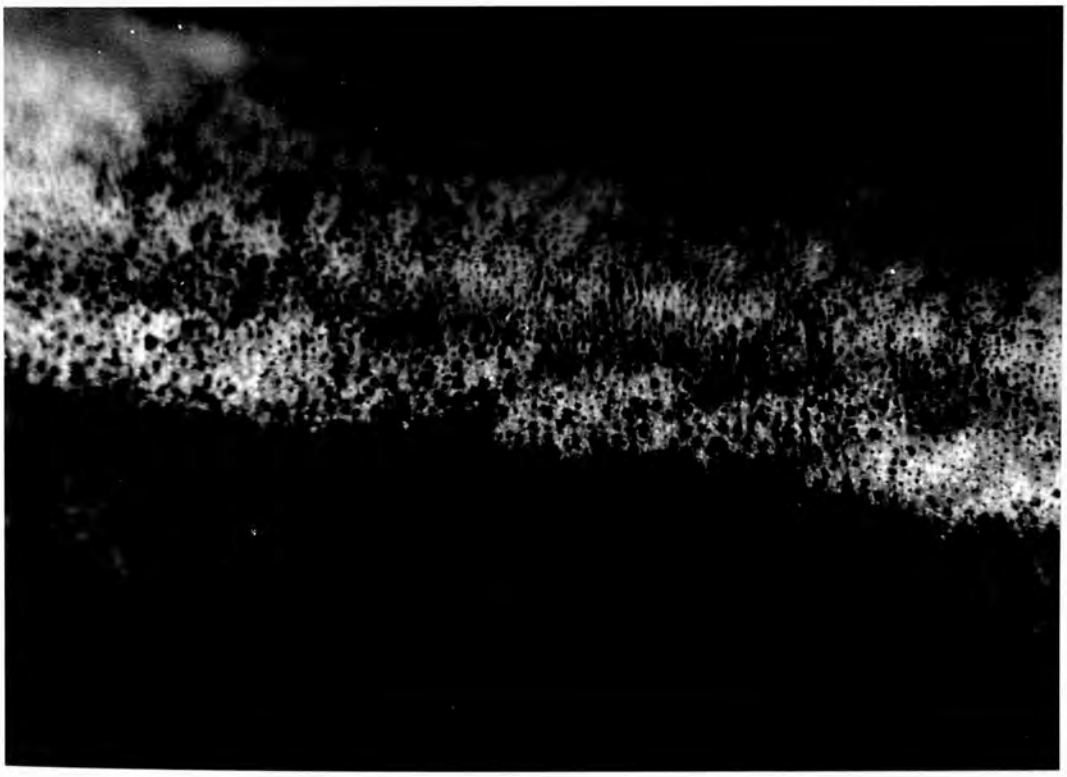
a - state of melanophores before the injection

b - state of melanophores 30 minutes after the injection (3.13×10^{-5} moles/kg).

Mag. X 15.



a



b

by the injection.

Plate V-3 page 160 shows the response of melanophores in the area affected by spinal nerve section and the neighbouring melanophores with intact innervation.

From the above results, it is evident that pigment dispersion following administration of bretylium is a nervously controlled response which is probably due to specific impulses arriving at the periphery from the centre. The failure of the pigment granules to disperse in the melanophores of chromatically spinal fish and in melanophores affected by spinal nerve section, treated with bretylium is probably due to the interruption of the chromatic fibres from the centre. However, one might ask the following question: Does bretylium act peripherally to impair the transmission at the level of the neuro-melanophore junction? To answer the above question the following experiments were undertaken.

5.3.5. Effects of electrical stimulation of the spinal cord on chromatically spinal white-adapted fish pretreated with bretylium

5.3.5.1. Preliminary experiments on untreated fish

Negative square pulses (5 - 10 V, 10 - 50 Hz., 2 ms wide, 30 - 60 sec.) produced complete pigment aggregation over the whole body surface in chromatically spinal black-adapted fish and intensified the degree of pigment aggregation in melanophores of chromatically spinal white-adapted fish. Cessation of the electrical

Plate V-3a, b

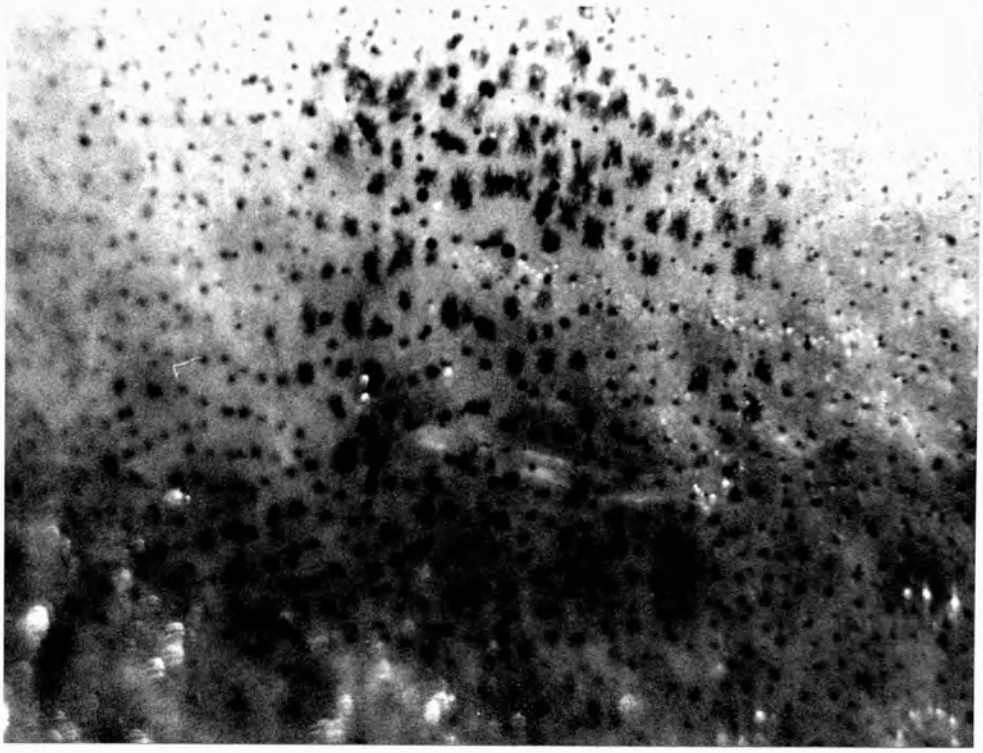
Effects of bretylium on melanophores affected by spinal nerve section and in which pigment granules are aggregated in response to prolonged white adaptation.

a - state of melanophores before treatment

b - state of melanophores after treatment (30 minutes), 3×10^{-5} moles/kg.

While the bretylium has caused full pigment dispersion in melanophores outside the area, it has failed to result in any pigment dispersion in melanophores within the affected area.

Mag. X 20.



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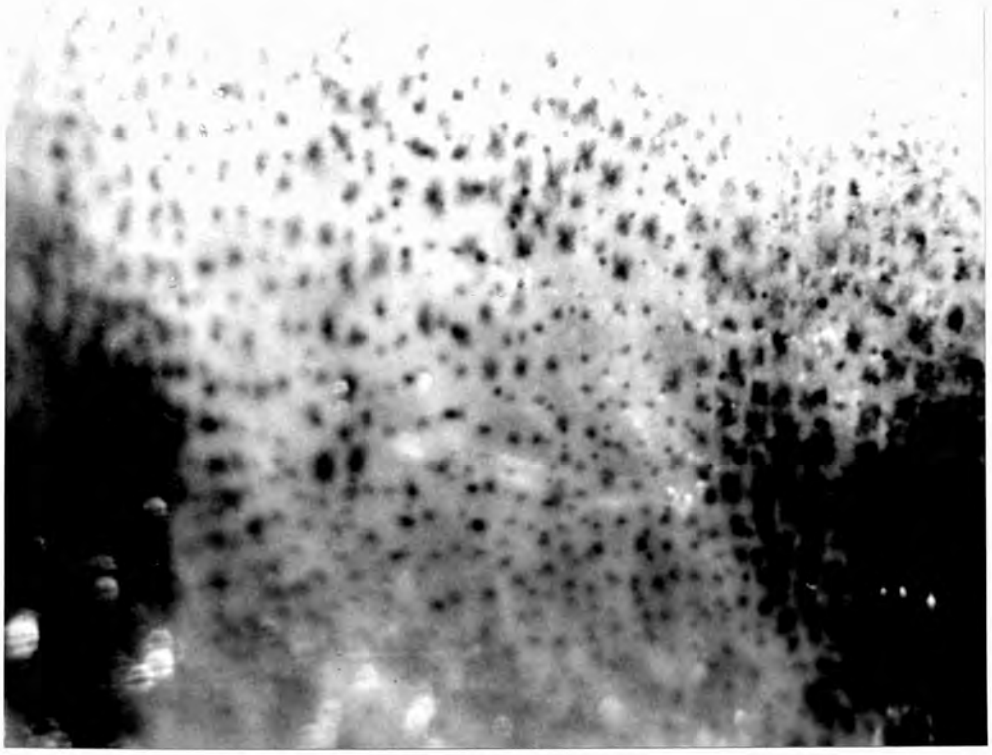


b

stimulation in the chromatic fibres, by removing the active electrode from the spinal cord and placing it on the muscle tissue at the side of the wound, resulted in redispersion of the pigment granules to their original states in both groups of fish.

By altering the parameter, it was observed that the magnitude and speed of the pigment aggregation are dependent on the frequency of the repetitive pulses. At 50 Hz. (5 V, 2 ms wide) pigment aggregation was fast (few seconds) and complete. At 10 Hz. (5 V, 2 ms wide) the pigment aggregation although still complete was very much slower (60 seconds). By decreasing the frequency still further, to 1 Hz. only slight pigment aggregation was observed and this was restricted to those melanophores outside the barred region of the skin.

The above results are in agreement with the results reported by Pye (1964a) on the effects of electrical stimulation on melanophores in *Phoxinus phoxinus*.

5.3.5.2. Spinal fish treated with bretylium

A chromatically spinal white-adapted fish was injected with a dose of 3.13×10^{-5} moles/kg of bretylium. Consistent with the previous observation, no significant pigment dispersion was found to follow the injection. Fifteen minutes after the injection, the fish was prepared for electrical stimulation of the spinal cord. It was found that all the parameters used in the previous experiment and which resulted in complete or incomplete pigment aggregation, depending on the rate of the pulse repetition, resulted in a

remarkable pigment dispersion in chromatically white-adapted fish pretreated with bretylium. However, the dispersion evoked by the electrical stimulation was persistent and was not followed by pigment aggregation, neither during electrical stimulation nor after cessation of the stimulation. Furthermore, the dispersion evoked by electrical stimulation in the chromatically white-adapted fish pretreated with bretylium was slower than the aggregation of pigment in melanophores of chromatically spinal black-adapted fish in response to electrical stimulation. The former was found to take 2 - 3 minutes to complete and the intensity of the darkening appeared not to depend on the frequency of the stimulation within the applied range (1 ~ 50 Hz.).

Control experiments were performed on fish injected with Ringer solution alone. Electrical stimulation of the spinal cord in these fish resulted in maximum pigment aggregation.

All the experiments described above were repeated at least 3 times using different fish and the results obtained were consistent.

5.3.6. Discussion of the action of bretylium

The above results clearly indicate that both the mechanisms, aggregation of pigment granules and the dispersion of these granules, are active mechanisms and nervously controlled. Also, the above results provide good reason to suggest that the site of action of bretylium in minnows, which results in pigment dispersion, is not at the centre but is probably at the periphery (postganglionic fibres), as has been shown to be the case in mammalian adrenergic nerves.

However, the nature of the mechanism mediating the active pigment dispersion remains unanswered.

How can the dispersion which follows the administration of the adrenergic blocking agent bretylium, after exposure of the fish to an illuminated black background, and sectioning in the chromatic fibres be accounted for? Several explanations have been put forward by various workers in an attempt to answer this question (Chapter IV, p. 142). In the present study the following experiments were designed to clarify the situation further.

5.4. Effects of adrenoceptor agonists and antagonists on the melanophores of chromatically normal and chromatically spinal minnows

5.4.1. Alpha and beta adrenoceptors agonists and antagonists

The term adrenoceptor is an abbreviation of adrenoreceptor and was first introduced by Bowman *et al.* (1968) to replace the term "adrenergic receptors". This new terminology has gained general acceptance and is widely used in recent publications. The terms adrenoceptor agonists and adrenoceptor antagonists are applied to those agents which stimulate and block the receptors respectively. Agents which stimulate adrenoceptors are catecholamines and other synthetic monoamines. Depending on their relative potencies, Ahlquist (1948) classified adrenoceptors into two main groups, Alpha-adrenoceptors and Beta-adrenoceptors. Adrenoceptor antagonists are available and these antagonists can specifically block one of

the above receptors. These antagonists, especially beta antagonists have a similar chemical structure to their corresponding agonists. Therefore, some of the adrenoceptor antagonists exert a slight intrinsic activity in addition to their main blocking effects.

5.4.2. Effects on chromatically normal spinal fish of alpha- and beta-adrenoceptor agonists: noradrenaline, adrenaline and isoproterenol

5.4.2.1. Noradrenaline

Noradrenaline has a great affinity to interact with alpha-adrenoceptors, but its affinity to interact with beta-adrenoceptors is very low and varies in different organs, e.g. noradrenaline stimulates beta-adrenoceptors in the heart but fails to stimulate beta-adrenoceptors of skeletal muscle vessels even after blockade of the alpha-adrenoceptors (Ginsburg and Cobbold, 1960). However, in the absence of the neuronal uptake, it has been shown that noradrenaline is only three times less active than isoprenaline (isoproterenol) in interacting with beta-adrenoceptors of guinea-pig heart and duodenum (Furchgott, 1967).

Studies on the effects of noradrenaline on teleost melanophores started soon after von Euler (1946) demonstrated that noradrenaline is the transmitter generally involved at the neuro-effector junctions in mammals. This monoamine is generally considered to have potent pigment aggregating effects. However, there is one exception to the above. Enami (1955) reported

that noradrenaline had a dispersing effect on the melanophores of *Parasilurus asotus*. This observation has recently been confirmed by Fujii and Miyashita (1976). Fujii and Miyashita further reported that pigment aggregation in this fish is also exceptionally mediated by acetylcholine.

As far as the effect of noradrenaline on minnow melanophores is concerned, it has always been reported to be a potent pigment-aggregating agent (Pye, 1964a; Healey and Ross, 1966; Grove, 1969a). In the present study, the above observations on the effect of noradrenaline on minnow melanophores was confirmed. Doses of 1.42×10^{-5} and 2.85×10^{-6} moles/kg injected into black-adapted fish were found to cause almost full pigment aggregation of pigment granules in about 15 minutes. The fish started to recover in about 45 minutes after the injection. Lower doses, 2.85×10^{-7} and 1.42×10^{-7} moles/kg caused incomplete pigment aggregation, melanophores of the lateral patches of the skin did not show complete pigment aggregation. This is probably due, as was mentioned earlier (page 126) to a higher threshold of neurotransmitter required by these melanophores to show complete pigment aggregation. Similar doses to those above and a lower dose of 2.85×10^{-8} moles/kg were found not to have any dispersing effect on chromatically normal white-adapted fish. Fig. V-3 page 167 shows the effect of various doses of noradrenaline on chromatically normal black-adapted minnows.

5.4.2.2. Adrenaline

Adrenaline interacts with both alpha- and beta-adrenoceptors.

Its potent aggregating effect on teleost melanophores has been reported long ago (Chapter I, p. 34). However, as has already been described for noradrenaline, there are some exceptions to the responses of teleost melanophores to adrenaline. Breder and Rasquin (1955), working on *Chaetodipterus* and Enami (1940, 1955), Fujii and Miyashita (1976) working on *Parasilurus asotus* reported that adrenaline had pigment dispersing effects on the melanophores of both these fish. On minnow melanophores, adrenaline is known to have a highly potent aggregating effect (Bray, 1918; Abolin, 1925; Pye, 1964a) and has been reported to be almost as potent as noradrenaline in causing pigment aggregation (Healey and Ross, 1966; Grove, 1969a).

In the present study, and in line with the results reported by the previous workers, adrenaline was found to be a potent aggregating agent. Doses of 1.42×10^{-5} moles/kg and 2.58×10^{-6} moles/kg were found to cause full pigment aggregation in 10 - 15 minutes, the fish started to recover in about 45 - 60 minutes after the injection. A dose of 2.85×10^{-7} moles/kg resulted in incomplete pigment aggregation, and a lower dose, 1.42×10^{-7} moles/kg, had no significant aggregation effect. Fig. V-4 page 167 shows the effect of various doses of adrenaline on chromatically normal black-adapted fish. The above doses injected into chromatically normal white-adapted fish did not result in any pigment dispersion.

The magnitude and duration of the response after treatment with adrenaline was found to be very similar to that after nor-

Fig. V-3

The effect of noradrenaline on chromatically normal black-adapted *Phoxinus phoxinus*.

○ 1.42×10^{-5} moles/kg; 18°C. (3 animals)

◒ 2.85×10^{-6} moles/kg; 18°C. (3 animals)

● 2.85×10^{-7} moles/kg; 17.5°C. (3 animals)

◑ 1.42×10^{-7} moles/kg; 17.5°C. (3 animals)

Fig. V-4

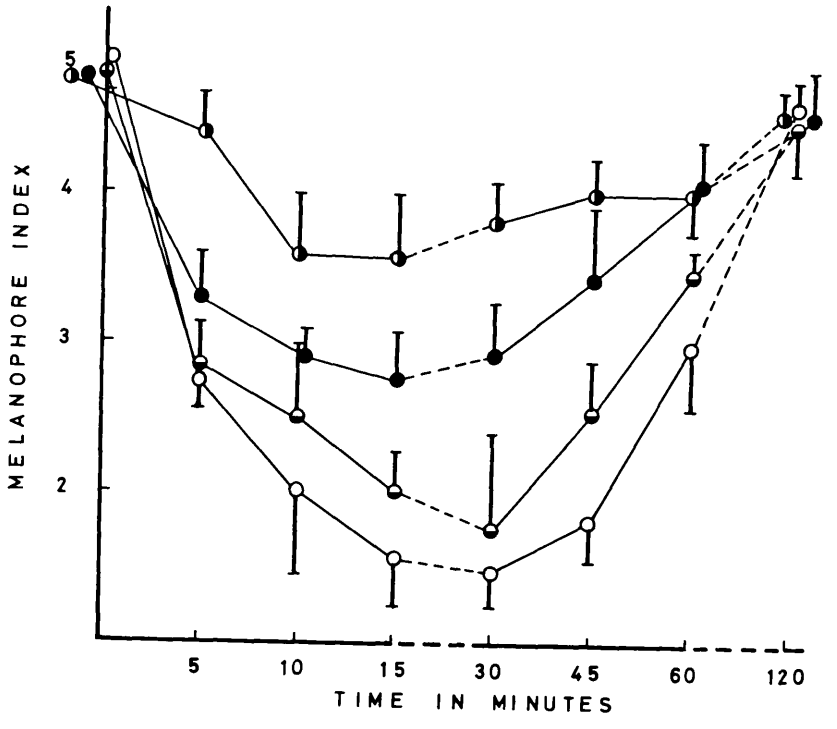
The effect of adrenaline on chromatically normal black-adapted *Phoxinus phoxinus*.

○ 1.42×10^{-5} moles/kg; 20°C. (3 animals)

◒ 2.85×10^{-6} moles/kg; 20°C. (3 animals)

● 2.85×10^{-7} moles/kg; 20°C. (3 animals)

◑ 1.42×10^{-7} moles/kg; 19°C. (3 animals)


MELANOPHORE INDEX
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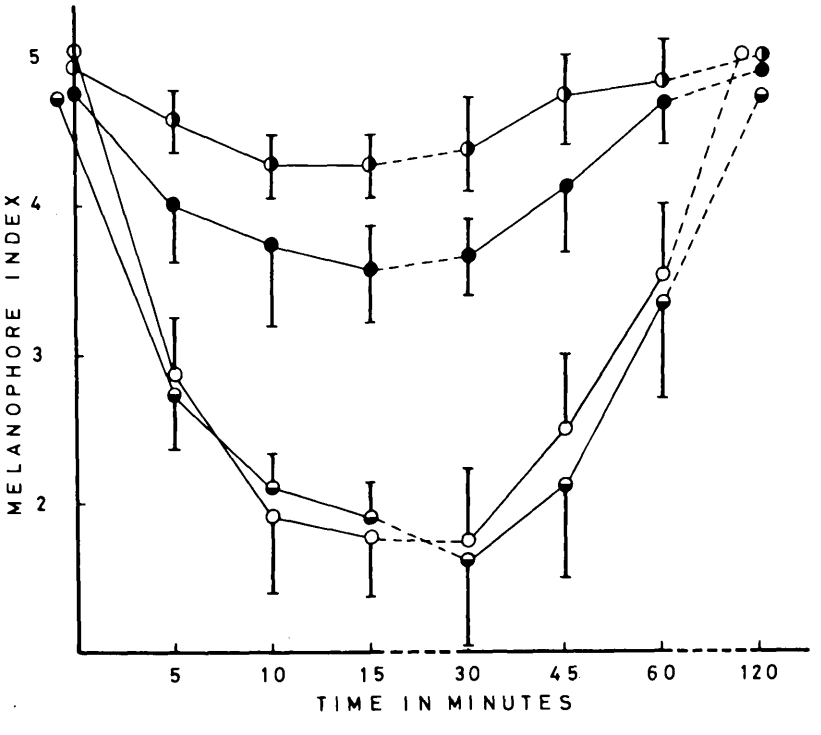
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TIME IN MINUTES

adrenaline, except in lower concentrations where noradrenaline appears to be more effective.

5.4.2.3. Isoproterenol (isoprenaline)

Isoproterenol is a synthetic monoamine. It is known to have a high affinity to interact with beta-adrenoceptors in mammalian tissues at nanomolar concentrations. Therefore, it is by far the most commonly used monoamine in classification of adrenoceptors along with noradrenaline. However, in much higher concentrations than those which initiate responses at beta-adrenoceptors, its (l)-isomer can activate alpha-adrenoceptors as well (Jenkinson, 1973).

Studies concerning the effects of isoproterenol on teleost melanophores are relatively recent. Healey and Ross (1966) reported that isoproterenol had an aggregating effect on black-adapted chromatically normal minnows. White-adapted fish were found not to be affected by isoproterenol chromatically. The above observation was confirmed by Grove (1967) working on the same species. Reed and Finnin (1972) and Miyashita and Fujii (1975) reported that isoproterenol had a dispersing effect on melanin granules of melanophores in *Pterophyllum eimekei* and *Lebistes reticulatus* respectively (Chapter I, p. 60). In the present study, various doses of isoproterenol were injected into the chromatically normal black-adapted and white-adapted fish. It was found that isoproterenol caused pigment aggregation in the melanophores of chromatically normal black-adapted fish. Doses of 1.42×10^{-5} and 2.85×10^{-6}

moles/kg were found to bring about incomplete pigment aggregation. Higher doses, 2.85×10^{-4} moles/kg however, resulted in almost full pigment aggregation. The above doses and lower doses of 2.85×10^{-7} and 2.85×10^{-8} moles/kg failed to bring about any pigment dispersion in chromatically normal white-adapted fish. Fig. V-5, page 170 shows the effect of various doses of isoproterenol injected into chromatically normal black-adapted minnows. Comparing the above mentioned figure to Figs. V-3 and V-4 page 167 on the effects of noradrenaline and adrenaline, it is evident that the doses of isoproterenol required to bring about pigment aggregation are much higher than those required of noradrenaline or adrenaline. The above results are in agreement with the results reported on the effects of this amine on the same fish (Healey and Ross, 1966; Grove, 1969a). The failure of isoproterenol in various doses to result in pigment dispersion in chromatically normal white-adapted fish does not indicate the presence of antagonistic beta-adrenoceptors mediating pigment dispersion. Grove (1967) suggested that adrenoceptors of minnow melanophores are either synergistic alpha- and beta-adrenoceptors or are primitive undifferentiated receptors.

However, the following alternative explanation can be put forward to explain the failure of isoproterenol to result in pigment dispersion. Exposure of chromatically normal minnows to an illuminated white background results in rapid and complete pigment aggregation. This aggregation is known to be mediated by alpha-adrenoceptors. In mammals, in tissues in which both the alpha- and beta-adrenoceptors are present and mediate antagonistic responses, the effect of one of

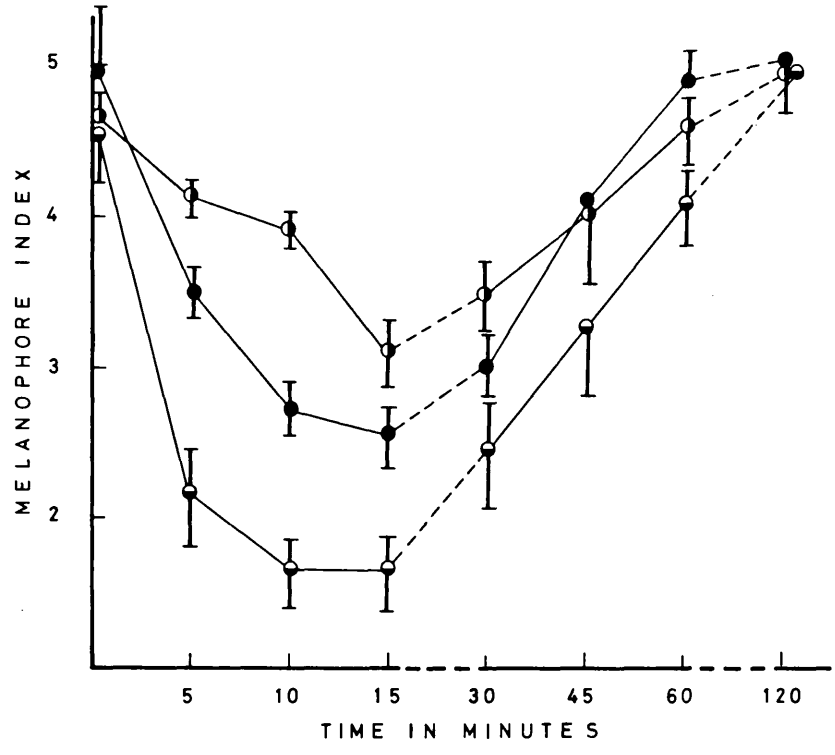
Fig. V-5

The effects of isoproterenol on chromatically normal black-adapted *Phoxinus phoxinus*.

● 1.42×10^{-5} moles/kg; 16°C. (3 animals,

◑ 2.85×10^{-6} moles/kg; 16°C. (3 animals)

◒ 2.85×10^{-4} moles/kg; 14°C. (3 animals)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the receptors predominates. For example, in regions of the alimentary tract where there are sphincters, alpha-excitatory adrenoceptors predominate over beta-inhibiting adrenoceptors (Furness and Burnstock, 1975). Should a similar situation be found at the neuro-melanophore junctions in minnows, then the failure of isoproterenol to result in pigment dispersion might well be due to the endogenously activated, predominant alpha-adrenoceptors mediating pigment aggregation. If the above explanation is true, then the assessment of melanophore antagonistic beta-adrenoceptor becomes complicated, because blocking of alpha-adrenoceptors by alpha-antagonists will result in the activation of the antagonistic beta-adrenoceptors by the endogenous neurotransmitter, once the predominant alpha-adrenoceptors are blocked. This will result in the full dispersion of pigment. The above situation is not, of course, suitable to test the dispersing effects of beta-agonists. To avoid the above complications, the following treatment can be suggested.

(1) Cessation of the impulses in the chromatic fibres in response to illuminated backgrounds surgically (chromatically spinal fish). Then, allowing the operated fish to adapt slowly to a white background, this results in varying degrees of pigment aggregation.

(2) Depletion of the stored neurotransmitter in the nerve endings at the neuro-melanophore junctions by an adrenergic depleter agent (reserpine).

(3) Using an adrenergic neuron blocking agent such as bretylium to block adrenergic transmission at the neuro-melanophore junctions.

The first suggestion of the above three might provide a situation in which assessment of the antagonistic beta-adrenoceptor could be possible, because adrenergic depletors and adrenergic neuron blocking agents by themselves result in pigment dispersion (Healey and Ross, 1966; Grove, 1967; page 154 of the present thesis). Therefore, based on the first suggestion, experiments were designed to test the possible presence of antagonistic beta-adrenoceptors in minnow melanophores.

However, before commencing the above experiments, the following question should be dealt with: How can the aggregating effects of isoproterenol be accounted for?

As was already described, the (l)-isomer of isoproterenol does interact with alpha-adrenoceptors in relatively high concentrations (page 168). It was found that for isoproterenol to cause pigment aggregation, relatively high doses were required (page 169). The above pigment aggregation might be as a result of its interaction with alpha-adrenoceptors of minnow melanophores. This can be tested by treating the fish with alpha-adrenoceptor antagonists, followed by isoproterenol in doses which cause significant pigment aggregation in untreated fish.

5.4.2.4. Effects of alpha-adrenoceptor blocking agents combined with alpha- and beta-adrenoceptor agonists

5.4.2.4.1. Yohimbine

Yohimbine was the second alpha-adrenoceptor blocking

agent to be discovered after ergot alkaloids by Raymond-Hamet (1925). This alpha-adrenoceptor antagonist blocks competitively the response of end organs to both sympathetic nerve stimulation and injected adrenaline (Nickerson, 1949).

Earlier workers have shown that yohimbine is one of the most effective agents to cause pigment dispersion in minnow melanophores. Injections of yohimbine were found to cause almost full pigment dispersion within 10 - 30 minutes and persisted for many hours (Healey and Ross, 1966; Grove, 1969a). However, while the latter worker found that injections of 3.9×10^{-8} moles/g of noradrenaline reversed the effect of yohimbine (injected one hour earlier) completely in 30 minutes, the former workers reported that injections of adrenaline in minnows with dispersed pigment as a result of treatment with yohimbine, did not cause significant pigment aggregation in the melanophores.

In the present study, injections of 2.85×10^{-5} moles/kg yohimbine were found to cause full pigment dispersion in chromatically normal white-adapted fish. The dispersion was rapid and was almost complete in 15 minutes. Macroscopic observations on fish treated with yohimbine revealed that the dispersion lasted for as long as 2 - 3 days. Fig. V-6 page 176 shows the effect of yohimbine on melanophores of white-adapted fish. In another series of experiments, 30 minutes after treatment of chromatically normal white-adapted fish with yohimbine (2.85×10^{-5} moles/kg) they were injected with one of the following adrenoceptor agonists, noradrenaline,

adrenaline or isoproterenol (Fig. V-7 p. 177 ; Plate V-4 p. 178). Of the above adrenoceptor agonists, noradrenaline was found to be the most potent in reversing the dispersing effects of yohimbine. Adrenaline was found to have slight reversing effects in relatively high doses. Isoproterenol failed completely to reverse the effect of yohimbine in doses up to 2.85×10^{-5} moles/kg. Doses of 2.85×10^{-4} moles/kg isoproterenol were found to bring about some pigment aggregation. The above results confirm the previous observations of Healey and Ross (1966) and Grove (1969a) on the effects of yohimbine on minnow melanophores and the effects of adrenaline and noradrenaline on melanophores of fish treated with yohimbine. Also, the above experiments indicate that the aggregating effects of isoproterenol are probably due to its interaction with alpha-adrenoceptors because its effects were antagonised by yohimbine. However, in order to gain more confidence in the above results, similar experiments to the above were repeated using another adrenoceptor blocking agent, tolazoline.

5.4.2.4.2. Tolazoline

Tolazoline is an imidazoline derivative and is known as a short-acting competitive alpha-adrenoceptor blocking agent which is easily antagonised by relatively large doses of noradrenaline. This blocking agent is chemically related to histamine and to the sympathomimetic agent naphazoline. Therefore, it exerts some histamine-like action and some adrenoceptor stimulating effects (see Lewis's Pharmacology, 1970; Ahlquist, 1976). The effect of

tolazoline on minnow melanophores has not yet been investigated and not many reports are available on the effect of this agent on the melanophores of other teleosts. Abbott (1968) reported that tolazoline has a pigment-dispersing effect on the melanophores of *Fundulus heteroclitus in vivo* and *in vitro*. This author has also reported that the dispersion caused by tolazoline can be reversed by adrenaline or noradrenaline. More recently, Fujii and Miyashita (1975) reported that tolazoline had antagonistic effects on pigment aggregation in response to nervous stimulation or perfused monoamines in split fin melanophores of *Lebistes reticulatus*.

In the present study the effect of tolazoline on minnow melanophores was investigated. A preliminary dose of 2.85×10^{-6} moles/kg in a number of free swimming white-adapted fish resulted in intense darkening of the fish in about 10 - 15 minutes. The fish started to recover from the effect of tolazoline in about one hour. Comparison of the duration of the dispersion caused by tolazoline with that caused by yohimbine draws attention to the evident short-acting effect of the former. A higher dose of tolazoline (2.85×10^{-5} moles/kg) was found to be lethal.

Fig. V-6 page 176 shows the responses of chromatically normal white-adapted fish to a dose of 2.85×10^{-6} moles/kg tolazoline. The dispersion evoked by the above dose of tolazoline was readily reversed by doses of noradrenaline or adrenaline. However, isoproterenol in doses up to 2.85×10^{-5} moles/kg failed to bring about significant pigment aggregation in fish pretreated with tolazoline (Fig. V-8 p. 177; Plate V-5 p. 179).

Fig. V-6

The effect of yohimbine and tolazoline on chromatically normal white-adapted *Phoxinus phoxinus*.

○ yohimbine 2.85×10^{-5} moles/kg; $12^{o}C$. (3 animals)

● tolazoline 2.85×10^{-5} moles/kg; $16^{o}C$. (3 animals)

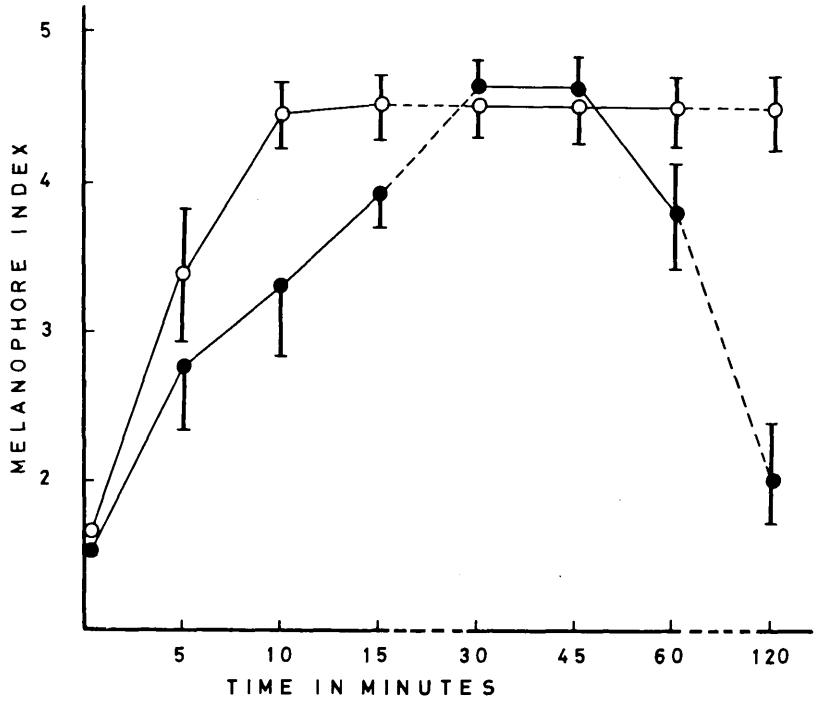

MELANOPHORE INDEX
5
4
3
2
5
10
15
30
45
60
120
TIME IN MINUTES

Fig. V-7

The effects of noradrenaline, adrenaline and isoproterenol on chromatically normal white-adapted *Phoxinus phoxinus* pretreated with yohimbine (2.85×10^{-5} moles/kg).

○ noradrenaline 2.85×10^{-6} moles/kg; 18^{o}C. (3 animals)

● adrenaline 2.85×10^{-6} moles/kg; 18^{o}C. (3 animals)

◒ isoproterenol 2.85×10^{-5} moles/kg; 18.5^{o}C. (3 animals)

Fig. V-8

The effects of noradrenaline, adrenaline and isoproterenol on chromatically normal white-adapted *Phoxinus phoxinus* pretreated with tolazoline (2.85×10^{-5} moles/kg).

○ noradrenaline 2.85×10^{-6} moles/kg; 14^{o}C. (3 animals)

● adrenaline 2.85×10^{-6} moles/kg; 14^{o}C. (3 animals)

◒ isoproterenol 2.85×10^{-5} moles/kg; 14^{o}C. (3 animals)

Fig.7

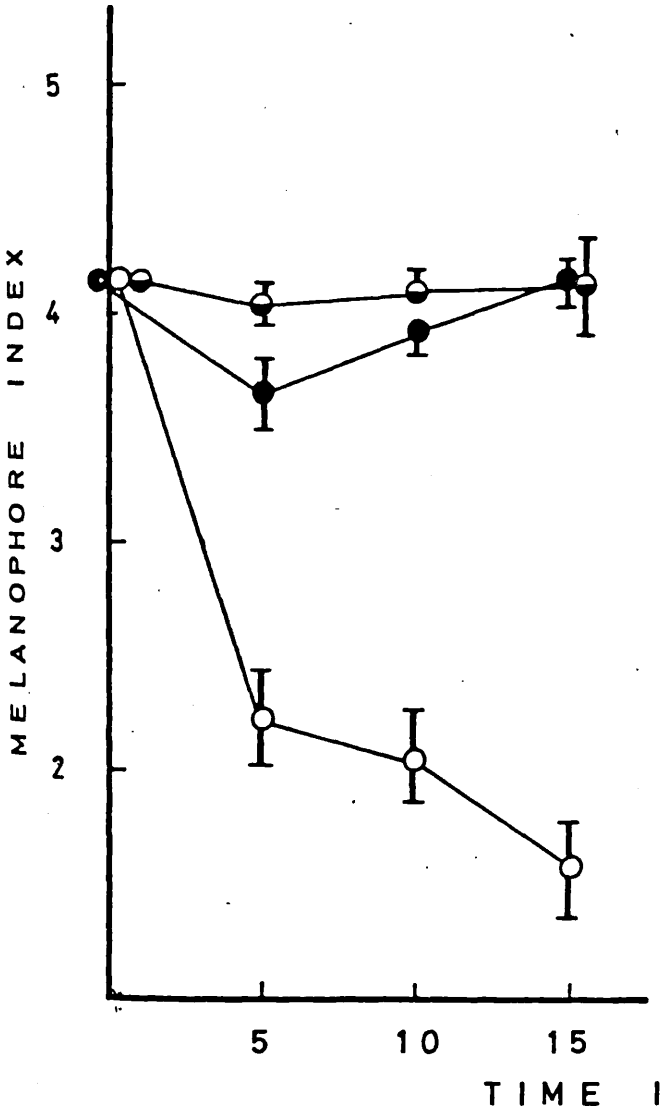


Fig.8

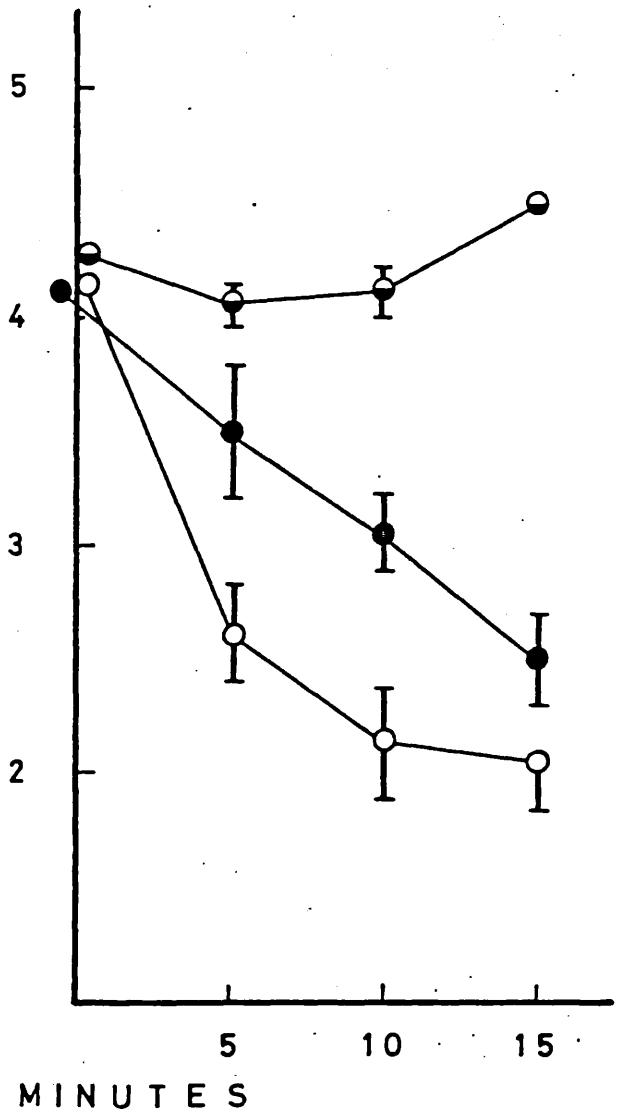


Plate V-4

Shows the dispersion effect of yohimbine (2.85 x 10^{-5} moles/kg) on a chromatically normal white-adapted *Phoxinus phoxinus* and the failure of isoproterenol to reverse the dispersion.

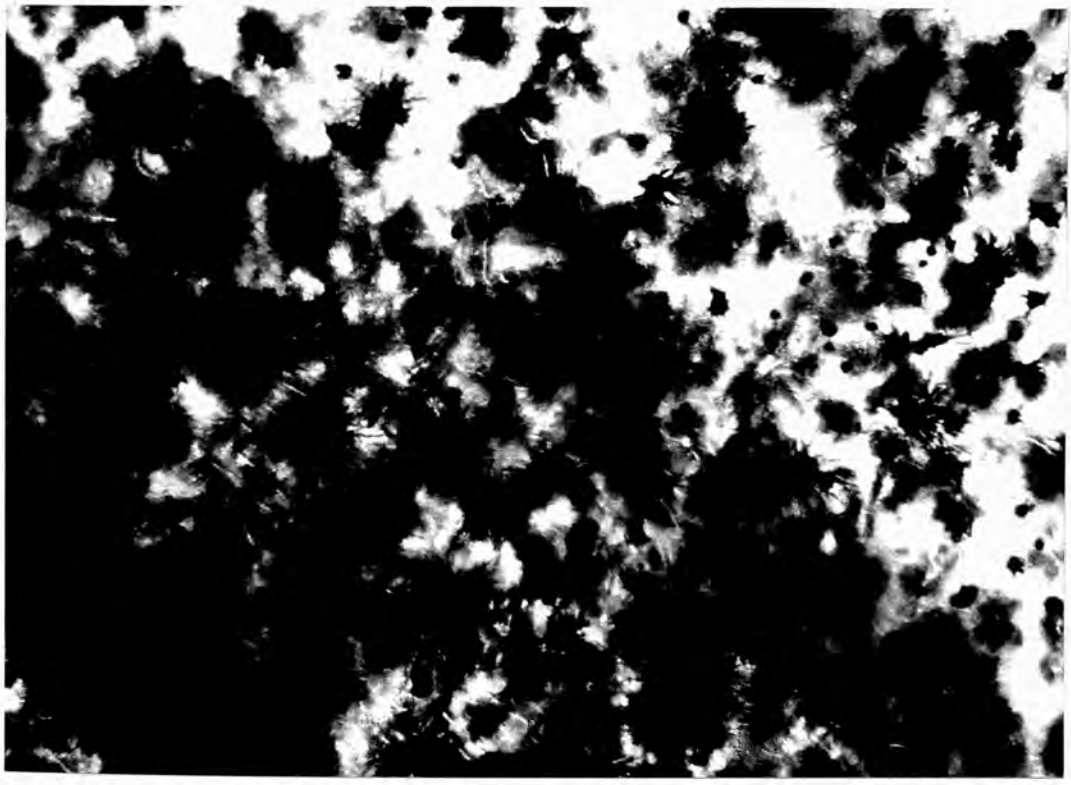
a - state of melanophores in the fish, in response to an illuminated white background in the continuous observation apparatus (30 minutes)

b - state of melanophores after treating the fish with the above dose of yohimbine (30 minutes)

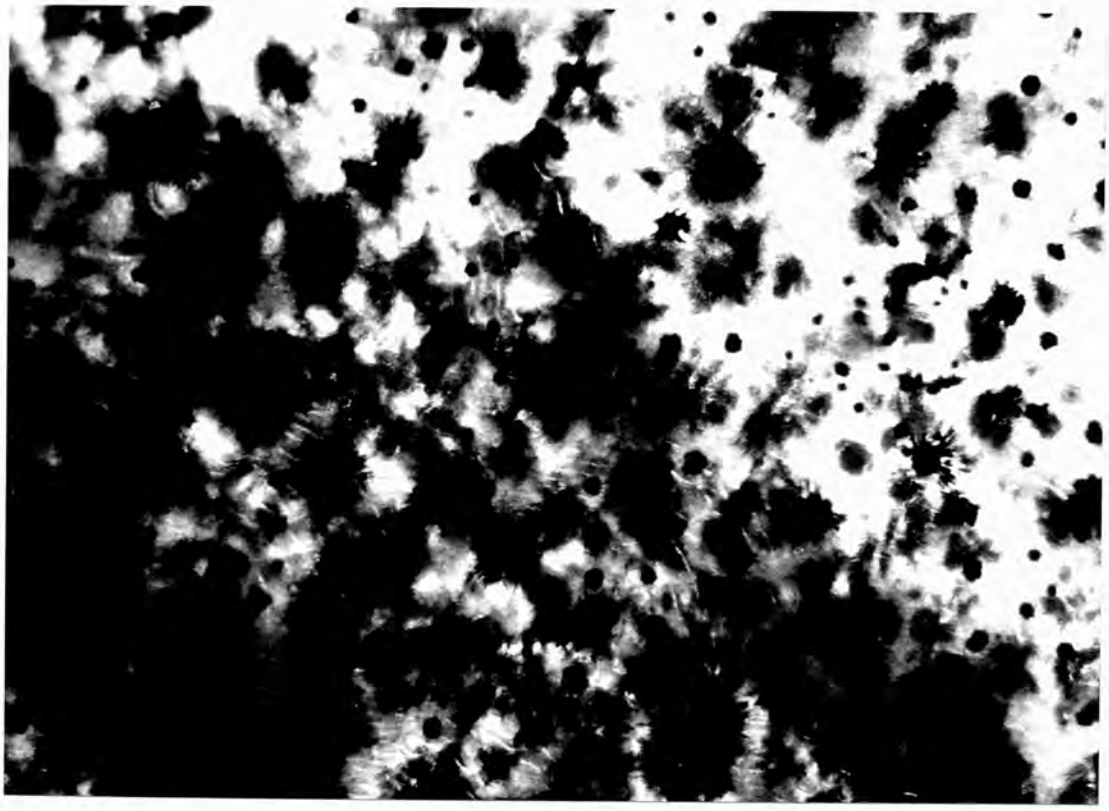
c - state of melanophores after a subsequent dose of isoproterenol; 2.85 x 10^{-5} moles/kg (30 minutes)

Mag. X 110.

a



b



c

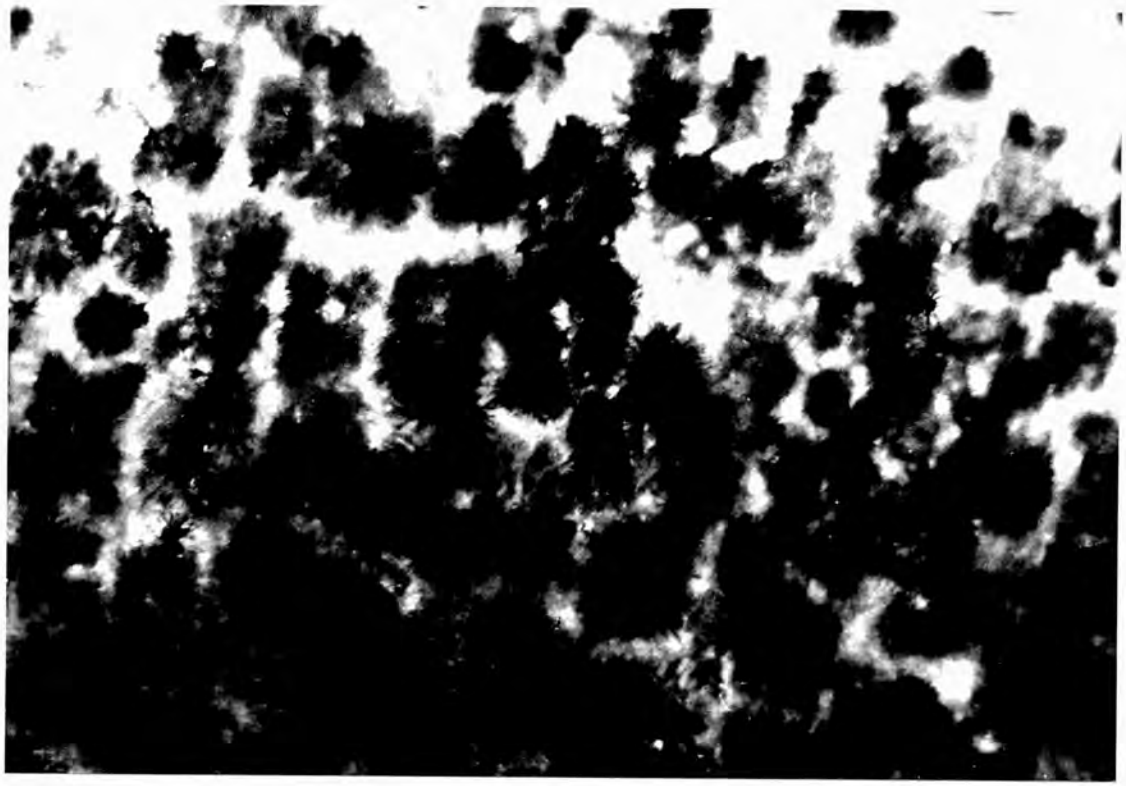
Plate V-5a, b

Shows the dispersing effect of tolazoline (2.85 x 10^{-5} moles/kg) on a chromatically normal white-adapted *Phoxinus phoxinus* and the failure of isoproterenol to reverse the dispersion.

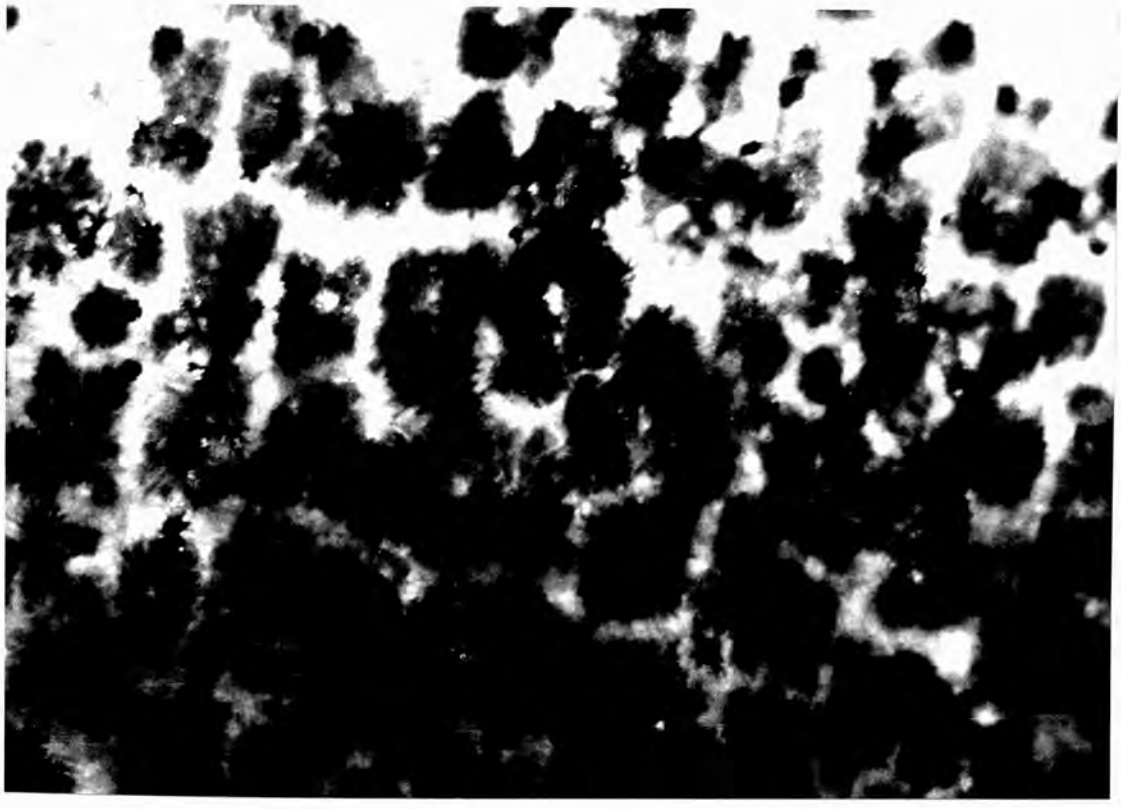
a - state of melanophores after treating the fish with the above dose of tolazoline (30 minutes)

b - state of melanophores after a subsequent dose of isoproterenol 2.85 x 10^{-5} moles/kg. (30 minutes)

Mag. X 110.



a



b

The above experiments with the alpha-adrenoceptor blocking agents yohimbine and tolazoline seem to provide an answer to the question regarding the pigment-aggregating effect of isoproterenol (page 172). The failure of isoproterenol to bring about significant pigment aggregation in fish pretreated with either of the alpha-adrenoceptor blocking agents strongly indicates that the pigment aggregation caused by isoproterenol is mediated by alpha-adrenoceptors.

5.4.3. Effects on chromatically spinal fish (decentralised melanophores) of alpha- and beta-adrenoceptor agonists

Chromatic spinal sectioning was performed on a group of fish which came from a white sink mottled with black according to the method already described (Chapter II, p. 69). The site of the operation was at the level of about vertebra 10. Some of the fish after the operation were transferred to illuminated white backgrounds and some others were transferred to illuminated black backgrounds. Three weeks after the operation the pigment granules in the melanophores of the white-adapted fish were found to be almost completely aggregated and the pigment granules in the melanophores of black-adapted fish appeared to be fully dispersed.

5.4.3.1. Preliminary macroscopic observations

At the commencement of these series of experiments, preliminary macroscopic observations on the effects of isoproterenol, adrenaline and noradrenaline on the melanophores of chromatically spinal, free-swimming fish were made.

A dose of 3.1×10^{-4} moles/kg isoproterenol injected into chromatically spinal black-adapted fish was found to cause pigment aggregation. The intensity and duration of the pigment aggregation as judged by macroscopic observation appeared to be similar to the effect of the same dose on chromatically normal black-adapted fish. Injection of the above dose into chromatically spinal white-adapted fish did not result in any significant macroscopic change in the state of pigment granules aggregated in response to the prolonged white background adaptation. However, an interesting observation was made when a lower dose of isoproterenol was injected into chromatically spinal white-adapted fish. A dose of 3.1×10^{-8} moles/kg of isoproterenol was found to cause significant pigment dispersion. The dispersing effect was observed about ten minutes after the injection and was maximal after an hour. It lasted for more than two hours and then slowly decreased. The fish were found to have recovered from the dispersal effect of isoproterenol by the following day. The above experiments were repeated to test the possible dispersing effects of noradrenaline and adrenaline on chromatically spinal white-adapted fish. A dose of 3.1×10^{-6} moles/kg of either adrenaline or noradrenaline was found to have a significant aggregating effect on the melanophores of chromatically spinal black-adapted fish, while lower doses of adrenaline 1.55×10^{-8} moles/kg and 3.1×10^{-8} moles/kg were found to have some dispersing effects on the chromatically spinal white-adapted fish, but similar doses of noradrenaline were not found to result in any change in the shade of fish with similar experimental conditions to those above. Based on

the above results, microscopic observation on the effects of the adrenoceptor agonists, isoproterenol, adrenaline and noradrenaline on chromatically spinal white-adapted fish was undertaken.

5.4.3.2. Microscopic observations

The preliminary observations on the effects of adrenoceptor agonists on chromatically spinal fish melanophores provided a guide line for the doses to be used to evoke pigment dispersion, except for noradrenaline which failed to result in pigment dispersion in the various doses used.

Fig. V-9 page 184 shows the effect of various doses of isoproterenol (in lower ranges) on chromatically spinal white-adapted fish. As is evident from the above mentioned figure, a dose of 3.1×10^{-8} moles/kg is the most effective in eliciting the dispersion of pigment granules within the melanophores. Plate V-6 page 186 also shows the dispersion of pigment granules, evoked by the most effective dose. Consistent with the visual observations in the preliminary experiments, dispersion of the pigment granules started about ten minutes after the injection and reached the peak in about 30 minutes. The dispersion evoked by the various doses differed in intensity. However, the responses were almost of the same duration and the fish were found to return to their preinjected shade by the following day. The latter observation was based on the macroscopic appearance of the fish.

Figs. V-10, 11 pages 184 & 185 show the effects of various doses of

noradrenaline and adrenaline on chromatically white-adapted fish. As is evident from the above figure, no dispersion has been evoked by noradrenaline at any of the various doses examined. However, consistent with the macroscopic observations, adrenaline in doses of 1.55×10^{-8} moles/kg and 3.1×10^{-8} moles/kg did bring about some pigment dispersion. The dispersion evoked by adrenaline is much less in magnitude than that brought about by similar doses of isoproterenol (Fig. V-10 p. 184; Plate V-7 p. 187). However, as far as the duration of the response is concerned, it is similar to the treatment with isoproterenol, i.e. the response was long lasting.

5.4.4. Indication of the presence of beta-adrenoceptors mediating pigment dispersion

The remarkable dispersion of the pigment granules following treatment of chromatically spinal white-adapted fish, with relatively low doses of isoproterenol and, to a lesser extent, following treatment with low doses of adrenaline, indicates the existence of beta-adrenoceptors mediating pigment dispersion in minnow melanophores. However, if the dispersion of pigment granules in chromatically spinal white-adapted fish following injections of beta-adrenoceptor agonists is as a result of their interaction with the beta-adrenoceptors, then it should be possible to antagonise this dispersion by beta-adrenoceptor antagonists. Therefore, experiments were carried out to study the effects of pretreatment with a beta-adrenoceptor antagonist on the potency of the adrenoceptor agonists in bringing about pigment dispersion.

Fig. V-9

The effects of isoproterenol in lower doses on chromatically spinal white-adapted *Phoxinus phoxinus*.

● 3.1×10^{-7} moles/kg; 14^{o}C. (3 animals)

○ 3.1×10^{-8} moles/kg; 14^{o}C. (3 animals)

◒ 9.3×10^{-9} moles/kg; 14^{o}C. (3 animals)

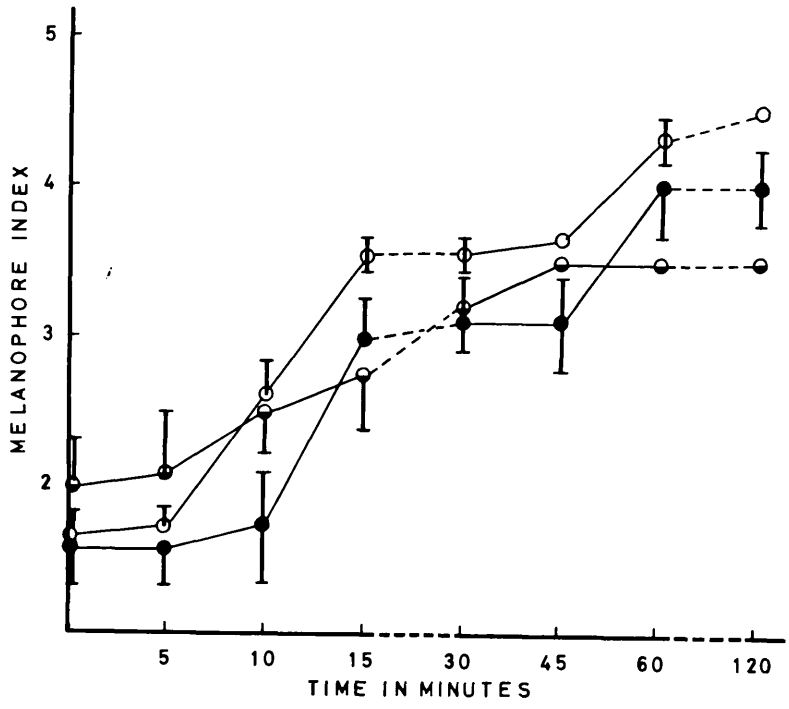
Fig. V-10

The effects of adrenaline in lower doses on chromatically spinal white-adapted *Phoxinus phoxinus*.

○ 3.1×10^{-7} moles/kg; 12^{o}C. (3 animals)

◒ 3.1×10^{-8} moles/kg; 12^{o}C. (3 animals)

● 1.55×10^{-8} moles/kg; 12^{o}C. (3 animals)


MELANOPHORE INDEX
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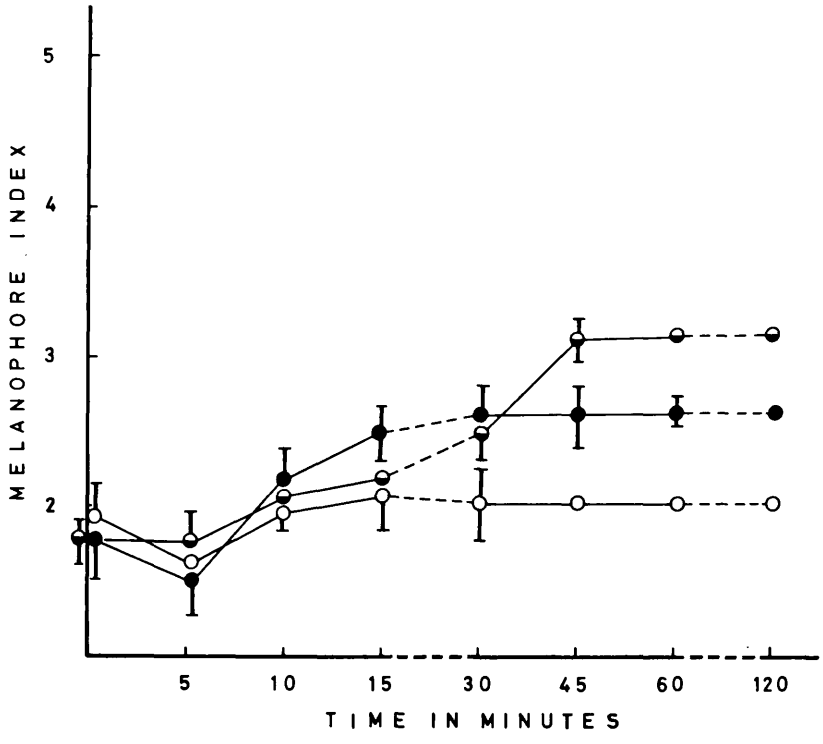
60
120
TIME IN MINUTES


MELANOPHORE INDEX
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45
60
120
TIME IN MINUTES

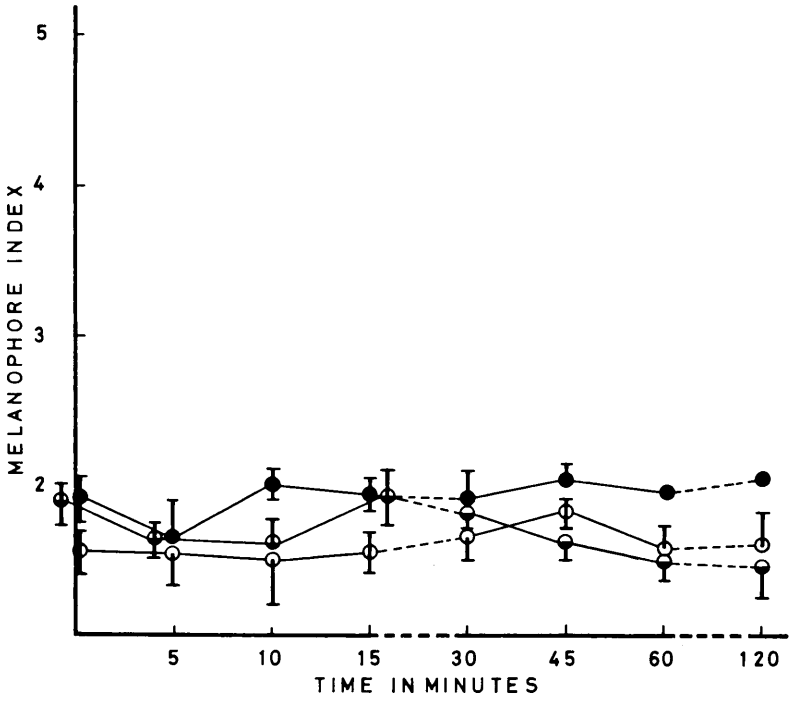
Fig. V-11

The effects of noradrenaline in lower doses on chromatically spinal white-adapted *Phoxinus phoxinus*.

○ 3.1×10^{-7} moles/kg; 12^{o}C. (3 animals)

◒ 3.1×10^{-8} moles/kg; 12^{o}C. (3 animals)

● 1.55×10^{-8} moles/kg; 12^{o}C. (3 animals)


MELANOPHORE INDEX
5
4
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2
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10
15
30
45
60
120
TIME IN MINUTES

Plate V-6a, b, c

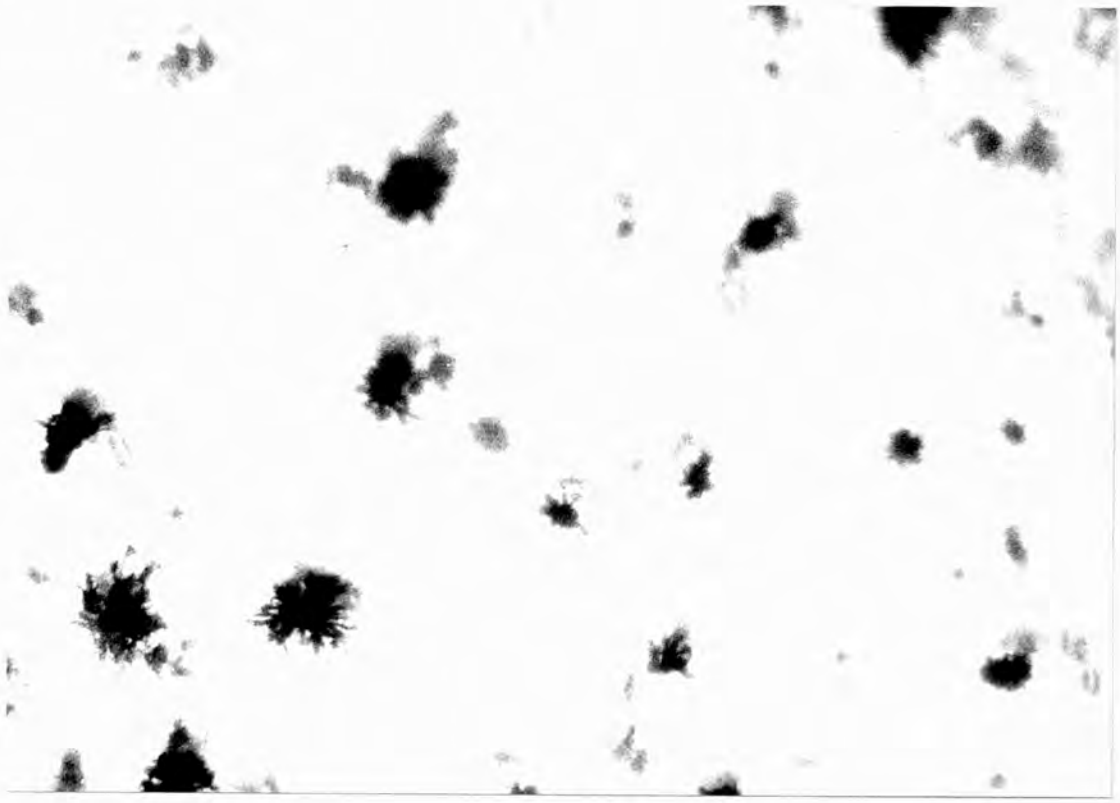
The dispersing effect of isoproterenol (3.1×10^{-8} moles/kg; $14^{o}C$) on melanophores of a chromatically spinal white-adapted *Phoxinus phoxinus*.

a - state of melanophores in the fish after being kept for two weeks on a white background and 30 minutes exposed to a white background in the continuous observation apparatus

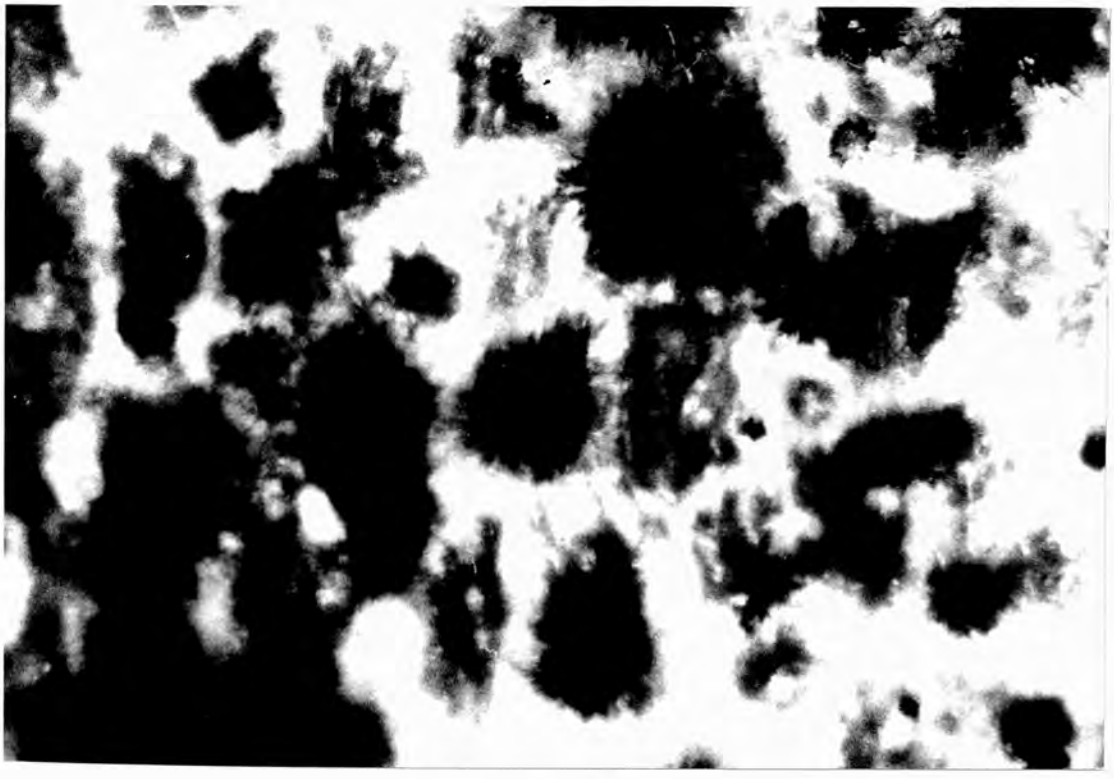
b - state of the melanophores ten minutes after injecting the fish with the above dose of isoproterenol

c - state of the melanophores 30 minutes after injecting the fish with the above dose of isoproterenol

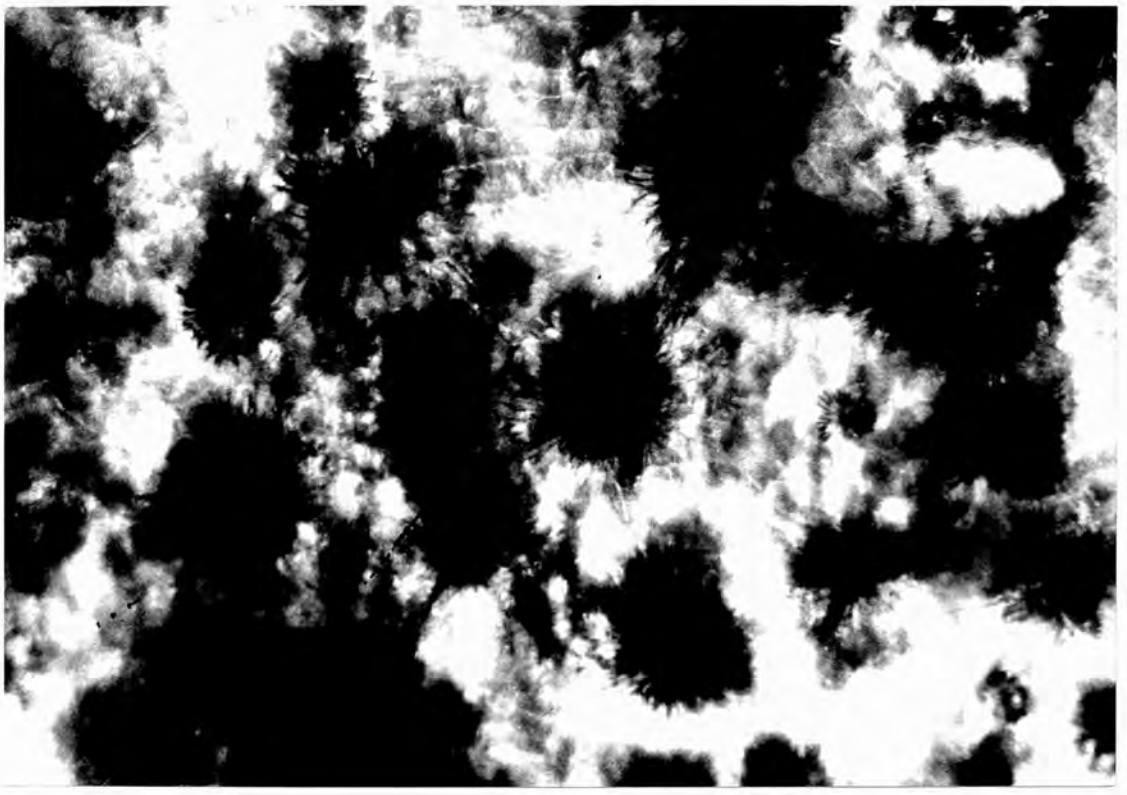
Mag. X 200.



a



b



c

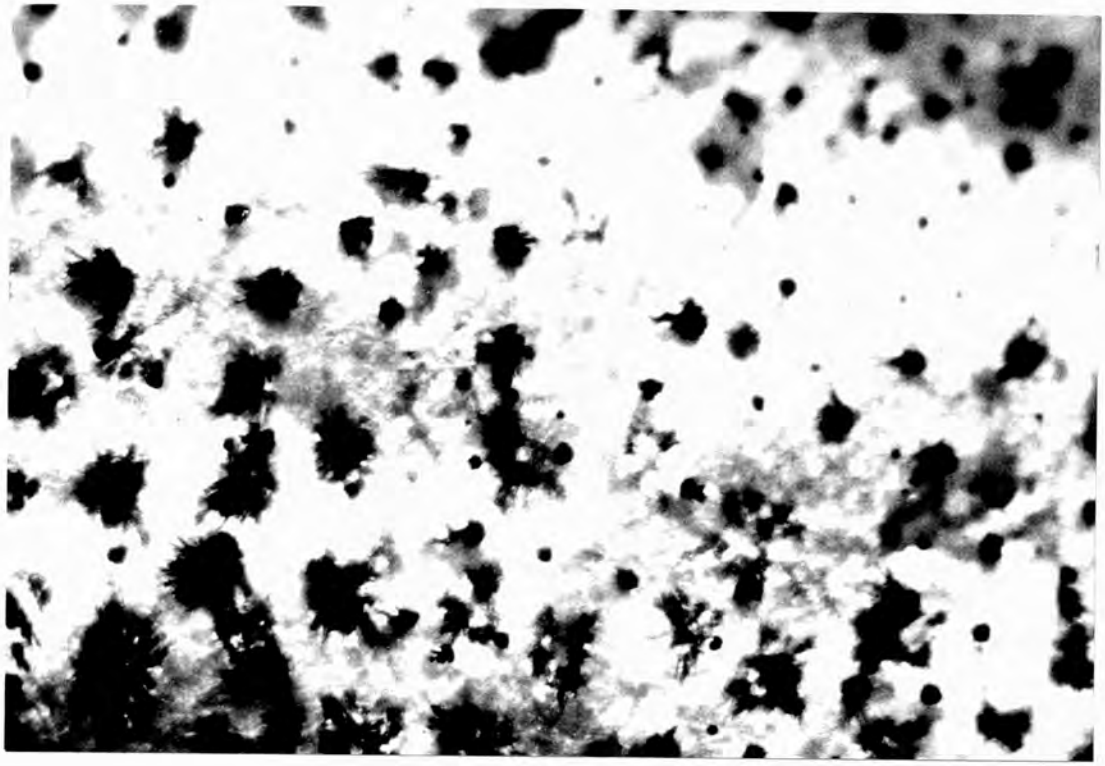
Plate V-7a, b

The dispersing effect of adrenaline (3.1×10^{-8} moles/kg; $12^{o}C$) on melanophores of a chromatically spinal white-adapted *Phoxinus phoxinus*.

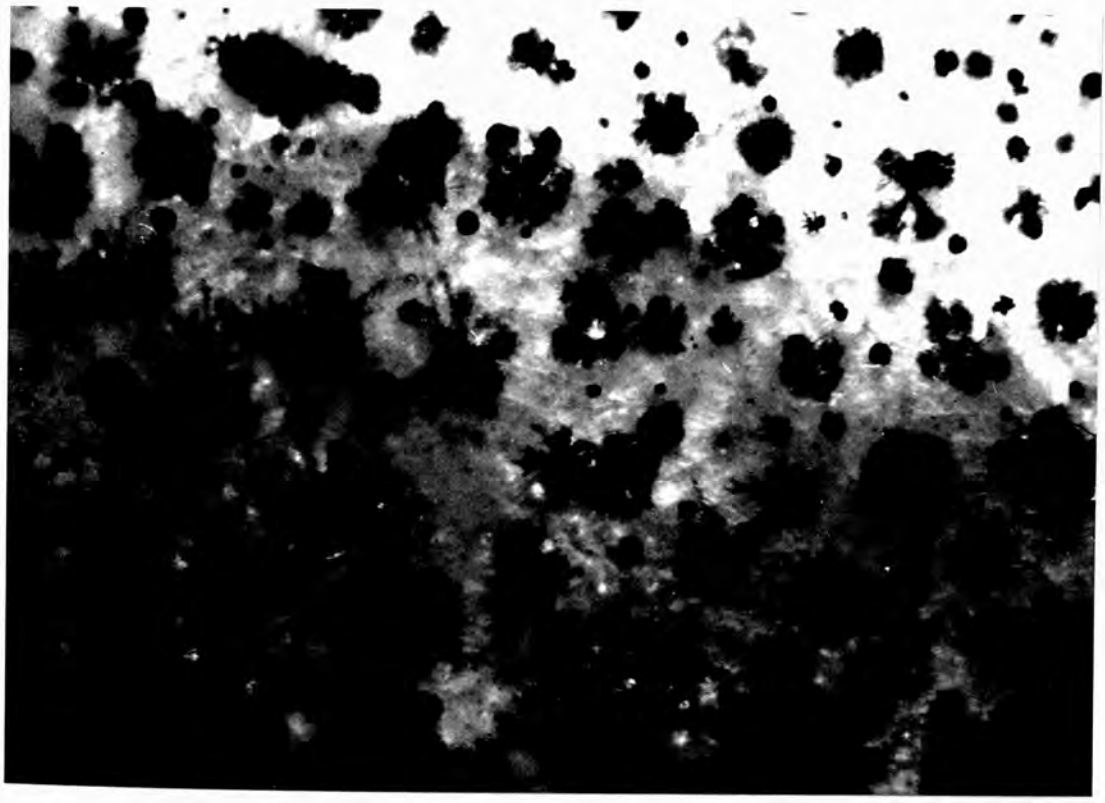
a - state of melanophores in the fish after being kept for two weeks on a white background and 30 minutes exposed to a white background in the continuous observation apparatus

b - state of the melanophores 30 minutes after injecting the fish with the above dose of adrenaline

Mag. X 120.



a



b

5.4.4.1. The effect of isoproterenol on pigment granules in melanophores of chromatically spinal fish long white-adapted and pretreated with a beta-adrenoceptor antagonist (propranolol)

Noradrenaline and isoproterenol are by far the most common drugs used in differentiation of alpha and beta classes of adrenoceptors, because the former has a high affinity to interact with the alpha-adrenoceptors and the latter has a high affinity to interact with the beta-adrenoceptors. The results presented here have shown that isoproterenol is the most potent agent in bringing about pigment dispersion in chromatically spinal white-adapted fish. Therefore, isoproterenol was selected to study the effects of pretreatment with a beta-adrenoceptor antagonist on the pigment dispersion caused by a beta-adrenoceptor agonist. Propranolol was selected among the various beta-adrenoceptor antagonists for the following reasons:

(1) It is almost devoid of the intrinsic sympathomimetic activity which is observed after treatment with other beta-adrenoceptor blocking agents such as dichloroisoproterenol (Moran and Perkins, 1958) and, to a lesser extent, pronethalol (Shanks, 1966).

(2) It is a potent beta-adrenoceptor antagonist and does not show any specific affinity to antagonise responses mediated by one subtype of the beta-adrenoceptor more efficiently than the other (β_1 and β_2 subtypes).

The fish under observation was treated with a dose of 3.1×10^{-6} moles/kg propranolol. Continuous microscopic observation of the melano-

phores revealed that, except for a further brief aggregation of almost fully aggregated pigment granules, propranolol appeared to have no significant effect on the melanophores. The brief pigment aggregation was of a similar nature to that which always results after a fish is injected.

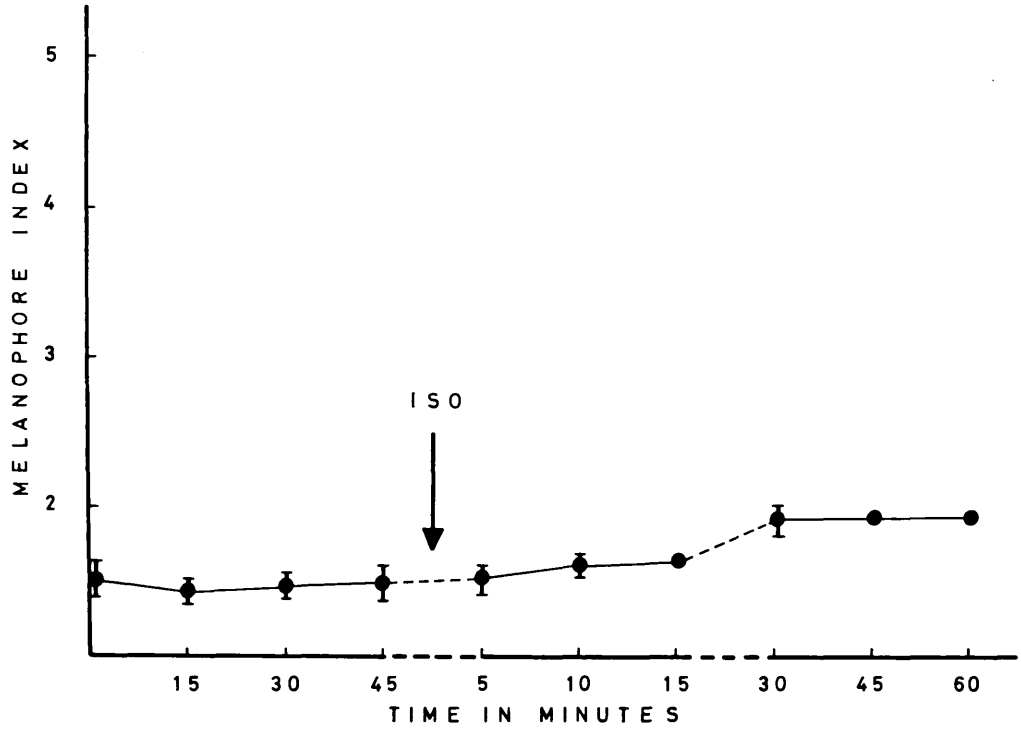
Forty five minutes after treatment with propranolol, the fish was injected with a dose of isoproterenol 3.1×10^{-8} moles/kg. This dose caused maximum pigment dispersion in the fish untreated with a beta-adrenoceptor blocking agent. Fig. V-12 page 190 and Plate V-8 page 191, show the effect of pretreatment with propranolol on the potency of isoproterenol in bringing about pigment dispersion. As is evident from the figure and the photographs, pretreatment with the beta-adrenoceptor antagonist propranolol almost completely antagonises the dispersing effect of the beta-adrenoceptor agonist isoproterenol. The above results are consistent with the previous suggestion (page 183) that pigment dispersion in melanophores of chromatically spinal white-adapted fish following injection of beta-adrenoceptor agonists is probably mediated by beta-adrenoceptors of minnow melanophores.

5.4.4.2. Further experiments with more specific beta-adrenoceptor agonists

The difference in the potencies of adrenoceptor agonists to interact with alpha and beta receptors is attributed to their structural formulae (Lands and Brown, 1967; Brittain *et al.*, 1970; Triggle and Triggle, 1976). With an increase in the size of the

Fig. V-12

The antagonistic effect of propranolol on the dispersing action of isoproterenol on melanophores of chromatically spinal white-adapted *Phoxinus phoxinus*.
Fish were injected with propranolol (3.1×10^{-6} moles/kg). After 45 minutes a dose of isoproterenol was injected (3.1×10^{-8} moles/kg; 15^{o}C. 3 animals).


MELANOPHORE INDEX
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45
60
TIME IN MINUTES

Plate V-8a, b, c

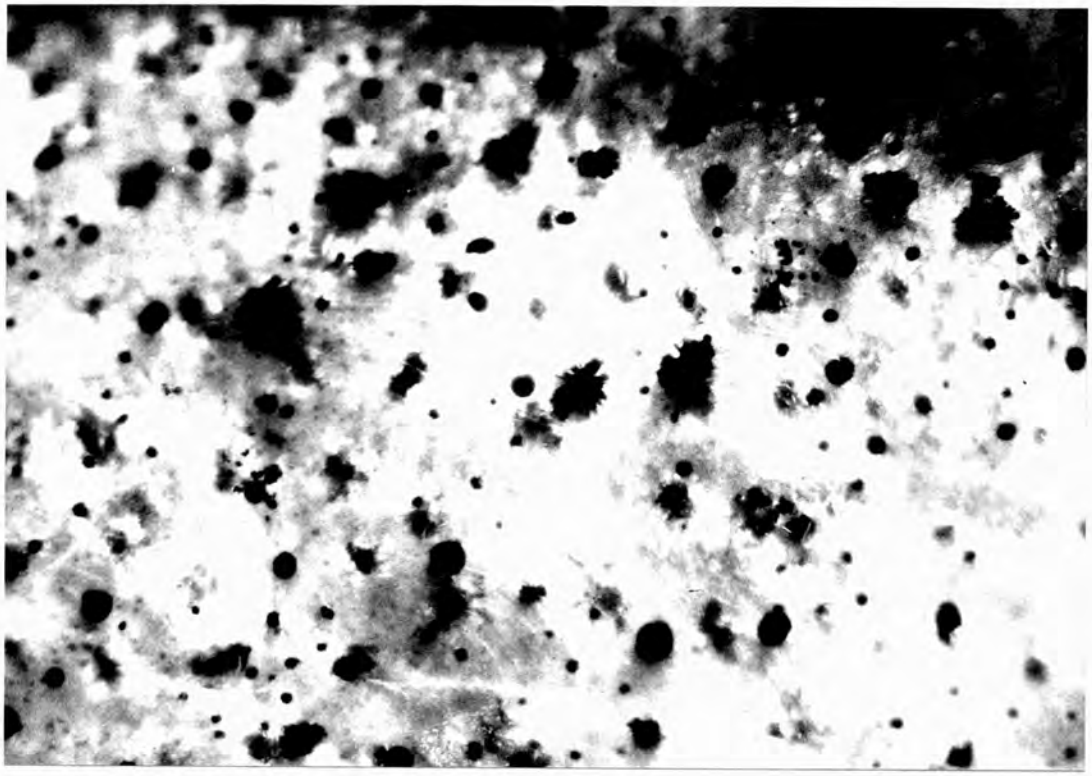
The antagonistic effect of propranolol (3.1×10^{-6} moles/kg; $15^{o}C$) on the dispersing by isoproterenol of melanophores of chromatically spinal white-adapted *Phoxinus phoxinus*.

a - state of melanophores in the fish after being kept for two weeks on a white background and 30 minutes exposed to a white background in the continuous observation apparatus

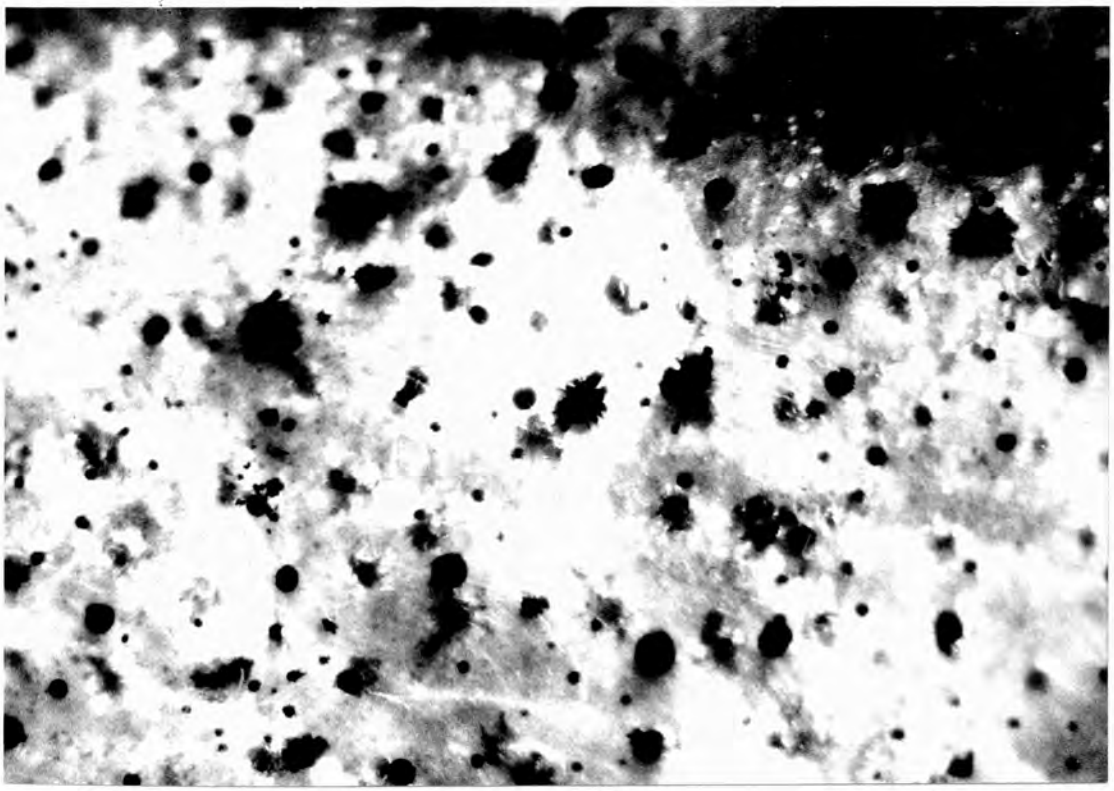
b - state of melanophores 45 minutes after the above injection of propranolol, then the fish was injected with a dose of isoproterenol (3.1×10^{-8} moles/kg).

c - state of melanophores 30 minutes after injecting the fish with isoproterenol

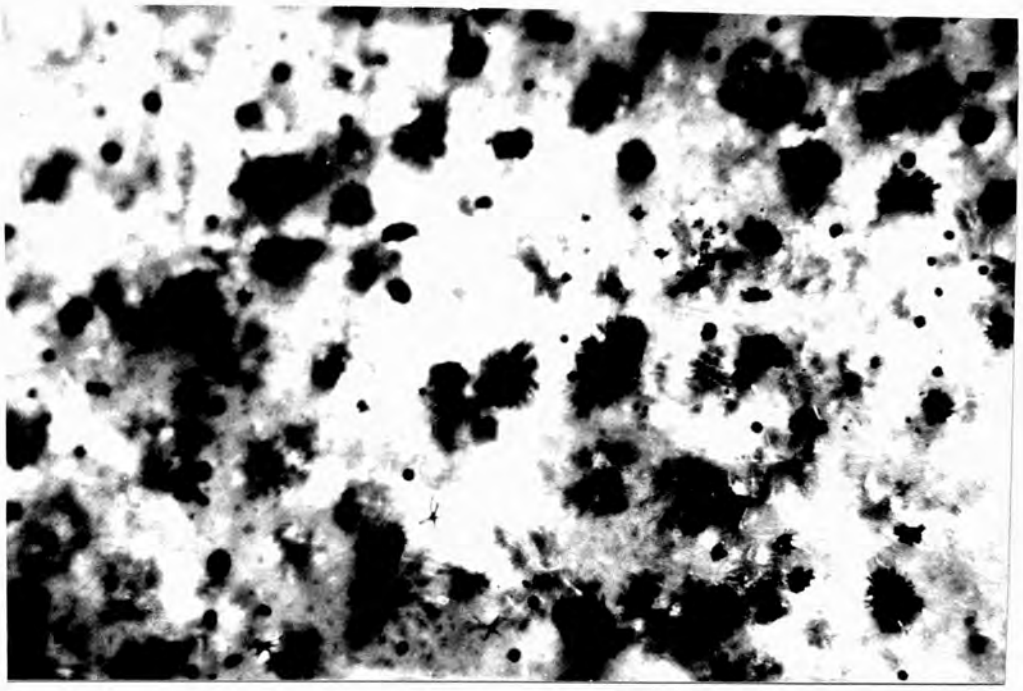
Mag. X 110.



a



b



c

substituent on the amino group the agonist affinity to interact with alpha receptors decreases while the affinity to interact with beta receptors increases. In other words, the more bulky the nitrogen-attached moiety on a catechol base, the more beta-specific the agent. The smaller the nitrogen-attached moiety, the more the activity shifts to non-specific alpha and beta stimulation until a point is reached, a total absence of a nitrogen-attached moiety, as in noradrenaline, where the molecule is highly alpha-specific. Therefore, it has been possible by manipulation of the size of the amino group substituent to synthesise various specific beta adrenoceptor agonists which have no significant activity at alpha receptor sites. The first compound of this nature to be produced was isoproterenol (Lands *et al.*, 1967). However, as has been mentioned (page 168), in relatively high concentrations this compound does interact with alpha adrenoceptors. Therefore, to provide a higher specificity, the size of the substituent on the amino group was further increased and/or hydroxy linkages of the phenol ring were modified. The various synthesized beta-specific agonists, with the exception of isoproterenol which stimulates both β_1 and β_2 adrenoceptors (page 58) to an equal degree, are mainly, if not exclusively, β_2 specific. From the various highly specific β_2 adrenoceptor agonists available, the effects of two, isoxsuprine and fenoterol, were investigated on minnow melanophores.

5.4.4.2.1. Isoxsuprine

Isoxsuprine was one of the initial modified forms of

isoproterenol to be reported. As can be seen from Table V-1 page 148 this compound has a larger substituent on the amino group than isoproterenol. Therefore it is more beta-specific. Moreover, the 3-4-dihydroxy substituent on the catechol ring is known to be in an adequate position for the enzyme catechol-o-methyl transferase (COMT) to cause degradation of monoamines. As can be seen in Table V-1 page 148 isoxsuprine is devoid of a 3-hydroxy substituent on its catechol ring. This might explain the longer duration of its action when compared to isoproterenol which has an unmodified 3-4-dihydroxy substituent. Isoxsuprine has been reported to be of clinical use by preventing premature labour. Bishop and Woutersz (1961) found that isoxsuprine could prevent threatened premature labour by causing relaxation of the uterus through beta-adrenoceptors. As far as the effect of isoxsuprine on teleost melanophores is concerned, Reed and Finnin (Chapter I p. 60) found that isoxsuprine produced marked pigment dispersion in melanophores of *Pterophyllum eimekei*. This dispersion, unlike the dispersion which followed the administration of isoproterenol, was not followed by a subsequent pigment aggregation. Also, Fujii and Miyashita (1975) and Miyashita and Fujii (1976), working on *Lebistes reticulatus* split fin preparation, reported that isoxsuprine, unlike isoproterenol, had no pigment aggregation effect on melanophores. However, in appropriate concentrations it was found to be a potent pigment dispersing agent.

In the present study, preliminary injections of isoxsuprine in a group of black-adapted (12 fish) and a group of white-adapted (10 fish) unoperated, free swimming minnows in doses of 3.1×10^{-5}

moles/kg, 3.1×10^{-6} moles/kg and 3.1×10^{-8} moles/kg were found to produce no significant change in the shade of the adapted fish. Injections of similar doses into chromatically spinal black-adapted and white-adapted fish did not cause any change in the shade of the former but evoked significant pigment dispersion in the latter. The dispersion was visible about 10 minutes after the administration of the drug and was maximal in about 45 minutes, remaining at a peak for as long as 4 hours after drug administration. Thereafter the pigment granules started to aggregate slowly and the fish were found to have returned to their original, pre-injection shade by the following day.

Based on the trial doses, the maximum pigment dispersion was evoked by 3.1×10^{-8} moles/kg. Fig. V-13 page 196 and Plate V-9 page 197 show the effect of this dose of isoxsuprine in chromatically spinal white-adapted fish.

5.4.4.2.2. Fenoterol (Th 1165a)

This compound, in addition to the large substituent on its amino group, unlike isoproterenol, has a 3,5 position for the dihydroxy of the phenol ring (Table V-1 p. 148). This modification in the ring, as was explained in the case of isoxsuprine, allows the compound not to be degraded by COMT. It is also speculated that the above modification might cause a greater β_2 specificity (Leifer and Wittig, 1975). In fact, it has been demonstrated in mammals that fenoterol has a very high affinity to evoke responses mediated by β_2 adrenoceptors. Its affinity to interact with β_1 adrenoceptors is

very low (Dreyer, 1971).

To the knowledge of the author, there have been no studies performed on the effects of this agent on melanophores. Preliminary injection of fenoterol in doses of 3.1×10^{-6} moles/kg; 3.1×10^{-7} moles/kg and 3.1×10^{-8} moles/kg into the body cavity of unoperated free swimming black-adapted fish (6 animals) and white-adapted fish (6 animals) did not produce any observable change in the shade of these fish on their respective backgrounds. However, the above doses resulted in pronounced pigment dispersion in chromatically spinal white-adapted fish. Pigment granules in melanophores of chromatically spinal black-adapted fish did not show any change in the state of their dispersion. Fig. V-13 page 196 and Plate V-10 a, b, c page 198 show the effect of fenoterol on melanophores of chromatically spinal white-adapted fish with aggregated pigment granules in response to prolonged white adaptation.

The time required for fenoterol to initiate pigment dispersion and the duration of the dispersion were very similar to those observed after treatment with isoxsuprine.

5.4.4.2.3. <u>The effect of pretreatment with propranolol on the dispersing actions of isoxsuprine and fenoterol</u>

Chromatically spinal white-adapted fish were injected with a dose of propranolol 3.1×10^{-6} moles/kg. Forty five minutes after the injection the effects of the most potent dose of isoxsuprine (3.1×10^{-8} moles/kg) or fenoterol (3.1×10^{-8} moles/kg) in

Fig. V-13

The effects of isoxsuprine and fenoterol on melanophores of chromatically spinal white-adapted *Phoxinus phoxinus*.

● isoxsuprine 3.1×10^{-8} moles/kg; $18^{o}C$. (3 animals)

○ fenoterol 3.1×10^{-8} moles/kg; $14^{o}C$. (3 animals)

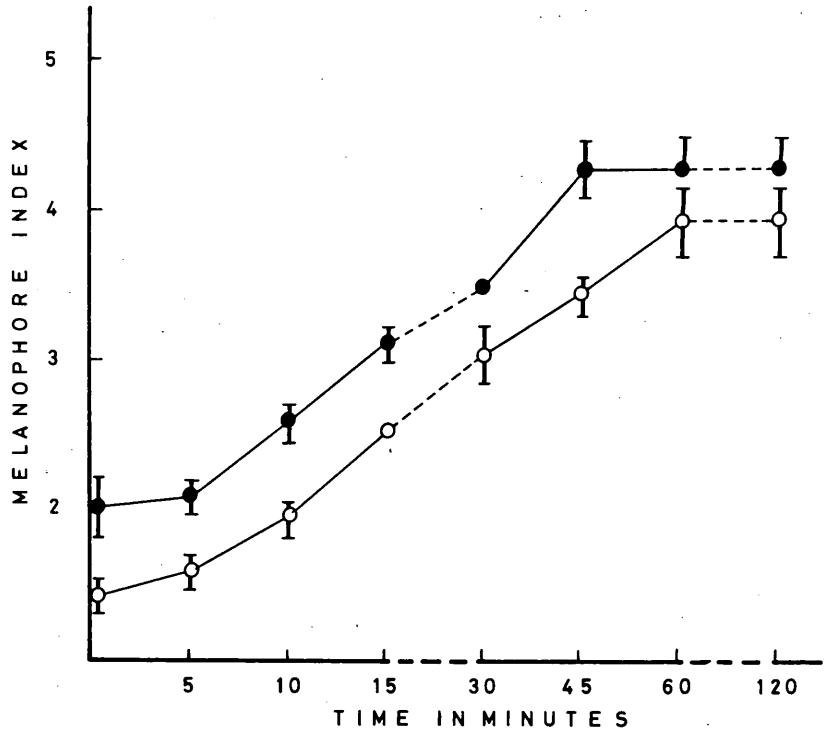

MELANOPHORE INDEX
5
4
3
2
5
10
15
30
45
60
120
TIME IN MINUTES

Plate V-9a, b, c

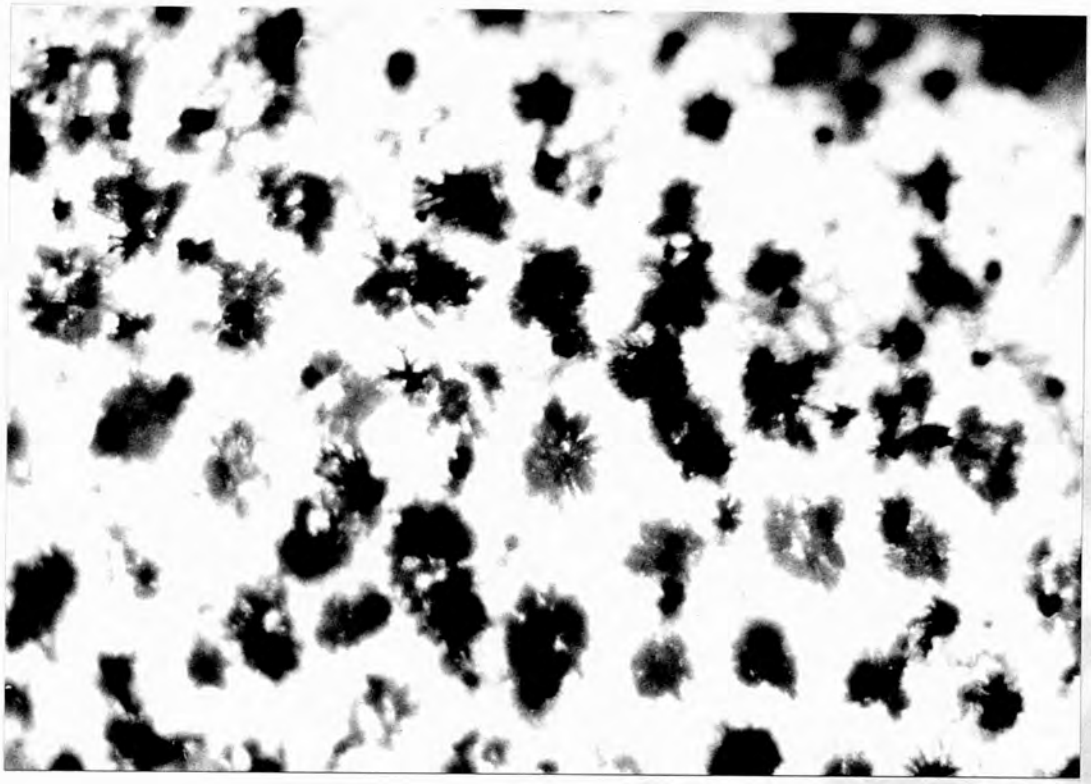
The dispersing effect of isoxsuprine (3.1×10^{-8} moles/kg; $18^{o}C$) on melanophores of a chromatically spinal white-adapted *Phoxinus phoxinus*.

a - state of melanophores in the fish after being kept for two weeks on a white background and 30 minutes exposed to a white background in the continuous observation apparatus

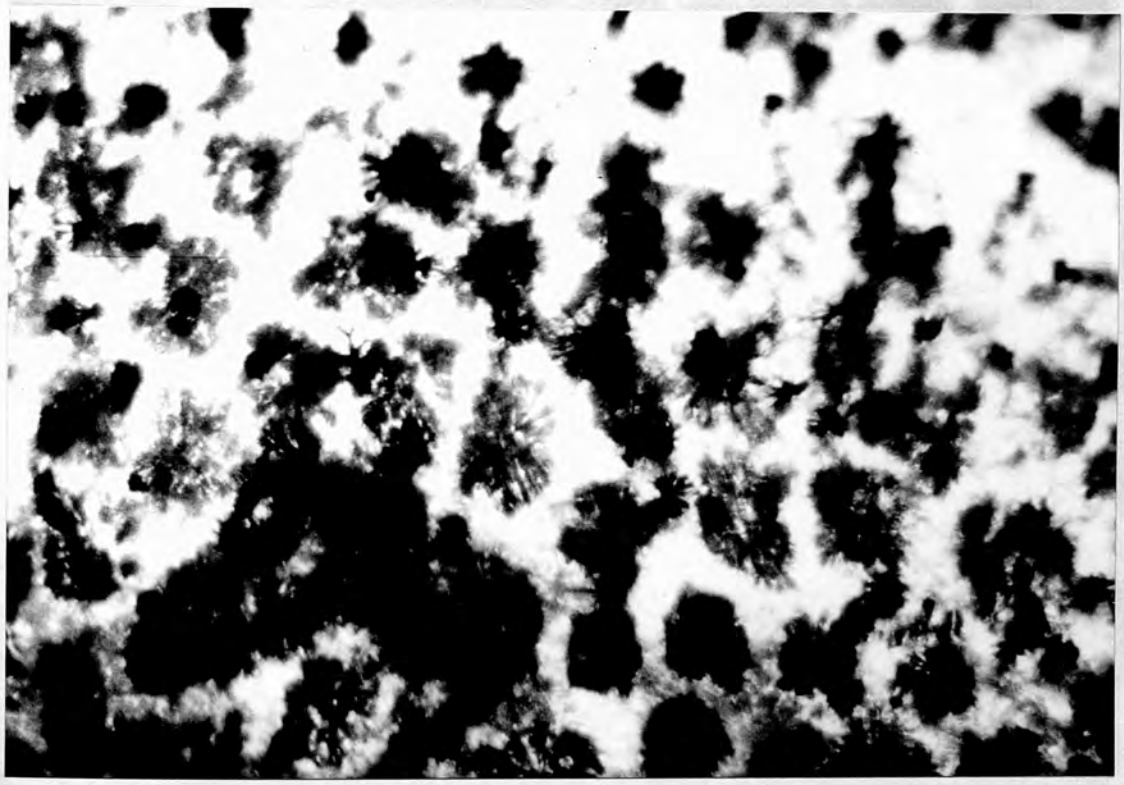
b - state of the melanophores ten minutes after injecting the fish with the above dose of isoxsuprine

c - state of the melanophores 30 minutes after injecting the fish with the above dose of isoxsuprine.

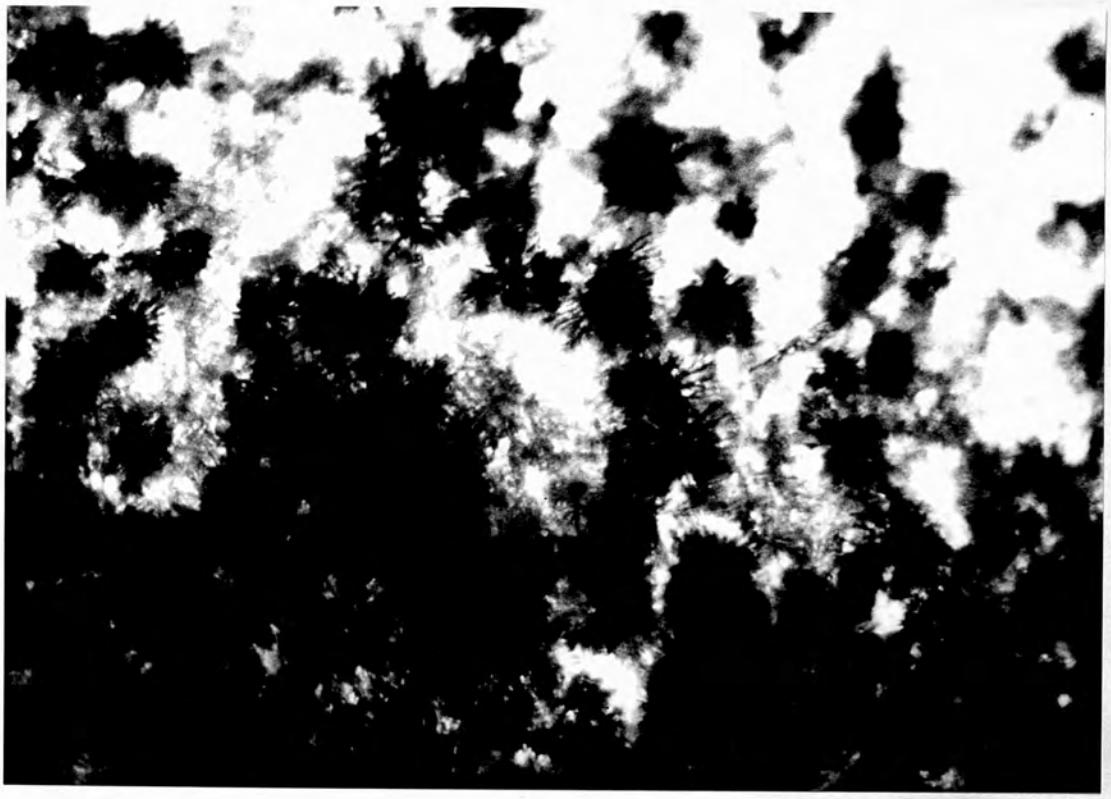
Mag. X 110.



a



b



c

Plate V-10a, b, c

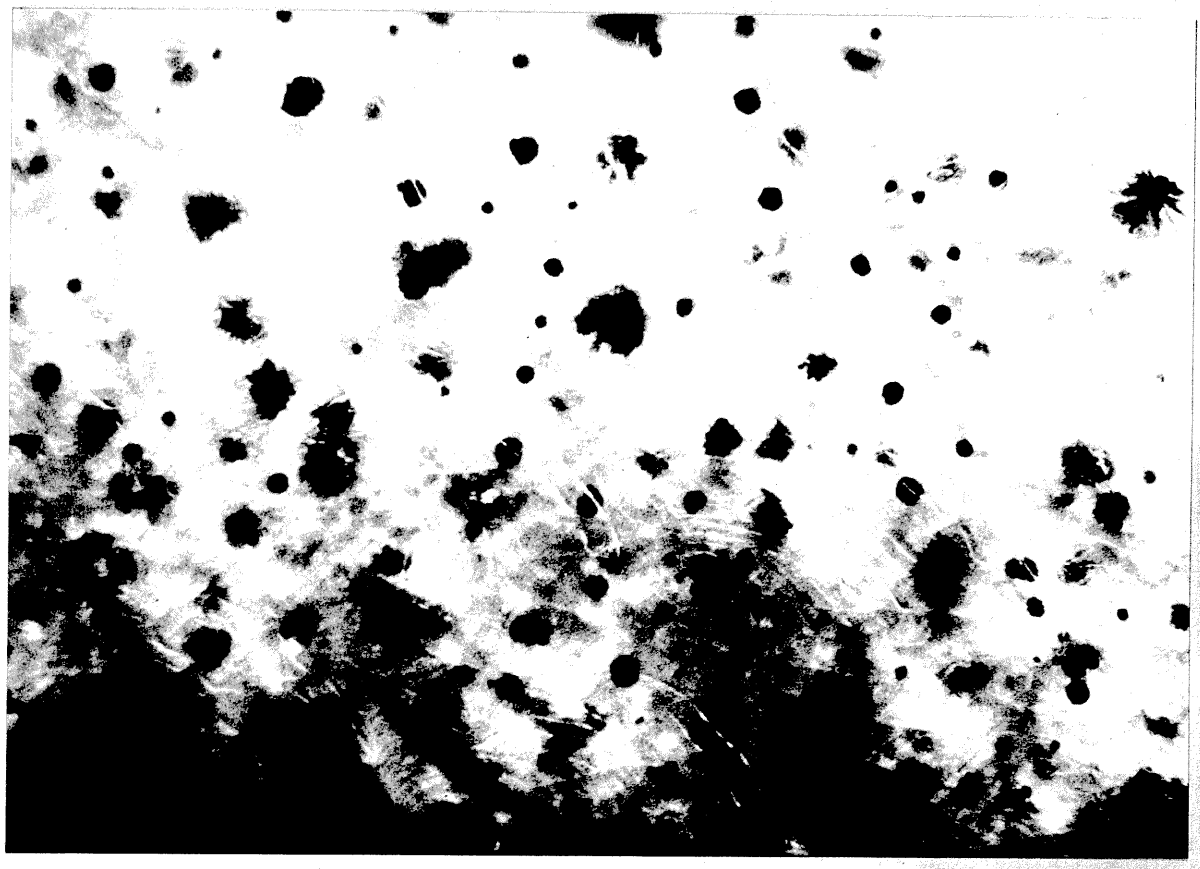
The dispersing effect of fenoterol (3.1 x 10^{-8} moles/kg; 18^{o}C) on melanophores of a chromatically spinal white-adapted *Phoxinus phoxinus*.

a - state of melanophores in the fish after being kept for two weeks on a white background and 30 minutes exposed to a white background in the continuous observation apparatus

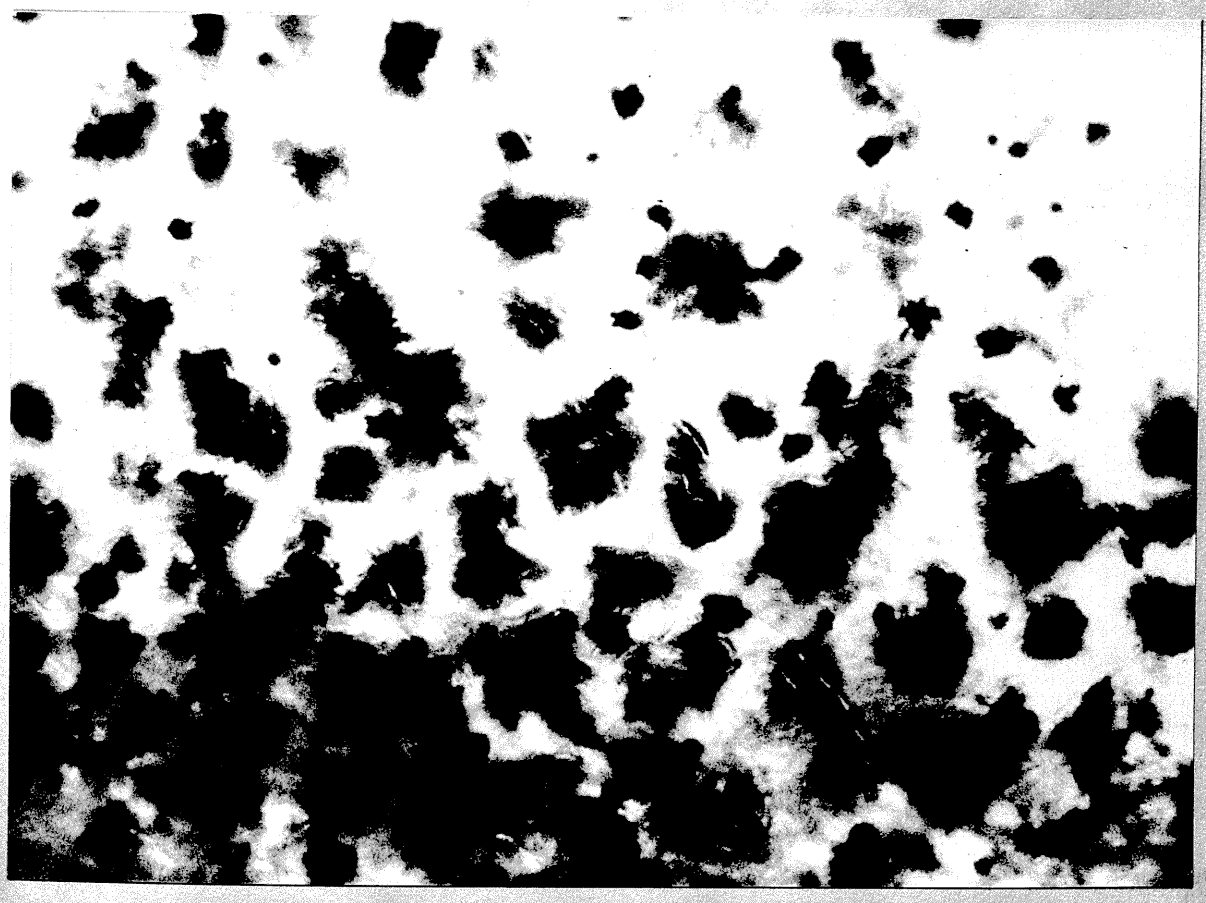
b - state of the melanophores ten minutes after injecting the fish with the above dose of fenoterol

c - state of the melanophores 30 minutes after injecting the fish with the above dose of fenoterol

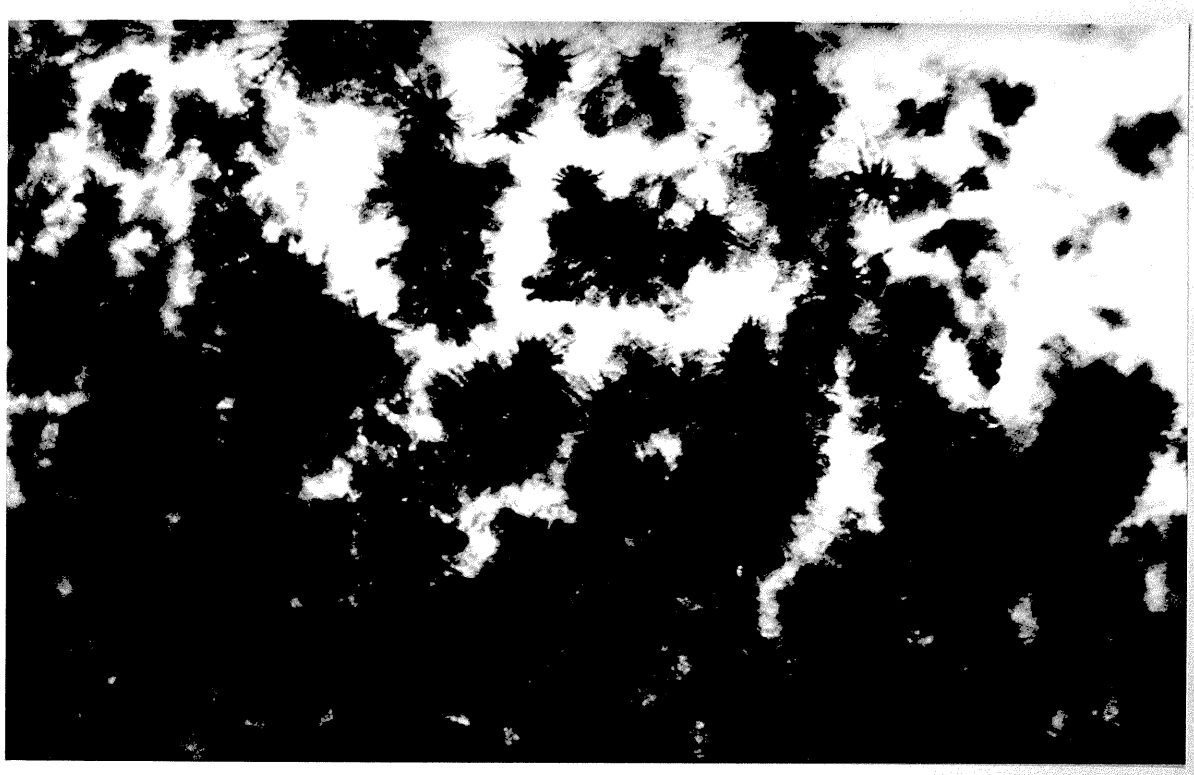
Mag. X 110.



a



b



c

Fig. V-14

The antagonistic effect of propranolol (3.1×10^{-6} moles/kg) on the dispersing action of isoxsuprine and fenoterol on melanophores of chromatically spinal white-adapted *Phoxinus phoxinus*.

● treatment with propranolol followed by a dose of isoxsuprine (3.1×10^{-8} moles/kg; $12^{o}C$. 3 animals)

○ treatment with propranolol followed by a dose of fenoterol (3.1×10^{-8} moles/kg; $12^{o}C$. 3 animals)

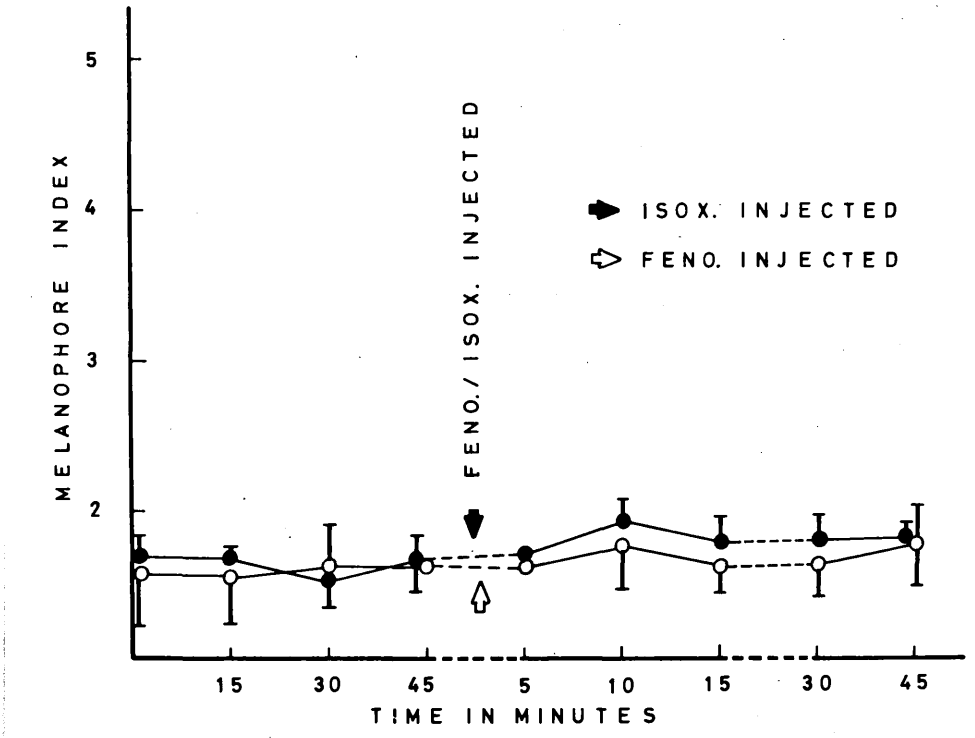

MELANOPHORE INDEX
5
4
3
2
FENO./ISOX. INJECTED
ISOX. INJECTED
FENO. INJECTED
15
30
45
5
10
15
30
45
TIME IN MINUTES

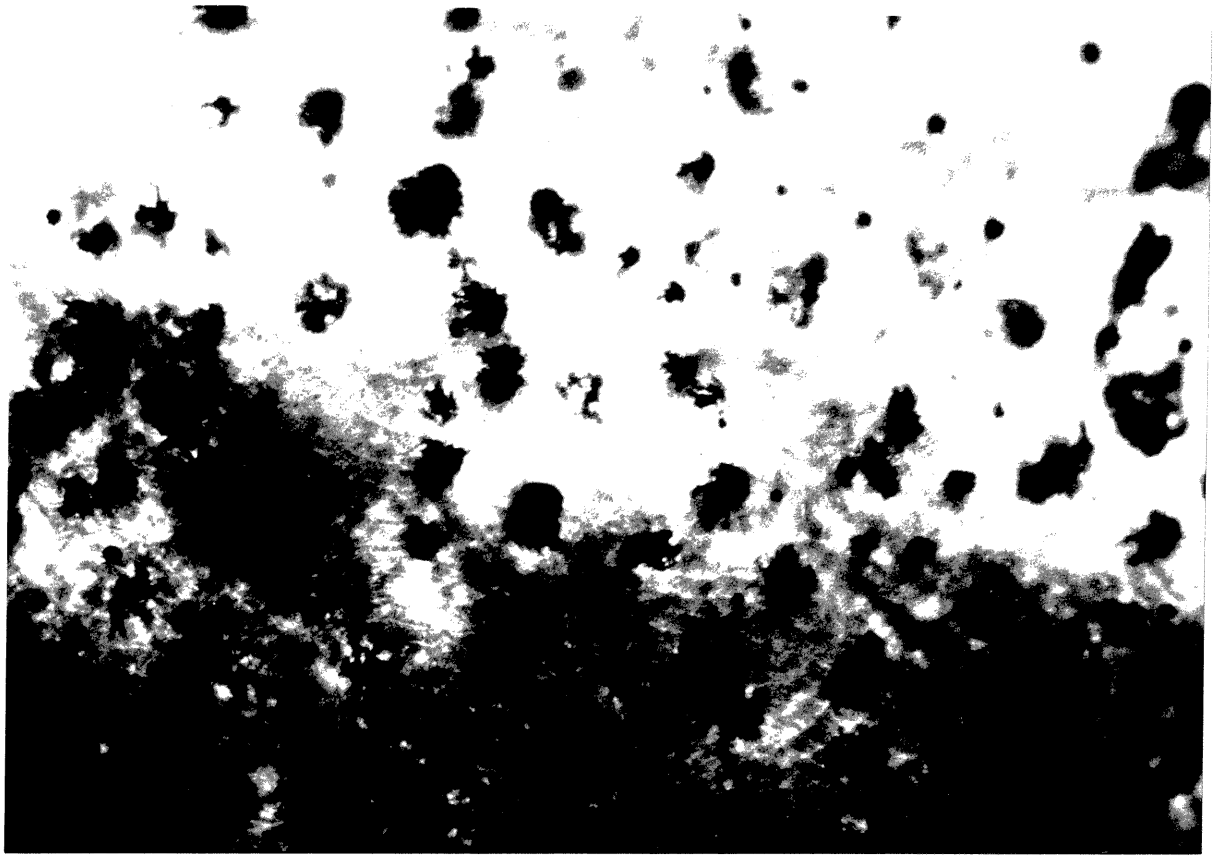
Plate V-11a, b

The antagonistic effect of propranolol (3.1×10^{-6} moles/kg; $12^{o}C$) on the dispersing action of isoxsuprine on melanophores of a chromatically spinal white-adapted *Phoxinus phoxinus*.

a - state of melanophores 45 minutes after the above injection of propranolol; the fish was then injected with a dose of isoxsuprine (3.1×10^{-8} moles/kg)

b - state of melanophores 30 minutes after injecting the fish with isoxsuprine

Mag. X 110.



a

b

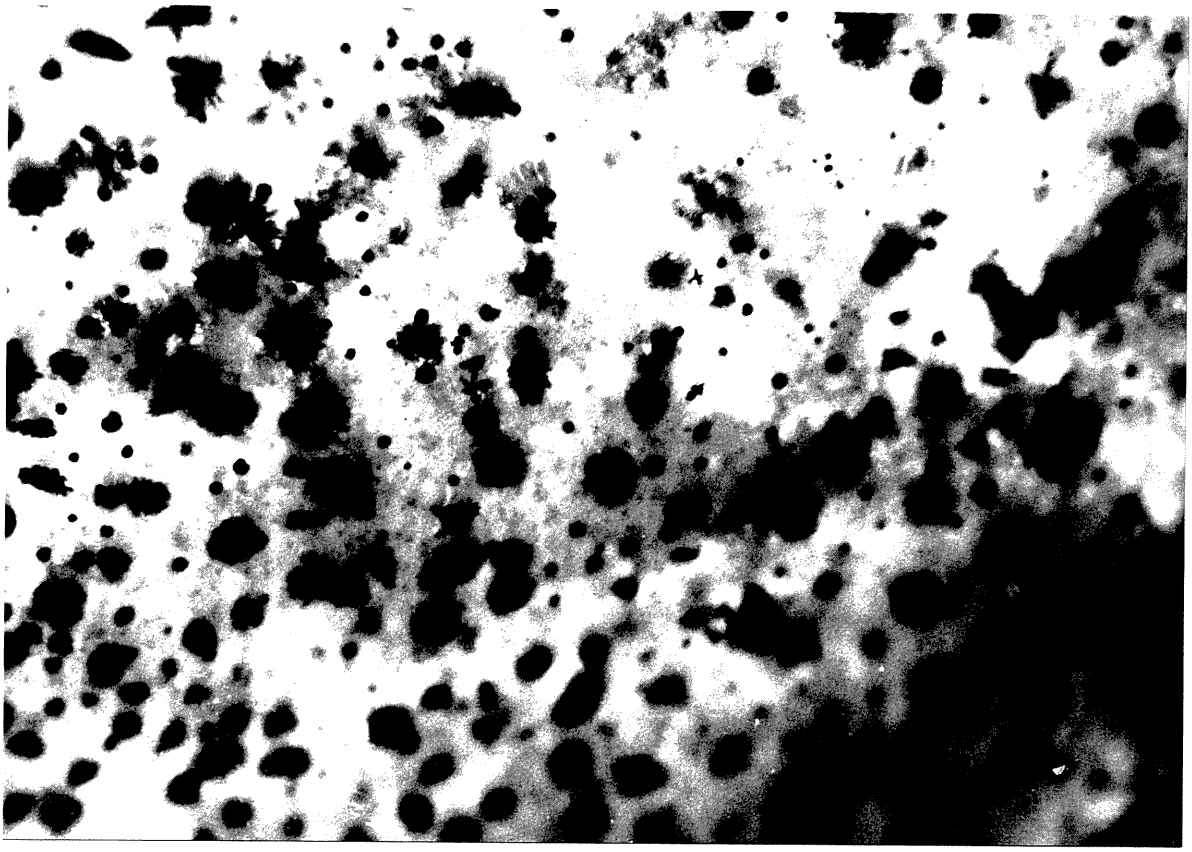
Plate V-12a, b

The antagonistic effect of propranolol (3.1×10^{-6} moles/kg; $12^{o}C$) on the dispersing action of fenoterol on melanophores of a chromatically spinal white-adapted *Phoxinus phoxinus*.

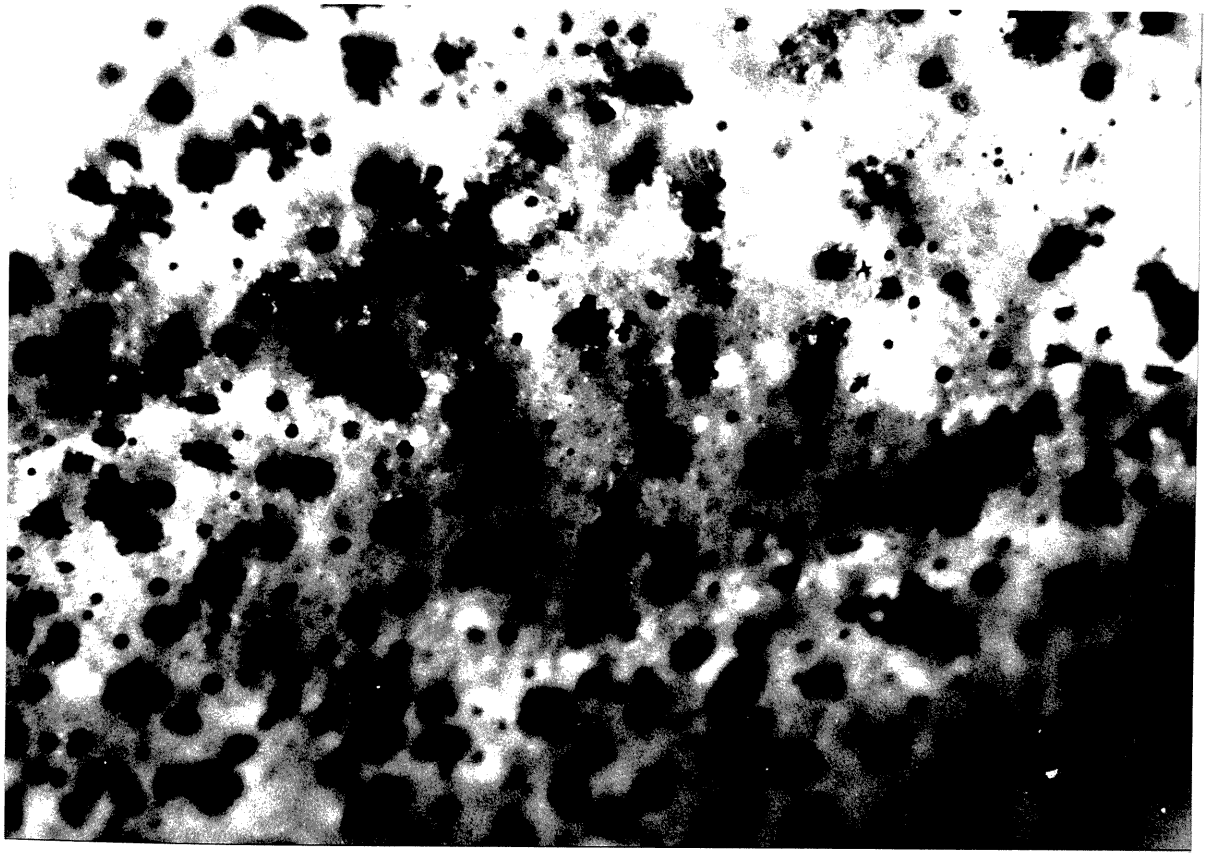
a - state of melanophores 45 minutes after the above injection of propranolol; the fish was then injected with a dose of fenoterol (3.1×10^{-8} moles/kg)

b - state of melanophores 30 minutes after injecting the fish with fenoterol.

Mag. X 110.



a



b

dispersing the pigment granules was tested. Fig. V-14 page 199 and Plates V-11, 12 pages 200, 201 show the results of the above experiments. As is evident, pretreatment with propranolol almost completely antagonised the dispersing effects of both agents.

5.5. Discussion

5.5.1. The pathway of the chromatic fibres

The pathways of the chromatic fibres in *Phoxinus* were first described by von Frisch (Chapter I, p.31), after which similar outflows from the spinal cord in other teleosts were demonstrated (Schaefer, 1921; Adelmann and Butcher, 1937). However, more recently Healey (1965) showed that the chromatic fibres leave the cord over several segments and that the degree of pigment dispersion which follows the transection in the spinal cord depends on the number of segments disconnected from the centre. The greater the number of the disconnected segments the greater the degree of the pigment dispersion. Since the degree of pigment aggregation depends upon nerve impulse frequency one may suppose that the impulse frequency in the chromatic fibres to the periphery depends on the number of intact segments in the outflow region of the spinal cord. The above observations of Healey were confirmed in the present investigation and it appears that the outflow segments bearing chromatic fibres cover the region roughly between vertebrae 10 and 15. The degree of pigment dispersion which follows the transection consequently depends on the impulse frequency in the chromatic fibres. This may come about in two ways.

Firstly, if one assumes single innervation of melanophores (von Frisch, 1910, 1911), one may suppose that the melanophores disperse their pigment granules passively as the impulse frequency travelling to the periphery decreases by decreasing the number of intact segments bearing chromatic fibres. Finally a point is reached where the total number of the concerned segments has been disconnected from the centre by more anterior transection of the cord (above vertebra 10). Now all the melanophores over the body surface will be disconnected. (Alternatively, by peripheral nerve transection a group of melanophores in the affected area will be disconnected).

The above assumption is challenged by a number of experimental results which suggest that the pigment dispersion is mediated by an active mechanism (Chapter I, p. 35 and Chapter IV of the present thesis). A second possibility therefore exists. If one assumes the presence of such a mechanism, i.e. a double innervation of melanophores (Parker, 1948), the frequency of impulses in the aggregating fibres is indirectly regulating the degree of pigment dispersion. That is, for the proposed dispersing fibres to exert their maximal activities, the rate of impulses in the aggregating fibres should be minimal. Results published by Healey (1954), Gray (1956) and Pye (1964a) indicate that if the dispersing fibres do exist, they follow the same pathways as the aggregating fibres. Therefore, the transection in the spinal cord should disconnect the melanophores from their central control along both sets of fibres. If that is the case, the only explanation available is Parker's hypothesis of

sustained injury discharge set up by cutting in the dispersing fibres but not in the aggregating fibres. However, as was earlier stated (Chapter I, p. 37), this hypothesis has been strongly criticized as having no evidence to support it elsewhere in our knowledge of neurophysiology. Therefore, since neither of the assumptions - passive dispersion of pigment granules by assuming single innervation of melanophores or active dispersion of pigment granules by assuming stimulation of only the dispersing fibres in a double innervation of melanophores - can be considered to be justified, interpretation of the melanophore responses after section of their nervous supply becomes difficult. This problem will be considered in more detail later in this discussion.

5.5.2. Effects of the adrenergic neuron blocking agent "bretylium" and electrical stimulation

The pigment dispersion which follows the administration of bretylium in chromatically normal white-adapted fish but not in chromatically spinal white-adapted fish suggested to Grove (1967) that the proposed dispersing fibres might themselves be adrenergic as well. The precise site of action of bretylium in lower vertebrates is not definitely known. Therefore, Grove thought that the failure of bretylium to produce pigment dispersion in chromatically spinal white-adapted fish might be a result of a central depressant effect of this drug which was not conveyed to the periphery in chromatically spinal fish.

As has been shown in the present experiments, chromatically

spinal fish in which the pigment granules have been aggregated in response to prolonged white adaptation can be made to demonstrate the normal dispersing effect of bretylium if the cord is stimulated electrically posterior to the site of the transection. This result clearly indicates that the site of action of this drug in the minnow is at least outside the central nervous system and probably similar to the site of its action in higher vertebrates. That is to say, it impairs adrenergic transmission at the neuro-effector junction by accumulating in adrenergic neurons and hence blocking the conduction.

The electrically evoked pigment dispersion in chromatically spinal white-adapted fish pretreated with bretylium can be given the following explanation. On the basis of double innervation it can be suggested that bretylium blocks the conduction in the adrenergic neurons mediating pigment aggregation, thus allowing the proposed pigment dispersing fibres to exert their effect. The failure of pigment granules to disperse in melanophores of chromatically spinal fish following injection of bretylium probably follows from the inability of the proposed dispersing fibres to exert their effect unless they are connected with the central nervous system (Grove, 1967). Therefore, electrical stimulation in the present experiment replaced the centre in chromatically spinal fish and activated the proposed dispersing fibres of preparations in which the aggregating fibres were blocked by bretylium. The above explanation appears to provide an argument in favour of double innervation. Parker's hypothesis to account for the dispersion which follows the trans-

ection of chromatic fibres is far from acceptable. However, an alternative interpretation can be suggested. Hughes (1972) demonstrated that the efficiency of bretylium in blocking adrenergic transmission is very much dependent on the concerned neuroeffector distance. The greater the neuroeffector distance the more efficient is the effect and the reverse is true. For example, treatment with bretylium lowers the output of noradrenaline from the nerve endings to a very low level in both the rat portal vein and *vas deferens*. However, while it blocks transmission in the portal vein efficiently (neuromuscular distance of about 150 nm), it fails to block transmission in the *vas deferens* (neuromuscular distance of about 20 nm). The probable explanation is that in the tissues with short neuromuscular distances the very low output of the transmitter in preparations treated with bretylium is still sufficient to build up a concentration which can elicit a response. Should the neuroeffector distance between the melanophores and the nerve endings be of a similar size it might be expected that bretylium at least will not abolish the transmission across the neuromelanophore junctions completely. Indeed, ultrastructural studies on melanophore innervation (Chapter III, p. 101) have clearly demonstrated a very close association between nerve endings and melanophores (neuromelanophore distance about 20 nm, Plate III-20 p. 100). Therefore it is possible and even likely that the level of the neurotransmitter available at the neuromelanophore junction is an important factor in determining the responses of melanophores.

5.5.3. A possible explanation to account for pigment aggregation and dispersion in minnow melanophores under the influence of the nervous system

Based on the above considerations, the author suggests the following explanation to account for pigment aggregation and pigment dispersion in minnow melanophores. The ultrastructural details of the nerve-endings associated with melanophores were found to be very similar to the well-established adrenergic nerve-ending ultrastructures in mammalian tissues (Chapter III, p. 98). Moreover, the positive interaction of 5-hydroxydopamine with the neurovesicles strongly suggests that the stored neurotransmitter is noradrenaline. Furthermore, since all the nerve endings observed to be associated with the melanophores were of the same nature anatomically and histochemically, it is justifiable to conclude that melanophore innervation is single and is adrenergic. On the other hand, experiments involving electrical stimulation of the chromatic fibres in the present study and in previous studies by other workers have indicated that the degree of pigment aggregation in response to electrical stimulation is directly proportional to the frequency of the stimulation, within a physiological range (Pye, 1964a; Healey, 1965; Kinosita and Ueda, 1970; p. 161 of this Chapter). It is known that the frequency-response curve is hyperbolic; the amount of transmitter released increases almost linearly with the frequency (Hughes, 1972). The hyperbolic shape of the frequency-response curve is attributed to the failure of the effector to respond rather than to the failure of the adrenergic nerve to release

transmitter (Langer, 1970; Hughes, 1972). Therefore it can be suggested that while the fish is exposed to an illuminated white background the frequency of impulses in the chromatic fibres is high. This will result in a relatively high concentration of the neurotransmitter at the melanophore receptors, which consequently results in pigment aggregation. Upon background reversal to an illuminated black background the impulse frequency travelling in the chromatic fibres decreases a great deal. This will result in a rapid decrease of neurotransmitter at the melanophore receptors with consequent pigment dispersion.

Similarly, transection in the chromatic fibres (e.g. chromatic transection of the spinal cord, spinal nerve section) results in a rapid decrease in the level of neurotransmitter at the melanophore receptors. This, likewise, will result in dispersion of the pigment granules in melanophores over the whole body surface in the case of chromatically spinal fish or in the area affected by peripheral nerve section in the case of spinal-nerve section. The pigment granules within the affected area remain almost fully dispersed for as long as the fish is exposed to an illuminated black background and for a considerable length of time if the fish is then exposed to an illuminated white background. The time required for the affected melanophores to aggregate in response to an illuminated white background varies and this variation depends on the nature of the operation. It takes hours to several days in the case of melanophores affected by peripheral nerve section and about 2 weeks in the case of melanophores affected by anterior spinal

cord section. The persistent state of dispersed pigment in the affected melanophores is probably due to a low level of the transmitter available to the postsynaptic receptors by circulation and/or by tissue fluids.

Prolonged exposure of the animal with affected melanophores to an illuminated white background results in slow and gradual pigment aggregation within the concerned melanophores. This slow aggregation of the pigment granules (particularly in the chromatically spinal fish) is caused by aggregating hormone from the pituitary (Healey, 1954). There is also sensitisation to the neurotransmitter of the effector (melanophores) which develops after denervation and decentralisation (Trendelenburg, 1963; Langer and Trendelenburg, 1966). Healey and Ross (1966), Grove (1969a) and Fernando and Grove (1974a, b) have shown that surgical sympathectomy and treatment with cocaine (blocks neuronal uptake) potentiate the effects of monoamines on teleost melanophores. However, pigment granules in the area affected by the nerve transection maintain their ability to respond to changes in the shade of the background. This response is much slower in melanophores affected by peripheral nerve section and very much slower in melanophores of chromatically spinal fish.

Mills (1932a, b), Parker (1948) and Gray (1956) explained the responses of melanophores in the area affected by nerve section on the basis of a double innervation concept (Chapter IV, p. 143). Based on the present alternative proposal, the responses of melanophores to illuminated backgrounds in an area affected by spinal nerve section can be given the following explanation.

The slow and gradual pigment aggregation on a white background of melanophores affected by spinal nerve section is probably due to a slow elevation in the level in the affected area of the neurotransmitter which has diffused from an adjacent area with intact nerve terminals. On reversal of the background to an illuminated black background there is a rapid decrease in the level of the neurotransmitter at the melanophore receptors with intact innervation and pigment dispersion is elicited as was described earlier (page 208). This decrease in concentration of neurotransmitter results in a slower decrease in the level of the neurotransmitter in the affected area and likewise results in a slower dispersion of the pigment granules within this area. The much slower rate of dispersion of the pigment granules in the melanophores chronically separated from the spinal cord (one week) if the fish is exposed to an illuminated black background (Chapter IV, p. 138) can be explained in line with the denervation supersensitivity which develops after sympathectomy and was described earlier (p. 136). Degeneration of the distal part of the severed fibres results in the abolition of the neuronal uptake of transmitter which is the most important factor in the neurotransmitter inactivation mechanism (Chapter I, p. 55). This will result in a higher concentration of the neurotransmitter at the melanophore receptors. Therefore, for this relatively high level of the neurotransmitter in the affected area to decrease in response to an illuminated black background a longer time will be required. Once the concentration of the neurotransmitter in the affected area has reached a level which allows pigment dispersion, pigment

granules in melanophores of the concerned area continue to disperse for some time despite the fish being exposed to an illuminated white background (Chapter IV, p. 138; Plate IV-7c p. 139). Mills (Chapter IV, p. 140) observed similar responses but she interpreted them by assuming that the antagonistic dispersing agent released from the neighbouring intact nerve terminals, while the fish was exposed to an illuminated black background, continues to spread in the affected area despite the change in the shade of the background.

5.5.3.1. Adrenoceptors of *Phoxinus* melanophores

Although Ahlquist's (1948) dual receptor concept is relatively recent, as early as 1906, when Dale noted that the normal pressor effect of adrenaline was converted to a depressor effect in the ergotized cat, the phenomenon of adrenaline "reversal" attracted the attention of workers in the field of chromatic responses. As already stated (p. 145) Barbour and Spaeth (1917) were first to show adrenaline "reversal" effects on the ergotized scale melanophores of *Fundulus*. However, Wyman (1924) was unable to demonstrate the above reversal in intact *Fundulus*. On the other hand, Barbour and Spaeth's observation on adrenaline "reversal" in ergotized preparations has been confirmed by other workers in different species (Fujii, 1961). Giersberg (1930) and Pye (1964) examined the effect of adrenaline in ergotized preparations of *Phoxinus* and they found that adrenaline did not evoke any pigment dispersion in these preparations. Therefore, they were unable to confirm Barbour and Spaeth's concept of adrenaline "reversal" to account for pigment

dispersion in this teleost. Healey and Ross (1966), however, did observe some dispersion in melanophores of ergotized *Phoxinus* after injection of adrenaline (p.146). Grove (1969a, b) experimented with ganglionic blocking and adrenergic neuron blocking agents which did indicate to him the possibility that both mechanisms, pigment aggregation and pigment dispersion, are adrenergic, since pretreatment with the ganglionic blocking agent hexamethonium and chronic treatment with bretylium and guanethidine antagonised the darkening which usually follows the chromatic transection of the spinal cord. However, his results with isoproterenol (beta agonist) and pronethalol (beta antagonist) did not indicate that the pigment dispersion is likely to be mediated by beta-adrenoceptors.

In Chapter III of the present thesis, by means of ultrastructural studies, strong evidence is presented in favour of a direct adrenergic innervation of *Phoxinus* melanophores. This and the results obtained from the adrenergic drugs used in the present work are in agreement with the conclusion of earlier workers (p.59) that the pigment aggregation mechanism in *Phoxinus* is adrenergic and is mediated by postsynaptic adrenoceptors of alpha nature. The order of potency of adrenergic agonists to bring about pigment aggregation appears to be NA > A > ISO. This is based on the fact that isoproterenol was found to be clearly the least potent aggregating agent and in lower concentrations noradrenaline appeared to be more potent than adrenaline. The aggregating effects of these three adrenergic agonists were also studied in animals pretreated with either of the alpha-adrenergic antagonists, yohimbine or tolazoline. Again, while

the dispersing effect of yohimbine or tolazoline was easily reversed by noradrenaline, adrenaline was found to be less potent in this regard and isoproterenol was not effective at all. This order of potency is well in agreement with the results of Healey and Ross (1966) and Grove (1969a).

Moreover, the present experiments on chromatically spinal long white-adapted fish to investigate the effects of agonists known to have a high affinity to interact with beta-adrenoceptors in mammals, have suggested the existence of adrenoceptors of a beta type mediating pigment dispersion in *Phoxinus*. This is in line with the relatively recent reports on the effects of the same agents on melanophores of *Petrophyllum eimekei* by Reed and Finnen (1972) and on the melanophores of *Lebistes reticulatus* by Miyashita and Fujii (1975). This is based on the marked dispersing effects of isoproterenol (in relatively low concentrations), isoxsuprine, fenoterol and to a lessor extent adrenaline, in chromatically spinal white-adapted *Phoxinus*. The ability of propranolol (a beta antagonist) to antagonise the dispersing effects of isoproterenol further supports the conclusion. In relatively high concentrations, isoproterenol resulted in pigment aggregation in both chromatically normal and chromatically spinal fish. However, since this aggregating effect of isoproterenol was antagonised by the alpha-adrenoceptor antagonists, yohimbine and tolazoline, the effect is in line with similar effects of this drug on mammals (Jenkinson, 1973) and can be attributed to its interaction with alpha-adrenoceptors. Similar explanations were given by Fujii and Miyashita (1975) and Miyashita and Fujii (1975) for the aggregating

effects of isoproterenol.

Although isoproterenol is by far the most common agent used to test the existence of a beta-adrenoceptor in an effector, it might in some cases lead to misinterpretation of the results through the above reasoning, especially in tissues where both alpha- and beta-adrenoceptors are present and mediate antagonistic responses.

Isoproterenol is known to interact very efficiently with beta-adrenoceptors at nanomolar concentrations (Jenkinson, 1973). However, in the present work it was found that even in very low concentrations isoproterenol failed to evoke any pigment dispersion in chromatically intact white-adapted fish. This failure of isoproterenol to evoke dispersion in intact fish appears to be due to the predominant alpha-adrenoceptor mediating pigment aggregation activated by endogenous neurotransmitter in response to the illuminated white background (p. 171). This probably also applies to the failure of the other more specific beta agonists to evoke pigment dispersion in such intact fish. Evidence for this predominance can also be drawn from the following observations. While isoproterenol and the other beta agonists failed to evoke pigment dispersion in chromatically normal white-adapted fish, noradrenaline and adrenaline readily resulted in pigment aggregation in black-adapted fish. An interesting interpretation has been suggested for the above by Miyashita and Fujii (1975). That is, the alpha-adrenoceptors are more efficient at interacting with the endogenous neurotransmitter, probably noradrenaline, at relatively higher concentrations. On the other hand, at lower

concentrations of the neurotransmitter, the beta-adrenoceptors are more efficient at interacting and the dominance of the alpha-adrenoceptors is lost. This interpretation is well in agreement with the present proposal that the level of the neurotransmitter at neuromelanophore distances determines the degree of the pigment migration (p. 206).

The marked dispersing effects of the β-2 specific agonists, isoxsuprine and fenoterol, which were almost equivalent to the dispersing effects of isoproterenol on the melanophores of chromatically spinal white-adapted fish, suggest that the beta-adrenoceptors mediating pigment dispersion are probably of the β-2 type. Clearly, more detailed experiments are required to establish the point. It would be interesting to study the antagonistic effects of specific beta antagonists, practolol (β-1 specific) and butoxamine (β-2 specific) on the efficiency of isoproterenol to elicit pigment dispersion, adopting the dose ratio method suggested by Arunlakshana and Schild (1959), Furchgott (1972) and Schild (1973). Such experiments can be carried out at best *in vitro*, where complications such as binding of the drugs to plasma proteins and their metabolism in blood can be avoided, so reducing side effects.

C H A P T E R VI

LIGHT INTENSITY AND CHROMATIC ADAPTATION IN THE MINNOW *Phoxinus phoxinus* (L.) AND THE PLAICE *Pleuronectes platessa* (L.)

6.1. Introduction

Studies concerning the chromatic adaptation of animals have been generally restricted to the presence or to the total absence of light. As a rule, such animals are known to acquire an intermediate shade in total darkness and in the light their response is determined by the reflectivity of the backgrounds. Thus, on a background which almost fully absorbs light i.e. a black background, melanophores show maximum pigment dispersion. On a background which almost fully reflects light i.e. a white background, melanophores show maximum pigment aggregation. These responses are referred to as background responses and there is general agreement that the state of pigment granules in the integumentary melanophores is determined by the ratio of incident and reflected light striking the eye of the animal (see Chapter I, p. 29). However, as already mentioned, these animals assume an intermediate shade in complete darkness irrespective of the shade of their background. Therefore, by lowering the intensity of the incident light gradually, a point should exist where the melanophores tend to aggregate their pigment granules on a black background and disperse them on a white background.

Brown (1936) and Danielson (1938) investigated the above mentioned point on *Ericymba buccata* and *Nocomis biguttatus* respectively. These authors, working independently, concluded that at certain lower limits of light intensity the degree of pigment dispersion on a black background was proportional to the intensity of the incident light. Brown found that pigment granules in melanophores of *Ericymba buccata* dispersed completely in response to a black background if the incident light intensity was at and above 1.75 f-c (18.83 lux). However, at intensities lower than 1.75 f-c (18.83 lux) the magnitude of dispersion was dependent on the intensity of the incident light. Similar observations were recorded by Danielson on *Nocomis biguttatus*, excepting that the lower limit of incident light intensity for this fish to show maximum pigment dispersion was found to be 1 f-c (10.764 lux).

As far as the methods used by the above authors are concerned, Brown (1936) killed the adapted fish in boiling water, then dissected a piece of the integument from a given area of the fish and calculated the diameters of 30 to 80 melanin masses in that area. Although this method is a direct approach to the study of the state of melanophores, it has the following shortcomings:-

1. There is great individual variation as far as the size of the melanophores is concerned. Therefore, quantitative comparisons of melanin masses cannot provide an accurate picture unless specimens from very many individuals are examined.
2. The experiment cannot fully be conducted using the same animal.

3. Killing the fish in boiling water causes serious damage to the tissue and might cause changes in the state of pigment granules within the melanophores.

On the other hand, results reported by Danielson (1938) were merely based on the macroscopic appearance of the fish. This, as he admitted, can be very deceptive, especially at lower light intensities.

Therefore, in the present study, it was desirable to investigate the chromatic adaptation of the fishes used, adopting an alternative technique in recording the results. This technique is fully described in the following subsection (6.2. Methods). Although the previous workers had considered that incomplete pigment dispersion in response to a black background at the lower light intensities might be due to incomplete stimulation of the visual receptors in the retina by such low illumination (Danielson, 1938), no histological work had been carried out on the retina under such conditions. It is therefore equally desirable to study the fine structure of the retina adapted to low ranges of light intensity.

The main experiments to be described were carried out on *Phoxinus phoxinus*. However, some preliminary observations were made on the responses of a flatfish, the plaice *Pleuronectes platessa*, to patterned backgrounds in different degrees of light intensity. Flatfishes have long been known to have the remarkable capacity not only to change shade in response to backgrounds with different reflectivity but also to "match" the pattern of their surroundings

(Sumner, 1910, 1911; Mast, 1916; Osborn, 1939a, b).

Mast (1916) clearly demonstrated pattern changes of the flatfish *Paralichthys albiguttus*. He found that if the fish was successively placed on white and black chequerboards with squares of different sizes, although the total area of black and white of the backgrounds was equal, the fish exhibited different patterns. The fish was finely mottled on the fine chequerboard and coarsely mottled on the coarse one. Moreover, Mast's experiments also indicated that for these fish to adapt to the patterns of the backgrounds, overhead illumination is essential. Mast employed an apparatus in which the background could be illuminated from below without having illumination from above. He found that under such conditions the fish became pale and completely ignored the pattern of the background (sheet spotted heavily with indian ink). The same background was found to result in a spotted pattern upon the fish under normal conditions of illumination (illumination from above). However, no work has been performed to study the minimum overhead illumination required by these fish to respond to the pattern of their background. Therefore, in the present investigation, as already mentioned, preliminary experiments were carried out to study the responses of the fish in the above respects-

6.2. Methods

6.2.1. The experimental apparatus

The apparatus shown diagrammatically in Fig. VI-1 page 221

was built to produce a light source as homogeneous and constant as possible. The apparatus consisted mainly of a Vickers high intensity lamp complete with iris and condenser (a). The lamp was clamped on an adjustable retort stand (not shown in the diagram). A light-proof housing made of black cardboard was placed over the lamp (b). At position (c) a piece of opaque ground glass was placed to produce even illumination. Below the glass and at the position (d) the light-proof housing was hinged to provide a convenient entry for the positioning of neutral density filters, through which the beam passed. The hinge and all the other junctions in the apparatus were light-proofed with black masking tape. In order to stabilise the fluctuations in the mains voltage, a voltage stabiliser was installed between the main current and the lamp (e).

6.2.2. Calibrating the light-source for experiments on minnows

For calibrating the light intensity, an Eel Lightmaster Photometer (Evans Electroselenium Ltd., Halstead, Essex, England) was used. By placing the probe of the photometer in various positions of the illuminated field and by means of the neutral density filters with different transmission values and combinations of these filters, transmitted light was measured. The mean transmitted light of each filter or their combinations was calculated. The distance at which the intensities of the illuminated field was measured corresponded exactly with the distance at which the experimental animal's head would be positioned.

Minnows used in these experiments were chromatically normal

Fig. VI-1

The apparatus used to produce a homogeneous and constant light source.

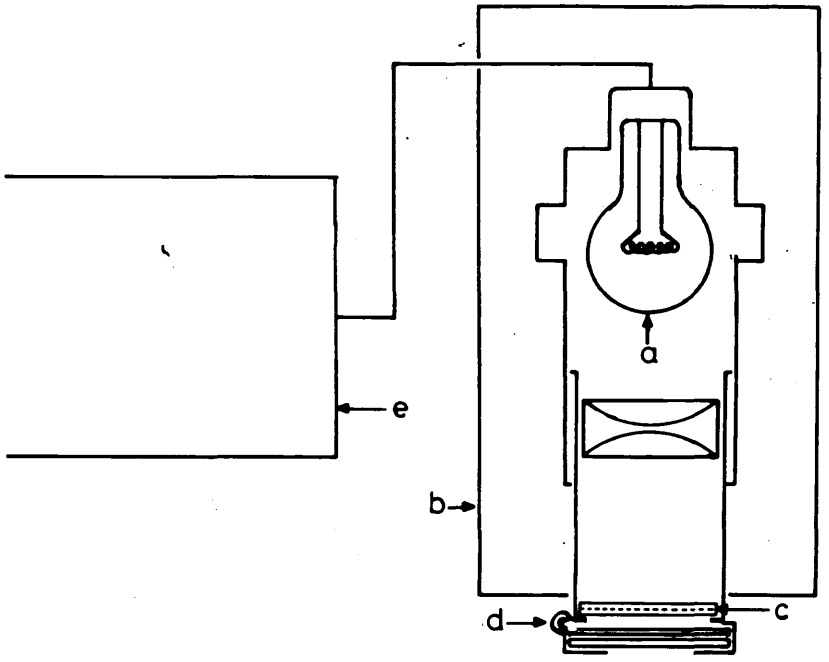
a - Vickers high intensity lamp.

b - Light-proof housing made of black cardboard.

c - Opaque glass.

d - Position where the light proof housing was hinged to conveniently allow the positioning of neutral density filters.

e - Voltage stabiliser.


a
b
c
d
e

spinal fish and were confined in the continuous observation tank already described (Chapter II, p. 71). For the method of confinement and the operating procedures, refer to pages 68, 71. To study the responses of the fish to a black background and a white background illuminated by different light intensities, photographs of the fish were taken using the Nikon camera with bellows mounted on a horizontal and vertically adjustable base (Chapter II, page 171.

A slight movement of the confined fish from its focused position during the experiment resulted in out-of-focus photographs. To avoid these, another apparatus was designed to provide a spot of light bright enough to facilitate focusing during the experiments (Fig. VI-2 p. 223). The light source of this apparatus was also supplied by a Vickers high intensity lamp (a). The light beam was intensified by a series of convex lenses (b). At the focal point of the lens (c), the emerging beam was transmitted through a flexible light-guide (Type LG5, Barr and Stroud Ltd., Caxton Street, Anniesland, Glasgow G13 1HZ, Scotland), 1250 mm in length and 1.75 mm in diameter to the site of the observation. This was done by accurate positioning of the proximal end of the light-guide in a piece of a square wooden board at the focal point (c). The distal end of the light-guide was clamped onto an adjustable stand for convenient positioning of the beam onto the site of observation. The focusing light was only switched on immediately before exposing the photographs. Thus the effect of local illumination and heating on the melanophores was reduced to a minimum. The set-up, as is shown in the figure, was totally light-proofed. The water temperature

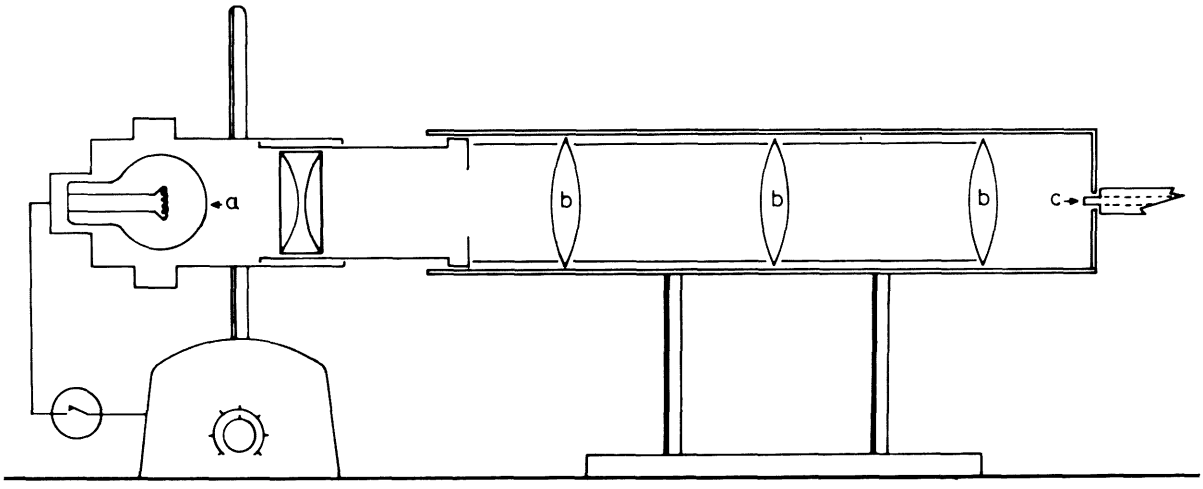
Fig. VI-2

The apparatus used to facilitate the focusing and reading of the MI in low light intensities.

a - Vickers high intensity lamp.

b - A series of convex lenses to intensify the light beam.

c - The position of the light guide to transmit the emerging beam to the site of observation.


a
b
b
b
c

was not controlled during the experiments and fluctuated between 14 - 16°C.

6.2.3. Calibration of the light-source and the equipment used for studying the responses of the plaice *Pleuronectes platessa*

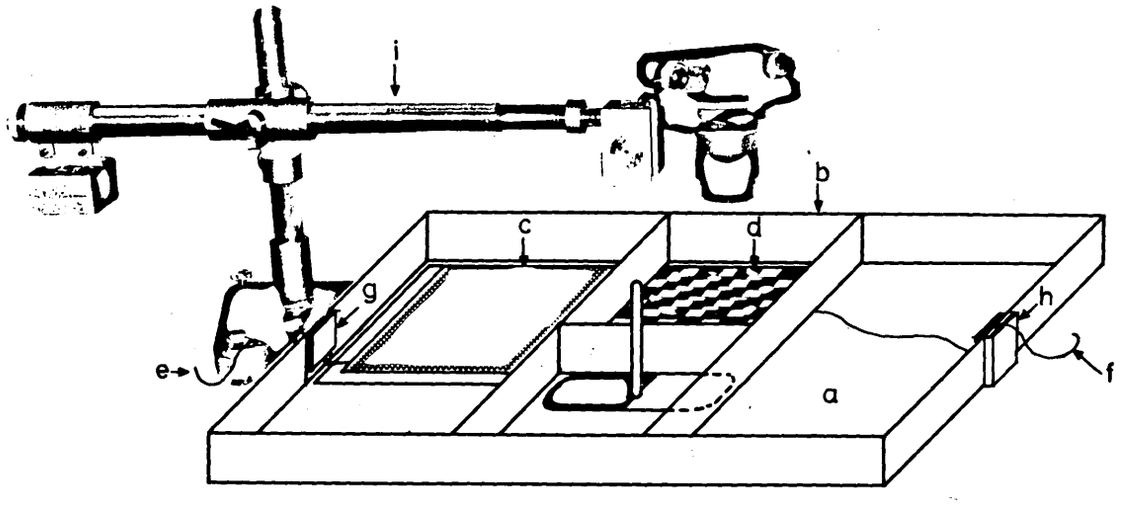
Flatfish *Pleuronectes platessa* (L.) were transported from Lowestoft and kept in the aquarium in 100% aerated sea water. The fish were gradually adapted to 50% sea water in tap water. They were fed on worms *Enchytraeus* and *Tubifex* three times a week and the tank was regularly washed and cleaned to prevent infection.

The equipment shown in Fig. VI-3 page 225 was used for exposing the fish to background reversal. This apparatus was designed by Healey (unpublished) and consisted of a main rectangular Perspex tank measuring 36 cm x 78 cm and 6 cm in height in which a second rectangular Perspex tank measuring 35 cm x 22 cm of the same height as the former could be slid at the centre and across the first tank (b). The floor of the second tank was made of clear Perspex. Bars of Perspex at the base and at the sides of the main tank provided a firm and rigid space for the second tank to slide in. The fish under study could be exposed to background reversal by using a white Perspex panel. On this panel two different backgrounds could be placed (c, d) and covered by a piece of transparent glass. The panel could be passed under the platform by means of a thin nylon thread which was attached centrally to the opposite side of it (e, f). The thread led out of the main tank through a small, water-tight sleeve at the opposite side of the main tank (g, h).

Fig. VI-3

The apparatus used for studying the responses of the plaice *Pleuronectes platessa* to patterned backgrounds.

a - The main rectangular Perspex tank in which a second rectangular Perspex tank (fish platform) is slotted in (b): c, d - The Perspex panel on which the different backings are placed: e,f - Thin nylon thread used to pass the panel under the platform: g,h - Positions of the water-tight sleeve through which the nylon thread leads out.


i
b
c
d
g
h
e
f
a

Therefore, exposure of the fish to background reversal could be done without the least disturbance to the fish, simply by pulling on the appropriate thread. The main tank was half filled with fresh water to prevent the water temperature (50% sea water) in the platform from rising. By means of the Nikon camera mounted on a base, adjustable horizontally and vertically (i), flash photographs were taken to study the responses of the fish. Once the fish was brought into focus the flat position of these fish on their background made further focusing unnecessary. There was therefore no need to use a light-guide.

For exposing the fish to different values of light intensity, the same apparatus and the same methods were adopted as were employed in experiments with minnows. The only difference was that the apparatus was tilted to a certain degree to avoid the beam being masked by the base on which the camera was mounted. The apparatus was recalibrated in its new position and the values of light intensities obtained by different neutral density filters or their combinations were measured. The fish, if undisturbed, usually settled on one position for a considerable length of time. Therefore, no attempt was made to confine the fish. However, in some of the experiments the fish was found to have moved from the focused area and the experiment had to be repeated.

6.2.4. Adaptation of the minnow eye to different values of light intensity and histological procedures

Fig. VI-4 page 227 shows the apparatus used to expose the

Fig. VI-4

The apparatus used to study the minnow eye exposed to different values of light intensity.

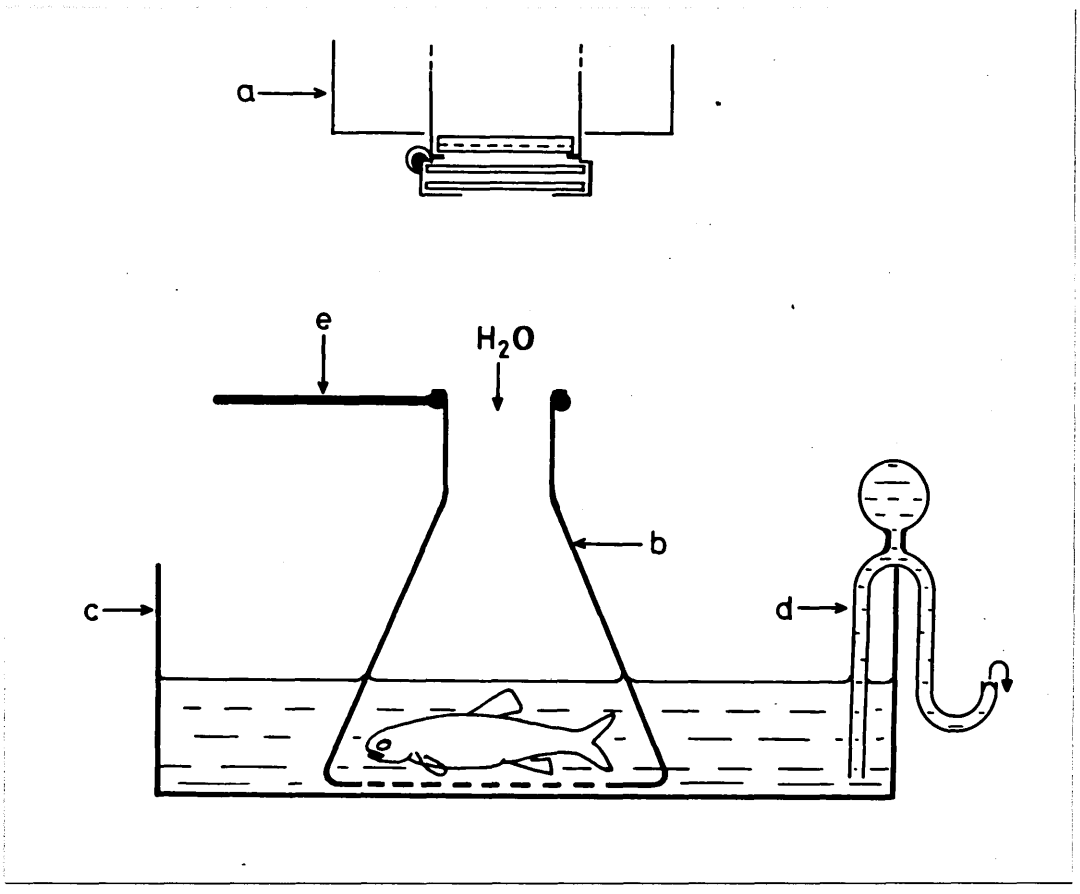
a - Homogeneous and constant light source.

b - Perforated conical flask.

c - Rectangular tray half-filled with water.

d - Glass siphon.

e - Aluminium wire.


a
e
H_2O
b
c
d

eye to different values of light intensity. The illumination was provided by the same means as that used in the previous experiments (a). For quick killing of the fish and fixing the attained response, the animal was placed in a 250 ml conical flask (b) with a perforated base to facilitate free circulation and rapid drainage of water. The flask was kept in a rectangular tray half-filled with water (c). A slow current of water was passed through the flask and the excess water in the tray was drained off by a glass siphon (d). A piece of aluminium wire was secured over the neck of the flask (e) by means of which the flask could be lifted from the tray and water could be drained off quickly. The flask containing the fish was then immediately placed in a vacuum flask containing liquid nitrogen and the fish so killed instantly. It was then transferred to Bouin's fluid for fixation. All the above procedures were carried out under experimental conditions of illumination and took about 30 seconds to 1 minute from the time the fish was lifted up until it was transferred to the fixative. All the experiments were carried out in an experimental dark room with black walls and the time recording was by means of an electronic digital watch. Fifteen minutes after the fish was transferred to the fixative the room light was switched on and the eyes were processed for histological examination.

(a) While in the fixative the fish was decapitated and the head was transferred to a specimen bottle containing fresh fixative with a label describing the experimental conditions.

(b) Four hours later the eye was transferred to a dissecting dish containing Bouin's fluid. The optic nerve and the eye muscles

were cut and by means of a very fine knife a superficial small section was made at the dorsal part of the eye. The latter indicated the dorso-ventral axis of the released eye ball.

(c) The eye was then transferred to the labelled specimen bottle for a further 48 hours, changing the fixative every 12 hours.

(d) Dehydration was carried out in alcohol: 70% alcohol overnight (4 changes), followed by 4 changes in 80%, 90% and 100% alcohol, 2 hours in each.

(e) The eye was cleared in xylene for 30 minutes.

(f) After clearing in xylene the eye was transferred to a bath of 54^{o}C paraffin wax in a vacuum embedding oven and the impregnation was carried out at reduced pressure to remove any trapped air in the eye ball. Wax impregnation was given 4 hours with 2 changes.

(g) After the impregnation the eye was embedded in a suitable mould with molten paraffin wax. A pair of hot dissecting needles helped the orientation of the eye in the mould. An identifying label was attached to the inside edge of the mould corresponding with the dorsal side of the eye and the mould was left to solidify in cold water for an hour.

(h) After solidification the block was removed from the mould, trimmed and mounted on the microtome. Sections were cut at 6μ and the ribbon was carefully detached from the knife and placed on a piece of black cardboard before being mounted on glass slides.

(i) The mounted sections were dried by keeping the slides in an incubator at 37^{o}C for 24 hours.

(j) The sections were stained with Heidenhain's iron haematoxylin and eosin.

6.2.5. Electron microscopy

Fixation, dehydration, embedding, sectioning and staining procedures for electron microscopy were identical with those described in Chaper III, pages 76-81.

To fix a retina, the eye was enucleated and dissected into two halves in a petri dish containing Young's freshwater teleost Ringer. The dissected parts were immediately transferred to the fixative for 60 minutes. The dissected retina was then cut into small pieces while in the fixative. These pieces were transferred to fresh fixative for a further 60 minutes.

6.3. Results

6.3.1. Background responses of chromatically normal minnows in different values of light intensity including complete darkness

A fish previously adapted to an intermediate background (white sink mottled with black) for about 3 months, was subjected to spinal cord section posterior to the 15th vertebra. This operation has no effect on the chromatic responses of the fish and, at the same time, reduces the stress of confinement (Chapter II, p. 68). After the operation the fish was transferred to an illuminated white aquarium. On the second postoperative day the fish was transferred

to the continuous observation tank and its responses to black and white backgrounds in different values of incident light were recorded. Due to the limitations of the apparatus (Chapter IV, p. 122), the experiments were arranged in such a way that continuous observation of the fish did not exceed a period of two hours, after which the fish was returned to the white background and recording was continued the day after. The responses of the fish to black and white background reversals were recorded under values of light intensities presented in Table VI-1 page 232.

It was observed that in incident light intensities of 12.5 lux (1.16 f-c) and above, the response of the fish to black and white backgrounds was constant both in the rate and the degree of pigment movement and was not influenced by the intensity of the incident light. Plate VI-1a, b page 233 show the response of the fish to black and white background reversals. However, in intensities of 1.25 lux (0.116 f-c) and lower, the background response of the fish was incomplete. Plate VI-2a, b page 235 show the responses of the fish to black and white backgrounds in the above incident light intensity. As is evident, the extent of pigment dispersion in dorso-lateral melanophores was decreased when compared with the extent of pigment dispersion when the illumination was 12.5 lux (Plate VI-1a p. 233). Similarly the extent of pigment aggregation on a white background decreased in the above light intensity when compared with the extent of pigment aggregation on a white background where the intensity of illumination was 12.5 lux (Plate VI-1b p. 233).

THE MEAN VALUES OF THE INCIDENT LIGHT INTENSITIES USED IN EXPERIMENTS ON *Phoxinus phoxinus*

LUX	FOOT CANDLE	DENSITY OF THE SCREENING FILTER(S)
2500	232.25	NONE
250	23.22	1
25	2.32	2
12.5	1.16	3.2
1.25	0.116	4.2
0.125	0.0116	5.2
0.0125	0.00116	6.2
0.00125	0.000116	7.2

THE MEAN VALUES OF THE INCIDENT LIGHT INTENSITIES USED IN EXPERIMENTS ON *Pleuronectes platessa*

LUX	FOOT CANDLE	DENSITY OF THE SCREENING FILTER(S)
3300	306.57	NONE
330	30.65	1
33	3.065	2
16.5	1.53	3.2
1.65	0.153	4.2
0.165	0.0153	5.2
0.0165	0.00153	6.2

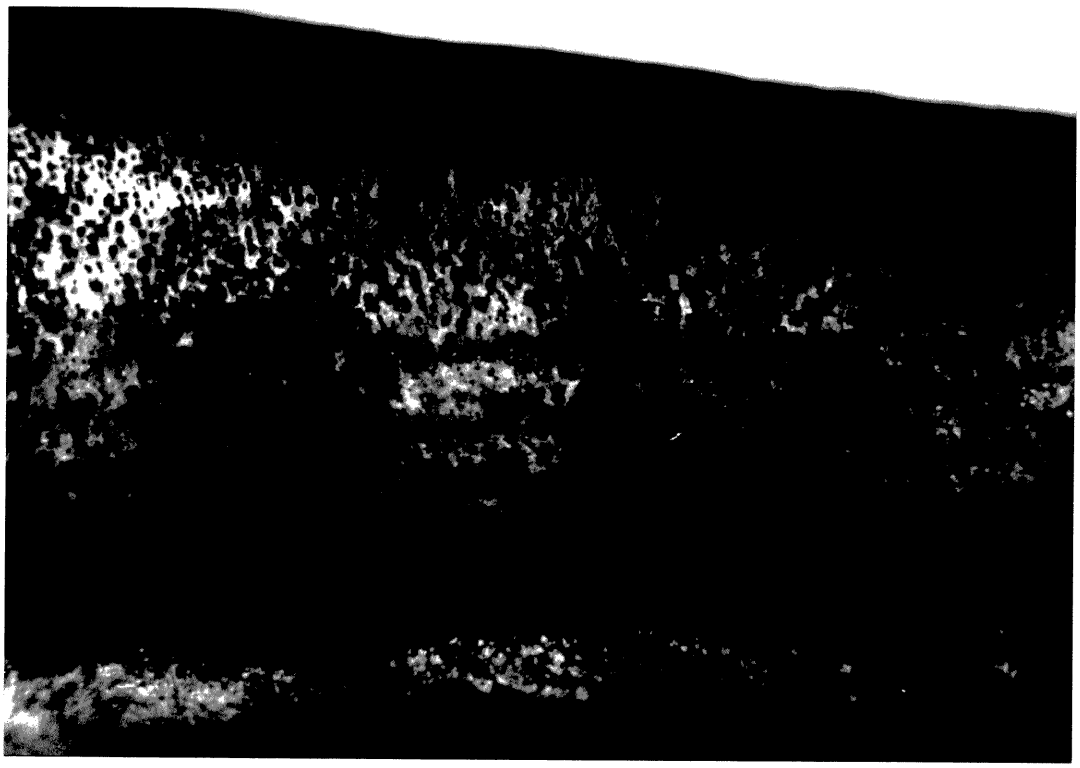
Plate VI-1a, b

Background responses of *Phoxinus phoxinus* in the incident light intensity of 12.5 lux (1.16 f-c).

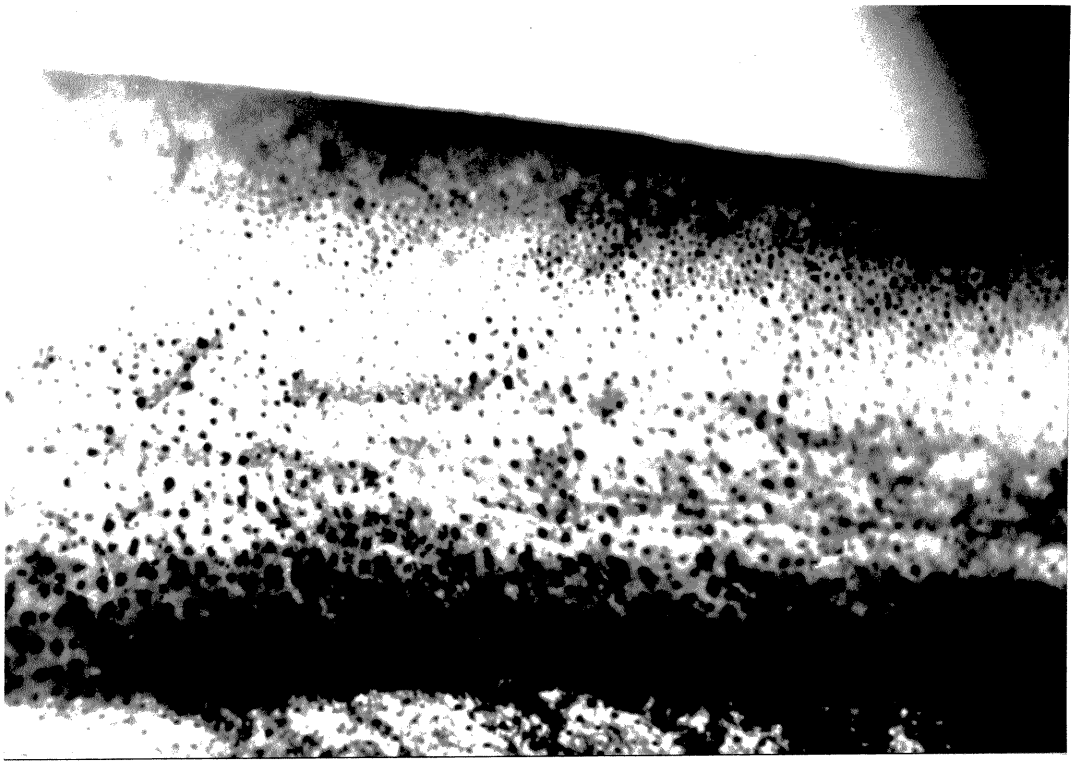
a - Black background response, 30 minutes after the background was presented

b - White background response, 30 minutes after the background was presented

Mag. X 15.



a



b

By exposing the fish to a still lower light intensity of 0.125 lux (0.0116 f-c), its ability to change its shade was reduced further. This is clearly demonstrated in Plate VI-3a, b page 236

Melanophores in the dorso-lateral area of the fish showed a greater tendency to aggregate their pigment granules in lower light intensities when the fish was exposed to a black background than did the melanophores of the lateral stripes. On the other hand, pigment granules in melanophores of the lateral stripe showed a greater tendency to remain dispersed in lower light intensities on a white background than did the melanophores of the dorso-lateral region (compare Plates VI-1a, b; 2a, b; 3a, b pp.233, 235, 236). An interesting observation was made on melanophores in between the macromelanophores of the lateral stripes. These melanophores showed some degree of pigment dispersion as the intensity of the incident light decreased. This dispersion might be ~~as~~ a result of the primary response of these melanophores to the light provided by the light-guide (Chaper I, p. 26).

In further experiments the response of the fish to complete darkness was recorded. The state of pigment granules within the melanophores at different time intervals in complete darkness was compared with the state of pigment granules within the melanophores when the fish was exposed to the lower ranges of light intensities against black and white backgrounds. In the initial series of the above experiments the fish was first adapted to a black background in an incident light intensity of 2500 lux (232.25 f-c). Plate VI-4a page 238 shows the state of pigment granules of the fish 30 minutes

Plate VI-2a, b

Background responses of *Phoxinus phoxinus* in the incident light intensity of 1.25 lux (0.116 f-c).

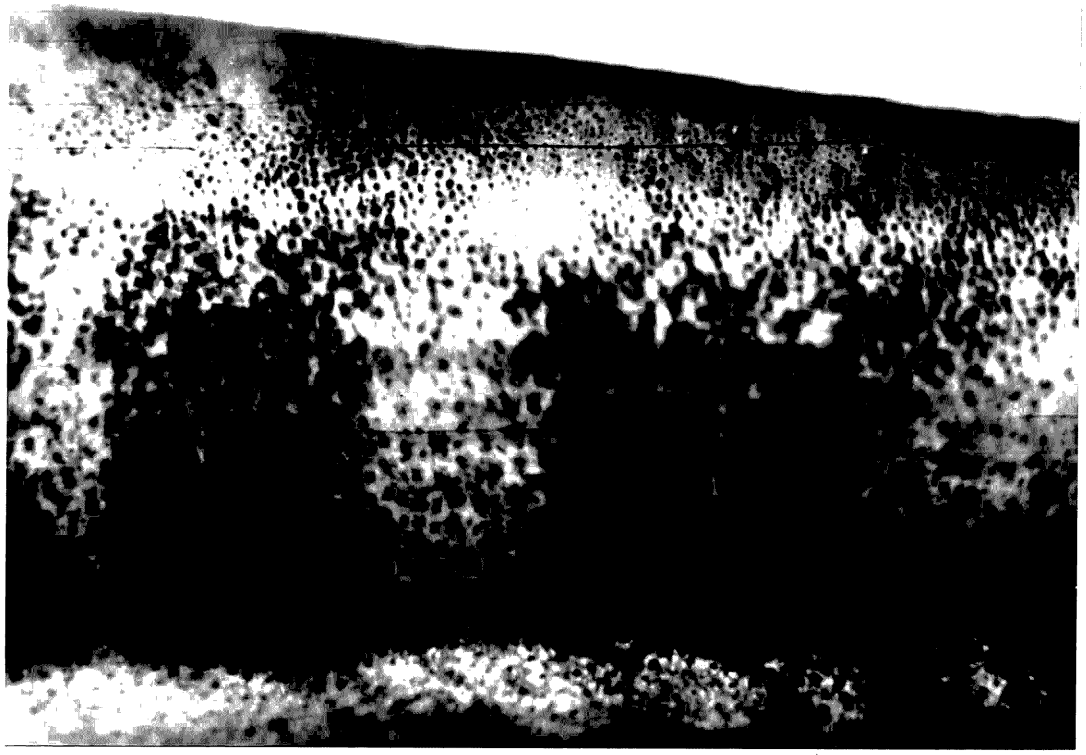
a - White background response, 30 minutes after the background was presented

b - Black background response, 30 minutes after the background was presented

Note : Large melanophores between the lateral stripe melanophores have shown some pigment dispersion in this lower light intensity when compared with their state in the higher intensity.

Mag. X 15.

a



b

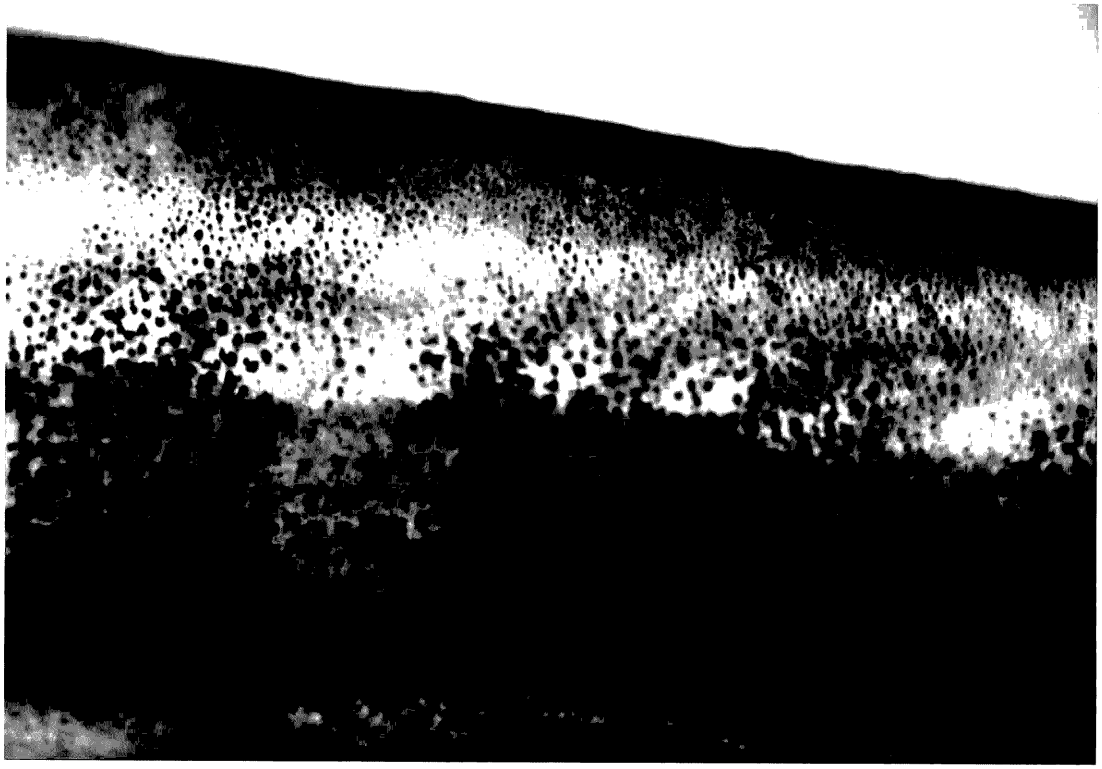
Plate VI-3a, b

Background responses of *Phoxinus phoxinus* in the incident light intensity of 0.125 lux (0.0116 f-c).

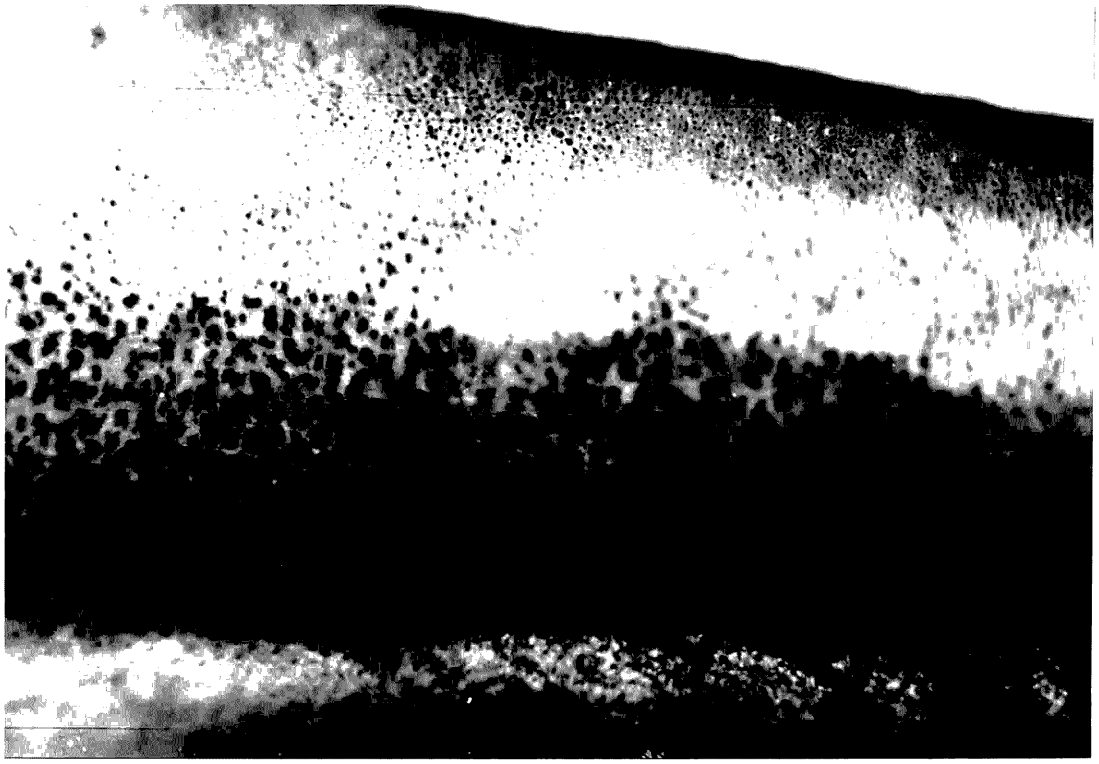
a - Black background response, 30 minutes after the background was presented

b - White background response, 30 minutes after the background was presented

Mag. X 15.



a



b

after this treatment. The overhead illumination was then switched off and the recording was made at different time intervals with the aid of the light-guide. Plate VI-4b, c, d, e page 238 show the state of the melanophores in complete darkness at 5, 10, 15, and 30 minutes respectively. It is evident that the dorso-lateral melanophores show almost complete pigment aggregation in darkness in as short a time as 5 minutes. Melanophores in the large dark lateral stripes show incomplete aggregation but a gradual increase of pigment aggregation in 30 minutes. Again, as in the case of the observations recorded at lower light intensities, melanophores in between the lateral stripe show some degree of pigment dispersion in the total absence of overhead illumination. This latter observation reinforced the speculation that pigment dispersion in the concerned melanophores might have been evoked in response to light illuminating them from the light-guide. Therefore, to find out more precisely whether or not this local illumination is the actual factor involved in pigment dispersion in lower light intensities and complete darkness, the following experiments were conducted. In the first series the light-beam from the light-guide was left on throughout the experiments and in the second series which served as the control, the light-beam was switched off throughout the experiments. Results indicated that under conditions when the light-beam from the light-guide was switched on throughout the experiment, the melanophores in the lateral stripes showed more intense pigment dispersion when compared with the results of the control experiment in which the beam was switched off and the fish remained in absolute darkness. Plate VI-5a, b page 239 shows the results of the above experiments.

Plate VI-4a, b, c, d, e

Responses of *Phoxinus phoxinus* to complete darkness.

a - Black background response (30 minutes), incident light intensity 2500 lux (232.25 f-c)

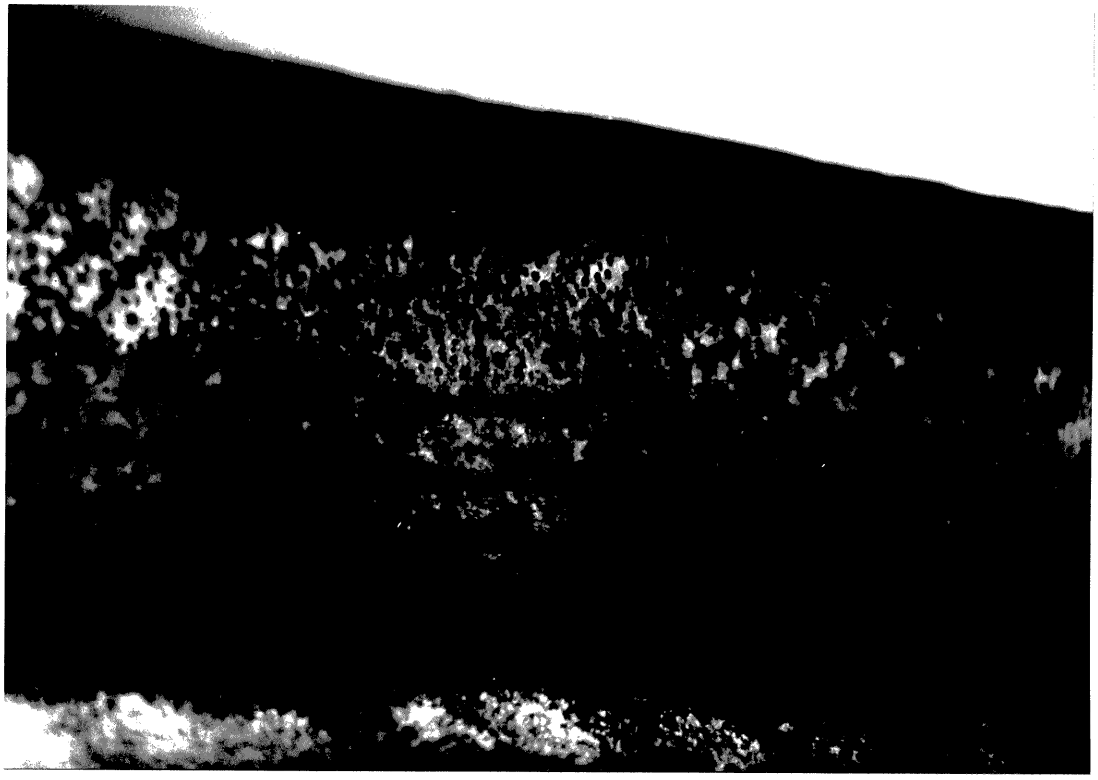
b - After five minutes in complete darkness

c - After ten minutes in complete darkness

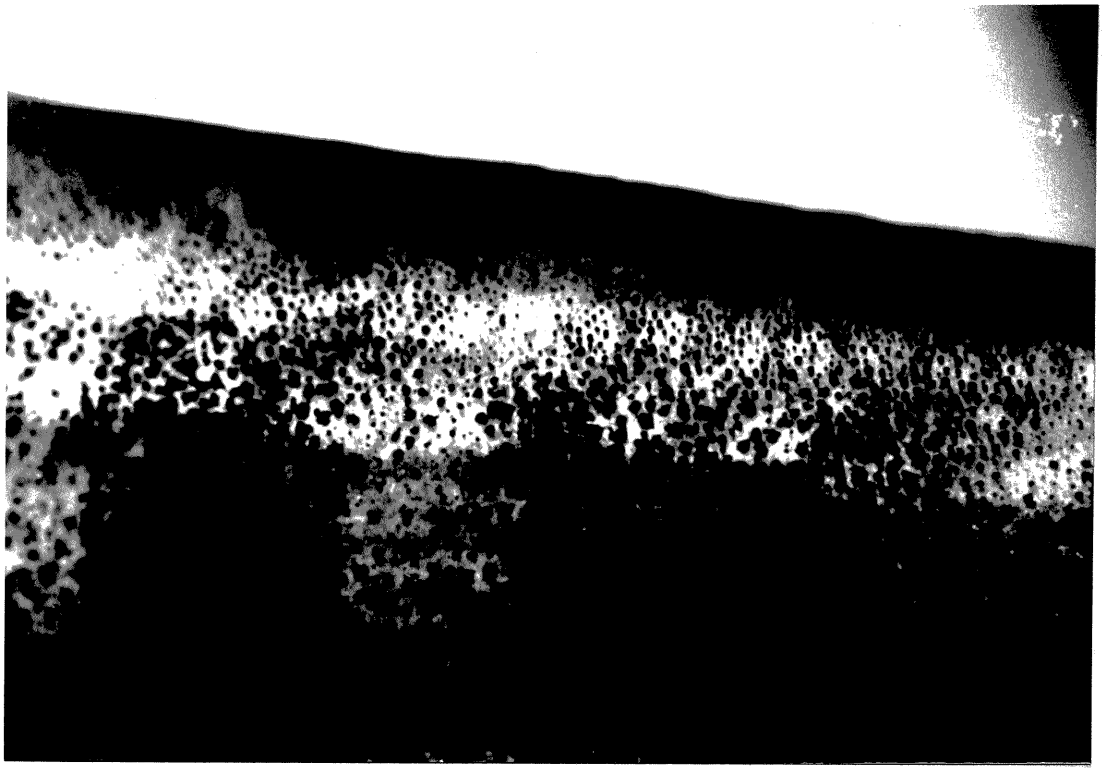
d - After fifteen minutes in complete darkness

e - After thirty minutes in complete darkness

Mag. X 15.



a



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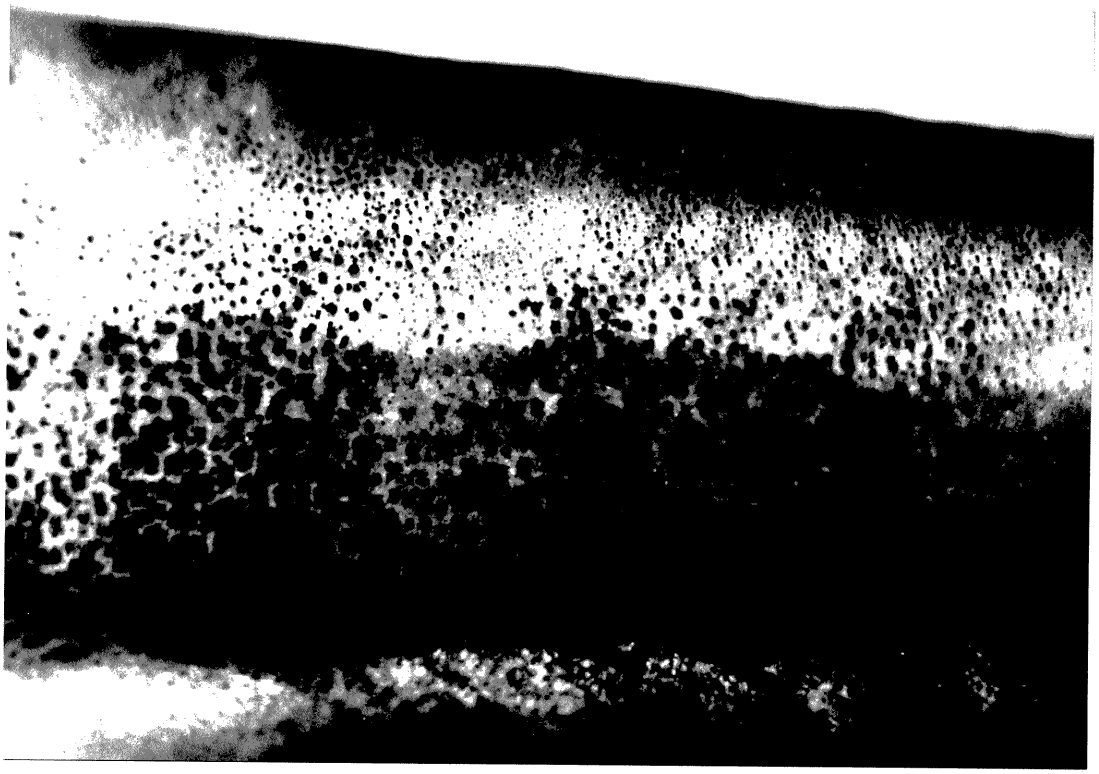
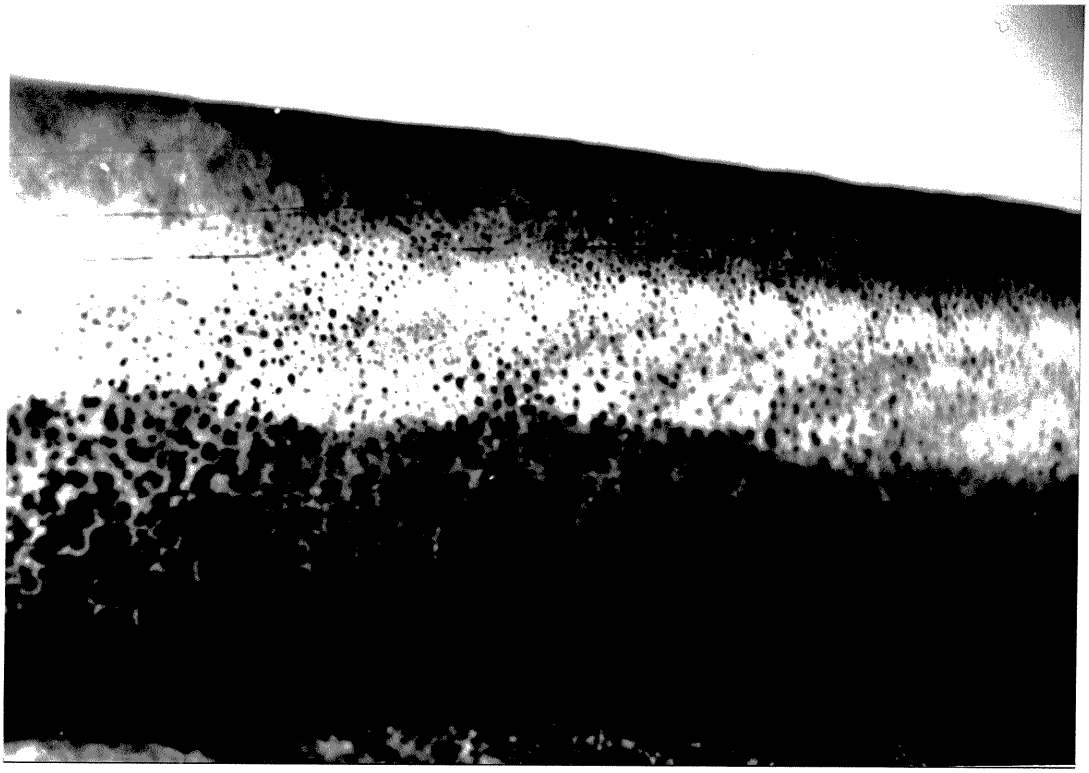


c



d

e

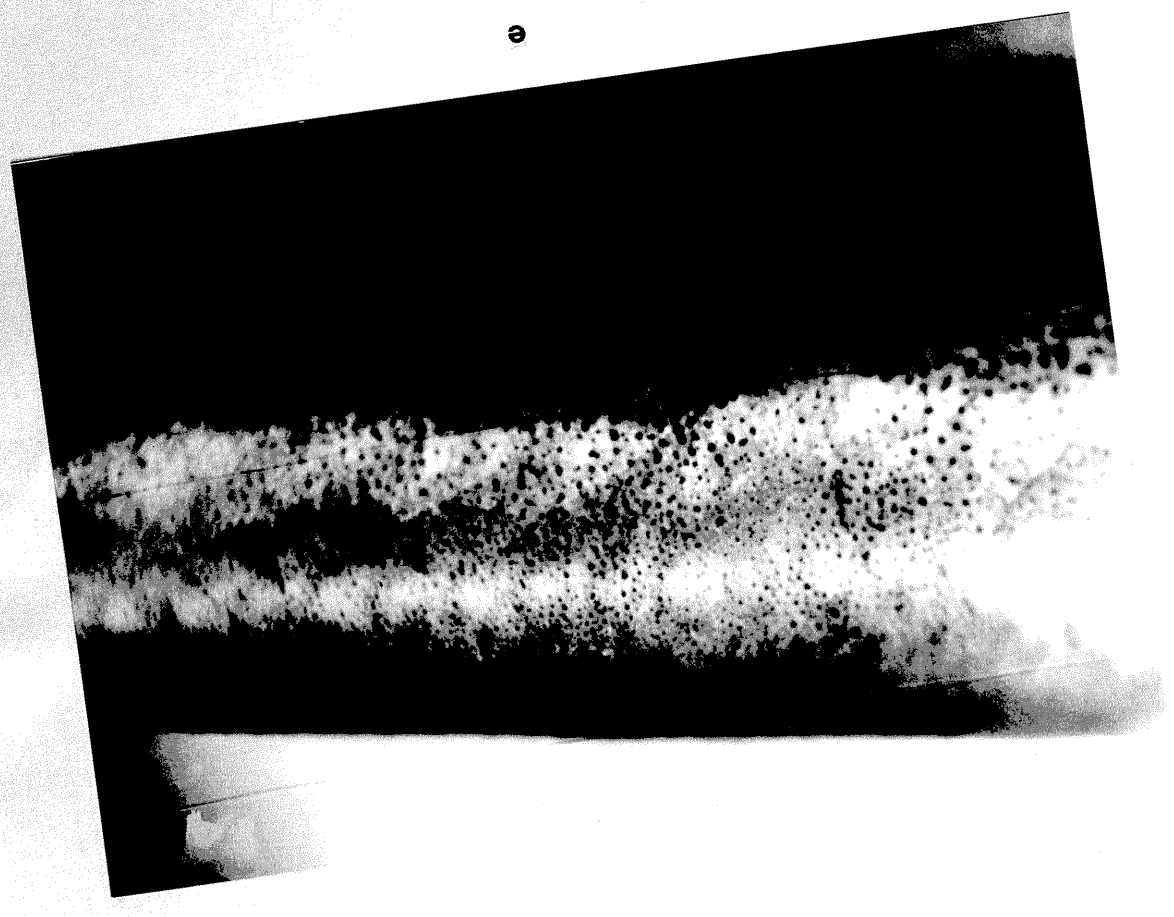


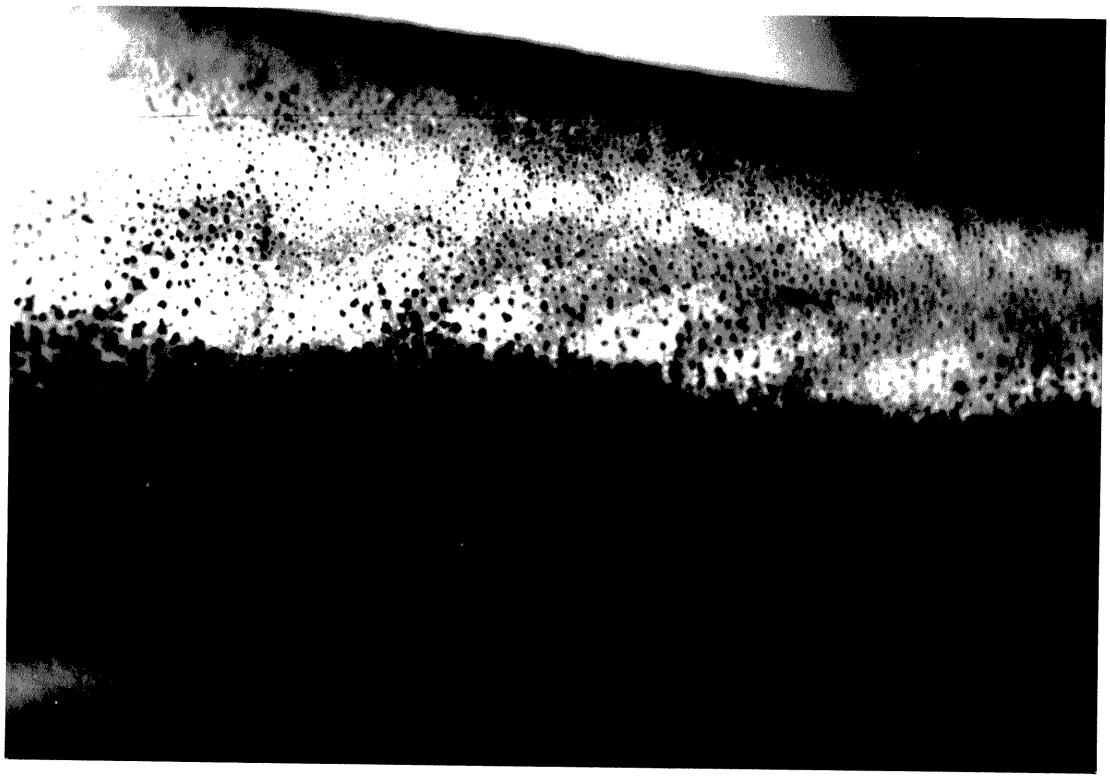
Plate VI-5a, b

Responses of *Phoxinus phoxinus* to complete darkness. The fish was first adapted to a white background for 30 minutes, then the light was switched off and the photographs were taken after the fish spent 30 minutes in darkness.

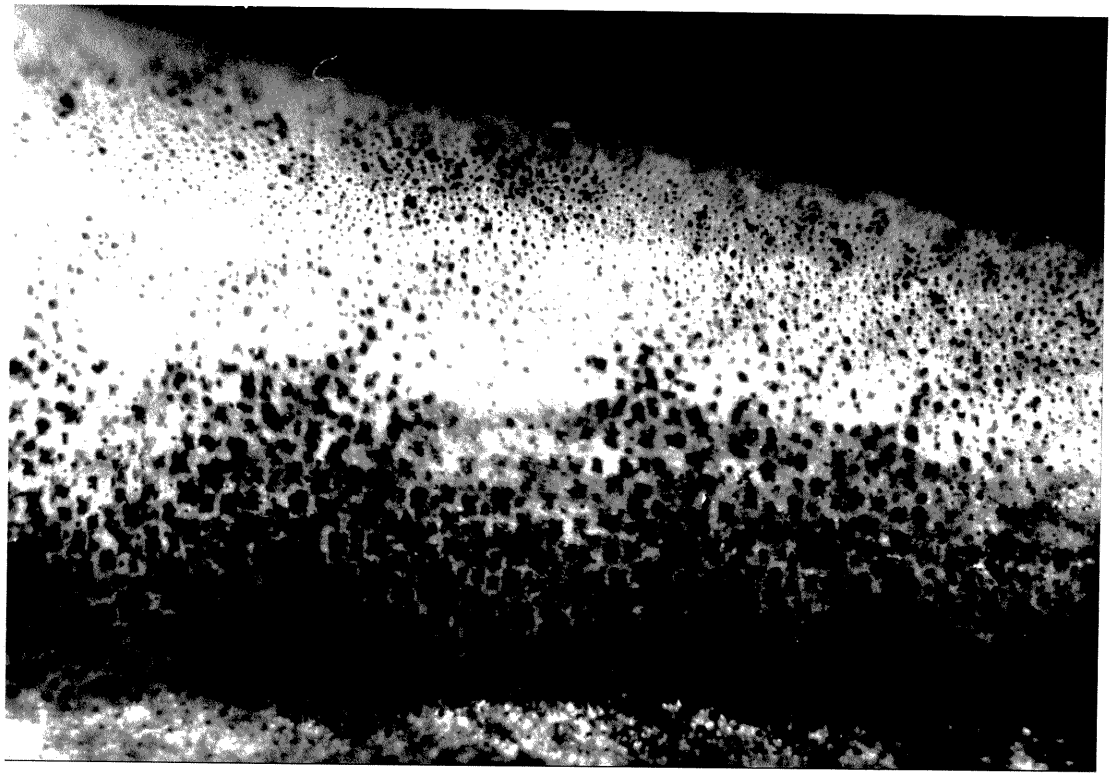
a - Local illumination from the light-guide was on throughout the experiment

b - Local illumination from the light-guide was switched off throughout the experiment.

Mag. X 15.



a



b

All the experiments were repeated on five other fish and identical results were obtained. Therefore, it seems that as the intensity of overhead illumination decreases towards total darkness, the melanophores in the lateral stripes do show some primary response to the local illumination which results in pigment dispersion. The remarkable pigment aggregation in the dorso-lateral melanophores when the fish is exposed to low ranges of overhead illumination and total darkness indicate that melanophores in these regions do not show any direct response to local illumination, since these melanophores remained with their pigment aggregated even under conditions where they were locally illuminated.

6.3.2. The fine structure of the retina of *Phoxinus phoxinus*

Examination of the retina under a Zeiss Universal photo-microscope revealed that the retina of the minnow is very well organised and its structure is very typical of the general structure of the vertebrate retina described by Cajal (1911) and McEwan (1938). As is evident from Plate VI-6a page 241 the retinal layers and the retinal cells are readily recognisable. The neural cells in the retina, as is the case in vertebrates generally, can be classified into five main types: photoreceptors, horizontal cells, bipolar cells, amacrine cells and ganglion cells. The cell bodies of the above cells are known to be in three nuclear layers, namely, the outer nuclear layer (which contains the cell bodies of the photoreceptors), the inner nuclear layer (which contains the cell bodies of horizontal, bipolar and amacrine cells) and the ganglion cell layer (which contains the cell bodies of the ganglion cells).

Plate VI-6a, b

a - A colour photomicrograph of a transverse section showing the retinal layers and the retinal cells in *Phoxinus phoxinus*.

P.R.C. Photoreceptor cells

H.C. Horizontal cells

B.C. Bipolar cells

A.C. Amacrine cells

O.N.L. Outer nuclear layer

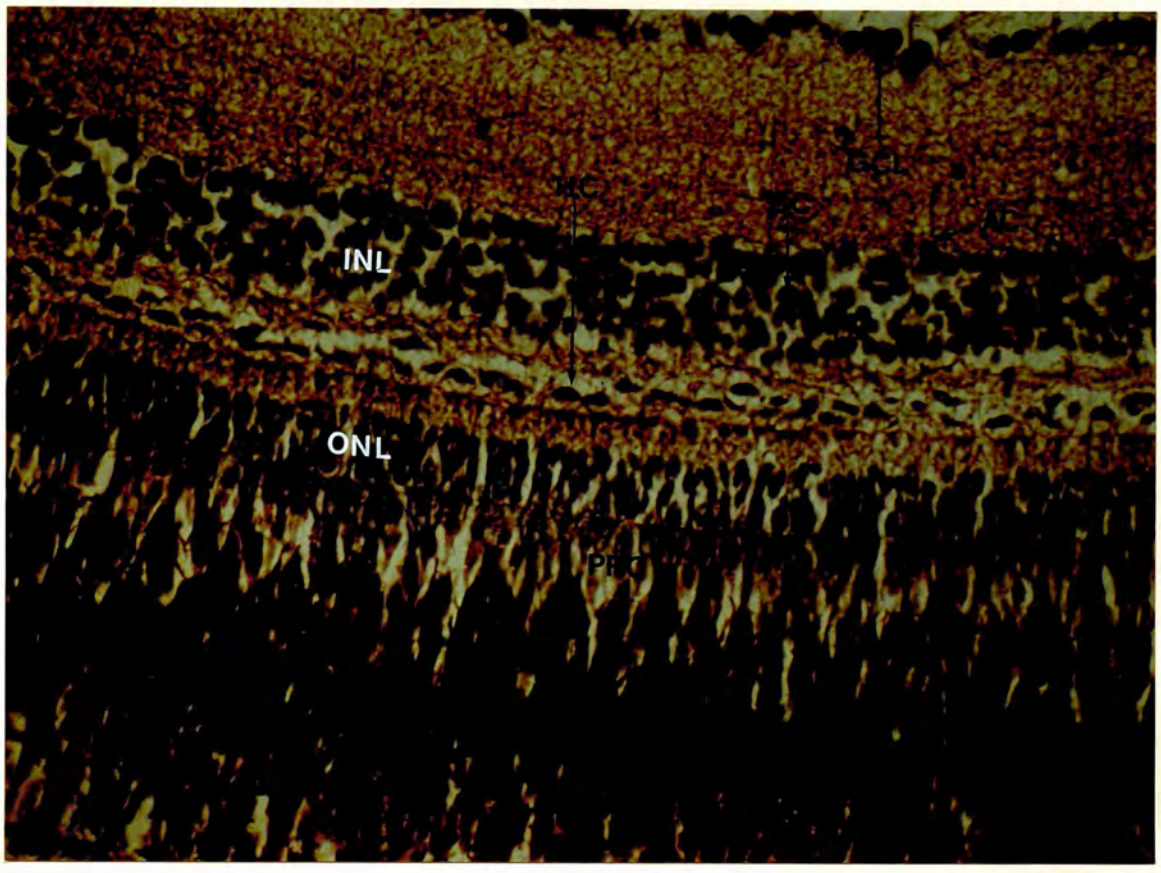
I.N.L. Inner nuclear layer

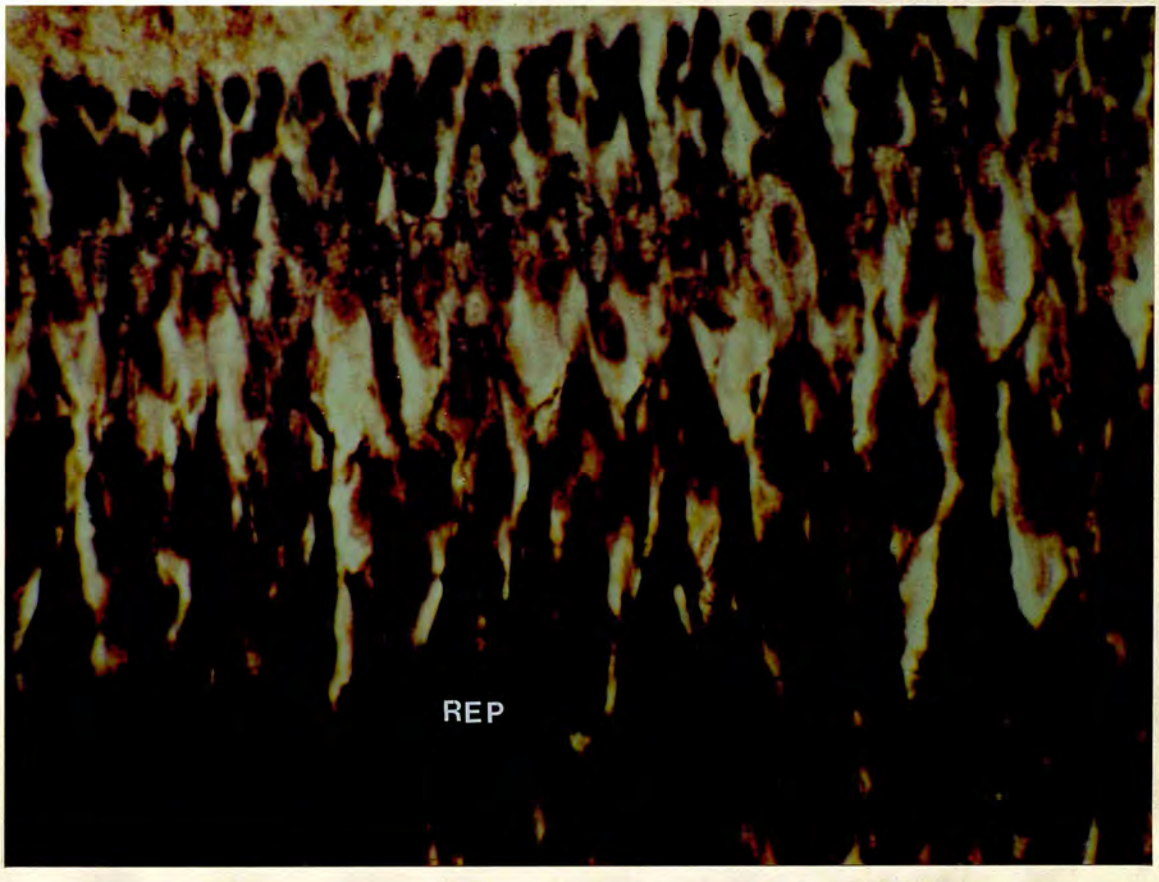
G.C.L. Ganglion cell layer

Mag. X 600.

b - A higher magnification to show the retinal epithelium pigment (REP) and extent of their migration in the light-adapted retina to protect the photoreceptors from the light.

Mag. X 1,600.


INL
ONL


REP

The photoreceptor layer in the retina of *Phoxinus* is known to be composed of cones and rods (von Frisch, 1925), the former being of various types (Lyall, 1956, 1957; Gentle, 1968). These observations are confirmed in the present study. The rods are very difficult to observe in the light-adapted retina because they are masked by the retinal epithelium pigment 'REP'. However, this difficulty was easily overcome by bleaching the epithelial pigment from the sections. The bleaching was carried out according to Engström's technique (1963) of oxidising the sections in a mixture of potassium permanganate and sulphuric acid and then bleaching them in sodium bisulphite (Plate VI-7 p. 243). As is evident from the plate, the treatment has bleached the masking pigment efficiently without affecting the fine detailed structure of the retina. In the bleached preparation, in addition to the rods, the various cone types are very well demonstrated. The photoreceptors, generally speaking, are found to consist of an outer segment, an inner segment and a cell nucleus.

The outer segments lie farthest from the light and their terminals, directed towards the pigment epithelium, are the light-sensitive part of the visual cell. They contain the visual pigment and under the electron microscope they appear to be filled with a lamellar membrane structure orientated perpendicularly to the photoreceptor axis (Plate VI-8 p. 244). This lamellar organisation of the outer segment in the vertebrate retina was first described by Sjöstrand (1948).

Plate VI-7

A photomicrograph of a transverse bleached section of the retina of *Phoxinus phoxinus* to show the various types of photoreceptor cells.

D.E. Depigmented epithelium cell

S.S.C. Short single cone

L.S.C. Long single cone

D.C. Double cone

T.C. Treble cone

R. Rod

C.I.S. Cone inner segment

C.O.S. Cone outer segment

R.I.S. Rod inner segment

R.O.S. Rod outer segment

Mag. X 1,250.

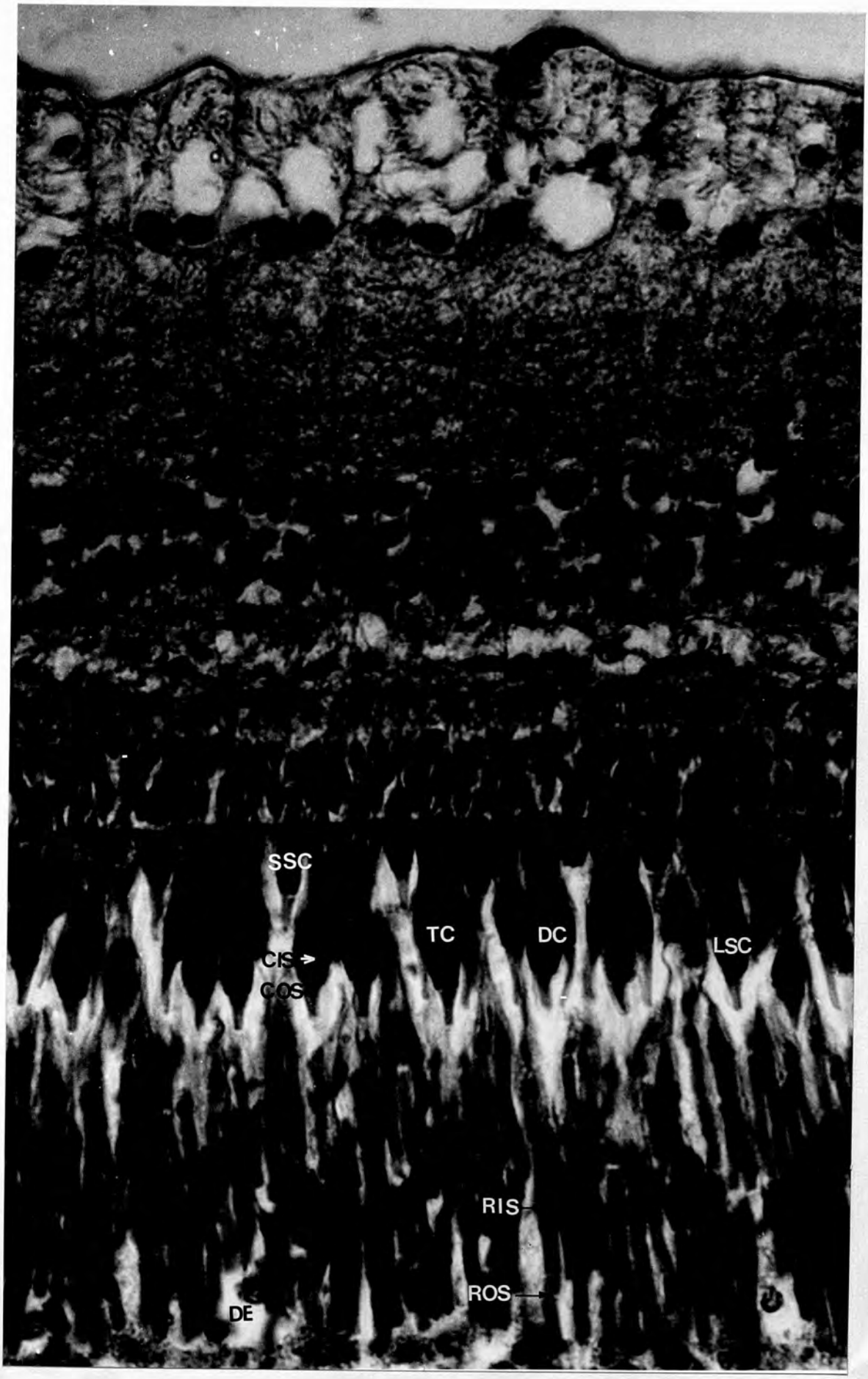

SSC
TC
DC
LSC
CIS
COS
RIS
ROS
DE

Plate VI-8

An electronmicrograph from a transverse section of the retina of *Phoxinus phoxinus* showing the distal part and the proximal part of the inner and outer segment of a cone.

D.I.S. Distal part of the inner segment

P.O.S. Proximal part of the outer segment

Mit. Mitochondria

L.M. The lamellar membrane of the outer segment

Note : The cytoplasmic bridges (S.B.) between the inner and outer segment.

Mag. X 18,000.

Plate VI-9

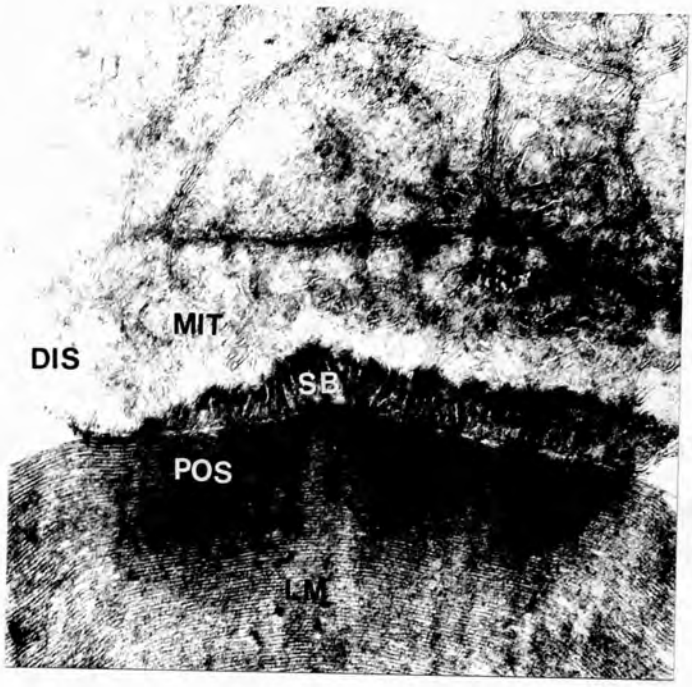
An electronmicrograph of a transverse section of the retina of *Phoxinus phoxinus* showing a part of the inner segment of a treble cone. As can be seen, the components of multiple cones are not fused.

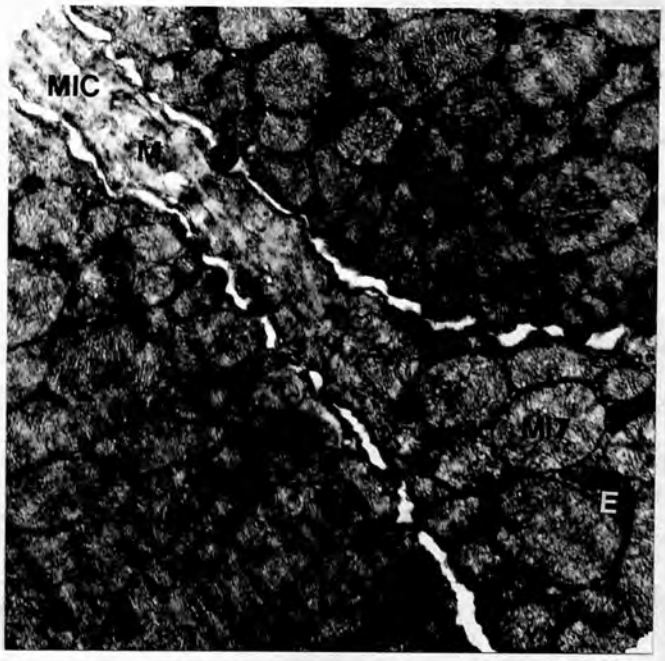
Note : The two portions of the inner segment can be seen in the middle cone (the ellipsoid, E; the myoid, M).

Mit : Mitochondria

Mic : Microtubules

Mag. X 10,800.


MIT
DIS
SB
POS
LM


MIC
M
MIT
E

The outer segment was found to be connected with the inner segment by cytoplasmic bridges (Plate VI-8 p. 244). Since these bridges were observed only in some of the sections, it remains unclear whether or not this is the normal structure or an artifact. The inner segment can be divided into two regions, a region containing a mass of densely packed mitochondria, termed the ellipsoid, which lies proximal to the outer segment and a region distal to it which contains granular endoplasmic reticulum and microtubules termed the myoid (Plates VI-9, 10 pp. 244, 246). The retinal epithelium cells appear under the light microscope as dark, pigmented cells. Their processes, as judged by the extent of the pigment granules that have migrated into them, in the light-adapted retina, reach almost as far as the external limiting membrane (Plate VI-6b p. 241). Under the electron microscope the cells contain masses of pigment granules spherical or cylindrically shaped. The cells also contain very many mitochondria and a nucleus . (Plate VI-11 p. 246). These pigment granules mask the outer segment and protect the visual pigment from excessive striking light.

The structural basis for rod and cone classification is the shape of their outer segment, which in the former is relatively long and cylindrical and in the latter is relatively short and conical. The above can clearly be seen in Plate VI-7 page 243.

In the light-adapted retina the rods are connected to the external limiting membrane by very thin filamentous myoids, whereas the cone myoids are contracted and are relatively thicker in diameter.

Plate VI-10

Electronmicrograph of a transverse section of the retina of *Phoxinus phoxinus* showing part of myoid packed with granular endoplasmic reticulum (GER).

Mag. X 27,000.

Plate VI-11

Electronmicrograph to show the component of a retinal epithelium cell of *Phoxinus phoxinus*.

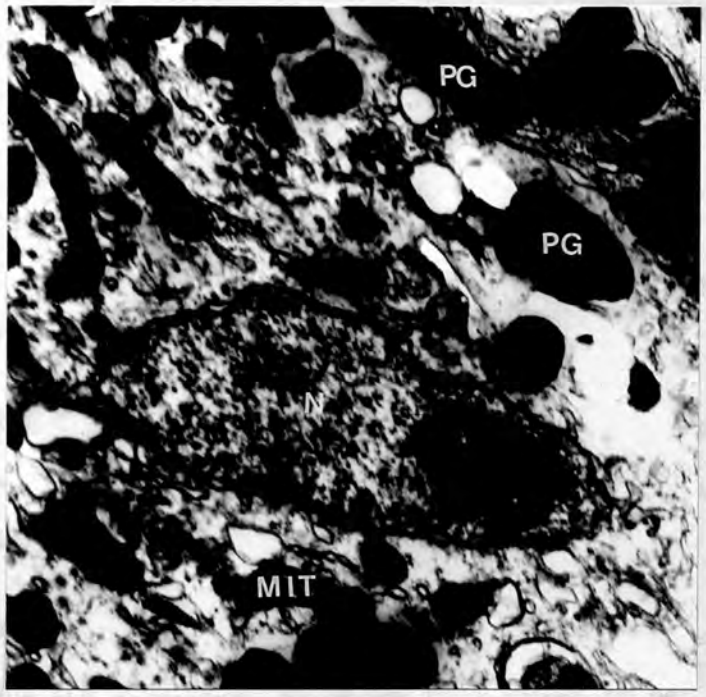
P.G. Pigment granules

Mit. Mitochondria

N. Nucleus

Mag. X 18,000.


GER


PG
PG
N
MIT

There are also some structural differences between the various types of cone present (Plate VI-7 p. 243). The ellipsoid in the short single cones is separated only by a short myoid from the external limiting membrane and the nucleus is always found inside this membrane. The long, single cones differ from the short, single cones in the following respects:-

(a) they have a relatively larger ellipsoid and longer myoid

(b) their nuclei are either partially inside or completely outside the external limiting membrane.

The two components of the double cones are of unequal size. The difference in their size is mainly due to the length of their myoids and the outer segment. The component with the shorter myoid appears to have a larger but thinner outer segment. Their nuclei, as was the case with the long single cones, are either partially inside or completely outside the external limiting membrane.

The treble cones, as appears in transverse section, are composed of a double cone and single long cone, closed together in a linear shape. The components of this multiple cone are not fused and do not appear to have any cytoplasmic linkages (Plate VI-9 p. 244).

No quadruple cones could be observed in the transverse sections, although their existence has been reported in tangental sections (Lyall, 1956, 1957; Gentle, 1968).

With regard to the distribution of the visual cells, some regional variations are observed. While the rods appear to be

uniformly distributed, different regions of the retina differ so far as the various types of cones are concerned. The dorsal and central regions of the retina are found to be more densely populated with treble cones and short single cones. The ventral part of the retina mainly consists of double and long single cones (Plate VI-12 p. 249). No specialised crescentic bridge containing more visual elements and more bipolar cells than any other part of the retina could be observed. This does not agree with the results reported by Butcher (1938) working on the retina of *Fundulus*.

6.3.3. Retinomotor responses in *Phoxinus phoxinus*

In the great majority of teleosts, retinomotor responses (also called "photomechanical") undertake the function of pupillary control of light intensity at the retinal level. A change from light to darkness and the reverse is accompanied by a change in the position of the visuals (rods and cones) and the retinal epithelium pigment. Plate VI-13 page 250 shows the position of the visual cells and the epithelium pigment in a retina adapted to complete darkness for a period of 60 minutes. The cones in complete darkness can be seen from the above plate to be elongated and the rods contracted. The epithelium pigment is aggregated and has withdrawn from the processes leaving the rods unmasked. These structural changes in complete darkness have been correlated with the situation when the illumination is very low or absent and the cones, no longer functional, become elongated and move out of the way. The other set of visual cells, the rods, with their lower threshold of stimulation than the cones, contract towards the light. The rods' contraction and the

Plate VI-12

Photomicrograph of a transverse section showing the ventral part of the retina of *Phoxinus phoxinus*. The cones in this region are mainly long single and double cones.

L.S.C. Long single cone

D.C. Double cone

Mag. X 800.

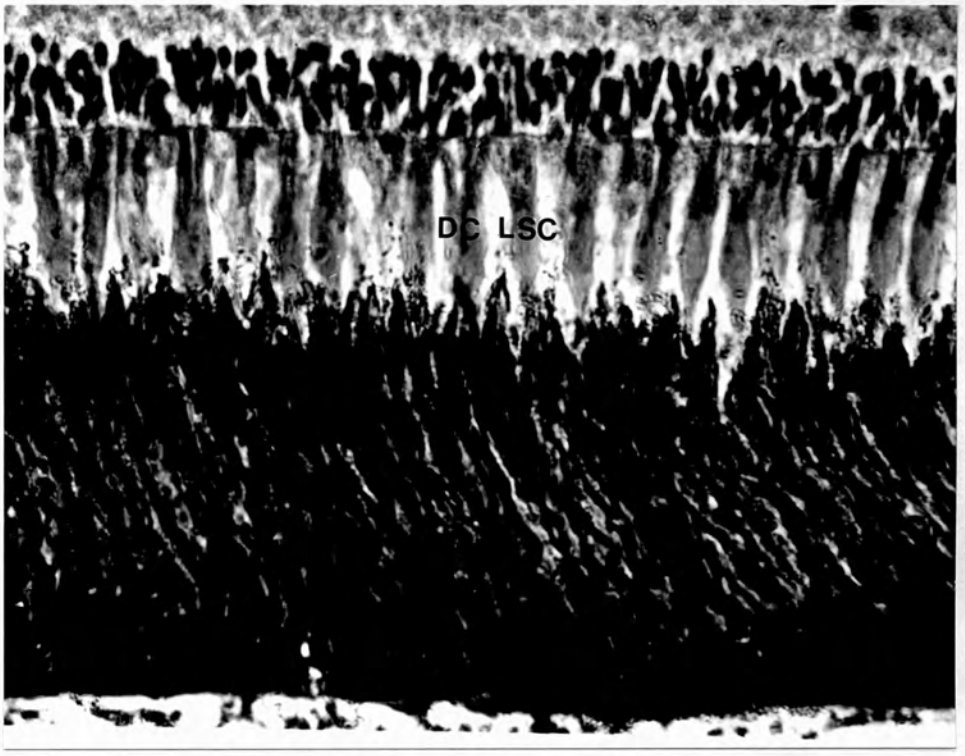

DC LSC

Plate VI-13

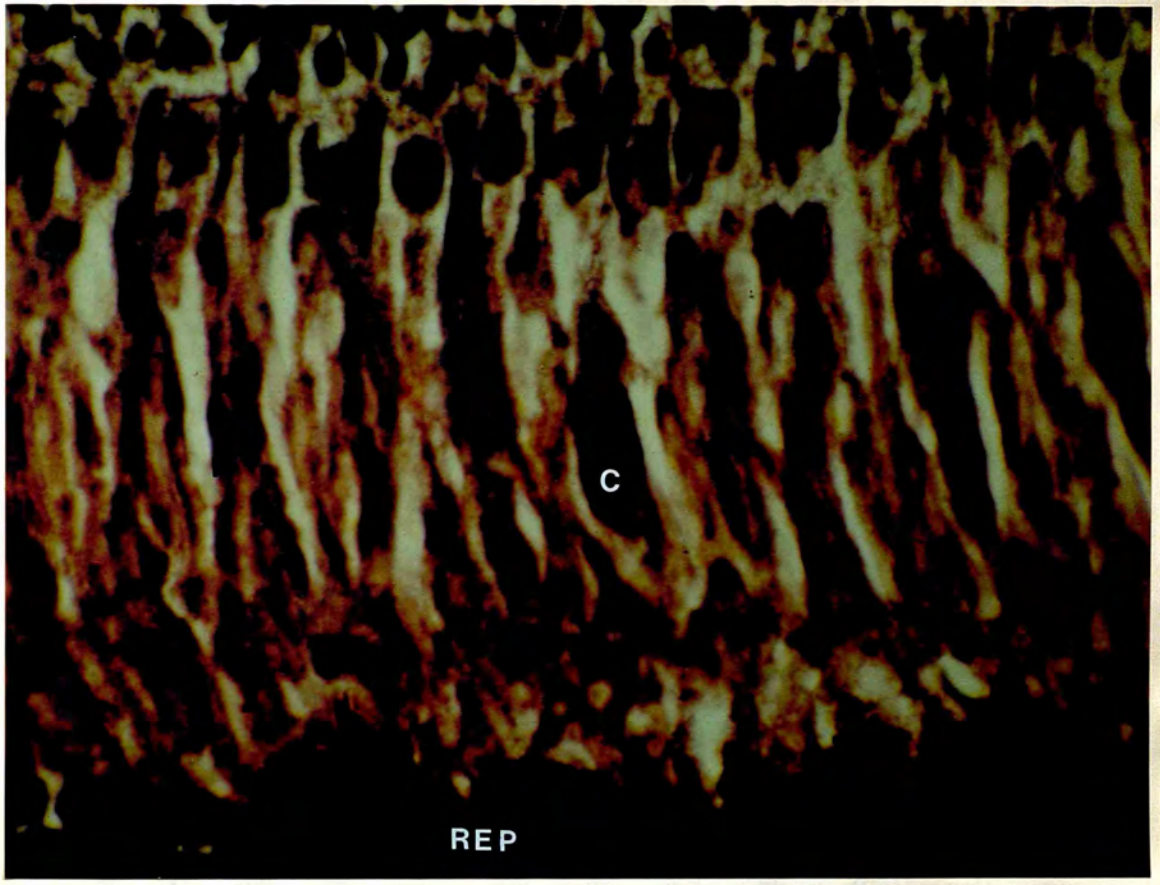
Photomicrograph of a transverse section showing a dark-adapted retina of *Phoxinus phoxinus*.

(R) - rod

(C) - cone

(REP) - retinal epithelium pigment

Mag. X 1250


C
REP

withdrawal of the masking pigment provides the optimum conditions for the former to be stimulated (Walls, 1942; Detwiler, 1943; Nicol, 1963; Blaxter, 1970; Ali, 1975). As the intensity of the light increases, the pigment granules disperse in the processes and so protect the visual pigment in the rods from light. At the same time this protection is reinforced by elongation of the rods themselves away from the light. Plate VI-14 page 252 shows the structure of the retina when the eye was first adapted to complete darkness for a period of 60 minutes following which light of an intensity of 12.5 lux was switched on for a period of 60 minutes. Under the above value of light intensity the retina appears to be fully light-adapted. The epithelial pigment has fully dispersed and the cones have contracted.

In another experiment, the eye was similarly adapted to complete darkness for 60 minutes after which it was exposed to a light intensity of 1.25 lux. Plate VI-15 page 252 shows the structure of the retina exposed to the above light intensity. As is evident from the above plate, the retina is not fully light-adapted nor is it fully dark-adapted. The pigment granules have not fully dispersed and the cones are not fully contracted. This intermediate position of the visual cells and the epithelial pigment was found to be uniform throughout the entire region of the retina and no regional differences could be observed as far as the retino-motor responses were concerned. In a further experiment, after 60 minutes dark adaptation, the fish was exposed to a light intensity of 0.0125 lux. At this intensity the retina appeared to be fully

Plate VI-14

Photomicrograph of a transverse section from a retina of *Phoxinus phoxinus* first adapted to complete darkness for 60 minutes then exposed to 12.4 lux light intensity. The retina appears as fully light-adapted.

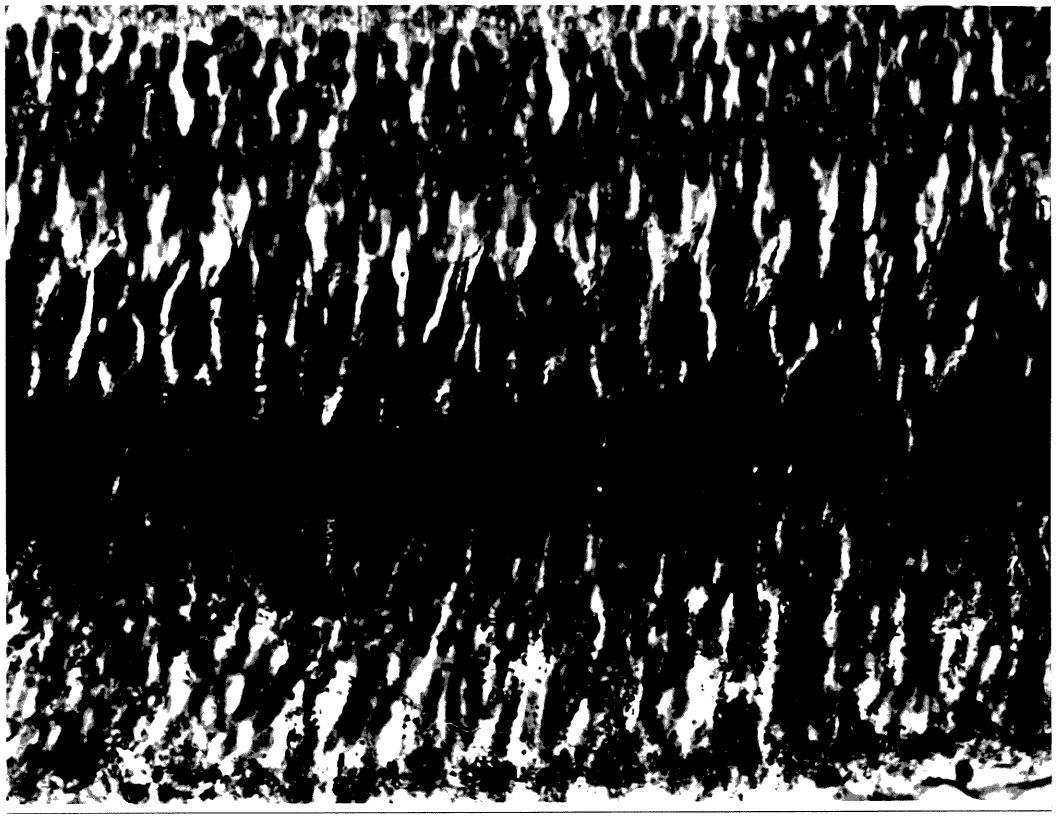
Mag. X 800.

Plate VI-15

Photomicrograph of a transverse section from a retina of *Phoxinus phoxinus* first adapted to complete darkness for 60 minutes then exposed to 1.25 lux light intensity.

Note : The cones have not fully contracted and the pigment granules are not fully dispersed.

Mag. X 800.



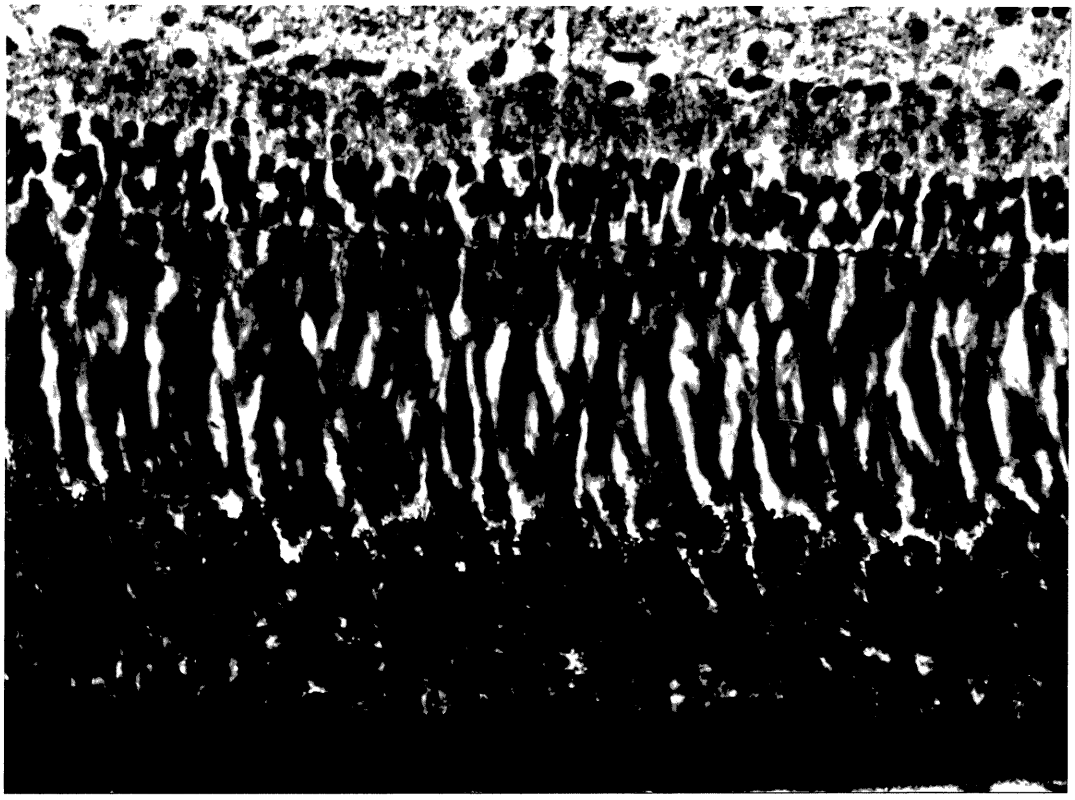


Plate VI-16

Photomicrograph of a transverse section from a retina of *Phoxinus phoxinus* first adapted to complete darkness for 60 minutes then exposed to 0.0125 lux light intensity.

Note: The cones have remained fully elongated and the rods fully contracted. The pigment granules are fully aggregated.

C. Cone

R. Rod

Mag. X 800.


R
C

dark-adapted (Plate VI-16 p.253). The above experiments were repeated and the fish were first adapted to light of 2,500 lux for 60 minutes. Then the intensity was reduced to 1.25 lux in one experiment and 0.0125 lux in another experiment. Examination of the retina revealed the same results as those obtained when the fish was first dark-adapted and then exposed to the above light intensities.

Therefore, based on the present experiments, it appears that the retinomotor responses are integrated responses and that the sensitivity of the visual cells and the epithelial pigment to light is equal throughout the retina. Thus, it would appear that any rigid concept of retinal differentiation is far too simple to account for chromatic adaptation of the fish.

6.3.4. Pattern changes in the plaice *Pleuronectes platessa* in different light intensities

On the pigmented side of plaice there are regions which differ among themselves with regard to the population of melanophores. The regions where melanophores are densely populated are called the dark patches and the regions where melanophores are less densely populated are called the pale patches (Hewer, 1927). The ability of this fish to match the pattern of the background depends upon the degree of contrast between these patches and their further subdivision. Plate VI-17a page 257 shows the response of the fish to a chequer-board with black and white squares of 1 cm side. As is evident, the two different patches of melanophores are very precisely distinguishable.

The distinction between the patches can be analysed in two respects, the difference in the number of melanophores per unit area in these patches (Hewer, 1927) and the state of pigment granules within the melanophores of the concerned patches. On this particular background, while the fish has shown a pronounced pigment dispersion in the dark patches, it has shown remarkable pigment aggregation in the pale patches. The above photograph was taken 60 minutes after the fish was placed on the background. The background was reversed to a different chequerboard with smaller black and white squares (2.5 mm). Plate VI-17b page 257 was taken 60 minutes after such a reversal. On this new background, melanophores in dark patches appear to show a certain degree of pigment aggregation. However, not all the melanophores of the dark patches appear to have shown the same extent of pigment aggregation. At the centre of each dark patch, a group of melanophores can be seen that show very slight, if any at all, pigment aggregation, while melanophores surrounding these groups show some degree of pigment aggregation. The melanophores in the pale patches on this background show a certain degree of pigment dispersion. Although changes in the patterns of the fish do not strongly match the patterns of the backgrounds, nevertheless, the plates do indicate that the fish shows some tendency in this line. As reported by earlier workers (Sumner, 1910, 1911; Mast, 1916) there is great individual variation among these fish in their capacity to match the pattern of their background. Unfortunately, in the present study, due to the limited number of specimens, there was no scope to be more selective as far as the pattern changes are

concerned. After exposing Plate VI-17b page 257 in order to study the pattern of the fish in complete darkness, the light was switched off and a photograph was taken after the fish had remained in total darkness for 60 minutes (Plate VI-17c p. 257). As is evident the fish has assumed an intermediate shade in complete darkness. This observation confirms the results reported by Sumner (1910, 1911) that flatfish assume an intermediate shade in complete darkness. The change is very pronounced in the dark patches. Melanophores in these patches showed a great degree of pigment aggregation. This pigment aggregation was again more intense in the melanophores surrounding those at the centre of the patch which almost remained fully dispersed. Melanophores in the pale patches, show some pigment aggregation in absolute darkness. This can be seen by comparing the state of these melanophores when the fish was on the chequerboard with smaller squares with their state in complete darkness (Plate VI-17b, c p. 257). But if the state of melanophores in the pale patches in complete darkness is compared with their state on an illuminated chequerboard with larger squares, then the melanophores in these paler patches appear to show some degree of pigment dispersion. This dispersion is more remarkable in the pale patches of the head region (compare Plate VI-17a and c page 257). As is evident from these plates, the pattern of the fish on an illuminated chequerboard with large squares and the pattern of the fish in complete darkness show the more pronounced changes. Therefore, this background was selected to find out what is the minimum incident light intensity required for the fish to keep the pattern.

Plate VI-17a, b, c

a - The pattern on *Pleuronectes platessa* on a chequerboard of side 1 cm.

D.P. dark patch

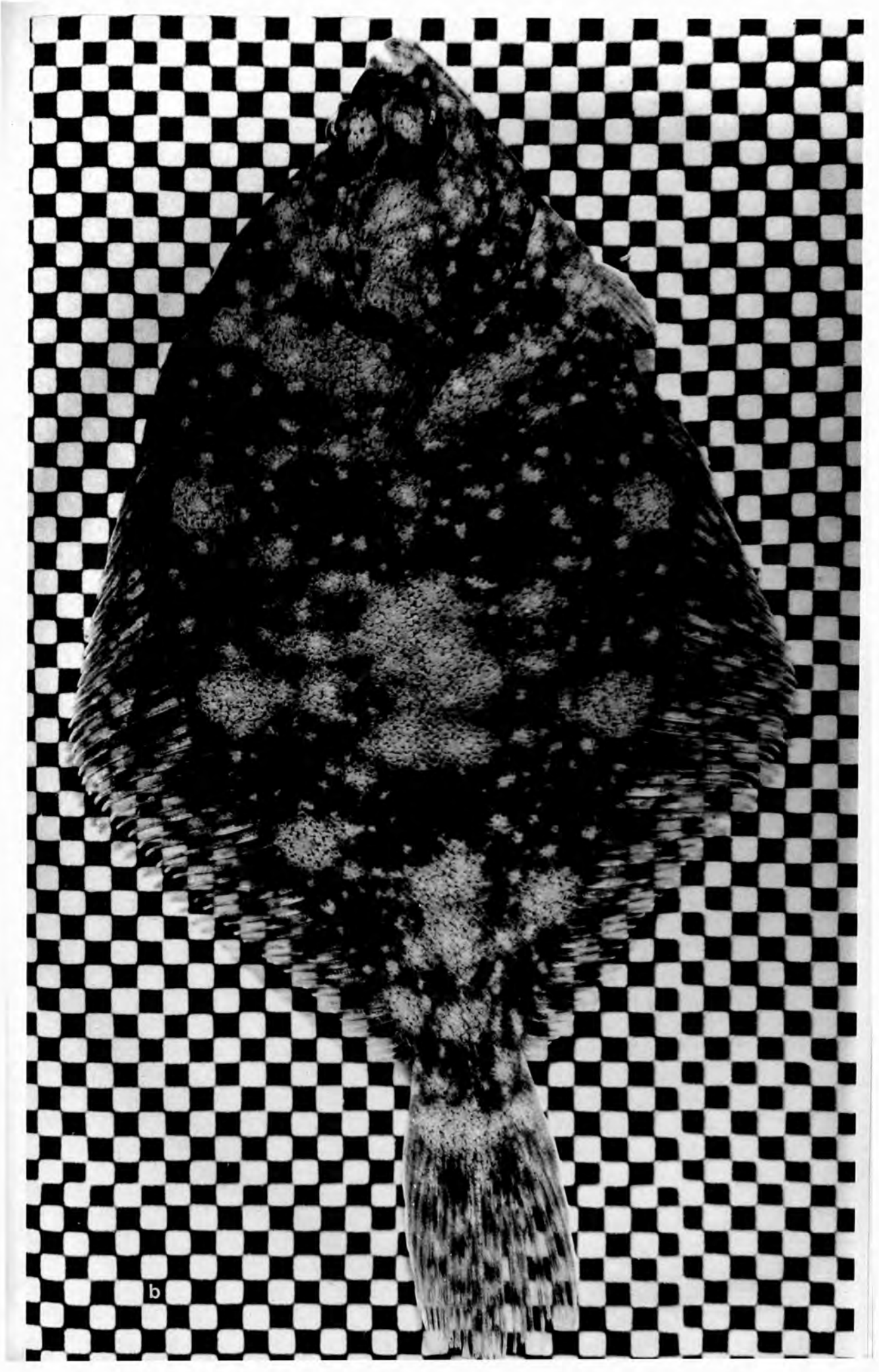
P.P. pale patch

b - The pattern of the fish on a chequerboard of side 2.5 mm.

c - The pattern of the fish in complete darkness.

Mag. X 2.


DP
PP
a


b

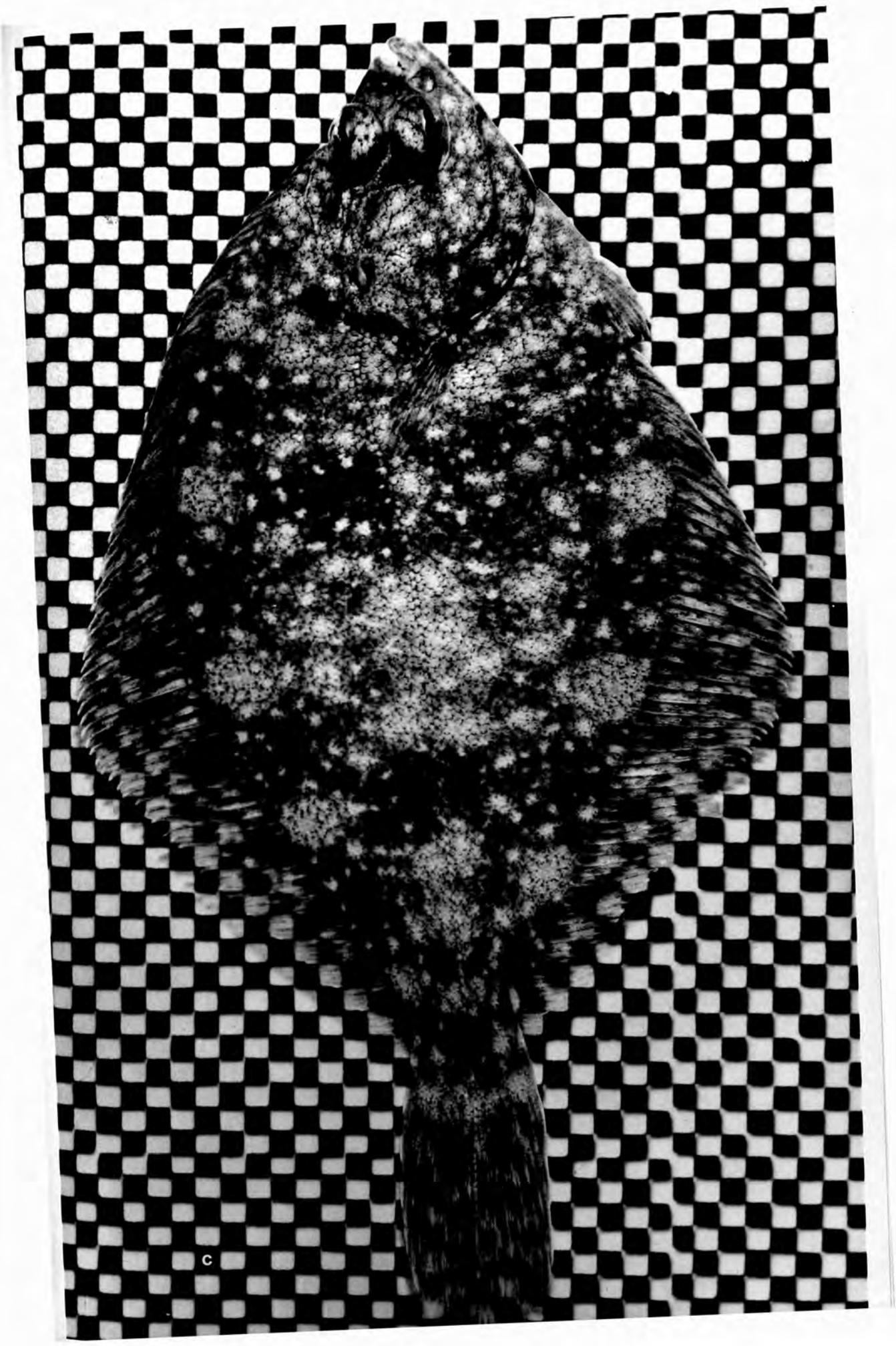

c

Plate VI-18a, b, c, d

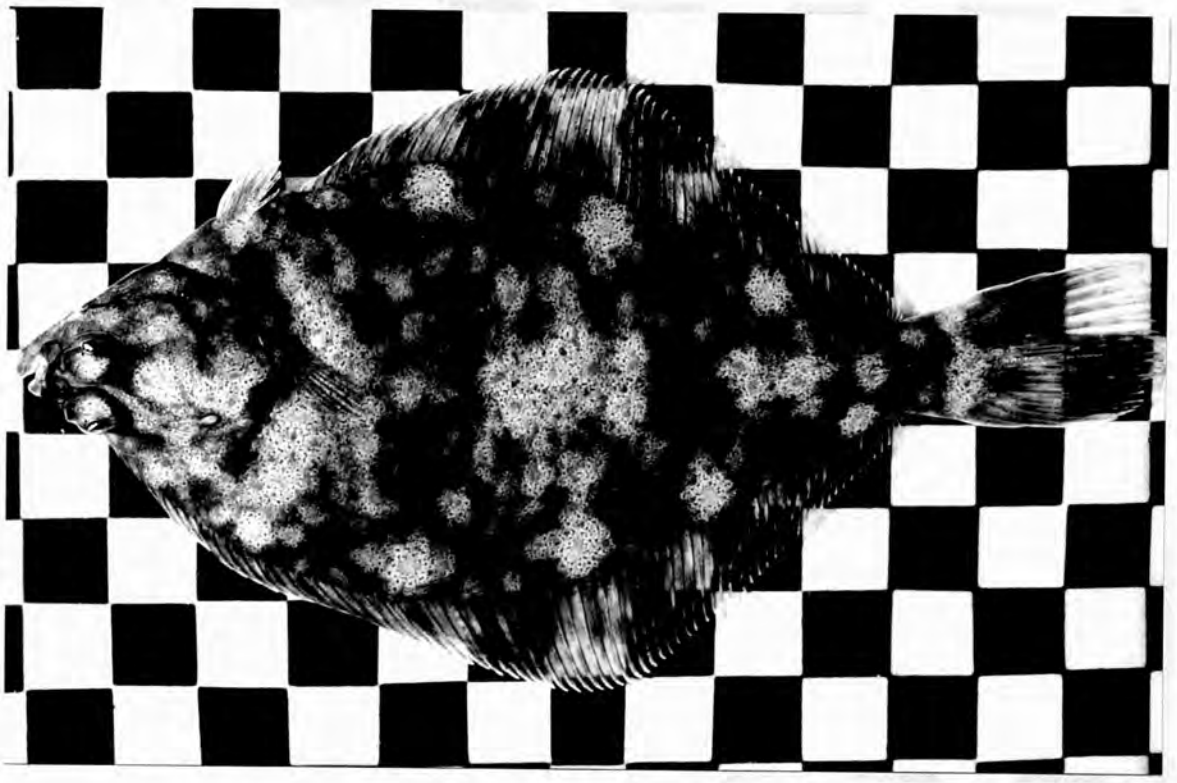
a - The pattern of *Pleuronectes platessa* on a chequerboard of side 1 cm. illuminated by an incident light of 16.5 lux.

b - The pattern of the fish on the same background but with the incident light intensity reduced to 1.65 lux.

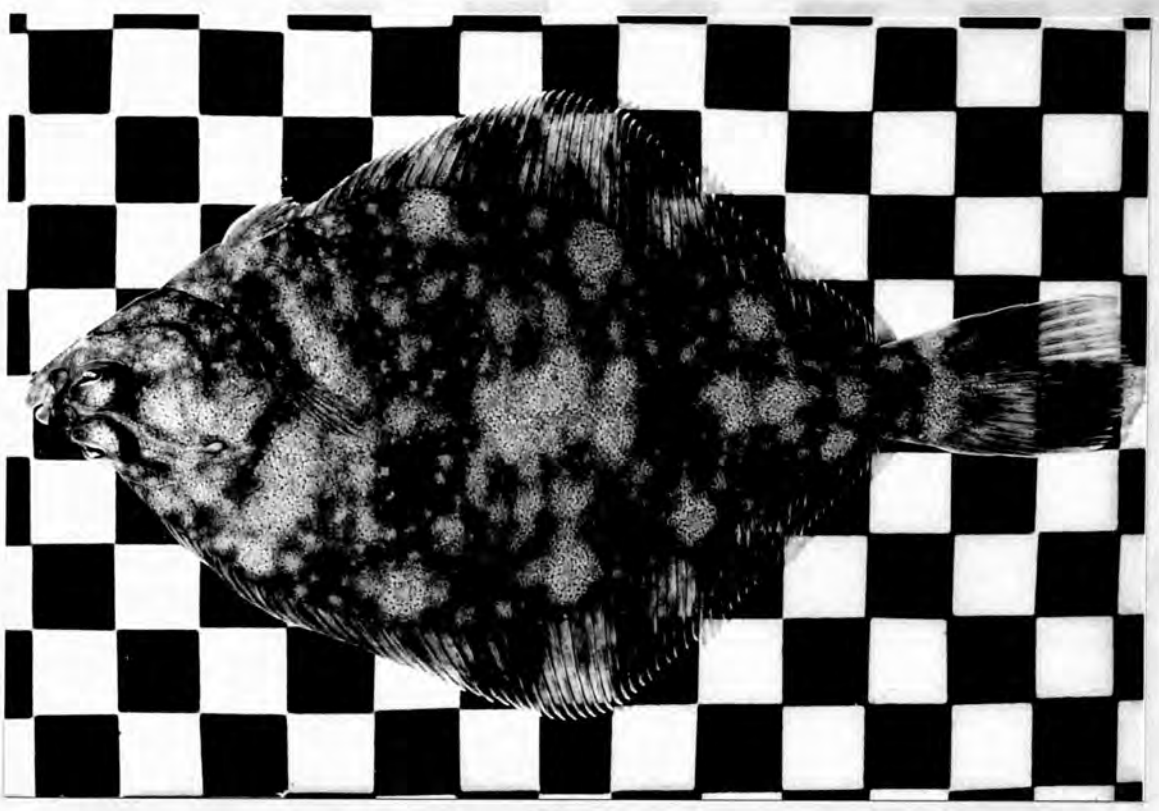
c - The pattern of the fish when the intensity was reduced to 0.165 lux.

d - The pattern of the fish when the intensity was reduced to 0.0165 lux.

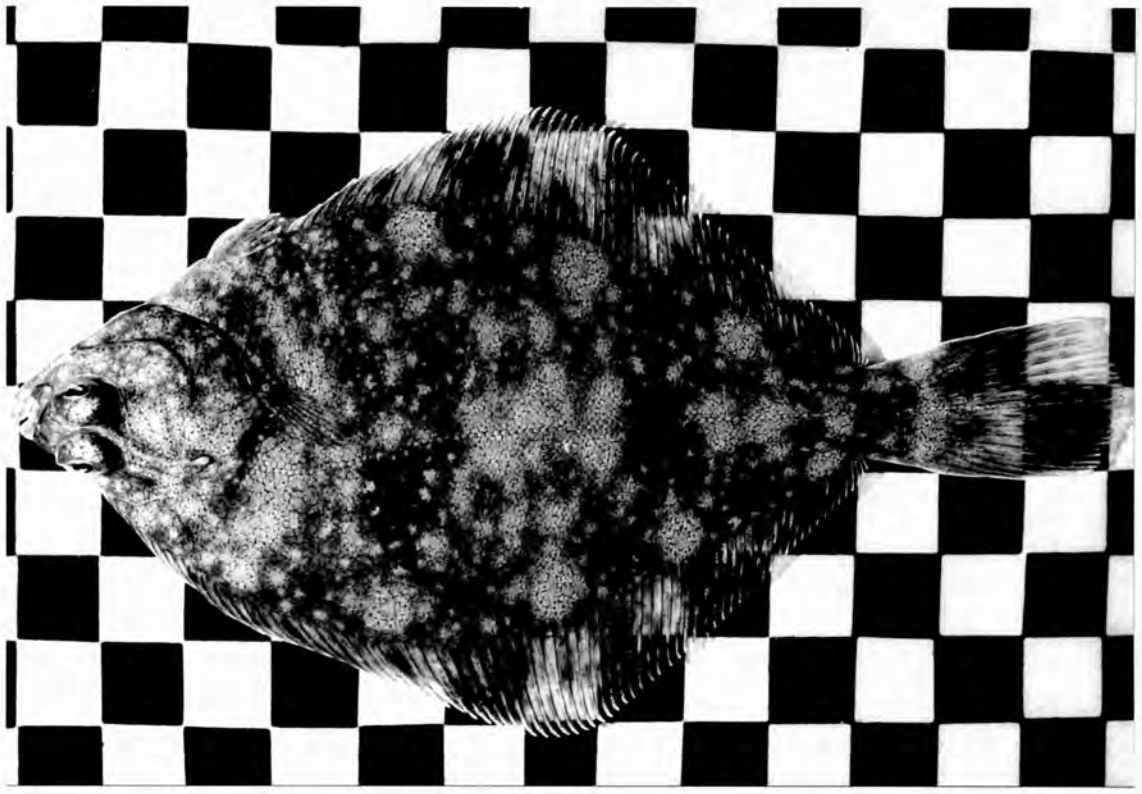
Actual size



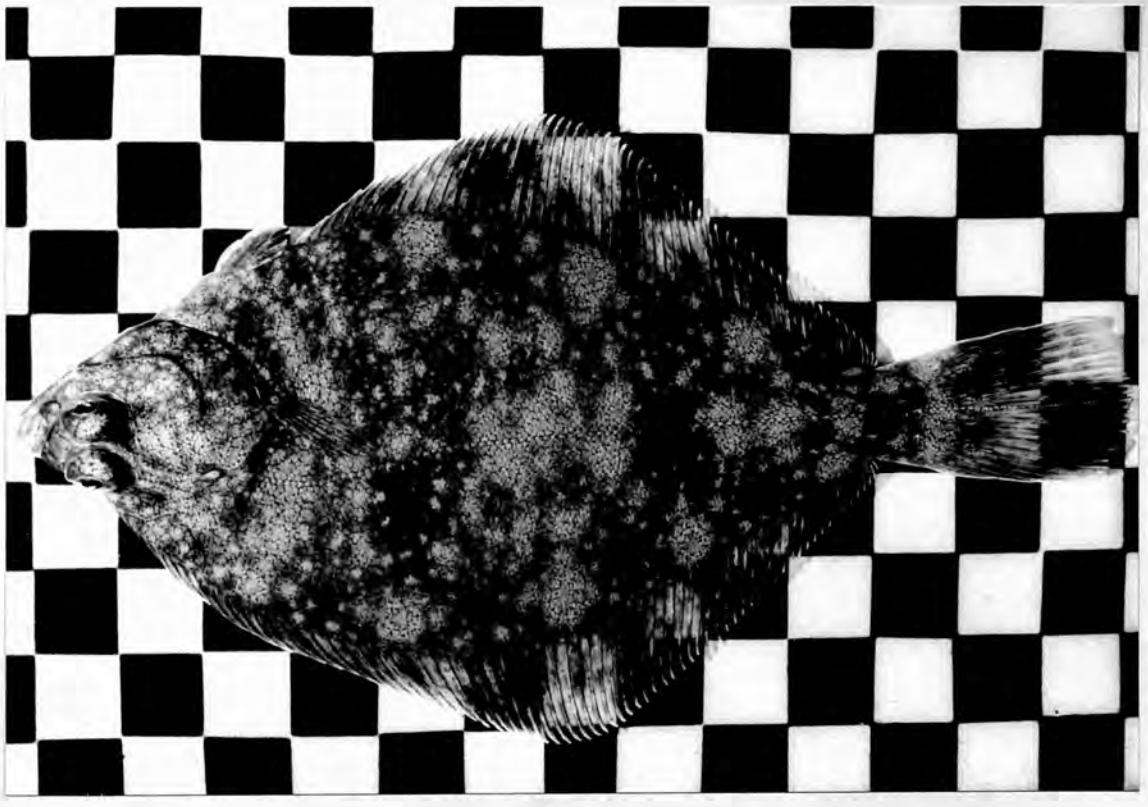
a



b



c



d

The fish was first adapted to the background for 60 minutes and photographed. Then, gradually, the incident light intensity was decreased according to the method described (page 220). The results of these experiments show that at an incident light intensity of 16.5 lux (1.53 f-c) and above, the fish maintained its pattern (Plate VI-18a p. 258). But under light intensities of values 1.65, 0.165 and 0.0165 lux, the fish gradually assumed the pattern it exhibited in complete darkness. Plate VI-18b, c, d page 258 shows the patterns of the fish at the above values of light intensities respectively.

6.4. Discussion

The fact that the intensity of illumination over very large ranges has no effect on the magnitude or the speed of response to background reversals in *Phoxinus* and the pattern of *Pleuronectes* on the variegated background, clearly indicates that light intensity has no effect on such responses. However, for the above responses to take place, the intensity of stimulation should not be below the threshold required to elicit such responses. This is in agreement with the results reported by previous workers on the effect of light intensity on the background responses in lower vertebrates (Sand, 1935; Brown, 1936; Danielson, 1938; Hogben and Landgrebe, 1940).

The mechanism which controls the striking phenomenon of pattern changes in flatfish is entirely unknown. Pharmacological studies on melanophore innervation in *Pleuronectes* have established an adrenergic innervation of melanophores in this flatfish and the pharmacological

properties of the pre- and post-synaptic receptors on the melanophores resemble those in *Phoxinus*.

Although, at this stage, one can say nothing with certainty about the nature of the mechanism mediating the responses of the flatfish to variegated backgrounds, nevertheless, the following line of experiments might shed some light on the problem.

Iwata and Fukuda (1973), in their studies on central control of colour changes in *Carassius carassius*, were able to localise the paling and the darkening areas in the tectum. They found electrical stimulation of the lateral side of the tectum caused paling, whereas electrical stimulation of the median part of the tectum caused darkening. Under continuous stimulation of the respected areas, they recorded impulses travelling along the sympathetic chain by means of an electrode connected to an oscilloscope screen through an amplifier. They observed that electrical stimulation given to the paling area of the optic tectum caused a burst of discharges of impulses in the sympathetic chain, and pigment aggregation followed. However, stimulation of the darkening area of the tectum decreased the discharge rate. Therefore, it will be very interesting to study the effect of electrical stimulation applied to different regions of the tectum in relation to the nature of the impulses travelling in the sympathetic chain and the pattern of the fish. In a further experiment, the tectum could be activated naturally by exposing the fish to various types of variegated background and the impulses travelling along the sympathetic chain could be recorded. These experiments would show

if the tectum in these fish are differentiated in such a way that the different retinal output in response to variegated backgrounds are discriminated and consequently, specific impulses would be sent to the periphery according to the pattern of the background. Also, it would be of great interest to study the neuro-melanophore relations in the dark and pale patches of these fish. This would help to find out whether the neuro-melanophore distances and the frequency of the intimate contact between nerve-endings and melanophores in the dark and pale patches are equal or not. Any differences between the two patches in the above respects might well account for the differential responses of their melanophores to the released neurotransmitter.

The dispersion evoked in the lateral stripe melanophores of *Phoxinus* by local illumination, under conditions where the overhead illumination was very low or totally absent, suggests that the melanophores in this region of the skin do show primary response to local illumination. The primary response of melanophores to light in chromatic animals has long been known (von Frisch in *Phoxinus*, 1911; Parker and Lanchner in *Fundulus*, 1922; Wykes in *Gobius*, 1937; Wykes in *Ameiurus*, 1938; Healey in *Phoxinus*, 1940, 1948). While the results reported are consistent, so far as the effect of local illumination on the state of pigment granules within the melanophores is concerned, whether or not the melanophores are acting as an independent receptor without the involvement of the central nervous system or local spinal reflex is uncertain. Von Frisch (1911) found that blinded minnows paled in darkness and darkened immediately when exposed to light.

Von Frisch first suspected the pineal to be the receptor. However, since pinealectomy failed to abolish the response and the response could not be evoked unless the light struck the head region, he concluded that the response might be coordinated through a deeper diencephalic structure. Wykes (1938) working on blinded *Ameiurus* confirmed the observations of von Frisch. However, she was unable to identify which part of the brain, if any, was involved in the concerned response. The observations made in the present study do not agree with the above, since the pigment dispersed in direct response to the light and without the head region being illuminated. This observation is, however, in agreement with those reported by Healey (1940, 1948) on minnows from Vienna. Healey investigated the effect of light on blinded spinal minnows. He found that illumination of the head resulted in pigment dispersion in melanophores of the concerned region only and the melanophores in the rest of the body were not affected. Similarly, illumination of the body excluding the head resulted in pigment dispersion within the melanophores of the body only. Moreover, he observed that if a small spot of light was directed onto any part of the fish, it resulted in pigment dispersion in the melanophores which were directly illuminated by the spot.

In experiments involving the response of fish to complete darkness or to a shade of background under low light intensity, macroscopic observation of the fish can be highly misleading and at most represents the assumed shade. Therefore it is essential to describe the state of the melanophores microscopically. At the same

time, microscopic observation can, at best, only provide information from melanophores in a very limited area of the body. Thus, any differential responses of melanophores in a different area of skin cannot be detected. However, the technique adopted in the present thesis appears to provide a method with a practicable precision that allows the study of melanophore responses over a large area of the body under different conditions of illumination.

Minnows are known to pale in a few minutes when put in complete darkness (von Frisch, 1911). This is confirmed in the present study. Photographs taken only 5 minutes after the fish was adapted to complete darkness show that the melanophores in the dorso-lateral region have aggregated their pigment granules fully. However, melanophores in the lateral stripes showed much less of a degree of pigment aggregation. Melanophores in the lateral stripes are known to have a higher threshold as far as pigment aggregation is concerned and a lower threshold as far as pigment dispersion is concerned (Chapter IV, p.126). This might also explain the response of these melanophores in complete darkness. Thus, the stimuli mediating the state of pigment granules within the melanophores in complete darkness, while of sufficient strength to bring about pigment aggregation in the dorso-lateral melanophores, are not sufficient to reach the threshold required by the lateral stripes melanophores to result in pigment aggregation.

It appears that white background response predominates the primary response of melanophores to light, because no pigment

dispersion can be observed in response to a spot of light directed on the body as long as the fish is exposed to an illuminated white background.

Since the eye is the receptor for background responses, the question to be answered is how does the eye relay the different information to the higher centre in order for appropriate stimuli to be sent to the periphery.

Over a wide range of light intensities, as previously stated, there is no change in the extent and rate of colour adaptation. This suggests that the background response observed is not solely an incident intensity effect, but is as a result of the ratio of the incident intensity to the reflected intensity from the background. The higher the value, the *darker* the fish and the lower the ratio the *paler* the fish. Von Frisch (1911) believed that the retina in the trout is differentiated, and that stimulation of the visual cells in the dorsal part resulted in pigment aggregation and stimulation of the visual cells in the ventral part resulted in pigment dispersion.

Many workers in this field agree with this view that the retinas of colour changing animals possess a certain degree of differentiation in relation to their ability to adapt chromatically. This has been reported in very many species by the following workers: Sumner (1933) in *Fundulus parripennis*; Brown (1936) in *Ericymba buccata*; Hogben and Slome (1936) in *Xenopus*; Butcher and Adelmann (1937) in *Fundulus heteroclitus*; Hogben and Landgrebe (1940) in *Gasterosteus*; Danielson (1941) in cyprinids and Gentle (1968) in *Phoxinus*. However, there

are some disagreements among the above authors regarding the interpretation of the differentiation in question. Butcher and Adelmann (1937) claimed that there is rigid physiological and anatomical differentiation in the retina of *Fundulus heteroclitus*. According to them the ventral part of the retina is solely involved in the darkening response of the fish and the dorsal part of the retina is solely involved in the paling response of the fish. Danielson (1941) suggested that although some differentiation might exist between the dorsal and the ventral part of the retina, background response did not depend upon the stimulation of one part or the other but "on the degree of contrast in the visual field as a whole".

Agreeing with the above interpretation, Gentle (1968) working on the visual system of *Phoxinus*, concluded that the retina most probably acted as a unit, relaying the total visual field to the brain where it is interpreted.

The results obtained in the present study on the fine structure of the retina and the retinomotor responses do not indicate any rigid anatomical or photomechanical differentiation in the retina. The retinomotor responses under the various intensities of illumination appeared to be uniform throughout the retina. The visual cells in the dorsal and ventral part of the retina were not found to have different thresholds towards light intensity. However, under a light intensity of 1.25 lux, where neither the black background response nor the white background response was complete, the structure of the retina adapted to this intensity also appeared in an inter-

mediate position (p.251)

Therefore, it can be suggested that for the full chromatic adaptation of *Phoxinus*, the light intensity striking the eye should not be below the threshold required for the cones to be fully functional. Thus, it is feasible to speculate that the cones are the receptors involved in conveying the shade of the background to the brain.

However, the structure of the retina and the structural changes induced by different values of illumination, cannot in any way be correlated directly with the phenomenon of chromatic adaptation. Therefore, it appears that the importance of the relationship between the direct and the reflected light lies in the total retinal output to the brain rather than in any rigid concept of retinal differentiation.

S U M M A R Y

*(new methods and observations)

1 - A general survey of chromatic adaptation in teleost fish is made and particular attention is given to the nervous control of melanophores.

(pp. 14-47)

2 - A brief account is given of the anatomy, physiology and pharmacology of adrenergic neurons.

(pp. 47-59)

3 - The source of the minnows *Phoxinus phoxinus* and their general treatment are described.

(p. 67)

4 - Operative techniques on the minnow *Phoxinus phoxinus* for various experimental preparations are described.

(pp. 67-71)

5 - An apparatus for continuous microscopic and photographic recording is described.

(pp. 71-74)

6 - Difficulties encountered in ultrastructural studies of the integument are described.

(p. 76)

*7 - Procedures which give satisfactory results for processing the integument for ultrastructure studies are described.

(pp. 76-81)

8 - The general structure of the integument of *Phoxinus phoxinus* was studied using both light and electron microscopes and was found to be very typical of teleost fish in general.

(pp. 81-87)

9 - The dermis consists of masses of collagen bundles, different types of chromatophores and bundles of myelinated and non-myelinated axons.

(p. 85)

*10 - Melanophores were found at varying depths beneath the basal lamella surrounded by dermal collagenous fibres. The epidermis was found to be generally free from melanophores.

(p. 85)

11 - Xanthophores and erythrophores were observed microscopically in living fish. Xanthophores were found to be present throughout the skin but erythrophores are only present at the base

of the paired fins and ventral skin.

(p. 87)

*12 - Iridophores were studied in whole-mount preparations using dark field microscopy. They were seen to be interspersed between melanophores.

(p. 87)

*13 - Electron microscope observations revealed that melanophores have a single plasma membrane, the cytoplasm inside being filled with numerous spherical highly electron-dense melanin granules. A well organised microtubule system is present in *Phoxinus phoxinus* melanophores and its relation to cell structure and the pigment granules is described.

(p. 92-96)

*14 - Melanophores were found to change their shape during pigment migration. In melanophores with their pigment granules in a state of complete dispersion their centrosphere appeared flat. In melanophores with their pigment fully aggregated their centrosphere was large and hemispherical.

(p. 96)

*15 - Nerve-endings about 0.6μ in diameter containing granular synaptic vesicles and mitochondria were found usually invaginating the melanophores both around the processes and at the

cell body.

(pp. 96, 98, 101)

*16 - The above nerve-endings showed a positive interaction with 5-hydroxydopamine indicating their adrenergic nature.

(pp. 101, 105)

*17 - Electron micrographs of iridophores revealed that their cytoplasm is totally occupied by groups of parallel lacunae with varying angles to each other.

(p. 105)

*18 - Electron micrographs of xanthophores revealed that their cytoplasm is occupied by round membrane-bound vesicles of low electron density.

(pp. 105, 108)

*19 - Electron micrographs of erythrophores revealed that the granules contained in these cells are non membrane bound.

(pp. 108, 111)

*20 - A possible role played by microtubules in the mechanism of pigment migration is discussed.

(pp. 112-115)

*21 - It is concluded that there is a direct and solely adrenergic innervation of melanophores in the minnow *Phoxinus phoxinus*.

(pp. 115-117)

*22 - The continuous observation apparatus was evaluated by studying the response of the confined fish to an illuminated black background, a white background and their reversal. The results obtained indicated that the apparatus is a valuable tool in experiments requiring continuous microscopic observation of melanophores not exceeding the period of two hours.

(pp. 121-127)

23 - Melanophores separated from the central control by spinal nerve section show dispersed pigment granules. These melanophores show slow and gradual pigment aggregation on an illuminated white background.

(pp. 130-131)

*24 - Responses of the above melanophores to black and white background reversal were studied and compared with responses of the neighbouring melanophores with intact innervation. Possible factors controlling these responses are discussed.

(pp. 131-144)

*25 - An apparatus was designed to study the effect of electrical stimulation on the spinal cord in living fish.

(p. 151)

26 - Preparation of the stimulating electrodes (Ag/AgCl non-polarizable electrodes) and the stimulation parameters

are described.

(pp. 153-154)

27 - The effects of bretylium (an adrenergic neuron blocking agent) on various minnow preparations were studied. This agent, which brought about considerable pigment dispersion in chromatically normal *Phoxinus phoxinus*, had no clear dispersing effects on melanophores of chromatically spinal fish or on melanophores separated by spinal nerve section.

(pp. 154-157)

*28 - It is suggested that the dispersion which follows administration of bretylium is a nervously controlled response and is probably due to specific impulses arriving at the periphery from the centre.

(p. 159)

29 - Electrical stimulation of the spinal cord in chromatically spinal black-adapted *Phoxinus phoxinus* resulted in full pigment aggregation. The magnitude and speed of pigment aggregation are dependent on the frequency of repetitive pulses.

(pp. 159, 161)

*30 - Electrical stimulation of chromatically spinal white-adapted *Phoxinus phoxinus* pretreated with bretylium resulted in a

remarkable pigment dispersion. This also indicated that both the mechanisms, aggregation of pigment granules and their dispersion, are active and nervously controlled.

(pp. 161-162)

31 - Noradrenaline, adrenaline and isoproterenol (in relatively higher concentrations) are found to be potent pigment aggregating agents in black-adapted *Phoxinus phoxinus*.

(pp. 164-168).

32 - Various doses of isoproterenol, adrenaline and noradrenaline failed to result in any pigment dispersion in chromatically normal white-adapted fish.

(pp. 164-168)

*33 - A possible explanation is given to account for the failure of isoproterenol to result in pigment dispersion in chromatically normal white-adapted *Phoxinus phoxinus*.

(pp. 169, 171, 172)

34 - Alpha-adrenoceptor blocking agents (yohimbine and tolazoline) caused full pigment dispersion in chromatically normal white-adapted *Phoxinus phoxinus*. This dispersion was easily reversed by noradrenaline and slightly reversed by adrenaline. Isoproterenol failed completely to reverse the effect.

(pp. 172-180)

*35 - It is concluded that aggregating effects of isoproterenol are probably due to its interaction with alpha-adrenoceptors, because its effects are antagonised by both the alpha blocking agents.

(p. 180)

*36 - In relatively low concentrations isoproterenol caused very considerable pigment dispersion in melanophores of chromatically spinal white-adapted *Phoxinus phoxinus*. Adrenaline caused slight and noradrenaline failed to evoke any pigment dispersion in the above preparation.

(pp. 181-188)

*37 - The dispersion effects of isoproterenol on chromatically spinal white-adapted *Phoxinus phoxinus* were easily antagonised by a dose of propranolol (a beta-antagonist).

(pp. 188-189)

*38 - The more specific beta-agonists, isoxsuprine and fenoterol, caused significant and long-lasting pigment dispersion in melanophores of chromatically spinal white-adapted *Phoxinus phoxinus*. The dispersion evoked was also antagonised by propranolol (a beta-antagonist).

(pp. 192-202)

*39 - It is concluded that pigment dispersion in melanophores of chromatically spinal white-adapted *Phoxinus phoxinus* following

injections of beta-adrenoceptor agonists is probably mediated by beta-adrenoceptors of *Phoxinus phoxinus* melanophores.

(pp. 202)

*40 - A possible explanation is given to account for pigment aggregation and pigment dispersion in *Phoxinus phoxinus* melanophores under the influence of the nervous system.

(pp 202-215)

*41 - An apparatus to produce a homogeneous and constant light source and a method for calibrating the light have been described.

(p.p. 219-220)

*42 - An apparatus to facilitate the focusing and reading of the MI in low light intensities is described.

(p. 222)

43 - An apparatus used for studying the responses of the plaice *Pleuronectes platessa* to patterned backgrounds is described.

(pp. 224-226)

*44 - An apparatus to study the minnow eye exposed to different values of light intensity together with histological procedures for processing the retina is described.

(pp. 226-227)

*45 - In incident light intensities of 12.5 lux (1.16 f-c) and above the response of *Phoxinus phoxinus* to black and white backgrounds was constant both in the rate and the degree of pigment movement and was not influenced by the intensity of the incident light.

(pp. 230-234)

*46 - Melanophores in the dorso-lateral area of the fish showed a greater tendency to aggregate their pigment in lower light intensities when the fish was exposed to a black background than did the melanophores of the lateral stripe.

(pp. 234-237)

47 - Melanophores in the lateral stripe of the skin showed some primary response to local illumination.

(pp. 237-240)

48 - The fine structure of the retina of *Phoxinus phoxinus* in its general structure is typical of the vertebrate retina.

(pp. 240-248)

49 - The photoreceptors were composed of cones and rods, the former being of various types.

(pp. 240-242)

50 - No anatomical or retino-motor differentiation was found

between the dorsal and ventral part of the retina of *Phoxinus phoxinus*. Based on the above it was concluded that any rigid concept of retinal differentiation is far too simple to account for chromatic adaptation of the fish.

(pp. 248-254)

51 - The pattern of the plaice *Pleuronectes platessa* on a chequer-board with black and white squares of 1 cm side and another chequerboard of 2.5 mm side was studied. It was found that melanophores in the various regions of the skin, respond differently on these two backgrounds.

(pp. 254-256)

*52 - The plaice was found to maintain its pattern at incident light intensities of 16.5 lux (1.53 f-c) and above. In lower light intensities down to complete darkness the fish assumed an intermediate shade.

(pp, 256-259)

A P P E N D I X

EFFECTS OF SOME ADRENERGIC DRUGS ON TELEOST MELANOPHORES AS REPORTED BY SOME PREVIOUS WORKERS

(* *in vivo*; + *in vitro*)

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Phenylephrine	Alpha-adrenoceptor agonist	Aggregation	*Lebistes*	Fujii and Miyashita (1975)+
			Petrophyllum	Reed and Finnin (1972)*
			Scophthalmus aquosus	Scott (1972)*
		Slight dispersion at lower concentrations in melanophores with pigment granules partially aggregated by high concentration of tolazoline	*Lebistes*	Miyashita and Fujii (1975)+

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Noradrenaline	Alpha-adrenoceptor agonist	Aggregation	*Chasmichthys*	Fujii (1961)+
			Fundulus	Abbott (1968)*,+
			Gadus	" Fange (1962)*
			Gambusia	Ueda (1955)+
			Ictalurus	Khokhar (1971)+
			Labrus	Scheline (1963)+
			Lebistes	" Fange (1962)+; Fujii & Miyashita (1975)+
			Phoxinus	Pye (1964)+; Healey & Ross (1966)*; Grove (1969a)*
			Pleuronectes	Fernando & Grove (1974a, b)*,+
			Pterophyllum	Reed & Finnin (1972)*
			Rasbora	Dwivedi (1978)*
			Scophthalmus	Scott *et al* (1962)*; Scott (1965, 1972)*

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Noradrenaline	Alpha-adrenoceptor agonist	Incomplete aggregation	*Phoxinus*	Healey & Ross (1966)+
		Slight dispersion at lower concentrations in melanophores with pigment granules partially aggregated by high concentration of tolazoline	*Lebistes*	Miyashita & Fujii (1975)+
		Dispersion	*Parasilurus*	Enami (1955)*
Adrenaline	Alpha- & beta-adrenoceptor agonist	Aggregation	*Abudefduf*	Rasquin (1958)*
			Ameiurus	Bray (1918)*; Bacq (1933b)*; Parker (1934d)*; Abramowitz (1936)*; Wykes (1938)*; Rasquin (1958)*
			Bathygobius	Rasquin (1958)*
			Carapus	Iwata *et al.* (1959a,b)+
			Chasmichthys	Fujii (1961)+

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Adrenaline	Alpha- & beta-adrenoceptor agonist	Aggregation	*Cyprinodon*	Rasquin (1958)*
			Epinephalus	Rasquin (1958)*
			Fundulus	Spaeth (1916)+; Barbour & Spaeth (1917)*,+; Wyman (1924)*
			Gadus	" Fange (1962)+
			Gambusia	Ueda (1955)+
			Ictalurus	Khokhar (1971)+
			Labrus	Scheline (1963)+
			Lebistes	" Fange (1962)+; Fujii & Miyashita (1975)+
			Opsanus	Rasquin (1958)*
			Oryzias	Ueda (1955)+
			Phoxinus	Abolin (1925)*; Giersberge (1930)*; Pye (1964)+; Healey & Ross (1966)*; Grove (1969a)*
			Petrophyllum	Reed & Finnin (1972)*

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Adrenaline	Alpha- & beta-adrenoceptor agonist	Aggregation	*Pleuronectes*	Fernando & Grove (1974a, b)*,+
			Pseudorasbora	Ueda (1955)+
			Rasbora	Dwivedi (1978)*
			Salmo	Robertson (1951)*,+
			Scophthalmus	Scott *et al.* (1962)*; Scott (1965, 1972)*
		Incomplete aggregation	*Phoxinus*	Healey & Ross (1966)+
		No effect on epidermal melanopheres	*Abudefduf*	Rasquin (1958)*
		No effect	*Angelichthys*	Rasquin (1958)*
			Pomacanthus	Rasquin (1958)*
			Acanthurus	Rasquin (1958)*
		Aggregation followed by dispersion	*Oryzias*	Watanabe *et al.* (1962)+

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Adrenaline	Alpha- & beta-adrenoceptor agonist	Slight dispersion at lower concentrations in melanophores with pigment granules partially aggregated by reserpine or high concentrations of tolazoline	*Lebistes*	Miyashita & Fujii (1975)+
		Dispersion	*Chaetodipterus*	Breder & Rasquin (1955)*
			Parasilurus	Enami (1940, 1955)*
Isoprenaline (isoproterenol)	Beta-adrenoceptor agonist	Aggregation	*Fundulus*	Abbott (1968)*,+
			Ictalurus	Khokhar (1971)+
			Lebistes	Fujii & Miyashita (1975)+
			Phoxinus	Healey & Ross (1966)*; Grove (1969a)*
			Pleuronectes	Fernando & Grove (1974a, b)*,+
			Rasbora	Dwivedi (1978)*

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Isoprenaline	Beta-adrenoceptor agonist	Dispersion followed by aggregation (in partially aggregated melanophores by a constant electrical stimulation at low pulse rates)	*Pterophyllum*	Reed & Finnin (1972)*
		Dispersion (at lower concentrations in melanophores with pigment granules partially aggregated by reserpine or high concentration of tolazoline	*Lebistes*	Miyashita & Fujii (1975)+
Isoxsuprine	Beta-adrenoceptor agonist	No effect	*Lebistes*	Fujii & Miyashita (1975)+
		Dispersion (in melanophores with pigment granules partially aggregated by reserpine or high concentration of tolazoline)	*Lebistes*	Miyashita & Fujii (1975)+

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Isoxsuprine	Beta-adrenoceptor agonist	Dispersion (in partially aggregated melanophores by a constant electrical stimulation at low pulse rates)	*Pterophyllum*	Reed & Finnin (1972)*
Metaproterenol	Beta-adrenoceptor agonist	No effect	*Lebistes*	Fujii & Miyashita (1975)+
		Dispersion (in melanophores with pigment granules partially aggregated by reserpine or high concentrations of tolazoline	*Lebistes*	Miyashita & Fujii (1975)+
Methoxphenamine	Beta-adrenoceptor agonist	Aggregation	*Lebistes*	Fujii & Miyashita (1975)+

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Methoxphenamine	Beta-adrenoceptor agonist	Dispersion (at lower concentrations in melanophores with pigment granules partially aggregated by reserpine or high concentration of tolazoline	*Lebistes*	Miyashita & Fujii (1975)+
Protokylol	Beta-adrenoceptor agonist	Aggregation	*Lebistes*	Fujii & Miyashita (1975)+
		Dispersion (at lower concentrations in melanophores with pigment granules partially aggregated by reserpine or high concentrations of tolazoline	*Lebistes*	Miyashita & Fujii (1975)+
Dibenamine	Alpha-adrenoceptor blocking agent	Dispersion	*Chasmichthys*	Fujii (1961)+
			Fundulus	Abbott (1968)*+

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Dibenamine	Alpha-adrenoceptor blocking agent	Dispersion	*Lebistes*	Fujii & Miyashita (1975)+
			Oryzias	Watanabe *et al.* (1962)+
			Pleuronectes	Fernando & Grove (1974b)+
			Phoxinus	Healey & Ross (1966)*,+
			Scophthalmus	Scott (1965)*
		Some dispersion	*Phoxinus*	Grove (1969a)*
Dibenamine followed by adrenaline		No effect	*Chasmichthys*	Fujii (1961)+
			Phoxinus	Healey & Ross (1966)+
		Aggregation	*Fundulus*	Abbott (1968)*
Dibenzyline	Alpha-adrenoceptor blocking agent	Dispersion	*Fundulus*	Abbott (1968)*
			Phoxinus	Healey & Ross (1966)*
			Scophthalmus	Scott (1965)*

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Dibenzyline followed by adrenaline	Alpha-adrenoceptor blocking agent	No effect	*Labrus*	Scheline (1963)+
			Phoxinus	Healey & Ross (1966)*
Ergotamine	Alpha-adrenoceptor blocking agent	Aggregation	*Ameiurus*	Bacq (1933b)*
			Chasmichthys	Fujii (1961)+
			Ictalurus	Khokhar (1971)+
		Slight aggregation	*Phoxinus*	Pye (1964)+; Healey & Ross (1966)+
			Salmo	Robertson (1951)*,+
		Aggregation followed by dispersion	*Fundulus*	Barbour & Spaeth (1917)*,+; Wyman (1924)*
		Dispersion	*Ameiurus*	Bacq (1933)*; Parker (1941b)
			Chasmichthys	Fujii (1961)+
			Lebistes	Fujii & Miyashita (1975)+

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Ergotamine	alpha-adrenoceptor blocking agent	Dispersion	*Scophthalmus*	Scott (1965)*
		No effect	*Ameiurus* (in denervated melanophores)	Parker (1941b)*
			Pleuronectes	Fernando & Grove (1974b)+
Ergotamine followed by adrenaline/ noradrenaline		Dispersion	*Chasmichthys*	Fujii (1961)+
			Fundulus	Barbour & Spaeth (1917)+
			Ictalurus	Khokhar (1971)+
			Phoxinus	Healey & Ross (1966)*
		No effect	*Fundulus*	Wyman (1924)*
			Phoxinus	Giersberg (1930)*; Pye (1964)*
Phentolamine (regitin)	Alpha-adrenoceptor antagonist	Dispersion	*Fundulus*	Abbott (1968)*,+
			Lebistes	Fujii & Miyashita (1975)+
			Pleuronectes	Fernando & Grove (1974b)+

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Phentolamine	Alpha-adrenoceptor antagonist	Dispersion	*Phoxinus*	Pye (1964)*; Healey & Ross (1966)*; Grove (1969a)*
			Pterophyllum	Reed & Finnin (1972)*
			Rasbora	Dwivedi (1978)*,+
		Negligible dispersion	*Ictalurus*	Khokhar (1971)+
Phentolamine followed by adrenaline/ noradrenaline		Aggregation	*Phoxinus*	Pye (1964)*; Healey & Ross (1966)*, Grove (1969a)*
Piperoxane	Alpha-adrenoceptor antagonist	Dispersion	*Pleuronectes*	Fernando & Grove (1974b)+
			Phoxinus	Healey & Ross (1966)
Tolazoline	Alpha-adrenoceptor antagonist	Dispersion	*Fundulus*	Abbott (1968)*,+
			Lebistes	Fujii & Miyashita (1975)+

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Tolazoline	Alpha-adrenoceptor antagonist	Dispersion	*Pterophyllum*	Reed & Finnin (1972)+
			Rasbora	Dwivedi (1978)*,+
Yohimbine	Alpha-adrenoceptor antagonist	Dispersion	*Fundulus*	Abbott (1968)*
			Pleuronectes	Fernando & Grove (1974b)+
			Phoxinus	Healey & Ross (1966)*,+; Grove (1969a)*
		Slight dispersion	*Ictalurus*	Khokhar (1971)+
Yohimbine followed by adrenaline		No effect	*Phoxinus*	Healey & Ross (1966)*
Yohimbine followed by noradrenaline		Aggregation	*Ictalurus*	Khokhar (1971)+
			Phoxinus	Grove (1969a)*
Dichloroisoproterenol	Beta-adrenoceptor antagonist	Aggregation (local	*Scophthalmus*	Scott (1972)*

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Dichloroisopro-terenol	Beta-adrenoceptor antagonist	Dispersion	*Lebistes*	Miyashita & Fujii (1975)+
Pronethalol	Beta-adrenoceptor antagonist	Dispersion	*Scophthalmus*	Scott (1972)*
		No effect	*Phoxinus*	Grove (1969a)+
Propranolol	Beta-adrenoceptor antagonist	Dispersion	*Scophthalmus* (local)	Scott (1972)*
		No effect	*Fundulus*	Abbott (1968)*
			Pleuronectes	Fernando & Grove (1974b)+
Bretylium	Adrenergic neuron blocking agent	Dispersion	*Fundulus*	Abbott (1968)*
			Pleuronectes	Fernando & Grove (1974b)*,+
		No effect	*Fundulus*	Abbott (1968)+

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Guanethidine	Adrenergic neuron blocking agent	Dispersion	*Pleuronectes*	Fernando & Grove (1974b)+
			Phoxinus	Healey & Ross (1966)*
		Aggregation	*Fundulus*	Abbott (1968)*
			Phoxinus	Healey & Ross (1966)+
Reserpine	Adrenergic depletor	Dispersion	*Betta*	Turner & Carl (1955)+
			Brachydanio	Turner & Carl (1955)+
			Corydorans	Turner & Carl (1955)+
			Fundulus	Abbott (1968)*
			Labrus	Scheline (1963)+
			Macropodus	Turner & Carl (1955)+
			Pleuronectes	Fernando & Grove (1974b)+
			Phoxinus	Healey & Ross (1966)*,+; Grove (1969a)*

DRUG	ADRENERGIC EFFECT	EFFECTS ON MELANOPHORE	FISH USED	SOURCE
Reserpine	Adrenergic depletor	Dispersion	*Rasbora*	Dwivedi (1978)*,+
			Trichogaster	Turner & Carl (1955)+
		No effect	*Fundulus*	Abbott (1968)+

REFERENCES

ABBOTT, F.S. (1968) - "The effect of certain drugs and biogenic substances on the melanophores of *Fundulus heteroclitus*." Can. J. Zool., 46, 1149-1161.

ABE, K., BUTCHER, R.W., NICHOLSON, W.E., BAIRD, C.E., LIDDLE, R.A. & LIDOLE, G.W. (1969) - "Adenosine 3',5'-monophosphate (Cyclic AMP) as the mediator of the actions of melanocyte stimulating hormone (MSH) and norepinephrine on the frog skin." Endocrinology, 84, 362.

ABOLIN, L. (1925) - "Beeinflussung des Fischfarbwechsels durch chemikalien. 1. Infundin-und Adrenalinwirkung auf die Melano- und Xanthophoren der Elritze (*Phoxinus laevis* Ag.)." Arch. mikr. Anat., 104, 667-669.

ABRAMOWITZ, A.A. (1935) - "Regeneration of chromatophore nerves." Proc. Natl. Acad. Sci., U.S.A., 21, 137-141.

ABRAMOWITZ, A.A. (1936a) - "The double innervation of caudal melanophores in *Fundulus*." Proc. Natl. Acad. Sci., U.S.A., 22, 233-238.

ABRAMOWITZ, A.A. (1936b) - "Physiology of the melanophore system in the catfish, *Ameiurus*." Biol. Bull., Woods Hole, 71, 259-281.

ADELMANN, H.B. & BUTCHER, E.O. (1937) - "Experiments on the nervous control of the melanophores in *Fundulus heteroclitus*." Bull. Mt Desert Isl. Biol. Lab., pp. 15-16.

AHLQUIST, R.P. (1948) - "A study of the adrenotropic receptors." Amer. J. Physiol., 153, 586-600.

AHLQUIST, R.P. (1976) - "Present state of alpha- and beta-adrenergic drugs. 1. The adrenergic receptor." Am. Heart J., 92, 661-664.

AHMAD, R.U. (1970) - "Investigations on the effect of chromatic spinal section on the so-called morphological colour changes in the minnow *Phoxinus phoxinus* (L.) with observation on the rate of pigment movement in the newly formed melanophores." Ph.D. Thesis, Univ. Lond.

ALI, M.A. (1975) - "Retinomotor responses." In: *Vision in Fishes* M.A. Ali, ed. Plenum Press, New York. pp. 313-355.

ANDERSSON, R.G.G. (1973) - "Role of cyclic AMP and Ca^{++} in mechánical and metabolic events in isometrically contracting vascular smooth muscle." Acta Physiol. Scand., 87, 84-95.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959) - "Some quantitative uses of drug antagonists." Brit. J. Pharmacol., 14, 48-58.

*BACQ, Z.M. (1933a) - "Récherches sur la physiologie du système nerveux autonome. III Les propriétés biologiques et physico-chimiques de la sympathine comparées à cells de l'adrénaline." Arch. int. Physiol., 36, 167-246. (Cited from Burnstock & Costa, 1975).

BACQ, Z.M. (1933b) - "The action of ergotamine on the chromatophores of the catfish *Ameiurus nebulosus*." Biol. Bull. Woods Hole, 65, 387-388.

BAGNARA, J.T. & HADLEY, M.E. (1973) - *Chromatophores and Colour Change* Prentice-Hall Inc., Englewood Cliffs, New Jersey.

BAKER, B.I. (1963) - "The effects of adaptation to black and white backgrounds on the teleost pituitary." Nature, Lond., 198, 404.

*BALLOWITZ, E. (1893a) - "Die Innervation der Chromatophoren." Verh. anat. Ges., 7, 71-76. (Cited in Parker, 1948).

*BALLOWITZ, E. (1893b) - "Die Nervenendigungen der Pigmentzellen, ein Beitrag zur Kenntnis des Zusammenhanges der Endverzweigungen der Nerven mit dem Protoplasma der Zellen." Z. wiss. Zool., 56, 673-706. (Cited in Parker, 1948).

BARBOUR, H.G. & SPAETH, R.A. (1917) - "Responses of fish melanophores to sympathetic stimulants and depressants." J. Pharmacol., 9, 356-357.

BARDELE, C.F. (1973) - "Struktur, Biochemie und Funktion der Mikrotubuli." Cytobiologie, 7, 442-448.

BIKLE, D., TILNEY, L.G. & PORTER, K.R. (1966) - "Microtubules and pigment migration in the melanophores of *Fundulus heteroclitus*." Protoplasma, 61, 322-345.

BISHOP, E.H. & WOUTERSZ, T.B. (1961) - "Isoxsuprine, a myometrial relaxant." Obstet. Gynecol., 17, 442.

BLAXTER, J.H.S. (1970) - "Light: Fishes." In: *Marine Ecology* O. Kinne, ed. Wiley – Interscience, Lond. – pp. 213-320.

BLOOM, F.E. (1972) - "Electron microscopy of catecholamine-containing structures." In: *Catecholamines* H. Blaschko & E. Muscholl, eds. Springer-Verlag, Berlin, Heidelberg, New York. pp. 46-78.

BOURA, A.L.A. & GREEN, A.F. (1959) - "The action of bretylium: Adrenergic neurone blocking and other effects." Brit. J. Pharmacol., 14, 536-548.

BOWMAN, W.C., RAND, M.J. & WEST, G.B. (1968) - *Textbook of Pharmacology* Blackwell Scientific Publications, Oxford.

BRAY, A.W.L. (1918) - "The reactions of the melanophores of *Ameiurus* to light and to adrenaline." Proc. Natl. Acad. Sci., U.S.A., 4, 58-60.

BREDER, C.M. & RASQUINN, P. (1955) - "Further notes on the pigmentary behaviour of *Chaetodipterus* in reference to background and water transparency." Zoologica, N.Y., 40, 85-90.

BRISTOW, M., SHERROD, T.R. & GREEN, R.D. (1970) - "Analysis of beta receptor drug interactions in isolated rabbit atrium, aorta, stomach and trachea." J. Pharmacol. exp. Ther., 171, 52-61.

BRITTAIN, R.T., JACK, D. & RITCHIE, A.C. (1970) - "Recent β-adrenoreceptor stimulants." Adv. Drug Res., 5, 197-253.

BROWN, F.A. Jr. (1936) - "Light intensity and melanophore response in the minnow, *Ericymba buccata* Cope." Biol. Bull. Woods Hole, 70, 8-15.

BROWN, G.L. & GILLESPIE, J.S. (1957) - "The output of sympathetic transmitter from the spleen of the cat." J. Physiol. Lond., 138, 81-102.

*BRÜCKE, E. (1852) - "Untersuchungen über den Farbenwechsel des afrikanischen Chamaleons." Denkschr. Akad. Wiss. Wien, Math. - nat. Kl. 4, 179-210. (Cited in Parker, 1948).

*BUCHHOLZ, R. (1862) - "Ueber die Mikropyle von *Osmerus eperlanus*." Arch. Anat. Physiol. wiss. Med., 1863, 71-81. (Cited in Parker, 1948).

BURGES, R.A. & BLACKBURN, K.J. (1972) - "Adenyl cyclase and the differentiation of β-adrenoceptors." Nature New Biol., 235, 249.

BURN, J.H. (1963) - *The Autonomic Nervous System* Blackwell Scientific.

BURNSTOCK, G. (1969) - "Evolution of the autonomic innervation of visceral and cardiovascular systems in vertebrates." Pharmacol. Rev., 21, 247-324.

BURNSTOCK, G. (1970) - "Structure of smooth muscle and its innervation." In: *Smooth Muscle* E. Bülbring, A. Brading, A. Jones & T. Tomita, eds. Edward Arnold Publ. Ltd., Lond. pp. 1-69.

BURNSTOCK, G. & COSTA, M. (1975) - *Adrenergic Neurons: Their Organization, Function and Development in the Peripheral Nervous System*. Chapman & Hall, Lond.

BUTCHER, E.O. (1938) - "The structure of the retina of *Fundulus heteroclitus* and the regions of the retina associated with the different chromatophoric responses." J. Exp. Zool., 79, 275-297.

BUTCHER, E.O. & ADELMANN, H.B. (1937) - "The effects of covering and rotating the eyes on the melanophoric responses in *Fundulus heteroclitus*." Bull. Mt. Desert Isl. Biol. Lab. pp. 16-18.

BYERS, H.R. & PORTER, K.R. (1976) - "Pigment migration in cultured erythrophores." J. Cell Biol., 70, 402a.

BYERS, H.R. & PORTER, K.R. (1977) - "Transformation in the structure of the cytoplasmic ground substance in erythrophores during pigment aggregation and dispersion. 1. A study using whole-cell preparations in stereo high voltage electron microscopy." J. Cell. Biol., 75, 541-558.

CAJAL, S.R. (1911) - *Histologie du Systeme neureau de l'Homme et des vertebres* A. Maloine, Paris.

CAMPBELL, G. (1970) - "Autonomic nervous supply to effector tissues." In: *Smooth Muscle*. E. Bülbring, A. Brading, A. Jones & T. Tomito, eds. Edward Arnold Publ. Co. Lond. pp. 451-495.

CANNON, W.B. & ROSENBLUETH, A. (1937) - *Autonomic Neuro-effector Systems*. Macmillan, New York.

DALE, H.H. (1906) - "On some physiological actions of ergot." J. Physiol. Lond., 34, 163-206.

DALE, H.H. (1937) - "Acetylcholine as a chemical transmitter of the effects of nerve impulses. I. History of ideas and evidence. Peripheral autonomic actions. Functional nomenclature of nerve fibres." J. Mt. Sinai Hosp., 4, 401-415.

DANIELSON, R.N. (1938) - "Light intensity and melanophore response in a cyprinid fish." Physiol. Zool., 11, 292-298.

DANIELSON, R.N. (1941) - "The melanophore response of fishes in relation to contrast in the visual field." Physiol. Zool., 14, 96-102.

DENTON, E.J. & NICOL, J.A.C. (1966) - "A survey of reflectivity in silvery teleosts." J. Mar. Biol. Ass. U.K., 46, 685-722.

DE POTTER, W.P. (1973) - "Release of amines from sympathetic nerves." In: *Frontiers in Catecholamine Research* E. Usdin & S. Snyder, eds. Pergamon Press, Oxford.

DE ROBERTIS, E. & PELLEGRINO DE IRALDI, A. (1961) - "A plurivesicular component in adrenergic nerve endings." Anat. Rec., 139, 299.

DETWILER, S.R. (1943) - *Vertebrate Photoreceptors*. The Macmillan Co., New York.

DREYER, A.C. (1971) - "The $beta_1$ and $beta_2$ adrenergic properties of some anti-asthmatic drugs." S.A. Pharmaceut. J., 38, 27-28.

DWIVEDI, D.K. (1978) - "The effects of drugs on the melanophores of teleost *Rasbora daniconius*." Aust. J. Pharm. Sci., 7(1), 29-31.

*EBERTH, C.J. & BUNGE, R. (1895) - "Die Nerven der Chromatophoren bei Fischen." Arch. mikr. Anat., 46, 370-378. (Cited in Parker, 1948).

EGNER, O. (1971) - "Zur Physiologie der Melanosomenverlagerung in den Melanophoren von *Pterophyllum scalare*." Cytobiologie, 4, 262-292.

ENAMI, M. (1940) - "Action mélanodilatatrice de l'adrénaline chez un silure chat (*Parasilurus asotus*)." Proc. Imp. Acad. Jap., 16, 236-240.

ENAMI, M. (1955) - "Melanophore-concentrating hormone (MCH) of possible hypothalamic origin in the catfish, *Parasilurus*." Science, 121, 36-37.

ENGSTROM, K. (1963) - "Cone types and cone arrangements in the retina of some flat fishes." Acta Zool., Stockh., 44, 119-129.

EULER, U.S. VON (1946) - "A specific sympathomimetic ergone in adrenergic nerve fibres (sympathin) and its relation to adrenaline and noradrenaline." Acta Physiol. Scand., 12, 73-97.

EULER, U.S. VON (1956) - *Noradrenaline* Charles C. Thomas, Springfield, Illinois, U.S.A.

FALCK, B., MUNTZING, J. & ROSENGREN, A.M. (1969) - "Adrenergic nerves to the dermal melanophores of the rainbow trout, *Salmo gairdneri*." Z. Zellforsch. Mikrosk. Anat., 99, 430-434.

FALK, S. & RHODIN, J. (1957) - "Mechanism of pigment migration within teleost melanophore." In: *Electron Microscopy* Proc. Stockholm Conf. F.S. Sjostrand and J. Rhodin, eds. Academic Press, New York. pp. 213-215.

FÄNGE, R. (1962) - "Pharmacology of poikilothermic vertebrates and invertebrates". Pharmacol. Rev., 45, 296-339.

FERNANDO, M.M. & GROVE, D.J. (1974a) - "Melanophore aggregation in the plaice (*Pleuronectes platessa* L.). I. Changes in *in vivo* sensitivity to sympathomimetic amines." Comp. Biochem. Physiol., 48(A), 711-721.

FERNANDO, M.M. & GROVE, D.J. (1974b) - "Melanophore aggregation in the plaice (*Pleuronectes platessa* L.). II. *In vitro* effects of adrenergic drugs." Comp. Biochem. Physiol., 48(A), 723-732.

FINGERMAN, M. (1959) - "The physiology of chromatophores." Internat. Rev. Cytol., 8, 175-210.

FINNIN, B.C. & REED, B.L. (1970) - "The continuous recording of melanophore responses in teleost fishes." Life Sci., 9, 321-333.

FOX, D.L. (1957) - "The pigments of fishes." In: *The Physiology of Fishes* M.E. Brown, ed. Vol. 2, Academic Press, New York. pp. 367-385.

FRISCH, K. VON (1910) - "Ueber die Beziehungen der Pigmentzellen in der Fischhaut zum sympathischen Nervensystem." Festschrift R. Hertwig, 3, 15-28.

FRISCH, K. VON (1911) - "Beiträge zur Physiologie der Pigmentzellen in der Fischhaut." Pflüg Arch. ges. Physiol., 138, 319-387.

FRISCH, K. VON (1925) - "Farbensinn der Fische und Duplizitätstheorie." Z. Vgl. Physiol., 2, 393-452.

*FRISCH, K. VON (1941) - "Über einen Schreckstoff der Fischhaut und seine biologische Bedeutung." Z. Vgl. Physiol., 29, 46-145.

FUJII, R. (1959a) - "Mechanism of ionic action in the melanophore system of fish. I. Melanophore-concentrating action of potassium and some other ions." Annot. Zool. Jpn., 32, 47-58.

FUJII, R. (1959b) - "Mechanism of ionic action in the melanophore system of fish. II. Melanophore-dispersing action of sodium ions." J. Fac. Sci. Univ. Tokyo. Section IV. 8, 371-380.

FUJII, R. (1961) - "Demonstration of the adrenergic nature of transmission at the junction between melanophore-concentrating nerve and melanophore in bony fish." J. Fac. Sci. Univ. Tokyo. Section IV. 9, 171-196.

FUJII, R. (1966a) - "A functional interpretation of the fine structure in the melanophore of the guppy, *Lebistes reticulatus*." Annot. Zool. Jpn., 39, 185-192.

FUJII, R. (1966b) - "Correlation between fine structure and activity in fish melanophore." In: *Structure and Control of the Melanocytes* G.D. Porta and O. Muhlbock, eds. Springer-Verlag, Berlin, Heidelberg, New York. pp. 114-123.

FUJII, R. (1969) - "Chromatophores and pigments." In: *Fish Physiology* W.S. Hoar and D.J. Randall, eds. Academic Press, New York. pp. 307-353.

FUJII, R. & FUJII, Y. (1966) - "An electron microscope study of the innervation of fish melanophores." Symp. Soc. Cellular Chem., 16, 87-100.

FUJII, R. & MIYASHITA, Y. (1975) - "Receptor mechanisms in fish chromatophores. I. Alpha nature of adrenoceptors mediating melanosome aggregation in guppy melanophores." Comp. Biochem. Physiol., 51(C), 171-178.

FUJII, R. & MIYASHITA, Y. (1976) - "Receptor mechanisms in fish chromatophores. III. Neurally controlled melanosome aggregation in a siluroid *Parasilurus asotus* is strangely mediated by

cholinoceptors." Comp. Biochem. Physiol., 55(C), 43-49.

FUJII, R. & NOVALES, R.R. (1968) - "Melanin movements in fish melanophores in response to electrical stimulation of their controlling nerves." Proc. Intern. Union Physiol. Sci., 7, 145.

FUJII, R. & NOVALES, R.R. (1969a) - "The nervous mechanism controlling pigment aggregation in *Fundulus* melanophores." Comp. Biochem. Physiol., 29, 109-124.

FUJII, R. & NOVALES, R.R. (1969b) - "Cellular aspects of the control of physiological color changes in fishes." Am Zool., 9, 453-4-3.

FUJII, R. & TAGUCHI, S. (1970) - "Ultrastructure of nerve-melanophore relationships in the guppy, *Lebistes reticulatus*." Annot. Zool. Jpn., 43, 123-131.

FURCHGOTT, R.F. (1967) - "The pharmacological differentiation of adrenergic receptors." Ann. N.Y. Acad. Sci., 139, 553-570.

FURCHGOTT, R.F. (1972) - "A classification of adrenoceptors (adrenergic receptors). An evaluation from the stand-point of receptor theory." In: *Catecholamines* H. Blaschko and J.E. Muscholl, eds. Springer-Verlag, Berlin, Heidelberg, New York. pp. 283-335.

FURNESS, J.B. & BURNSTOCK, G. (1975) - "Role of circulating catecholamines in the gastrointestinal tract." In: *Handbook of Physiology. Endocrinology* H. Blaschko and A.D. Smith, eds. Am. Physiol. Soc., (Washington).

FURNESS, J.B. & COSTA, M. (1974) - "Adrenergic innervation of the gastrointestinal tract." Ergebn. Physiol., 69, 1-51.

GABELLA, G. (1976) - *Structure of the Autonomic Nervous System* Chapman and Hall, Lond.

GEFFEN, L.B. & LIVETT, B.G. (1971) - "Synaptic vesicles in

sympathetic neurons." Physiol. Rev., 51, 98-157.

GEFFEN, L.B. & OSTBERG, G.A. (1969) - "Distribution of granular vesicles in normal and constricted sympathetic neurones." J. Physiol. Lond., 204, 583-592.

GELEI, G. VON (1942) - "Zur Frage der Doppelinnervation der Chromatophoren." Z. Vgl. Physiol., 29, 532-540.

GENTLE, M.J. (1968) - "The visual system of the minnow *Phoxinus phoxinus*, with special reference to its relationship to colour change and behaviour." Ph.D. Thesis, Univ. Lond.

GESCHWIND, I.I., HOROWITZ, J.M., MICKUCKIS, G.M. & DEWEY, R.D. (1977) - "Iontophoretic release of cyclic AMP and dispersion of melanosomes within a single melanophore." J. Cell Biol., 74, 928-939.

GIERSBERG, H. (1930) - "Der Farbwechsel der Fische." Z. Vgl. Physiol., 13, 258-279.

GINSBURG, J. & COBBOLD, A.F. (1960) - "Effects of adrenaline, noradrenaline and isopropylnoradrenaline in man." In: *Adrenergic Mechanisms* G.E.W. Wolstenholme and M. O'Connor, eds. Ciba Foundation Symposium. Churchill, Lond.

GOODMAN, L.S. & GILMAN, A. (1955) - *The Pharmacological Basis of Therapeutics* Macmillan, New York.

GOLDMAN, J.M. & HADLEY, M.E. (1969a) - "*In vitro* demonstration of adrenergic receptors controlling melanophore response of the lizard *Anolis carolinensis*." J. Pharmacol. exp- Ther., 166, 1-7.

GOLDMAN, J.M. & HADLEY, M.E. (1969b) - "The beta adrenergic receptor and cyclic 3',5'adenosine monophosphate: Possible roles in the regulation of melanophore responses of the spadefoot toad, *Scaphiopus couchi*." Gen. Comp. Endocrinol., 13, 151-163.

GRAHAM, J.D.P. (1961) - "The response to catecholamines of the melanophores of *Xenopus laevis*." J. Physiol. Lond., 158, 5-6.

GRAY, E.G. (1955) - "The control of melanophores in teleosts by nerves and hormones, with special reference to *Phoxinus phoxinus* (L.)." Ph.D. Thesis, Univ. of Wales.

GRAY, E.G. (1956) - "Control of the melanophores of the minnow *Phoxinus phoxinus* (L.)." J. exp. Biol., 33, 448-459.

GREEN, L. (1968) - "Mechanism of movements of granules in melanocytes of *Fundulus heteroclitus*." Proc. Natl. Acad. Sci. U.S.A., 59, 1179-1186.

GROVE, D.J. (1967) - "The physiology of colour changes in the minnow *Phoxinus phoxinus* with particular reference to the pharmacology of the melanophore-controlling fibres." Ph.D. Thesis, Univ. Lond.

GROVE, D.J. (1969a) - "The effects of adrenergic drugs on melanophores of the minnow *Phoxinus phoxinus* (L.)." Comp. Biochem. Physiol., 28, 37-54.

GROVE, D.J. (1969b) - "Melanophore dispersion in the minnow *Phoxinus phoxinus* (L.)." Comp. Biochem. Physiol., 28, 55-65.

HAMA, T. (1963) - "The relation between the chromatophores and pterin compounds." Ann. N.Y. Acad. Sci., 100, 977-986.

HAMA, T. & HASEGAWA, H. (1967) - "Studies on the chromatophores of *Oryzias latipes* (teleostean fish): Behavior of the pteridine, fat and carotenoid during xanthophore differentiation in the color varieties." Proc. Jap. Acad., 43, 901-906.

HAMA, T., MATSUMOTO, J. & MITSUMA, R. (1963) - "On the pterinosomes of swordtail fish." Zool. Mag. Tokyo, 72, 318.

HARRIS, J.E. & HUNT, S. (1973) - "The fine structure of iridophores

in the skin of the atlantic salmon *Salmo salar* L.". Tissue and Cell, 5(3), 479-488.

HEALEY, E.G. (1940) - "Ueber den Farbwechsel der Elritze (*Phoxinus laevis* Ag.)". Z. Vgl. Physiol., 27, 545-586.

HEALEY, E.G. (1948) - "The colour change of the minnow (*Phoxinus laevis* Ag.)". Bull. Anim. Behav., 6, 5-15.

HEALEY, E.G. (1951) - "The colour change of the minnow (*Phoxinus laevis* Ag.). I. Effect of spinal section between vertebrae 5 and 12 on the responses of the melanophores." J. Exp. Zool., 28, 297-319.

HEALEY. E.G. (1954) - "The colour change of the minnow (*Phoxinus laevis* Ag.). II. Effects of spinal section between vertebrae 1 and 15 and of anterior autonomic section on the responses of the melanophores." J. exp. Biol., 31, 473-490.

HEALEY, E.G. (1965) - "The effect of spinal cord sections on melanophore responses in the minnow (*Phoxinus phoxinus* L.)." J. Physiol. Lond., 178, 13-14.

HEALEY, E.G. (1967) - "Experimental evidence for the regeneration of nerve fibres controlling colour changes after anterior spinal section in the minnow (*Phoxinus phoxinus* L.)." Proc. Roy. Soc. B., 168, 57-81.

HEALEY, E.G. & ROSS, D.M. (1966) - "The effects of drugs on the background colour response of the minnow, *Phoxinus phoxinus* (L.)." Comp. Biochem. Physiol., 19, 545-580.

HERIKSON, R.C. & MATOLTSY, A.G. (1968) - "The fine structure of teleost epidermis." J. Ultrastruct. Res., 21, 222-232.

HEWER, H.R. (1927) - "Studies in colour changes of fish. II. An analysis of the colour pattern of the dab. III. The action of nicotine and adrenaline in the dab. IV. The action of caffeine in the dab, and a theory of the control of colour changes in fish." Philos. Trans. B, 215, 177-200.

HILL, A.V., PARKINSON, J.L. & SOLANDT, D.Y. (1935) - "Photoelectric records of the colour change in *Fundulus heteroclitus*." J. exp. Biol., 12, 397-399.

HITCHINGS, G.H. & FALCO, E.A. (1944) - "The identification of guanine in extracts of *Girella nigricans*." Proc. Natn. Acad. Sci. U.S.A., 30, 294-297.

*HOAR, W.S. (1955)"Phototactic and pigmentary responses of sockeye samon smolts following injury to the pineal organ." J. Fish. Res. Board, Canada, 12, 178-185.

HOGBEN, L.T. (1924) - "The pigmentary effector system. IV. A further contribution to the role of pituitary secretion in amphibian colour response." Brit. J. Exp. Biol., 1, 249-270.

HOGBEN, L.T. (1942) - "Chromatic behaviour." - Croonian Lecture. Proc. Roy. Soc. B, 131, 111-136.

HOGBEN, L.T. & LANDGREBE, F. (1940) - "The pigmentary effector system. IX. The receptor fields of the teleostean visual response." Proc. Roy. Soc. B., 128, 317-342.

HOGBEN, L.T. & SLOME, D. (1931) - "The pigmentary effector system. VI. The dual character of endocrine coordination in amphibian colour change." Proc. Roy. Soc. B, 108, 10.53.

HOGBEN, L.T. & SLOME, D. (1936) - "The pigmentary effector system. VIII. The dual receptive mechanism of the amphibian background response." Proc. Roy. Soc. B, 120, 158-173.

HOGBEN, L.T. & WINTON, F.R. (1922) - "Studies on the pituitary. I. The melanophore stimulant in the posterior lobe extract." Biochem. J., 16, 619-630.

HOGBEN, L.T. & WINTON, F.R. (1923) - "The pigmentary effector system. III. Colour responses in the hypophysectomised frog." Proc. Roy. Soc. B, 95, 15-31.

HÖKFELT, T. (1968) - "*In vitro* studies on central and peripheral monoamine neurons at the ultrastructural level." Z. Zellforsch. Mikrosk. Anat., 91, 1-74.

HÖKFELT, T. (1969) - "Distribution of noradrenaline storing particles in peripheral adrenergic neurons as revealed by electron microscopy." Acta Physiol. Scand., 76, 427-440.

HÖKFELT, T. (1973) - "Localization of catecholamines with special reference to synaptic vesicles." Life Sci., 13, 73-74.

HÖKFELT, T. & LJUNGDAHL, A. (1972) - "Application of cytochemical techniques to the study of suspected transmitter substance in the nervous system." In: *Studies of Neurotransmitters at the Synaptic Level* E. Costa, L.L. Iverson and R. Padetti, eds. Advances in Biochemical Psychopharmacology. 6, pp. 1-36.

HUGHES, J. (1972) - "Evaluation of mechanisms controlling the release and inactivation of the adrenergic transmitter in the rabbit portal vein and *vas deferens*." Brit. J. Pharmacol., 44, 472-491.

IGA, T. (1968) - "Action of catecholamines on the melanophores in the teleost *Oryzias latipes*." Zool. Mag. Tokyo, 77, 19-26.

IMAI, K. (1958) - "Extraction of melanophore concentrating hormone (MCH) from the pituitary of fishes." Endocrinol. Jpn., 5, 34-48.

IVERSEN, L.L. (1965) - "The inhibition of noradrenaline uptake by drugs." Adv. Drug Res., 2, 5-23.

IVERSEN, L.L. (1967) - *The Uptake and Storage of Noradrenaline in Sympathetic Nerves* Cambridge University Press.

IVERSEN, L.L. (1971) - "Role of transmitter uptake mechanisms in synaptic neurotransmission." Brit. J. Pharmacol., 41, 571-591.

IVERSEN, L.L. (1973) - "Catecholamine uptake processes." Brit. med. Bull., 29, 130-135.

IVERSEN, L.L. & LANGER, S.Z. (1969) - "Effects of phenoxybenzamine on the uptake and metabolism of noradrenaline in the rat heart and *vas deferens*." Brit. J. Pharmacol., 37, 627.

IWATA, K.S. & FUKUDA, H. (1973) - "Central control of colour change in fish." In: *Response of Fish to Environmental Changes* W. Chavin, ed, Thomas Springfield, pp. 316-341.

IWATA, K.S., WATANABE, M. & KURIHARA, T. (1959a) - "Changes of state and response of the fish scale melanophore during continuous immersion in Ringer's solution." Biol. J. Okayama Univ., 5, 185-194.

IWATA, K.S., WATANABE, M. & NAGAO, K. (1959b) - "The mode of action of pigment concentrating agents on melanophores in isolated fish scale." Biol. J. Okayama Univ., 5, 195-206.

JACOBOWITZ, D.M. & LATIES, A.M. (1968) - "Direct adrenergic innervation of a teleost melanophore." Anat. Rec., 162, 501-504.

JENKINSON, D.H. (1973) - "Classification and properties of peripheral adrenergic receptors." Brit. med. Bull., 29, 142-147.

JØRGENSEN, C.B. (1962) - "Effect of total hypophysectomy on the melanophores of *Xenopus laevis*." D. Gen. Comp. Endocrinol., 2, 610.

JØRGENSEN, C.B. & LARSEN, L.O. (1960) - "Control of colour change in amphibians." Nature, Lond. 186, 641-642.

JUNQUEIRA, L.C. & FARIAS, E.C. (1976) - "Ultrastructure and pigment transport in the xanthophores and erythrophores of 14 species of teleosts." J. Cell Biol., 70, 1a.

JUNQUEIRA, L.C. & PORTER, K.R. (1969) - "Pigment migration in *Fundulus* melanophores." Biophys., 9, A-152.

JUNQUEIRA, L.C., RAKER, E. & PORTER, K.R. (1974) - "Studies on pigment migration in melanophores of the teleost *Fundulus heteroclitus* (L.)." Arch. Histol. Jap., 36, 339-366.

JUNQUEIRA, L.C. REINACH, A. & SALLES, L.M.M. (1977) - "The presence of spontaneous and induced filaments in the melanophores of three species of teleosts." Arch. histol. Jpn., 40(5), 435-443.

KAMEI-TAKEUCHI, I. & HAMA, T. (1971) - "Structural change of pterinosome (pteridine pigment granule) during the xanthophore differentiation of *Oryzias* fish." J. Ultrastruct. Res., 34, 452-463.

KAMEI-TAKEUCHI, I. & KAJISHIMA, T. (1971) - "Fine structure of adult goldfish melanophores." Annot. zool. Japon., 44(1), 23-31.

KARNOVSKY, M.J. (1965) - "A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy." J. Cell Biol., 27, 137A.

KAWAGUTI, S. & KAMISHIMA, Y. (1966) - "Electron microscopy on the blue black of a clupeoid fish, *Harengula zunasi*." Proc. Japan Acad., 42, 389-393.

KEEBLE, F. & GAMBLE, F.W. (1904) - "The colour physiology of higher crustacea." Philos. Trans. Roy. Soc. Lond. B., 196, 295-388.

KENT, A.K. (1959) - "The significance of time relations of hormonally coordinated chromatic responses." Nature, Lond., 184, 2027-2028.

KENT, A.K. (1960) - "An investigation of the melanophore-aggregating principle of the pituitary of some teleost fishes." Ph.D. Thesis, Univ. Lond.

KHOKHAR, R. (1971) - "On the nervous and hormonal control of melanophores in the catfish *Ictalurus melas* (Rafinesque) and their differential reactions." Ph.D. Thesis., Univ. of Lond.

KINOSITA, H. (1953) - "Studies on the mechanism of pigment migration within fish melanophores with special reference to their electric potentials." Annot. Zool. Jpn., 26, 115-127.

KINOSITA, H. (1963) - "Electrophoretic theory of pigment migration within fish melanophores." Ann. N.Y. Acad. Sci., 100, 992-1004.

KINOSITA, H. & UEDA, K. (1970) - "Physiological studies of fish melanophores. I. Concentration and dispersion responses elicited by electric stimulation." J. Fac. Sci. Univ. Tokyo, Sect. IV, 12, 101-116.

KLEINHOLZ, L.H. (1935) "The melanophore-dispersing principle in the hypophysis of *Fundulus heteroclitus*." Biol. Bull., Woods Hole, 69, 379-390.

LANDS, A.M. ARNOLD, A., MCAULIFF, J.P., LUDUENA, F.P. & BROWN, T.G. (1967) - "Differentiation of receptor systems activated by sympathomimetic amines." Nature, Lond., 214, 597-598.

LANDS, A.M. & BROWN, T.G. (1967) - "Sympathomimetic 'Adrenergic' stimulants." In: *Drugs affecting the Peripheral Nervous System* A. Burger, ed., Dekker, New York.

LANGER, S.Z. (1970) - "The metabolism of ^{3}H-noradrenaline released by electrical stimulation from the isolated nictitating membrane of the cat and from the *vas deferens* of the rat." J. Physiol. Lond., 208, 515-546.

LANGER, S.Z. (1977) - "Presynaptic receptors and their role in the regulation of transmitter release." Br. J. Pharmac., 60, 481-497.

LANGER, S.Z., ALDER-GRASCHINSKY, E. & GIORGI, O. (1977) - "Physiological significance of the alpha-adrenoceptor mediated negative feed-back mechanism that regulates noradrenaline release during nerve stimulation." Nature, Lond., 265, 648-650.

LANGER, S.Z., ENERO, M.A., ALDER-GRASCHINSKY, E., DUBOCOVICH, M.L. & GIORGI, O. (1976) - "Regulation of transmitter release." In: *Vascular Neuroeffector Mechanisms* S. Karger, ed. AG Medical and Scientific Publishers, Basel. pp. 112-122.

LANGER, S.Z., STEFANO, F.J.E. & ENERO, M.A. (1972) - "Pre- and post-synaptic origin of the norepinephrine metabolites formed during transmitter release elicited by nerve stimulation." J. Pharmac. exp. Ther., 183, 90-102.

LANGER, S.Z. & TRENDELENBURG, U. (1966) - "The onset of denervation supersensitivity." J. Pharmac. exp. Ther., 151, 73-86.

LANGLEY, J.N. (1921) - *The Autonomic Nervous System* - Part I. Heffer, Cambridge.

LANZING, W.J.R. & WRIGHT, R.G. (1974) - "The ultrastructure of the

skin of *Tilapia mossambica*." Cell. Tissue Res., 154, 251-264.

LEE, A.S.K., VANSTONE, W.E., MARKERT, J.R. & ANTA, N.J. (1969) - "UV-absorbing and UV-fluorescing substances in the belly skin of Coho salmon (*Oncorhynchus kisutch*)." J. Fish. Res. Bd. Can., 26, 1185-1198.

LEFKOWITZ, R.J. (1975) - "Heterogeneity on adenylate cyclase-coupled β- adrenergic receptors." Biochem. Pharmacol., 24(5), 583-590.

LEIFER, K.N. & WITTIG, H.J. (1975) - "The beta-2 sympathomimetic aerosols in the treatment of asthma." Ann. Allerg., 35(2), 69-80.

LERNER, A.B. & CASE, J.D. (1960) - "Melatonin." Federation Proc., 19, 590-592.

LEVY, B. & WILKENFELD, B. (1969) - "An analysis of selective beta receptor blockade." Eur. J. Pharmacol., 5, 227-234.

LEWIS, J.J. (1970) - *Lewis's Pharmacology* Revised by James Crossland. E. & S. Livingstone, Edinburgh and London.

*LISTER, J. (1858) - "On the cutaneous pigmentary system of the frog." Philos. Trans., 148, 627-643. (Cited in Parker, 1948).

LYALL, A.H. (1956) - "Occurrence of triple and quadruple cones in the retina of the minnow *Phoxinus phoxinus*." Nature, Lond. 177, 1086-1087.

LYALL, A.H. (1957) - "Cone arrangements in teleost retinae." Quart. J. micr. Sci., 98, 189-201.

MALAWISTA, S.E. (1965) - "On the action of colchicine. The melanocyte model." J. Exp. Med., 122, 361-384.

MALAWISTA, S.E. (1971) - "The melanocyte model. Colchicine-like effects of other antimitotic agents." J. Cell Biol., 49, 848-855.

MARSLAND, D.A. (1944) - "Mechanism of pigment displacement in unicellular chromatophores." Biol. Bull. Woods Hole, 87, 252-261.

MAST, S.O. (1916) - "Changes in shade, color, and pattern in fishes and their bearing on the problems of adaptation and behavior, with special reference to the flounders *Paralichthys* and *Ancylopsetta*." Bull. U.S. Bur. Fish., 34, 173-238.

MATSUMOTO, J. (1965a) - "Studies on fine structure and cytochemical properties of erythrophores in swordtail *Xiphophorus helleri*, with special reference to their pigment granules (pterinosomes)." J. Cell Biol., 27, 493-504.

MATSUMOTO, J. (1965b) - "Role of pteridines in the pigmentation of chromatophores in cyprinid fish." Jpn. J. Zool., 14, 45-94.

MATTHEWS, S.A. (1931) - "Observation on pigment migration within the fish melanophore." J. Exp. Zool., 58, 471-486.

MATTHEWS, S.A. (1933) - "Color changes in *Fundulus* after hypophysectomy." Biol. Bull., Woods Hole, 64, 315-320.

McCORD, C.P. & ALLEN, F.P. (1917) - "Evidence associating pineal gland function with alteration in pigmentation." J. Exp. Zool., 23, 207-224.

McEWAN, M.R. (1938) - "A comparison of the retina of the mormyrids with that of various teleosts." Acta Zool., 19, 427-465.

MILLS, S.M. (1932a) - "The double innervation of fish melanophores." J. Exp. Zool., 64, 231-244.

MILLS, S.M. (1932b) - "Evidence for a neurohumoral control of fish melanophores." J. Exp. Zool., 64, 245-255.

MIYASHITA, Y. & FUJII, R. (1975) - "Receptor mechanism in fish chromatophores. II. Evidence for beta adrenoceptors mediating

melanosome dispersion in guppy melanophores." Comp. Biochem. Physiol., 51(C), 179-187.

MORAN, N.C. & PERKINS, M.E. (1958) - "Adrenergic block of the mammalian heart by a dichloro analogue of isoproterenol." J. Pharmacol. Exp. Ther., 124, 223.

MURPHY, D.B. & TILNEY, L.G. (1974) - "The role of microtubules in the movement of pigment granules in teleost melanophores." J. Cell Biol., 61, 757-779.

NEILL, R.M. (1940) - "On the existence of two types of chromatic behaviour in teleostean fishes." J. exp. Biol., 17, 74-95.

NICKERSON, M. (1949) - "The pharmacology of adrenergic blockade." Pharmacol. Rev., 1, 27-101.

NICOL, J.A.C. (1963) - "Some aspects of photoreception and vision in fishes." Adv. Mar. Biol., 1, 171-208.

NOVALES, R.R. & NOVALES, B.J. (1966a) - "Cytological and ultrastructural aspects of amphibian melanophore control." In: *Structure and Control of the Melanocyte* G.D. Porta & O. Mühlbock, eds. Springer-Verlag, Berlin, Heidelberg, New York. pp. 52-59.

NOVALES, R.R. & NOVALES, B.J. (1966b) - "Factors influencing the response of isolated dogfish skin melanophores to melanocyte-stimulating hormone." Biol. Bull., Woods Hole, 131, 470-478.

OBIKA, M. (1976) - "Pigment migration in isolated fish melanophores." Annot. Zool. Jpn., 49, 157-163.

O'BRIEN, T.P., FEDER, N. & McCULLY, M.E. (1964) - "Polychromatic staining of plant cell walls with toluidine blue O." Protoplasma, 59, 368-373.

ODIORNE, J.M. (1933) - "The effects of the pituitary hormones on the melanophores of fishes." Proc. Natl. Acad Sci. U.S.A., 19, 745-749.

OSBORN, C.M. (1938a) - "The role of the melanophore-dispersing principle of the pituitary in the color change of the catfish." J. Exp. Zool., 79, 309-330.

OSBORN, C.M. (1938b) - "The role of the melanophore-dispersing hormone of the pituitary in the color changes of the catfish." Proc. Nat. Acad. Sci., Wash., 24, 121-125.

OSBORN, C.M. (1939a) - "The physiology of color change in flatfishes." J. Exp. Zool., 81, 479-515.

OSBORN, C.M. (1939b) - "The experimental production of melanin pigment on the ventral surface of the summer flounder *Paralichthys dentatus*." Anat. Rec., 75, Suppl. p. 60.

PARKER, G.H. (1933) - "The cellular transmission of neurohumoral substances in melanophore reactions." Proc. Natl. Acad. Sci. U.S.A., 19, 175-177.

PARKER, G.H. (1934a) - "Cellular transfer of substances especially neurohumors." J. exp. Biol., 11, 81-88.

PARKER, G.H. (1934b) - "The prolonged activity of momentarily stimulated nerves." Proc. Natl. Acad. Sci. U.S.A., 20, 306-310.

PARKER, G.H. (1934c) - "Color changes in the catfish, *Ameiurus* in relation to neurohumors." J. Exp. Zool., 69, 199-233.

PARKER, G.H. (1935a) - "The electric stimulation on the chromatophoral nerve-fibres in the dogfish." Biol. Bull. Woods Hole, 68, 1-3.

PARKER, G.H. (1935b) - "The disappearance of primary caudal bands

in the tail of *Fundulus* and its relation to the neurohumoral hypothesis." Proc. Am. Phil. Soc., 75, 1-10.

PARKER, G.H. (1941a) - "The method of activation of melanophores and the limitation of melanophore responses in the catfish, *Ameiurus*." Proc. Am. Phil. Soc., 85, 18-24.

PARKER, G.H. (1941b) - "The response of melanophores to ergotamine." Biol. Bull. Woods Hole, 81, 163-167.

PARKER, G.H. (1948) - *Animal Colour Changes and their Neurohumours* Cambridge University Press.

PARKER, G.H. & LANCHNER, A.J. (1922) - "The responses of *Fundulus* to white, black and darkness." Am. J. Physiol., 61, 548-550.

PARKER, G.H. & PORTER, H. (1933) - "Regeneration of chromatophore nerves." J. Exp. Zool., 66, 303-309.

PARKER, G.H. & ROSENBLUETH, A. (1941) - "The electric stimulation of the concentrating (adrenergic) and the dispersing (cholinergic) nerve fibres of the melanophores in the catfish." Proc. Natl. Acad. Sci. U.S.A., 27, 198-204.

PEARSON, J.F.W. (1930) - "Changes in pigmentation exhibited by the fresh-water catfish, *Ameiurus melas*, in response to differences in illumination." Ecology, 11, 703-712.

PORTER, K.R. (1973) - "Microtubules in intracellular locomotion." In: *Locomotion of Tissue Cells* Ciba Foundation Symposium 14, Associated Scientific Publishers, Amsterdam. pp. 149-166.

POUCHET, G. (1872) - "Du rôle des nerfs dans les changements de coloration des poissons." J. Anat. Physiol., 8, 71-74.

POUCHET, G. (1876) - "Des changements de coloration sous l'influence des nerfs." J. Anat. Physiol., 12, 1-90, 113-165.

POWELL, C.E. & SLATER, I.H. (1958) - "Blocking of inhibitory adrenergic receptors by a dichloro analog of isoproterenol." J. Pharmacol. exp. Ther., 122, 480-488.

PYE, J.D. (1961) - "An investigation of the effects of temperature on the melanophores of some teleost fishes with special reference to chromatic nervous control in *Phoxinus phoxinus* (L.)." Ph.D. Thesis, Univ. Lond.

PYE, J.D. (1964a) - "Nervous control of chromatophores in teleost fishes. I. Electrical stimulation in the minnow *Phoxinus phoxinus* (L.)." J. exp. Biol., 41, 535-541.

PYE, J.D. (1964b) - "Nervous control of chromatophores in teleost fishes. II. The influence of certain drugs in the minnow *Phoxinus phoxinus* (L.)." J. exp. Biol., 41, 525-534.

RALL, T.W., SUTHERLAND, E.W. & BERTHET, J. (1957) - "The relationship of epinephrine and glucagon to liver phosphorylase. IV. Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates." J. Biol. Chem., 224, 463-475.

RASQUIN, P. (1958) - "Studies in the control of pigment cells and light reaction in recent teleost fishes." Bull. Am. Mus. Nat. Hist., 115, 1-68.

*RAYMOND-HAMET (1925) - "Sur un nouveaucas d'inversion des effets adrénaliniques." Compt. rend. Acad. d. Sci., 180, 2074-2077. (Cited in Goodman, L.S. & Gilman, A. (1955) - *The Pharmacological Basis of Therapeutics* Macmillan, New York.)

REED, B.L. & FINNIN, B.C. (1972) - "Adrenergic innervation of melanophores in a teleost fish." In: *Pigmentation: Its Genesis and*

Biological Control V. Riley, ed., Appleton-Century-Crofts, New York.

REYNOLDS, E.S. (1963) - "The use of lead citrate at high pH as an electron-opaque stain in electron microscopy." J. Cell. Biol., 17, 208-212.

RICHARDSON, K.C. (1966) - "Electron microscopic identification of autonomic nerve endings." Nature, Lond., 210, 756.

ROBERTS, K. (1974) - "Cytoplasmic microtubules and their functions." Progr. Biophys. molec. Biol., 28, 371-420.

ROBERTS, R.J., YOUNG, H. & MILNE, J.A. (1972) - "Studies on the skin of plaice (*Pleuronectes platessa* L.). I. The structure and ultrastructure of normal plaice skin." J. Fish Biol., 4, 87-98.

ROBERTSON, O.H. (1951) - "Factors influencing the state of dispersion of the dermal melanophores in rainbow trout." Physiol. Zool., 24, 309-323.

ROBINSON, G.A., BUTCHER, R.W. & SUTHERLAND, E.W. (1970) - "On the relation of hormone receptors to adenyl cyclase." In: *Fundamental Concepts in Drug-Receptor Interactions* J.F. Danielli, J.F. Moran, and D.J. Triggle, eds. Academic Press, London and New York.

ROSENBLUETH, A. (1950) - *Transmission of Nerve Impulses of Neuro-effector Junction and Peripheral Synapses.* I. Transmission at autonomic neuro-effector junctions. Chapman and Hall, London.

SAND, A. (1935) - "The comparative physiology of colour response in reptiles and fishes." Biol. Rev., 10, 361-382.

SCHAEFER, J.G. (1921) - "Beitrage zur Physiologie des Farbenwechsels

der Fische. I. Untersuchungen an Pleuronectiden. II. Weitere Untersuchungen." Pflüg. Arch. ges. Physiol., 188, 25-48.

SCHELINE, R.R. (1963) - "Adrenergic mechanisms in fish: Chromatophore pigment concentration in cuckoo wrasse, *Labrus ossifagus* L." Comp. Biochem. Physiol., 9, 215-227.

SCHILD, H.O. (1973) - "Receptor classification with special reference to β - adrenergic receptors." In: *Drug Receptors* H.P. Rang, ed. Macmillan, London.

SCHLIWA, M. (1976) - "Fine structure of nerve-melanophore contacts in the angelfish *Pterophyllum scalare*." Cell Tiss. Res., 171, 381-388.

SCHLIWA, M. & BEREITER-HAHN, J. (1973) - "Pigment movements in fish melanophores: Morphological and physiological studies. II. Cell shape and microtubules." Z. Zellforsch. Mikrosk. Anat., 147, 107-125.

SCHLIWA, M. & BEREITER-HAHN, J. (1975) - "Pigment movements in fish melanophores: Morphological and physiological studies. V. Evidence for a microtubule-independent contractile system." Cell Tiss. Res., 158, 61-73.

SCHLIWA, M. & EUTENEUER, U. (1978) - "A microtubule-independent component may be involved in granule transport in pigment cells." Nature, Lond., 273, 556-558.

SCOTT, G.T. (1965) - "Physiology and pharmacology of color change in the sand flounder *Scophthalamus aquosus*." Limnol. Oceanogr., 10, R230-R246.

SCOTT, G.T. (1972) - "The action of psychoactive drugs on pigment

cells of lower vertebrates," In: *Pigmentation: Its Genesis and Biological Control* V. Riley, ed., Appleton-Century-Crofts, New York.

SCOTT, S.T., CLARK, R.L. & HICKMAN, J.C. (1962) - "Mechanism of chromatophore control in the common sand flounder *Scophthalamus aquosus*. Drugs causing localized lightening and darkening of the common sand dab, *Scophthalamus aquosus*." Biol. Bull., Woods Hole, 123, 486-511.

SECEROV, S. (1909) - "Farbenwechselversuche an der Bartgrundel *(Nemachilus barbatula* L.)" Arch. Entw. Mech., 28, 629-660.

SETOGUTI, T. (1967) - "Ultrastructure of guanophores." J. Ultrastruct. Res., 18, 324-332.

SHANKS, R.G. (1966) - "Methods for the evaluation of adrenergic beta-receptor antagonists." In: *Methods in Drug Evaluation* Mantegazza and Piccinni, eds., North Holland Publ. Co., Amsterdam. pp. 183-198.

*SIEBOLD, K.T.E. VON (1863) - *Die Süsswasserfische von Mitteleuropa* Leipzig. pp. 431. (Cited in Parker, 1948).

SJÖSTRAND, F.S. (1948) - "An electron microscope study of the retinal rods of the guinea pig eye." J. Appl. Physics, 19, 1188.

SMITH, A.D. (1971) - "Secretions of proteins (chromogranin A and dopamine β-hydroxylase) from a sympathetic neuron." Phil. Trans. Roy. Soc. Lond., Ser. B., 261, 363-370.

SMITH, A.D. (1973) - "Mechanisms involved in the release of noradrenaline from sympathetic nerves." Br. med. Bull., 29, 123-129.

SMITH, A.D. & WINKLER, H. (1972) - "Fundamental mechanisms in the

release of catecholamines," In: *Catecholamines* H. Blaschko and E. Muscholl, eds., Springer-Verlag, Berlin, Heidelberg, New York. pp. 538-617.

SMITH, D.C. (1931) - "The influence of humoral factors upon the melanophores of fishes, especially *Phoxinus*." Z. Vgl. Physiol., 15, 613-636.

SMITH, D.C. (1936) - "A method for recording chromatophore pulsations in isolated fish scales by means of a photo-electric cell." J. Cell. Comp. Physiol., 8, 83-87.

SMITH, P.E. (1916a) "Experimental ablation of the hypophysis in the frog embryo." Science, 44, 280-282.

SMITH, P.E. (1916b) - "The effect of hypophysectomy in the early embryo upon the growth and development of the frog." Anat. Rec., 11, 57-64.

SPAETH, R.A. (1913) - "The physiology of the chromatophores of fishes." J. Exp. Zool., 15, 527-585.

SPAETH, R.A. (1916) - "The responses of single melanophores to electrical stimulation." Am. J. Physiol., 41, 577-596.

SPAETH, R.A. (1918) - "Concerning a new method for the biological standardization of pituitary extract and other drugs." J. Pharmacol., 11, 209-219.

*STARK, J. (1830) - "On changes observed in the colour of fishes." Edinb. New Phil. J., 9, 327-331. (Cited in Parker, 1948).

STARKE, K. (1972) - "Influence of extracellular noradrenaline on the stimulation-evoked secretion of noradrenaline from sympathetic nerves: Evidence for an α-receptor-mediated feed-back inhibition of noradrenaline release." Naunyn Schmied. Arch.

exp. Path. Pharmak., 275, 11-23.

STJÄRNE, L. & BRUNDIN, J. (1976) - "β_2-adrenoceptors facilitating noradrenaline secretion from human vascoconstrictor nerves." Acta physiol. Scand., 97, 88-93.

SU, C. & BEVAN, J.A. (1970) - "The release of H^3-norepinephrine in arterial strips studied by the technique of superfusion and transmural stimulation." J. Pharmacol. exp. Ther., 172, 62-68.

SUMNER, F.B. (1910) - "Adaptive color changes among fishes." Bull. Zool. Soc. New York, 42, 699-701.

SUMNER, F.B. (1911) - "The adjustment of flatfishes to various backgrounds. A study of adaptive color change." J. Exp. Zool., 10, 409-505.

SUMNER, F.B. (1933) - "The differing effects of different parts of the visual field upon the chromatophore responses of fishes." Biol. Bull. Woods Hole, 65, 266-282.

SUMNER, F.B. (1943) - "A further report upon the effects of the visual environment on the melanin content of fishes." Biol. Bull. Woods Hole, 84, 195-205.

SUMNER, F.B. & KEYS, A.B. (1929) - "The effects of differences in the apparent source of illumination upon the shade assumed by a flatfish on a given background." Physiol. Zool., 2, 495-504.

SWINGLE, W.W. (1921) - "The relation of the *pars intermedia* of the hypophysis to pigmentation changes in anuran larvae," J. Exp. Zool., 34, 119-194.

TAKEUCHI, I.K. (1975) - "Electron microscopic study on erythrophores of the guppy, *Lebistes reticulatus* Peters." Annot. Zool. Jpn.,

84(4), 242-251.

TAKEUCHI, I.K., EGUCHI, G. & HAMA, T. (1968) - "Ultrastructure of the pteridine pigment granules of the larval xanthophore and leucophore in *Oryzias latipes*." J. Sci. Hiroshima Univ., Ser. B., DIV I (Zool.), 25, 259-270.

TAKEUCHI, I.K. & KAJISHIMA, T. (1972) - "Fine structure of goldfish xanthophore." J. Anat., 112, 1-10.

TAYLOR, J.D. (1969) - "The effects of intermedin on the ultrastructure of amphibian iridophores." Gen. Comp. Endocrinol., 12, 405-416.

TAYLOR, S.E. & TEAGUE, R.S. (1976) - "The beta adrenergic receptors of chromatophores of the frog *Rana pipiens*." J. Pharmacol. exp. Ther., 199(1), 222-235.

THOENON, H. (1972) - "Surgical, immunological and chemical sympathectomy. Their application in the investigation of the physiology and pharmacology of the sympathetic nervous system." In: *Catecholamines*. H. Blaschko and E. Muscholl, eds. Springer-Verlag, Berlin, Heidelberg, New York. pp. 813-844.

TRANZER, J.P. (1972) - "A new storing compartment in adrenergic axons." Nature, New Biol., 237, 57-58.

TRANZER, J.P. (1973) - "New aspects of localization of catecholamines in adrenergic neurons." In: *Frontiers in Catecholamine Research* E. Usdin and S. Snyder, eds. Pergamon Press, Oxford. pp. 453-458.

TRANZER, J.P. & THOENEN, H. (1967) - "Significance of 'empty vesicles' in post-ganglionic sympathetic nerve terminals." Experientia, (Basel), 23, 123-124.

TRENDELENBURG, U. (1963) - "Supersensitivity and subsensitivity to

sympathomimetic amines." Pharmacol. Rev., 15, 225-276.

TRIGGLE, D.J. (1972) -"Adrenergic receptors." Ann. Rev. Pharmacol., 12, 185-196.

TRIGGLE, D.J. & TRIGGLE, C.R. (1976) - *Chemical Pharmacology of the Synapse* Academic Press, London, New York, San Francisco.

TURNER, W.J. & CARL, A. (1955) - "Effect of reserpine on the melanophores of fish." Science, N.Y., 121, 877-878.

UEDA, K. (1955) - "Stimulation experiments on fish melanophores." Annot. Zool. Jpn., 28, 194-205.

UMRATH, K. (1957) - "Über den physiologischen und den morphologischen Farbwechsel des Bitterlings, *Rhodeus amarus*." Z. Vgl. Physiol., 40, 321-328.

UMRATH, K. & WALCHER, H. (1951) - "Farbwechsel Versuche an *Macropodus opercularis* und ein Vergleich der Geschwindigkeit der Farbänderung bei Macropoden und Elritzen." Z. Vgl. Physiol. 33, 129-141.

VEIL, C. (1938) - "Evaluation de la quantité d'intermédine contenue dans l'organisme du poisson-chat." C.R. Soc. Biol., Paris, 127, 42-43.

*VOGT, C. (1842) - "Embryologie des salmons." In: *Histoire naturelle des poissons d'eau douce de l'Europe centrale* Neuchatel, L. Agassiz, ed. (Cited in Parker, 1948).

VOLICER, L. & HYNIE, S. (1971) - "Effect of catecholamines and angiotensin on cyclic AMP in rat aorta and tail artery." Eur. J. Pharmacol., 15, 214-220.

WALLS, G.L. (1942) - *The Vertebrate Eye and its Adaptive Radiation* Cranbrook Institute of Science. Bull. No. 19.

WARING, H. (1942) - "The co-ordination of vertebrate melanophore responses." Biol. Rev., 17, 120-150.

WARING, H. (1963) - *Color Change Mechanisms of Cold-Blooded Vertebrates* Academic Press, New York.

WARING, H. & LANDGREBE, F.W. (1950) - *The Hormones, Vol II* G. Pincus and K.V. Thimann eds, Acad. Press. New York.

WATANABE, M., KOBAYASHI, M. & IWATA, K.S. (1962) - "The action of certain autonomic drugs on the fish melanophore." Biol. J. Okayama Univ., 8, 103-114.

WHITEAR, M. (1952) - "The innervation of the skin of teleost fishes." Quar. J. Mic. Sci., 93, 487-496.

WHITEAR, M. (1970) - "The skin surface of bony fishes." J. Zool. Lond., 160, 437-454.

WHITEAR, M. (1977) - "A functional comparison between the epidermis of fish and of amphibians." Symp. Zool. Soc. Lond., No. 39, 291-313.

WIKSWO, M.A. & NOVALES, R.R. (1969) - "The effect of colchicine on migration of pigment in the melanophores of *Fundulus heteroclitus*." Biol. Bull. Woods Hole, 137, 228-237.

WIKSWO, M.A. & NOVALES, R.R. (1972) - "The effect of colchicine on microtubules in the melanophores of *Fundulus heteroclitus*." J. Ultrastruct. Res., 41, 189-201.

WRIGHT, P.A. (1955) - "Physiological responses of frog melanophores *in vitro*." Physiol. Zool., 28, 204-218.

WYKES, U. (1937) - "The photic control of pigmentary responses in teleost fishes." J. exp. Biol., 14, 79-86.

WYKES, U. (1938) - "The control of photo-pigmentary responses in

eyeless catfish." J. exp. Biol., 15, 363-370.

WYMAN, L.C. (1924) - "Blood and nerve as controlling agents in the movements of melanophores." J. Exp. Zool., 39, 73-132.

YASUTOMI, M. & HAMA, T. (1972) - "Electron microscopic study on the xanthophore differentiation in *Xenopus laevis*, with special reference to their pterinosomes." J. Ultrastruct. Res., 38, 421-432.

YOUNG, J.Z. (1931) - "On the autonomic nervous system of the teleostean fish *Uranoscopus scaber*." Quart. J. mic. Sci., 74, 491-535.

YOUNG, J.Z. (1933) - "The preparation of isotonic solutions for use in experiments with fish." Pubbl. Staz. Zool. Napoli, 12, 1-7.

YOUNG, J.Z. (1950) - *The Life of Vertebrates* Clarendon Press, Oxford.

(*Original not seen)

خلاصـــة البحـــــث

Arabic Abstract

يدور هذا البحث حول الخلية اللونية التي تحتوى على حبيبـــات الميلانين (اللون الاسود) .

التجارب العملية اجريت على السمك النهرى الاروبي (Minnow) لأن جلد هذه السمكة تحتوى على مجموعات كبيرة من خلايا الملانين . تسمى هـــذه الخلايا ميلانوفورز (Melanophores) اذا كانت حبيبات الملانين فيها قابلة للانتشار والتجميع . خلايا الملانين في هذه السمكة وفي بعـــض الاسماك الاخرى وبعض الحيوانات الفقرية الاولية لها ذلك القابلية .

البحوث السابقة اكدت ان هذه الخلايا في معظم الاسماك العظميـــة تحت تحكم عصبي مباشر وان عملية تجميع حبيبات الملانين في هذه الخلايـــا تتم عن طريق الجهاز العصبي السمبتاوى (Sympathetic) .

ولكن فأن آراء العلماء قد تضاربت عن نوعية الاعصاب التي عن طريقها تتم انتشار الحبيبات اللونية .

الدراسات العملية في هذا البحث تضمنت المجهر الالكتروني ، الهيستو-كمسترى والادوية الخاصة بالاعصاب الادرونرجيك (هذه الأعصاب تتصـــل بالجهاز المركزى بواسطة مجموعة الاعصاب السمبتاوية عن طريق العقـــد العصبية) .

من النتائج الهامة التي تم التوصل اليها هي التاليـــة :

١- التأكيد على ان هذه الخلايا تحت تحكم عصبي مباشر وذلك لوجـــود نهايات الاعصاب بالقرب منها (دراسات بالمجهر الالكتروني) .

٢- الدراسات الهيستو-كيميائية والمجهرية اشرت الى ان نهايات الاعصاب القريبة من هذه الخلايا لها صفات الأعصاب الادرونرجيـــــــــــــك (Adrenergic Neurons) .

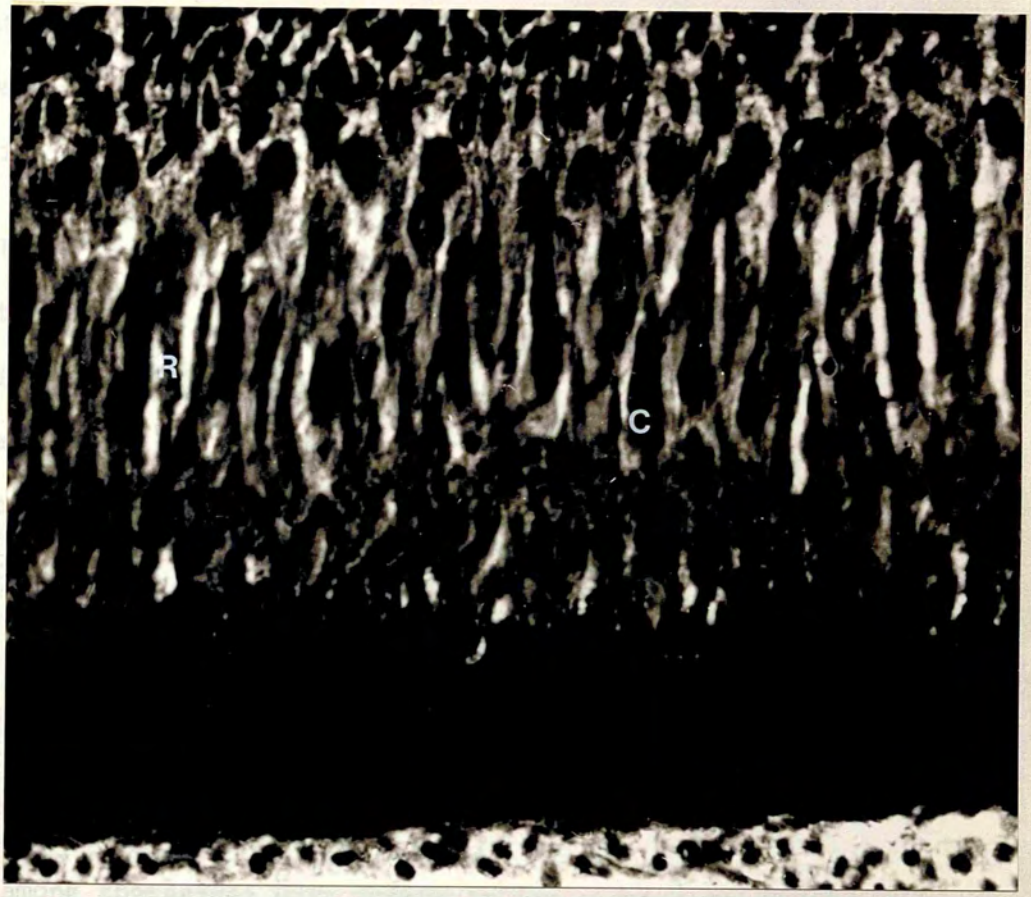
٣ - الدراسات الفرماكولوجية بالاضافة الى انها اكدت نتائج الدراســـات المجهرية والهيستو - كيميائية أن نهايات الاعصاب القريبة من هذه الخلايـــا لها صفات الادرونرجيك ، اقترحت ان هذه الخلايا ، من ناحية التحـــكم العصبي ، تحت تأثير الاعصاب الادرونرجيك فقط وهناك مراكز استقبـــال مختلفة (متضادة) في الخلية للأفراز الادرونرجيك . اثارة احداهما ينتـــج عنها تجميع حبيبات اللونية ، واثارة الاخرى ينتج عنها انتشارها . وبنـــاء على ذلك اقترح تفسير جديد لعملية انتشار وتجميع حبيبات الملانين فـــي

الخـــلايـــا اللونيـــــة .

تمت هذه الدراسة في كلية بدفورد / جامعة لنـــدن لهـــــدف الحصول على شهادة الدكتوراه في العلوم البيولوجية عام ١٩٧٩م .

مقدمـــــــــــــــه

محمد هـــادى امـــيرى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