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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.)

by

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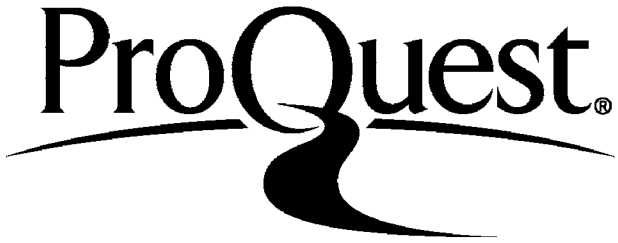


Summary

The effects of locally applied high and low temperatures on the melanophores of Phoxinus phoxinus (L) are investigated. The results of v.Frisch (1911) on Phoxinus and of Smith (1928) on Fundulus are in general confirmed and several new features of the responses are described. The effects are found to occur in several species of teleost fishes but are absent in others of the 31 species which are newly investigated. Reflex mediation of the responses from sensory endings in the skin is rejected and a new theory is put forward to account for the phenomena observed. This requires no novel anatomical elements but demands double reciprocal nervous control of melanophore action.

The question of double innervation is reinvestigated by means of selective electrical accommodation in a nerve-melanophore preparation. The experiments of v.Gelei (1942) involving the use of ergotamine as a selective blocking agent are confirmed but the arguments put forward by that author for the pathways of melanophore dispersing fibres are shown to be false. An alternative theory, consistent with the results of other authors, is proposed to explain these results. The possibility that ergotamine reverses the responses of melanophores to sympathomimetic agents is examined.

The effects of certain other selective blocking and stimulating drugs on the responses of melanophores to background colour, extreme temperatures and electrical

stimulation are examined.

No conclusive solutions to the initial problems are reached but several new questions are raised and suggestions for further experimental work are put forward.

Contents

CHAPTER I. INTRODUCTION

a. Colour Change in Teleosts.

The phenomenon of colour change in bony fishes has been known for many centuries. The development of the microscope showed that the skin pigments are often not uniformly distributed but are restricted to branched cells called chromatophores. Movements of the pigment granules within these chromatophores, either aggregated towards the centre or dispersed among the many fine branches, causes a change of tint when the skin is viewed macroscopically. The most common type of chromatophore is the melanophore, bearing the black pigment melanin. In many fishes the dispersion of melanin within many neighbouring melanophores tends to mask a deeper reflective layer of guanin causing the fish to darken, while aggregation of the pigment in each melanophore reveals the reflective layer and the fish appears pale.

Many workers have studied the physiological mechanisms by which the melanophores are controlled but despite much experiment and published literature many fundamental issues remain controvertible. A general review of the subject has been published by Parker (1948). A very brief summary of the present position is given here and a fuller discussion of some aspects is presented later in the text of this thesis.

It is well known that many chromatophore-bearing animals adapt their colour to a greater or lesser extent to match that of their background. The receptor involved is the eye.

Pouchet (1876) working on the turbot (Scophthalmus maximus L.) found that section of the spinal nerves peripheral to the rami communicantes caused a darkening of the denervated area of skin. v.Frisch (1911a) found similar effects in the minnow (Phoxinus phoxinus L.) followed by very slow colour changes to match the rest of the body (which responded normally to background). By a series of nerve sections he was able to trace the path of the melanophore motor nerves as shown in Figure 1a. From the brain the motor tract passes down the spinal cord as far as the level of the fifteenth vertebra and thence to the sympathetic chain, just before this enters the haemal canal. Then the fibres run anteriorly and posteriorly emerging through each ramus to the appropriate spinal nerve and also passing into the trigeminal (fifth) cranial nerve to serve the skin of the head. (These diagrams and those of Figures 2, 33 and 34 are greatly simplified to show only the nerve tracts concerned with chromatic control. Rami other than those in the region of vertebra 15, and the cranial roots of the trigeminal nerve have therefore been omitted). The fibres mapped in this way were shown to cause melanophore aggregation and paling of the skin. v.Frisch claimed that these fibres were controlled by a paling centre in the medulla and probably extending down the spinal cord, and that this centre was probably inhibited by a darkening centre somewhere in the midbrain or forebrain. Similar conclusions were reached by Adelman and Butcher (1937) on Fundulus heteroclitus and

Figure 1.

Paths of Chromatic Fibres in the Minnow.
A. Aggregating Fibres after von Frisch (1911).
B. Dispersing Fibres after von Gelei (1942).

by Schaeffer (1921) on Pleuronectes platessa although the nerve fibres pass from the spinal cord at the 10th and about the 6th vertebrae respectively in these species.

Many writers have suggested the presence of an opposing, darkening, or melanophore-dispersing set of nerve fibres within the autonomic nervous system and v.Gelei (1942) claimed to have followed their pathways in the minnow as shown in Figure 1b. So far however there is little direct evidence for double innervation and the problem is discussed more fully in Chapter V.

The much slower colour changes in response to background shown by denervated areas or by fishes which had been completely chromatically denervated were found by Smith (1931b) and Healey (1948) to be due to hormonal control (see Special Note Chapter Ie). This has been shown to be mediated by the pituitary gland, since denervated, hypophysectomised fish are incapable of any background adaptation. Injection of crude extracts of minnow pituitaries into normal minnows causes paling of the entire skin, suggesting the presence of a melanophore aggregating principle. Healey (1948), using refined techniques for nerve section, made careful analyses of the time relations of colour changes in both intact and spinal-sectioned minnows. To these results he applied the arguments of Hogben and Slome (1931) for the existence of a second, melanophore dispersing hormone opposed to the action of the first. This was also done by Waring (1942) for the slow humoral colour changes of Anguilla.

The bihumoral hypothesis and the logical basis of Hogben and Slome's arguments have been criticised recently by Kent (1959) and the possibility of a melanophore dispersing principle is still open. Further discussion of these problems is beyond the scope of the present study.

Chromatophores have also been shown to act as independent effectors in response to a variety of stimuli such as light, temperature, oxygen tension and ionic balance. A review of these effects has been published by Smith (1939).

Spaeth (1916a) discovered that melanophores of isolated scales of Fundulus are very sensitive to adrenalin which causes them to aggregate their pigment. It is now well known that injection of adrenalin causes paling in many species of teleost. Whether this hormone plays any part in normal background adaptation is unknown, but it is often assumed to be responsible for the quick paling which many fishes show when strongly excited. Further evidence on this point is difficult to obtain since the diffuse nature of adrenal tissue in fishes makes it impossible to remove its influence by surgical means.

b. Temperature and Colour Change.

One of the earliest observations of a change of colour with temperature (cited by Smith, 1928, from Brucke, 1852) is that of Goddard in an early volume of the Philosophical Transactions of the Royal Society (1678, see Appendix V).

He described studies on a chameleon and since then there has been a large number of similar observations, mainly on reptiles but also on amphibians, crustaceans and insects (Appendix V). The majority report that paling occurs at high environmental temperatures and darkening at low ones. Hogben (1924) regards this reaction as a universal one for amphibia. According to Smith (1928) the phenomenon led Krehl and Soetbeer (1899) to suggest a thermoregulatory function in the reptile Uromastix acanthinurus. Fuchs (1912-14) is said to have been so impressed with this idea that he assumed it to be a universal one and the primary function of colour change. While this may be so in some cases (e.g. desert-living reptiles) it was pointed out by Bauer (1914) that it could be of little use to fishes, whose temperature is much more rigidly controlled by that of the surrounding water.

Similar temperature responses have been reported in some teleosts. Cole and Schaeffer (1936, 1937) and Cole (1939), working with the killifish Fundulus heteroclitus, found that the rate of colour reversal in response to white-to-black or black-to-white background changes is quicker at higher temperatures. Between 5°C. and 30°C. the rate varied according to Arrhenius' equation, μ being 9,700 for paling and 10,900 for darkening in sea-water, but 11,400 for both reactions in fresh-water. Presumably μ was given in the unit °C. although this was not specified. Below these limits the

fish remained dark, regardless of background colour, while at higher temperatures they remained pale. By a photo-electric method for observing skin colour Hill, Parkinson and Solandt (1935) found that colour reversal is quicker at 30°C. than at 11.5°C. in Fundulus. According to v.Frisch (1911b) and Smith (1928), Franz (1908) found that fish killed by immersion in warm water turned pale and this was interpreted as an independent response by the melanophore cells. Fries (1927) showed that xanthophores of Fundulus are controlled independently of the melanophores and that denervated xanthophores aggregate at high temperatures, but no responses for innervated xanthophores were described. Foster (1933) reported that heat had no effect on the iridosomes of Fundulus although a greater number reacted to stimulation by light (as independent effectors) at higher temperatures.

According to v.Frisch (1911b) and Smith (1928), Knauthe (1891) exposed Cyprinus carpio, Carassius vulgaris, Rhodeus amarus, Perca fluviatilis, Tinca vulgaris, Gobio fluviatilis, Leucuspus delineatus, Leuciscus (= Phoxinus) phoxinus, Misgurnus fossilis and Nemacheilus barbatulus to cold conditions (0°C. to -2°C.). He found that in all cases the melanophores dispersed, the circulation stopped (at least peripherally) and the fishes went into a coma. The melanophores were said to remain responsive to light and to touch, but since their reaction to both these stimuli is by dispersion, the temperature effect cannot have been total.

The same reviewers mention the following similar instances. Leydig (1892) said that the phosphorescent spots of the shad (Alosa sp.) disappear in cold water due to dispersion of the overlying melanophores. Mayerhofer (1909) found that the melanophores of Esox lucius aggregated when changed suddenly from 11°C. to 30°C. or vice-versa. The 'normal' colour was soon resumed and it is probable that these were excitement reactions due to the sudden change.

Parker (1948) reported that Muzlera (1934) noted melanophore aggregation at high temperatures in Jenynsia lineata but that the cells redispersed at 35°C.

Pierce (1941) investigated the colour changes of Mollienisia latipinna (Le Seur). The fish became comatose below 9°C. and between 9°C. and 12°C. complete adaptation could not be attained on a white background. At high temperatures normal responses were obtained up to the lethal limit of 35°C. Wykes (1938) stated that below 9°C. the colour changes of Ameiurus were in complete abeyance and the fish remained dark in colour regardless of the background.

c. Local Temperature Effects in Phoxinus.

v.Frisch (1911b) commented on the general agreement on the effects of temperature when other aspects of colour change were so rich in contradiction. He pointed out, however, that in all cases the whole animal had been exposed to an extreme temperature and that profound physiological effects would be

expected. In particular he suspected that paling at high temperatures might be due to reduced respiratory efficiency and consequent shortage of oxygen, a factor known to cause melanophore aggregation. In order to determine the responses of the melanophores alone he performed a set of experiments which will be reported in detail here since they form the basis of the present work.

Transference of minnows from water at 15°C. to water at 25°C. generally gave a darkening, while a change to colder water usually produced paling. Frequently however the effect was indistinct and in many fishes reversed. If the temperature of the water in which the fishes swam were raised or lowered very slowly (14½ - 25°C. and 14½ - 11½°C. in 3½ hours) no changes in colour were detectable. v.Frisch was therefore unable to support the majority of previous results for Phoxinus.

He next experimented with newly decapitated, pithed minnows which he supported on a plasticine block with a microscope cover-glass resting against each flank. Onto each of these glass slips he played a gentle jet of water, one at 14°C., the other at 30 - 35°C. After 7 - 25 minutes the warmer flank became fully pale and remained so while the cooler flank did not pale until about 1½ hours after the start of the experiment. v.Frisch explained this as an acceleration by heat of postmortal changes due to oxygen deficiency in the tissues ('Anämieaufhellung'). He supposed that the oxygen

available in any region at the time of decapitation was exhausted more quickly at high temperatures.

Living fish were then mounted in the same apparatus with a tube in their mouths through which cool water was supplied for breathing. The fish lay quietly and respiratory stress was eliminated. When jets of water were played as before onto glass strips resting against the flank of the fish, a surprising reaction was produced. The area warmed to 35°C. quickly became darker while that at 14 - 16°C. showed no change. Cooled water at 3 - 5°C. produced the opposite effect, a pronounced paling which sometimes included complete fading of the skin pattern. These responses were quite independent and purely local since both could be produced together, either on opposite flanks or on adjacent areas of the same side. Neither effect could be attributed to an influence of temperature on the central nervous system since this would be equal in both regions. These results were confirmed on Phoxinus by Giersberg in 1930.

In order to eliminate vasomotor responses, v. Frisch then severed the sympathetic chain and dorsal aorta at a point anterior to the 15th vertebra. Following this the fish became maximally dark anteriorly due to denervation but posteriorly the chromatic motor-fibres remained intact. In this region however the circulation ceased completely as seen by observing the fin capillaries. Local temperature effects in this posterior region were entirely normal.

The next step was to eliminate the influence of the nervous system. This was done by crude cuts through the sympathetic chain and aorta either anterior to the 15th vertebra or posterior to it in the haemal canal. Of ten fishes tested as before, immediately after nerve section, half showed a slight paling in the denervated region on cooling. Heating had no effect since the region was already maximally dark in all cases. However 6 hours to 2 days after the operation the responses to locally applied temperatures were completely reversed in the denervated regions. 35°C. caused paling and 5°C. darkening. At this stage opposite effects could be obtained anterior and posterior to the point of sympathetic chain section by either high or low temperatures. The denervated responses were stated to be frequently indistinct or limited to small areas.

v.Frisch then suggested that a central reflex might be mediating the normal or 'innervated' response. To test this he performed a section of the spinal cord posterior to the 15th vertebra (which has no effect on colour change) and even complete destruction of the spinal cord in this region. Neither of these procedures altered the temperature responses of the tails of the minnows when tested by local application. Other possible explanations put forward were a reflex entirely within the autonomic nervous system or a direct response by the melanophores. v.Frisch in his discussion appears to have considered the latter more likely although he was unable to explain why the responses of the

melanophores as independent effectors should be reversed some time after denervation. He also pointed out that the strong innervated effect is unlikely to be of importance to minnows in their natural surroundings since it does not occur when the fishes are completely immersed at any temperature. In conclusion he appears almost apologetic at having discovered yet another contradiction in the field of colour change.

To summarise, Phoxinus shows little or no chromatic response when immersed in water at different temperatures. Local heating or cooling of fish, kept in air but supplied with normal water for breathing, produced strong responses which were reversed on denervation.

d. Temperature Effects in Fundulus.

Spaeth (1913) adopted a rather different approach in his studies on Fundulus heteroclitus (L.). In order to eliminate complex influence within the body of the fish he removed single scales bearing melanophores to isotonic solutions. By varying the experimental conditions he was able to examine a large number of physiological responses of the melanophores as independent effectors.

Immersion in 0.1M sodium chloride solution caused continuous and complete dispersion. When the available oxygen was reduced by putting the solution under an

atmosphere of pure hydrogen, the melanophores aggregated but redispersed on the introduction of air or oxygen. This reaction corresponds to the 'Anämieaufhellung' of v.Frisch. When the temperature was raised to 29 - 30°C. aggregation again ensued with redispersion at 19 - 23°C. This response was quick, reversible and occurred over a wide range of oxygen tensions. In one experiment Winkler's determination showed the amount of dissolved oxygen under oxygen at 30°C. to be four times that under air at 20°C.

Having thus demonstrated a temperature response which was quite independent of the available oxygen, Spaeth criticised v.Frisch's interpretations of his results on decapitated minnows. It must be mentioned however that the paling observed by v.Frisch was irreversible and did not occur immediately. Also a similar paling occurred on an unwarmed region at a later time, so that a direct response to temperature on the part of the melanophore appears to be an unacceptable explanation.

Smith (1928), also working on Fundulus heteroclitus, repeated some of the experiments of both Spaeth and v.Frisch, and was able to confirm the findings of both these workers. Isolated scales removed to 0.2N sodium chloride solution showed maximum melanophore dispersion in 2 - 3 minutes. Upon raising the temperature to between 32°C. and 41°C. the melanophores aggregated partially. The time required to complete this reaction was found to vary with the temperature

to which the melanophores were exposed. Typical values were 60 seconds at 40°C., 135 seconds at 36°C. and 300 seconds at 32°C. Smith does not state whether these reactions were reversible but the descriptions of his experimental method do not suggest that anoxic conditions could have been produced so quickly.

Smith further found that scales immersed in fresh-water at 19°C. soon showed complete melanophore aggregation. Transference to fresh-water at 1°C. produced a partial dispersion. Immersion of scales from a dark-adapted fish in fresh-water below 5°C. caused no dispersion at all. Thus it seemed that the melanophores reacted to cold as well as to heat and in the opposite direction. These responses are in the same direction as those of amphibians and reptiles.

Smith then repeated the experiments of Knauthe by immersing live fish in water of different temperatures. Fundulus acclimatised at 18 - 22°C., when put into water at 33°C., went into convulsions leading to a coma in which the fish were a silvery-white colour regardless of background colour. No fish survived this treatment. Below 10°C. the fish showed no signs of discomfort but went quietly into a coma in about ten minutes, assuming a rather dark colour even on pale backgrounds. The latter reaction was reversible. Smith stressed the point that no colour change occurred without the comatose state and suggested that once central nervous influences were removed the melanophores were free to

act as independent effectors. However he agreed with v.Frisch that the situation was so complex that little could be learned from such experiments. Further tests showed that some transitory colour changes could be produced at less extreme temperatures after thermal conditioning in various ways, but the normal responses to background colour further complicated these observations.

So far all results on Fundulus showed temperature responses in one direction only. Smith then proceeded to perform v.Frisch's experiment of removing the fish from water and locally warming or cooling regions of the body. Water was supplied by a tube in the mouth for respiration as before. Fundulus treated in this way became pale in all cases, presumably a case of excitement pallor. Water at 2°C. had no effect but warming to 38°C. produced reversible melanophore dispersion as v.Frisch had found. Smith also performed the experiment without the pieces of glass used by v.Frisch and found no difference except for a quicker response. Further experiments showed that jets up to 31°C. produced no effect but that from 32°C. to 40°C. the response occurred after the same 'exposure time' (about 30 seconds). Thus not only were such responses opposite in direction to those of isolated scale melanophores but they exhibited an all-or-nothing character with no temperature coefficient.

Regions of the body were denervated by section of some of the spinal nerves and an undefined period was

allowed for the initial complete darkening to fade. Melanophores in these areas then aggregated when warmed and dispersed when cooled. The time for this reaction varied with the temperature and the curves were similar to those for isolated scale melanophores but with a rather shorter time scale. The reversal of response when denervated, as described by v.Frisch for the minnow, was thus confirmed in another species of teleost. Smith does not suggest that the response of such denervated melanophores was in any way uncertain or weak as those of the minnow were said to be. In the caudal fin both innervated and denervated melanophores reacted in the same way as denervated melanophores on the flank.

A further important experiment performed by Smith was to test the temperature responses after bilateral blinding, in order to remove background stimuli from the eyes while leaving the motor innervation intact. In all cases the melanophores of the whole body slowly reacted as if denervated. Little was made of this surprising result. After remarking that actual denervation of the melanophores was unnecessary to reverse the response, Smith suggested that removing the eyes may have liberated a hormone causing the melanophores to act in the opposite way (with reference to the decapod crustacea). Recent experiments by Parry and Holliday (1960) on the chromatic influence of the pseudobranch in teleosts show that this idea was not as preposterous as it might have seemed. The efferent

blood supply of the pseudobranch passes to the choroid gland of the eye and interruption of this pathway causes strong darkening in some teleosts. This result cannot be confirmed in Phoxinus (Kent, personal communication) and in any case it is difficult to see how it could produce Smith's result. The effect of blinding is discussed further in Chapter IIIe.

In conclusion Smith regarded the denervated responses as independent reactions of melanophores removed from the control of the nervous system. The innervated responses he attributed to the nervous system, due to the all-or-nothing nature of the response. As v.Frisch had done much to reduce the likelihood of a central nervous reflex, Smith inclined to the idea of an axon reflex on the pattern of the vasodilator response in mammals. That is, that two branches of the same neuron serve a temperature sense-ending and a melanophore respectively. A further discussion of these ideas and results is given in Chapter II. The reactions of melanophores on the caudal fin suggested that they were not served by the reflex arrangement.

e. The Source and Treatment of Fish.

The present work attempts to shed some light on the phenomena which have been described. The principal experimental species was Phoxinus phoxinus (L.). At

Aberystwyth specimens were obtained by trapping from Blaen Melindwr Lake near Plynlimmon and were maintained in well aerated water in large grey slate stock tanks. When kept for an appreciable time the fish were fed on minced raw beef. At Bedford College, London, fish were obtained by a reputable dealer from a pond in Hertfordshire and later from a stream at Gravetie in the Ashdown Forest. Immediately on arrival at the dealer's they were collected and transferred to stock tanks consisting of white sinks.

Aberystwyth water was occasionally and unpredictably chlorinated at levels lethal to fish and all water used in the stock tanks was first treated by vigorous aeration for a few days. The more constant level of chlorination of the water supply in London allowed running water to be used with less danger of pollution in the tanks. Smaller numbers of fish were also obtained as required. This rendered feeding unnecessary and minimised the reduction in numbers of melanophores due to slow morphological changes in white vessels. Difficulties in maintaining a healthy stock of fish were encountered only in summer when the temperature of the London water supply rose dangerously high.

No attempt was made to acclimatise all fish at the same temperature. Fish showing breeding coloration were kept in the stock tanks for a short time until these patterns disappeared. The average weight of a minnow was 3.5 gm. The sources and treatment of other species used are

described individually in Chapter IV.

The operative techniques used were those of Healey (1948) with only slight modification. Fine nylon thread sold for repairing stockings was found to be preferable to cotton strands for sutures since it is easier to use and less liable to rot. The Ringer solution used in these operations was changed from Steinhausen's formula (1928) to that of Young (1933) for reasons explained in Appendix IV. The techniques for operations other than those described by Healey are dealt with in the relevant parts of the text.

Urethane (ethyl carbamate) was generally used as an anaesthetic in the way described by Healey; the fish were immersed in 0.5% solution until unconscious as judged by lack of response to light pressure on the caudal fin, and anaesthesia was maintained by 0.25% solution flowing over the gills. In some cases MS222 (Sandoz Metacaine or Tricaine methansulphonate) was found to be preferable. This was used almost exclusively for the larger marine teleosts. Ball and Cowen (1959) have warned against the danger of urethane to the experimenter, as carcinogenic doses may be absorbed through the skin. They urged that MS222 be generally used as an alternative anaesthetic for fishes in concentrations between 1/500 and 1/2000 (for small teleosts). Concentrations between 1/20,000 and 1/12,500 were found to be quite adequate in the present experiments and higher concentrations often produced

irreversible coma.

The apparatus and techniques used for the various experiments are described in the appropriate section of the text, but apparatus of general use has been referred to the Appendices for convenience.

In all cases fish taken from the stock tanks for experimentation were first placed on black and white backgrounds. Any individuals which were judged macroscopically to attain only incomplete adaptation were rejected.

Special Note.

The word "denervated" is used throughout this thesis to denote only an interruption of the melanophore motor-nerve tracts as shown in Figure 1a. It does not imply section of the terminal motor fibres with consequent degeneration as far as the chromo-neural junction. There is at present little evidence for the positions of synapses in the motor tracts and the experiments of Chapter Ve indicate that even after section of the autonomic chain anterior to vertebra 15 the motor fibres in the superficial ophthalmic nerve do not degenerate. Denervation therefore only implies surgical paralysis of melanophores regardless of the level of section. This is done for convenience since the level at which section is performed appears to

have no effect on the subsequent thermal responses of the melanophores.

CHAPTER II

EXPERIMENTS ON THE TEMPERATURE RESPONSES IN PHOXINUS

a. Preliminary Experiments.

At the commencement of this work some of the experiments performed by other workers and described in Chapter I were repeated. Whole minnows were subjected to a wide range of high and low temperatures on both black and white backgrounds. Above 30°C. the fish very quickly became comatose and sometimes showed signs of paling but recovery at normal temperatures was never quick and in many cases the treatment proved fatal. At low temperatures darkening could not be produced. In one experiment one dark and one pale fish were put in water between -1°C. and 0°C. on an intermediate coloured background. Both became sluggish after some time but showed no signs of coma. The pale fish appeared to darken somewhat but the dark fish

paled slightly; both recovered quickly at 11°C. At no other temperatures did any colour changes occur. Fish placed in freezing solutions between -5°C. and -13°C. quickly became stiff but showed no signs of darkening. Such temperatures were always fatal. It was therefore not possible to confirm the intense darkening under cold conditions reported by Knauthe. Background adaptation was little affected by temperature.

Experiments on freshly decapitated corpses, as performed by v.Frisch, yielded confirmatory results. If, after decapitation, the spinal cord was destroyed for some distance with a fine needle, paling followed after a time determined by the local temperature of the skin. No further responses followed at any temperature once paling was complete. The typical sequence was as follows. Immediately after decapitation the body of the fish darkened considerably (probably due to nerve section). An area washed by water at 35°C. commenced to pale in a few minutes and paling was usually complete within 25 minutes. A similar area under ice-water at 0°C. stayed dark for 30 - 60 minutes and paling generally became complete between 80 and 90 minutes. These responses were irreversible and complete.

In some cases pithing of the spinal cord was not performed and the ensuing responses followed a more complex sequence, attributed by v.Frisch (1911a) to postmortal

changes of nerve centres in the anterior part of the spinal cord. Such responses were by no means so stereotyped although final paling occurred more quickly in warm areas. However it seems that such experiments are of little help in understanding the phenomena shown by the living fish and v.Frisch's theory of 'Anämieaufhellung' appears to be perfectly satisfactory.

b. Local Responses in Living Fish.

v.Frisch's experiments upon living fish were repeated using the original method. In most cases the fish became pale when placed upon the plasticine support. If the rate of flow of water into the mouth were carefully controlled, some fish could be persuaded to lie quietly while the experiment was performed, but many became very agitated and had to be rejected. In every case which allowed observation, water at 35°C. on one flank, either with or without the glass slip, caused very strong local darkening. Ice-water caused paling which was generally slight owing to the initial pallor of the fish. Under a low power binocular microscope this reaction could be seen clearly since all the small melanophores always became completely aggregated when cooled. In many fish the skin pattern disappeared completely due to complete aggregation of the larger dermal melanophores, a condition described by

v.Frisch as "besonders schön".

No exceptions to these responses were seen and in all cases the reactions were well defined provided that the fish lay quietly. Melanophore changes occurred within a few seconds of heating or cooling and were complete within a minute. The effects were fully reversible and extremely localised since by manipulation of the water jets, both responses could be obtained close together on the same side of a fish. Thus the strong innervated responses were found to be in every way as described by v.Frisch.

Some spinal-sectioned fish were then prepared. Using the operative method of Healey (1948) the spinal cord was removed from two or three vertebrae posterior to the fifteenth vertebra. By this technique the haemal canal, aorta and sympathetic chain remain undamaged. The wound soon heals if kept free from infection and the fish survive for an indefinite period. Normal colour change in response to background colour is unaffected but swimming is impeded to a greater or lesser extent depending on the level at which section is performed. This technique represents a great improvement upon the original method of v.Frisch whereby the whole vertebral column was severed by a single knife-thrust, with consequent circulatory disturbances.

When such fishes were tested for temperature responses they were found to be normal both anterior and posterior to the site of operation and entirely indistinguishable from

intact fishes. During the course of later experiments this operation was performed many times and at a wide variety of levels, while temperature testing was performed at widely varying times after operation. No exceptions to the normal response were found. The spinal cord posterior to the 15th vertebra appears to play no part whatsoever in the local temperature responses of the minnow. This again confirms the findings of v.Frisch.

Another operative technique developed by Healey (1948) permits section of the sympathetic chain within the body cavity and anterior to the 15th vertebra, without damaging the aorta. When this is done the fish becomes maximally dark anterior to the point of section. The denervated region slowly becomes pale on a white background under humoral influences but nervous control is not reestablished, at least for a considerable time. Posterior to the point of section the colour changes are completely normal. The operated fish appear to be unaffected in other respects.

Several such sympathectomised fish were allowed to swim over a white background for varying periods so that the anterior region could pale somewhat. They were then tested for temperature responses. The region posterior to the point of section reacted in every way as in the intact fish. Anterior to this point however the response was reversed as described by v.Frisch; the melanophores cooled by ice-water now dispersed their pigment, while those warmed to 35°C.

became aggregated to produce a pale area. These denervated reactions appeared to be rather slower in appearance than the normal ones but were often just as marked when completed. A beautiful effect was obtained by playing the jets onto the body at the level of nerve section since opposite responses were given before and behind this point. The line of demarcation between innervated and denervated regions could be accentuated in either combination of light and dark by application of water at the right temperature, even when it was initially invisible. Such responses could be evoked as long as the fish survived, at least for several weeks.

Since the phenomena described by v.Frisch and Smith were in general confirmed, a further experiment which does not appear to have been performed by either of these workers was suggested. Healey's technique for spinal cord section can be performed at any level, but if anterior to the 15th vertebra the whole surface of the fish is chromatically denervated. Healey has shown (1951) that minnows so treated darken initially but after some days will respond slowly to background changes by humoral control alone. Since nervous control is thus eliminated from the entire fish it was decided to examine the temperature responses in this state.

Fish were prepared and allowed to recover on a white background for several days until they assumed an intermediate colour. When tested as before all fishes showed

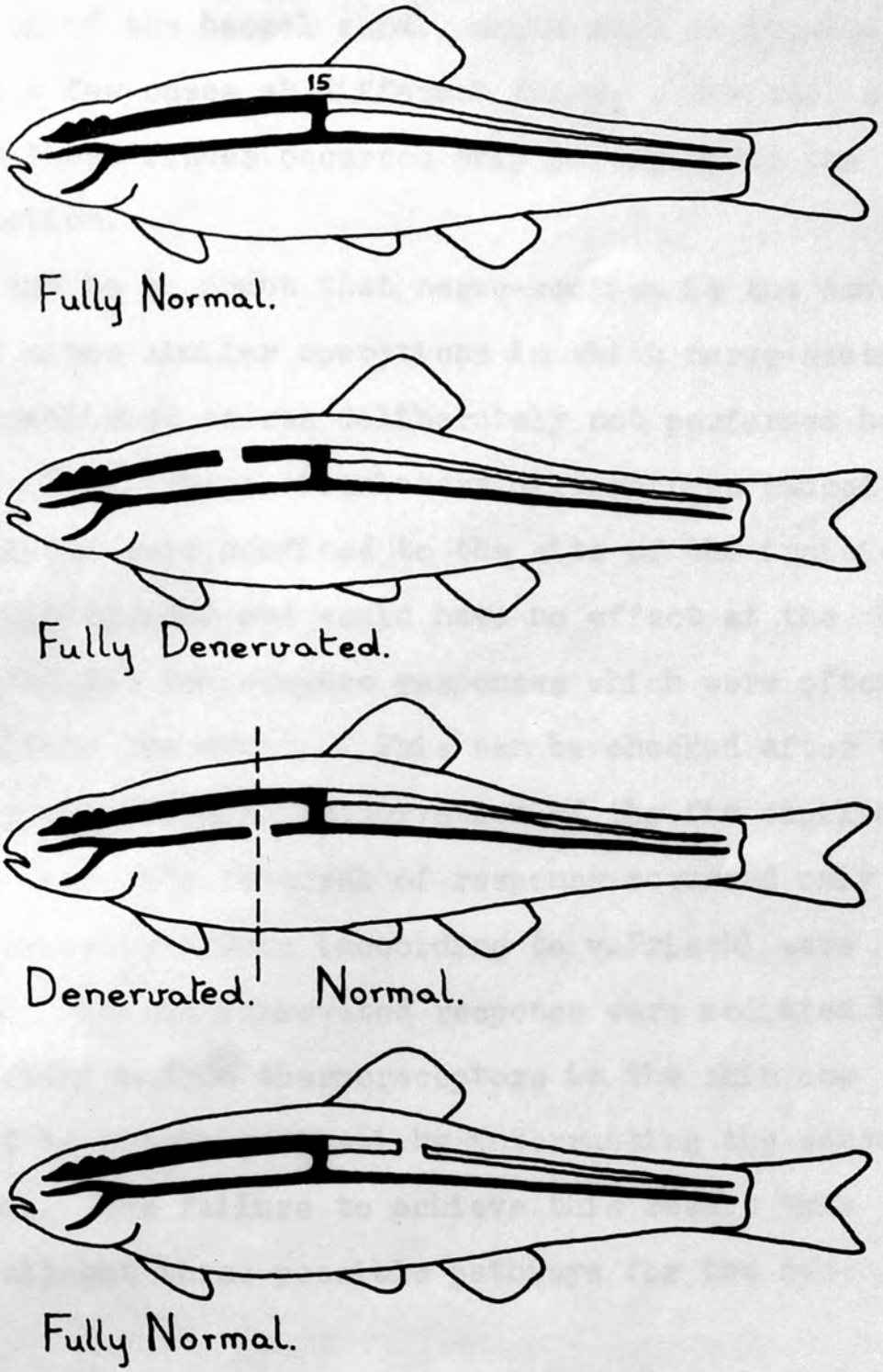
the typical denervated responses, that is the whole surface of each fish responded exactly as the anterior half of sympathectomised fish. The melanophores dispersed when cooled and aggregated when warmed. No differences could be found between fish whose spinal cords had been sectioned at different levels, provided that all sections were anterior to the 15th vertebra.

c. The Reflex Theory.

The question whether reflex control is involved in the normal innervated responses may now be re-examined. The experiments described so far were performed a very large number of times. No actual numbers are given since many repetitions were involved in the course of later experiments, but the reactions were not found to vary apart from certain exceptions described in Chapters IIe and IIIf.

These results are summarised in Figure 2 which shows the paths of chromatic fibres according to v.Frisch. Section of the spinal cord at any level anterior to the 15th vertebra causes complete reversal of response while section at any level posterior to the 15th vertebra has no effect. Section of the sympathetic chain anterior to the 15th vertebra causes reversal of response only anterior to the point of section. It is perhaps relevant to note here that section of the sympathetic chain between the 15th vertebra and

Figure 2.



The Effect of Nerve Section at Different Levels on the Temperature Response in the Minnow.

the beginning of the haemal canal, while more difficult, was achieved in a few cases at different times. Reversal of response in these fishes occurred only posterior to the point of section.

There can be no doubt that nerve-section is the cause of reversal since similar operations in which nerve-section was not accomplished or was deliberately not performed had no effect. Furthermore circulatory disturbances caused by these operations were confined to the site of the incision by the techniques used and could have no effect at the regions tested for temperature responses which were often far removed from the wound. This can be checked after the operation by microscopical observation of the fin capillaries.

In all cases the reversal of response occurred only for regions whose motor tracts (according to v.Frisch) were interrupted. If the innervated response were mediated by a reflex mechanism from thermoreceptors in the skin one might expect to obtain reversal by interrupting the sensory tracts alone. The failure to achieve this result thus eliminates all but three possible pathways for the sensory fibres.

(1) Sensory fibres might follow exactly the same paths as the motor fibres, running in the autonomic chain and entering the spinal cord at the 15th vertebra. Thus nerve section could not interrupt one pathway alone. This system is thought to be extremely unlikely since the temperature

response is strictly local in action and such a long reflex pathway would have to involve point-to-point connections within the brain. The reaction is not a natural one shown by minnows when completely immersed and the high temperature effect occurs at temperatures lethal to immersed fish. In view of this apparent lack of any adaptive function, any theory postulating a complex neural apparatus appears to have little to commend it.

(2) The axon reflex theory of Smith (see Chapter Id). This postulates that the terminal motor neuron to the melanophore also has a branch to a sensory cell in the skin. Sensory impulses could run first antidromically and then orthodromically to reach the effector cell. An analogy was drawn with the vasodilator reflex of mammals. Kuntz (1953) describes both ganglionic and postganglionic axon reflexes in mammals but states that both are purely experimental phenomena and the extent of their normal influence has yet to be determined. Celander and Folkow (1953) investigating the vasodilator axon reflex of cats found that only pain fibres were provided with this arrangement. "The increase of cutaneous blood flow which follows on moderate heating of the skin is not due to an activation of axon reflexes connected to temperature fibres but to a direct effect on the vascular smooth muscles". "...it follows that the function of the axon reflex is solely confined to evoke a local increase of blood flow through superficial tissues exposed to noxious

influences". An example quoted is flare around a cut or weal. Since the arrangement is more specific than was formerly supposed it does not appear to lend itself quite so readily to the explanation of other cutaneous reactions, such as the melanophore response. Again the lack of obvious adaptive significance makes the 'invention' of a new and specific apparatus undesirable. A further drawback is that it is difficult to see how section of the anterior end of the spinal cord, or even the optic nerves, could interfere with a peripheral three cell mechanism of the type postulated.

(3) Sensory fibres may run to the brain by an entirely different route, not involving the spinal cord or sympathetic chain. Only one such route can be suggested, that via the lateralis system. Although the same arguments apply as in (1) and point-to-point connections in the brain must be postulated, this theory appeared to be of sufficient interest to be considered in further detail.

d. <u>The Role of the Lateralis System</u>.

The question of temperature reception in fishes is still much debated. Some authors claim that temperature is the controlling influence in annual sexual development but others maintain that light is the operative factor.

Reviewers of the subject (Allen, Danforth and Doisy, 1939; Burrows, 1949; Marshall, 1942; Rowan, 1938) tend to treat it as an open question or to suggest that both factors are influential. They variously quote Courrier (1922) and Craig-Bennett (1930) on Gasterosteus, and Turner (1919) on Perca, in favour of temperature and denying the effect of light, although no sensory origin is suggested. The following authors are said to regard light as important: Bullough (1940) on Phoxinus, Hoover (1937) and Hoover and Hubbard (1937) on Salvelinus, Spaul (1938) on Phoxinus and Tinbergen (1938) on Gasterosteus.

Electrophysiological experiments on lateral line organs by Sand (1938) on Raia, by Hoagland (1933a and b) on Ameiurus and Salvelinus, and by Murray (1955, 1956) on Xenopus, and on ampullae of Lorenzini by Sand (1938) and Murray (1959), show a relation between the rate of spontaneous discharge from these organs and their temperature. Both adapting and non-adapting components of opposite sign were usually present. Thus cooling caused a quick rise in the discharge rate which then fell to a lower level than before, while heating caused opposite responses. The sensitivity of lateral line organs was low and Hoagland did not observe the initial responses, probably because his temperature changes were slow. However these effects may be fundamental to all spontaneously discharging receptors for Sand (1938) found that muscle stretch receptors respond in the same way if under slight

tension. Bernhard and Granit (1946) showed that under certain conditions hyperexcitable nerve fibres will act in this way and Kerkut and Taylor (1956) obtained similar results on the spontaneous activity of ganglia from the slug, cockroach and crayfish. Furthermore Bullock (1953) has warned that a response to a given stimulus does not necessarily imply specificity for that stimulus and central interpretation must be taken into account.

Bull (1928, 1936), by means of conditioned behavioural experiments on Blennius and several other species of teleost, demonstrated a temperature sensitivity to changes of 0.03 - 0.10°C. Dijkgraaf (1940) obtained similar responses from Phoxinus and Ameiurus. Rubin (1935) found that if the lateral line nerves of several teleosts were cut, the fish were no longer disturbed by high temperatures (27°C.) until the lethal level was attained. Andrews (1946, 1952) observed that Carassius auratus became insensitive to bright light at high temperatures, but that higher temperatures were needed if the lateral line nerves were cut. However Sullivan (1954) maintained that temperature reception in Salvelinus fontinalis was not mediated by the lateral line. Dijkgraaf (1940), by means of nerve section experiments, concluded that thermo-sensory impulses pass up the spinal cord, but Hoagland had earlier been unable to detect such impulses electrically.

To summarise, fishes are very sensitive to temperature;

the lateral line is potentially capable of acting as a receptor but may only play an effective part at extreme temperatures. The present experiments do employ extreme temperatures and the possibility of sensory pathways within the spinal cord has been ruled out. It thus appears interesting to investigate the effect of eliminating the action of the lateralis system on the responses obtained.

An examination of the minnow showed that the lateral line is poorly developed and seldom extends over more than two-thirds of the length of the body. Externally it is apparent as a wavy line rather ventral in position. Dissection of the tenth cranial nerve, however, revealed that the lateral cutaneous branch is very well developed. As shown in Figure 3 this nerve runs straight along the body in a mid-lateral position and is much thicker than the lateralis branch. Although there is no canal system associated with this nerve, segmental branches appear to serve most of the body surface.

Just posterior to the operculum at the positions shown in Figure 3 both nerves lie just below the skin. Using a technique similar to that of Gray (1955a) for caudal band section, a small chisel-like piece of razor blade, 0.5 - 1.0 mm. wide, was used to cut these branches. The fish were anaesthetised and laid on wax. Then the blade, held in artery forceps, was slipped under the posterior borders of the scales before being directed downwards below the skin.

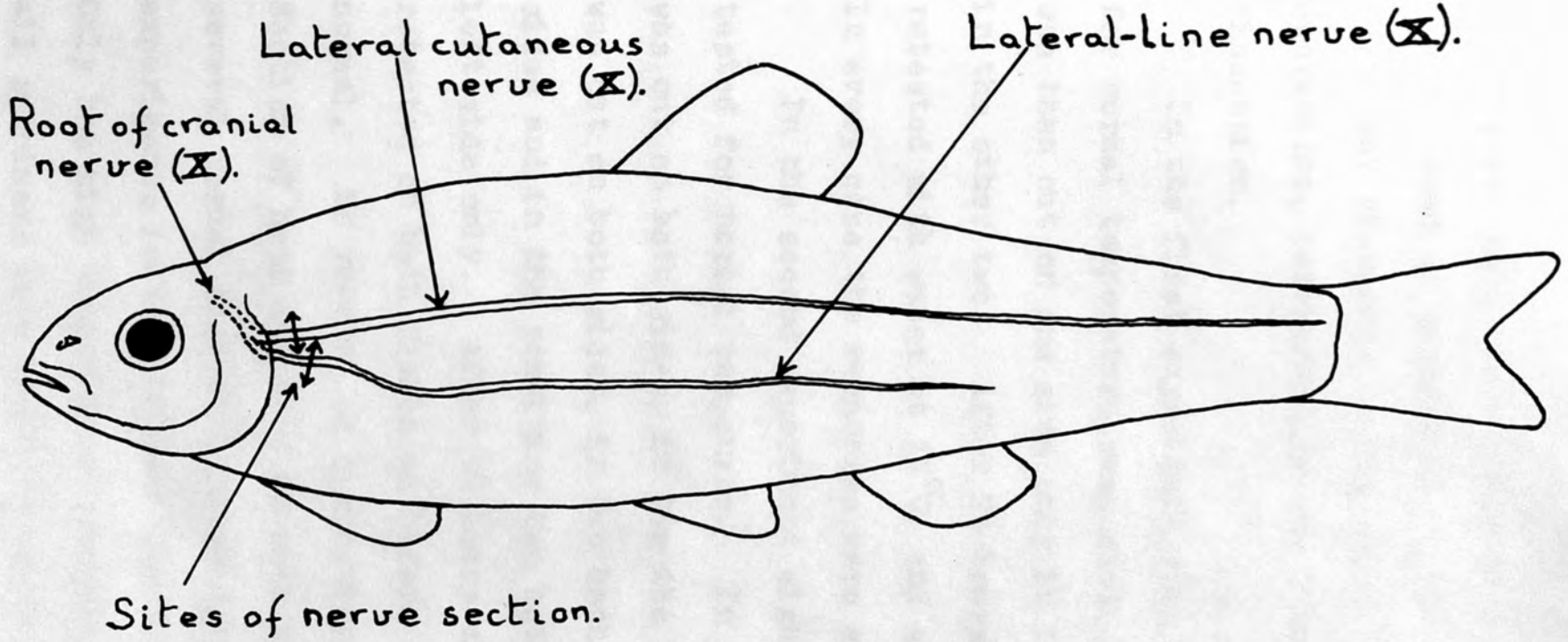


Figure 3.

Diagram of <u>Phoxinus</u> showing the positions and relative development of the Lateralis and Lateral Cutaneous nerves.

In this way only light pressure was required and the clean wound closed on withdrawal of the blade. The fish appeared in no way disturbed by the wound. As for all other operations, nerve-section was later checked by post mortem dissection.

In the first experiment four fish were taken and tested for normal temperature responses. The lateralis branch was then cut on one side only in two fishes and on both sides in the other two. After 24 hours all the fish were retested with water at 35°C. and at 0°C. on both flanks. In every case the responses were entirely normal.

In the second experiment eight fish were taken and tested for normal responses. In two the lateralis branch was cut on both sides, in two the lateral cutaneous branch was cut on both sides, in two both nerves were cut on both sides and in the remaining two both branches were cut on the left side only. After 48 hours all the fishes were retested on both flanks as before and each appeared fully normal. No reversal or diminution of response was found. Section of both branches on both sides was repeated in several normal fish at a later date in connection with experiments in the constant observation tank (Chapter IIIb). Only the high temperature responses were then checked but all specimens gave complete melanophore dispersion.

A point arising from these experiments deserves mention in relation to the literature described. When placed on

v.Frisch's apparatus many minnows refused to lie quietly. When the hot or cold jets were played upon the skin, or when the temperature was reversed from one extreme to the other, many quiet minnows became excited and struggled, even if they had been subjected to spinal section. Such experiments were always abandoned. All the fishes in which both lateral nerves were cut gave little or no trouble when once installed on the apparatus. Reversal of extreme temperatures rarely caused any disturbance, and this was the reason for repeating the procedure in the constant observation tank experiments (see Chapter IIIb).

Thus although the lateralis system may have a thermosensory function, it appeared to play no part whatsoever in mediating the normal responses to locally applied temperatures in the minnow. Only two possible reflex paths remain and these appear to be incapable of being put to experimental test at this stage. However, they are considered to be extremely unlikely for the reasons already mentioned. The reflex theory was therefore rejected and a search made for an alternative theory.

e. <u>The Denervated Response</u>.

The responses which v.Frisch reported for denervated melanophores when tested 6 hours to 2 days after the

operation were said to be weak and often patchy. It is presumed that longer periods were precluded by vascular damage to the fish. The experiments so far described here were performed at Aberystwyth and in all cases a clear denervated response was observed although microscopical examination often showed the reaction to be rather patchy. It was found that 48 hours after the operation some fish responded as described by v.Frisch but that within a week all gave definite responses. It appeared that the innervated effect gave way to the denervated one more or less slowly in different individuals.

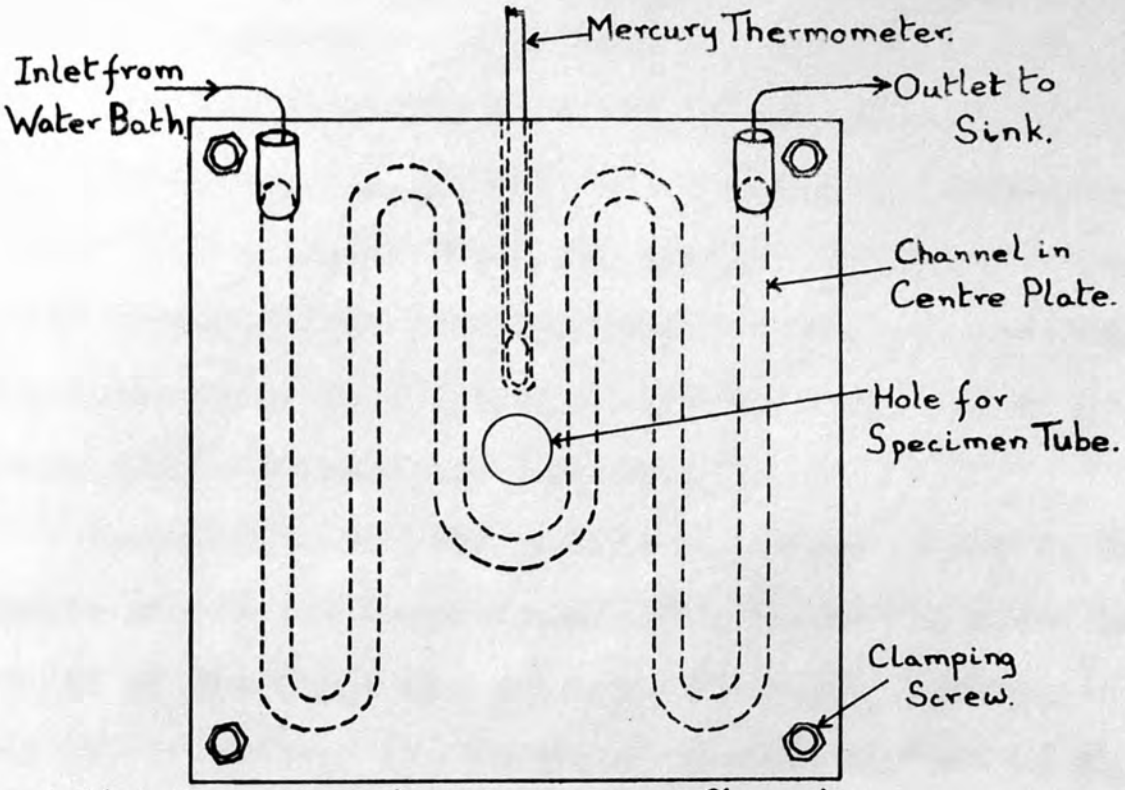
At a later date this development was reinvestigated on fishes from Gravetie, Surrey. Now no clear-cut responses could be obtained after spinal section anterior to vertebra 15. Six fish were taken and their responses to high temperature were checked. Each fish was marked by clipping the fins and subjected to anterior spinal section. After 48 hours and again after 7 days each was retested with jets up to 35°C. In another experiment five fish were tested several weeks after spinal section. In none of these eleven fish was a consistent response observed. Melanophore reactions were either absent or apparently haphazard. Smith and Smith (1935) also state that they were unable to obtain reversed responses after denervation in Phoxinus.

Spaeth (1913a and b) and Smith (1928) reported strong responses of the denervated type in melanophores attached to isolated single scales of Fundulus. It was decided to

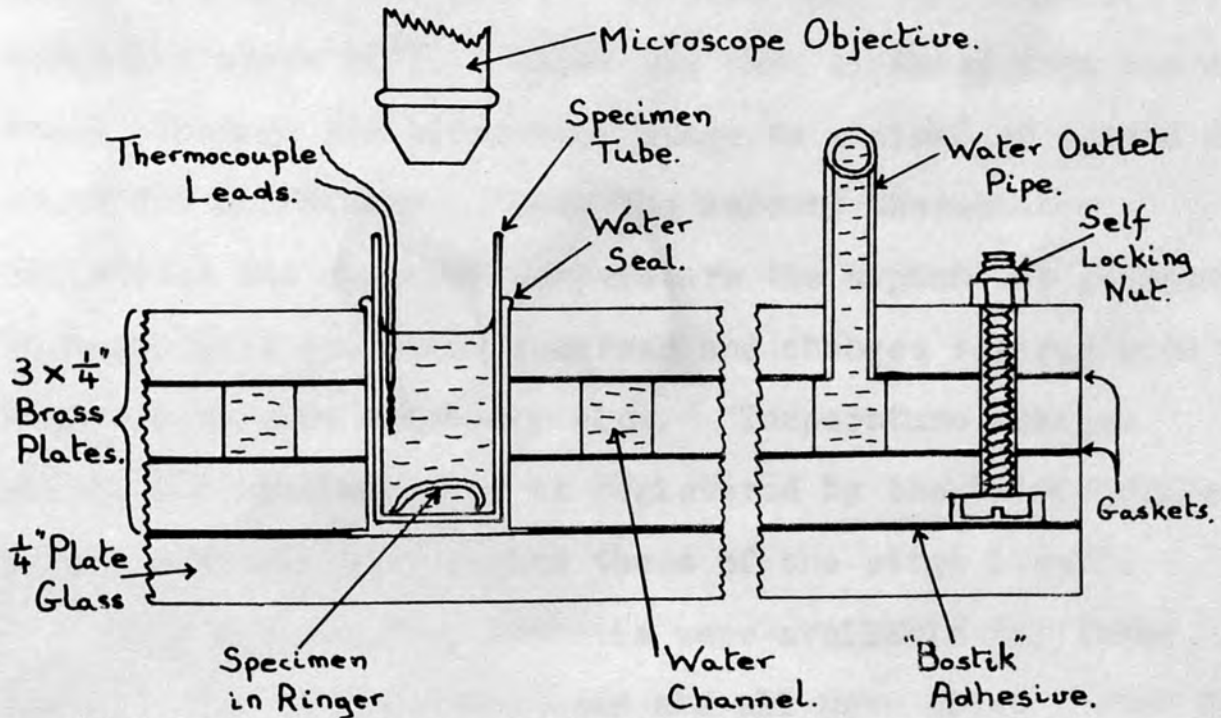
investigate whether similar responses could be obtained from Phoxinus. The scales of the minnow are extremely small and if removed individually do not bear any melanophores with them, so that larger pieces of skin had to be used. A selected minnow was completely anaesthetised in 0.5% urethane solution and laid on a wax dissecting plate. Two deep longitudinal incisions were made in the skin, one dorsally, the other mid-ventrally, from the operculum to the caudal fin. The anterior end of the strip of skin thus defined was gently raised with forceps and freed from the body musculature by a scything action, moving posteriorly. Once free, the strip of skin was immersed in Young's Ringer solution (Appendix IV) and divided up with sharp scissors. The whole process could be accomplished in 10 - 15 seconds. Knives were made from new razor blades and each was discarded after one experiment. With care very little subcutaneous tissue was retained and the melanophores appeared to be undamaged. The anterior end of the strip, held in forceps, was always discarded. The fish was always decapitated after sufficient skin had been removed.

A temperature-controlled microscope stage was constructed from three sheets of brass 4" x 4" x ¼", sandwiched together with gaskets of paper and shellac. The middle piece was cut as shown in Figure 4 to form a channel through which water could be passed and the whole was cemented to a piece of plate glass. A second channel

Figure 4.



(a) View from above showing course of Channels.



(b) Diagrammatic Cross Sections (not strictly to scale).

The Temperature Stage.

allowed a mercury-in-glass thermometer to be inserted into the block and a central hole accepted a square-ended glass tube containing the specimen which was illuminated from below. In practice this hole was half filled with water which formed a heat junction when the tube was inserted. The temperature in the tube itself was monitored by a thermocouple thermometer (Appendix I).

Temperature control of this apparatus proved to be very simple due to the large thermal inertia of the block (the weight of the brass was 2.3 kgm., thermally equivalent to 211 ml. of water; the volume of the channel was 15 ml.). One thermostat tank (Appendix II) was filled with ice and water, and the other was run at some high temperature, generally about 40°C. Water was then siphoned from one of these, through the microscope stage to a sink, at a rate of about 500 ml./minute. When the mercury thermometer registered the required temperature the siphon was stopped. No measurable overshoot occurred and changes towards room temperature were extremely slow. Temperature changes within the specimen tube as registered by the thermocouple lagged only slightly behind those of the stage itself.

Only minnows from Gravetie were available for these tests. Twelve fish were used and all were first tested for normal responses to background reversal. In turn a small undamaged piece of skin from each fish was immersed in a tube of Ringer and inserted into the stage.

Each specimen was subjected to extreme temperatures of 2 - 4°C. and 34 - 35°C. and both quick and slow temperature changes were tried. At the end of each experiment a little adrenalin was added to the tube at 20°C.; quick and complete melanophore aggregation demonstrating that the melanophores were still physiologically active.

Rather variable results were obtained. Seven of the twelve preparations gave clear responses in the denervated direction but the response appeared to deteriorate with time. At the beginning of each experiment responses were obtained only from patches of melanophores. In particular the melanophores around the periphery of the preparation appeared to be inert, possibly due to damage during dissection and manipulation. As time went on more and more melanophores failed to respond, the speed of reaction decreased and in some cases the responses became weaker. At first most preparations gave complete responses almost as quickly as the temperature could be changed but after half an hour several minutes at a sustained extreme temperature were required to produce a partial response. These preparations all reacted to the addition of adrenalin and in most cases even the inert melanophores responded normally by quick aggregation.

Three preparations showed no change at all to temperature variations, whether quick or slow, or to prolonged extreme temperatures. All responded completely

to adrenalin. The remaining two preparations did not respond to adrenalin; one showed no response to temperature variations, while the other responded strongly at first (in the denervated direction) but later became inert. In no case was a response in the innervated direction (as in the intact fish) observed even in a single melanophore.

These results are not so well defined as those described by Spaeth and Smith for isolated scales of Fundulus, but they are to a large extent confirmatory. The lack of uniformity and patchiness of response appear to some extent to match the response of live denervated minnows. According to the descriptions of Smith (1928) the response of denervated areas of Fundulus is much stronger and more definite than that observed in Phoxinus. There appears to be no evidence for opposing the view held by both v. Frisch and Smith that the response of denervated areas is that of the melanophores acting as independent effectors. The lack of denervated response in the live Gravetie minnows is therefore puzzling. Possibly endocrine factors maintained such rigid control in these fish that the melanophores were not free to respond independently until removed to a tube of Ringer solution. This argument may be supported by the comparison between Phoxinus and Fundulus, where endocrine control by the pituitary is very slight and possibly absent altogether (Mathews, 1933). Experiments on hypophysectomised fish were indicated but could not be undertaken at that time.

f. The Peripheral Interference Theory.

From the results so far described it is clear that the nervous system plays a part in mediating the temperature responses in the intact minnow as in Fundulus. In the absence of any evidence to the contrary it is supposed that the denervated response is due to independent action by the melanophore cells. Thus in the intact fish the nervous system must impose an overriding influence. The theory of mediation by a reflex pathway has been rejected. V.Frisch suggested as an alternative that both responses are due to independent action by the melanophores but that the responses are in some way affected by nerve degeneration. This again seems unlikely as spinal cord section just posterior to the medulla oblongata produces reversal of response over the whole surface of the fish, whereas one would expect at least one synapse between this level and that of the melanophore. Also Smith found reversal in Fundulus after optic nerve section, which would not be expected to cause degeneration of chromatic motor fibres. Furthermore it has been observed that the reversal of response does not occur immediately after denervation, yet denervated responses were found in skin preparations almost immediately after removal from the body when the nerve endings would not have time to degenerate. A direct test of this theory cannot be designed however.

A new theory proposed here does not require the

presence of any nervous elements other than those employed in normal chromatic responses to background colour or illumination. It assumes the following points:-

(1) The chromatic motor fibres in the intact fish carry a tonic discharge whose frequency controls the state of the effector cell. This appears to be well supported by studies of the control of other autonomic effectors (Kuntz, 1953).

(2) Peripherally the fibres run in the skin, close to the surface of the fish, where they may be susceptible to external influences. This is supported by the microscopical studies of Ballowitz (1893) on Perca and of Whitear (1952) on Phoxinus. Ueda (1955) obtained experimental evidence of nerve ramification in the skin of Gambusia sp. and Pseudorasbora sp. by electrical stimulation of isolated scale preparations.

(3) Water of extreme temperatures applied to the surface of the fish may sufficiently modify the nervous activity passing to the melanophores to produce a complete imbalance in the effector cell.

There are several ways in which this interference might be effected since it could occur at several levels. The motor nerve-fibre, the chromo-neural junction and the effector itself each involve a number of factors which could be influenced by applied temperatures. All such possibilities depend on the fact that each melanophore has

numerous nerve-endings applied to it (Ballowitz, 1893). Presumably each branch responds to the level of stimulation it receives from the motor endings. In support of this postulate, Gray (1955a and b) has recently shown that the response of a single melanophore may be asymmetrical; the cell may be "doing one thing in one half and a different thing in the other". This would not be possible if the cell produced a propogated response to every motor impulse. The findings of Kinosita (1953) also suggest this mode of action.

If only a single innervation is admitted, difficulties arise in the explanation of this proposed temperature influence, and the theory must be rejected. It could be that low temperatures increase the influence of paling fibres, so causing melanophore aggregation. Conversely, high temperatures could decrease their influence and produce melanophore dispersion. But at very high temperatures the unopposed aggregating influence would be so far suppressed that the melanophores would be free to respond independently. They would then aggregate their pigment, but this has not been observed. The acceptance of a double antagonistic innervation could eliminate these difficulties by permitting differential effects. Thus high temperatures may antagonise paling influence or accentuate darkening influences while low temperatures could have the opposite effects.

The majority of temperature effects which could produce these responses are continuous and would produce graded responses over the whole temperature range involved. It will be shown later that, as suggested by Smith (1928), the temperature responses of innervated melanophores are discontinuous and only occur at extreme threshold temperatures, with no temperature coefficient (Chapter IIIa and f). The excitation and blocking of nerve fibres by temperature are examples of discontinuous phenomena, both of which could explain the observed effects. But nerve excitation must be rejected for the same reasons as v.Frisch's nerve degernation theory and also because no accommodation is obtained during extended heating or cooling (see Dodt, 1953; Granit, 1955; Granit and Lundberg, 1947). Selective blocking of a double system of nervous control at extreme temperatures thus remains as the only acceptable suggestion.

The operation of this mechanism could be as follows. As the temperature is raised the paling fibres become blocked but the darkening fibres are affected less or not at all. Regardless of the original balance of tonus the affected region of skin will then become maximally dark. At low temperatures the reverse will occur so that the darkening fibres will be selectively blocked and the skin will become maximally pale. The theory demands that both heat and cold block occur at higher temperatures in the

dispersing fibres than in the aggregating fibres. While the high and low temperature responses may be brought about by quite different means, there is no evidence for this and the above theory appears adequately to account for both.

The slow appearance of the denervated responses is explicable on the basis of Parker's theory that the section of chromatic nerve fibres produces repetitive discharges for some time. While this theory has experienced much opposition it has been supported for the minnow by Gray (1955a) and is here considered to be the only acceptable explanation at present for some phenomena (Chapter Va). As the repetitive discharges die away, the melanophores would be expected to become more and more independent and direct responses to stimuli would appear gradually.

Physiological literature provides considerable circumstantial support for the peripheral interference theory. According to Kuntz (1953) double synergistic innervation of autonomic effector organs, whose activity is determined by the balance of tonus, was first proposed by Eppinger and Hess in 1909 and now appears to be the rule for many such organs in mammals. In a detailed review Kuntz states that sympathetic and parasympathetic tonus bear an inverse relation to each other. There is little evidence that this applies to similar organs in teleosts where there is no clear division of the autonomic tracts into sympathetic and parasympathetic systems, for example cardiac accelerator fibres are absent in teleosts

(Kisch, 1948; Nicol, 1952). Nevertheless if double innervation of melanophores can be demonstrated, it does not seem unreasonable to propose that the control mechanism may be similar to that found in mammals. A complete discussion of the case for double innervation of melanophores, upon which this theory depends, is given in Chapter V.

Several papers on selective thermosensitivity of nerve fibres show a remarkably consistent relation to fibre diameter. Bremer and Titeca (1934) found that the large fibre component disappeared when a mixed nerve was uniformly warmed. Later (1946) the same authors showed that the α and β elevations of frog sciatic nerve disappeared in turn at about 31°C. leaving only the γ activity. Lundberg (1952) showed conversely that at 10°C. the δ elevation disappeared from the action potentials of the saphenous nerve of the cat, leaving only the fast fibre activity. Lundberg (1948) had previously stated that both heat and cold block occur about 15°C. higher in mammals than in non-tropical frogs but the acclimatisation temperature of the latter was important. Cold block occurred at +5°C. to -7°C. in normal frogs and heat block at about 33°C.

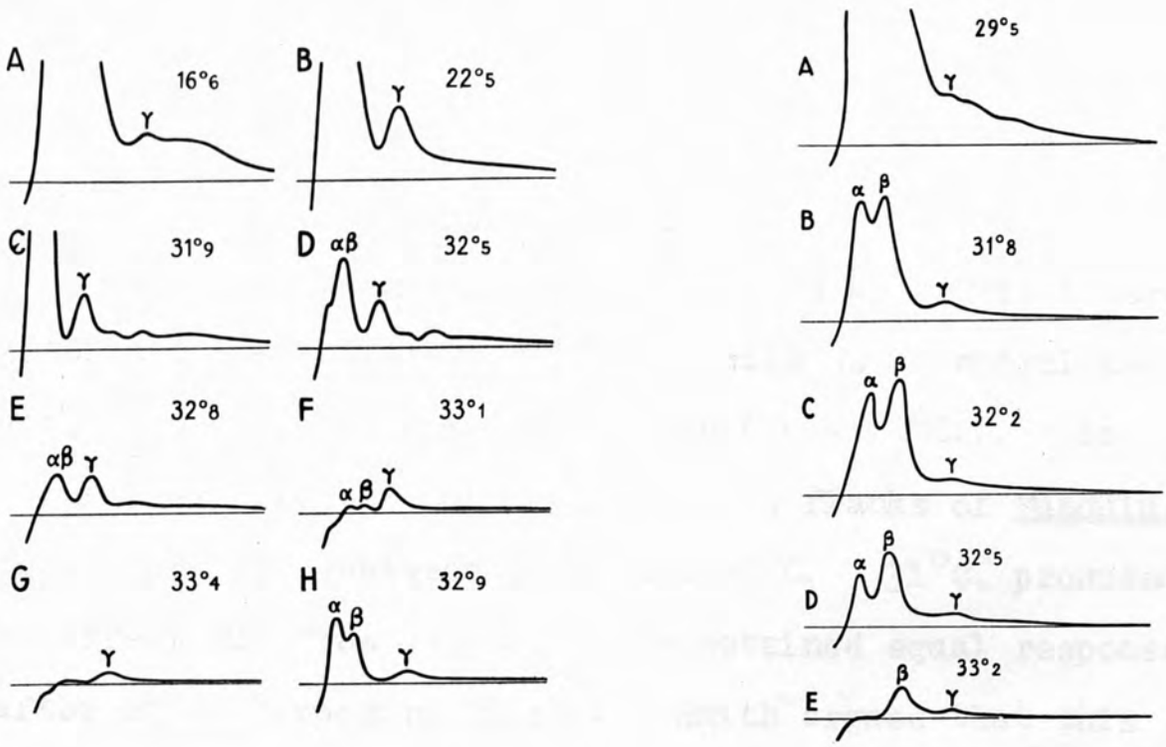
Dodt (1953) also working on cats confirmed that blocking temperatures were related to fibre diameter as follows:-

Fibre type.	β.	δ.
Diameter.	8-14μ.	1.5-3.0μ.
Cold Block.	below 10-15°C.	below 16-22°C.
Heat Block.	above 45-48°C.	above 50-52°C.

Oscillograms produced by Bremer and Titeca are shown in Figure 5. These authors stated that heat block was practically instantaneous at the critical temperature and was rapidly reversible, depending only on the thermal level and fibre diameter. If any pair of these fibre groups had opposite effects on an effector organ the system would be a perfect model of the innervated response of minnow melanophores. There appears to be no evidence concerning the types of fibre involved in chromatic control but if double innervation exists it seems likely that each set of fibres has different physiological (i.e. thermal) properties. Since electrical stimulation always produces paling, one might expect that the aggregating fibres have greater diameters than the, at present hypothetical, dispersing fibres. This agrees with the interpretation given above.

The remainder of this thesis attempts to test this theory of peripheral interference and in particular to re-examine the nature of the innervation of melanophores in the minnow.

Figure 5.



Compound action potentials of frog (Rana temporaria L.) sciatic nerves, from Bremer and Titeca (1946), showing selective blocking of the larger fibre components at high temperatures.

CHAPTER III

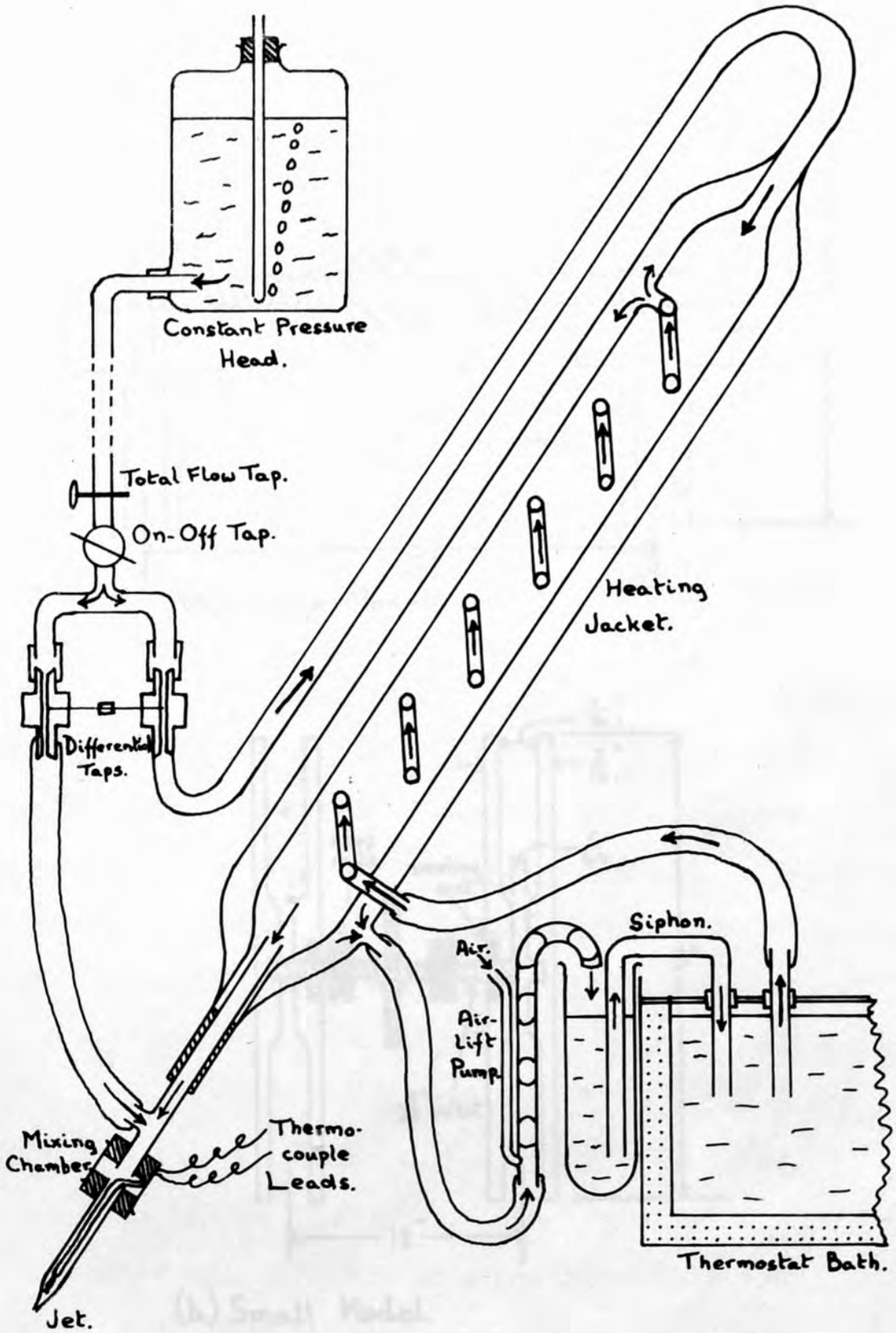
FURTHER EXPERIMENTS ON THE TEMPERATURE EFFECT.

a. The High Temperature Response.

The high and low temperatures used by v.Frisch were 35°C. and 3 - 5°C. respectively, while as a control he employed water at room temperature (14 - 16°C.). In similar experiments Smith exposed the flanks of Fundulus to temperatures between 31°C. and 40°C. 31°C. produced no effect but from 32 - 40°C. he obtained equal responses after equal 'exposure times'. Smith argued that this all-or-none response indicated reflex activity although the melanophore is obviously not an all-or-none effector. Only water at 2°C. appears to have been used for the cold effect.

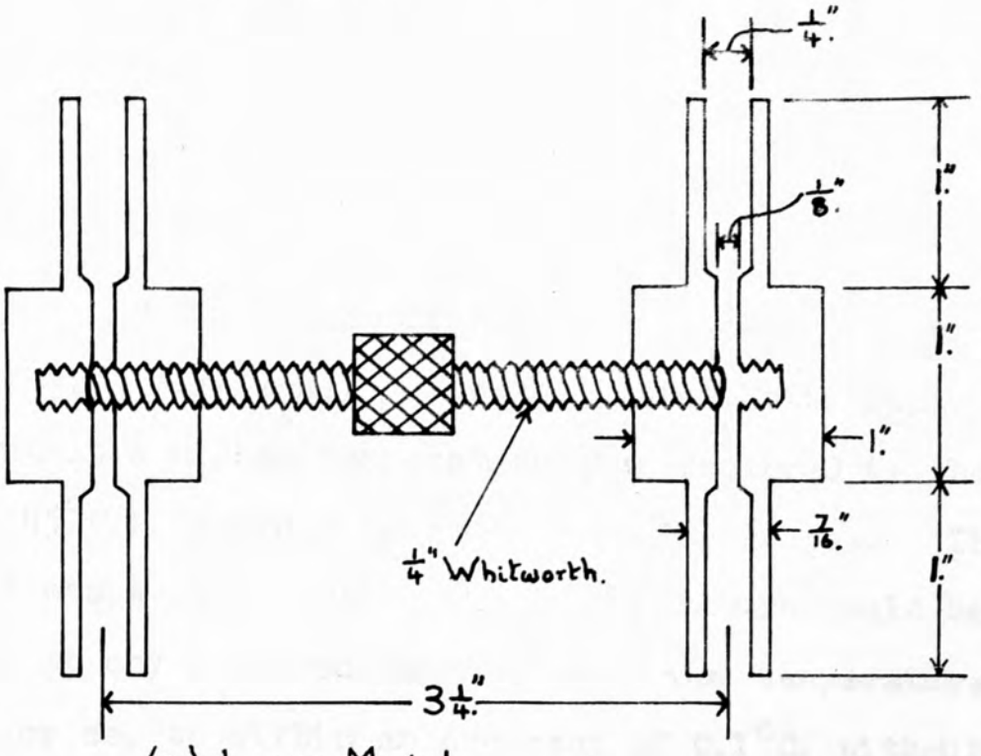
The conclusions reached in Chapter IIf made it of interest to examine whether definite threshold temperatures exist for the innervated responses of the minnow. The apparatus developed for this purpose is shown in Figure 6. Water from a constant pressure reservoir was fed straight to a jet and through a heating jacket made from a double jacket chemical condenser. The two flows were remixed in proportions controlled by the differential tap (shown in detail in Figure 7a), to provide any desired high temperature. By this means the total flow remained

Figure 6.

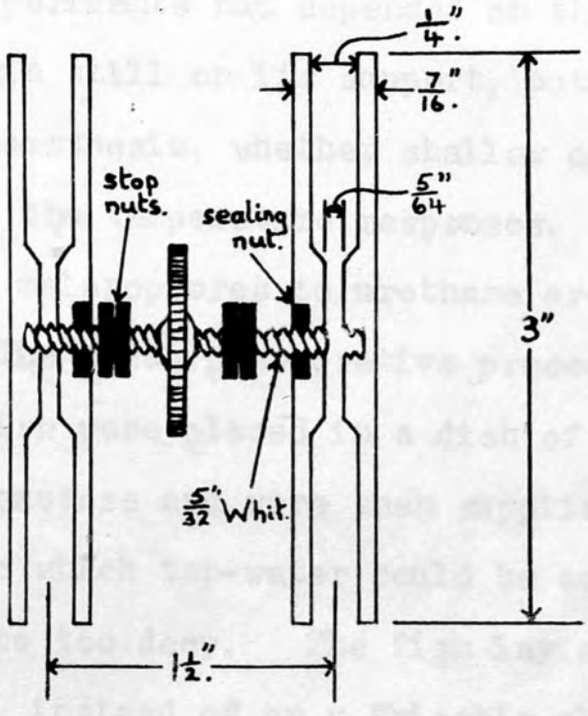


The Variable Temperature Jet Apparatus.

Figure 7.



(a.) Large Model.



(b.) Small Model.

The Differential Taps.

constant and thermal inertia was extremely low. The temperature of the jet was measured by a thermoelectric thermometer (Appendix I). Polyvinyl chloride tubing was used for its good heat insulating properties and its transparency which greatly assisted in detecting and eliminating air-locks. The thermostat bath (Appendix II) was run at a higher temperature than required in the jet (40 - 45°C.) in order to compensate for losses. The result was a jet of water whose temperature could be varied at any required rate between room temperature and 38°C. or so, to within an accuracy of 0.1°C. without any change in the mechanical stimulus.

Previous experiments had depended on the 'goodwill' of the fish to remain still on its support, but tests showed that urethane anaesthesia, whether shallow or very deep, did not suppress the temperature responses. The direct reactions of the melanophores to urethane are discussed in detail below. The usual preoperative procedure was followed; the fish were placed in a dish of 0.5% urethane solution until comatose and were then supplied orally with 0.25% solution to which tap-water could be added if anaesthesia became too deep. The fish lay on their sides on a dish of wax, instead of on v.Frisch's plasticine support, with the urethane tube placed lightly in their mouths. A second tube drained all waste fluids to a sink. With the fish thus under control, microscopical examination

of the melanophores became possible. The wax dish was supported so that an ordinary microscope, with the stage and substage removed, could be brought to focus on the skin and moved independently. The jet was supported separately by holding the heating jacket in a retort stand and the glass slips used by v.Frisch were dispensed with. This became the standard technique for all later experiments.

In the first experiment six fish were used in turn. The jet temperature was raised by steps of one or two degrees Centigrade. In all cases the entire response was elicited over a very narrow temperature range whose value was somewhat lower than expected. The six values were:- 26 - 28°C., 26 - 28°C., 25 - 27°C., 24 - 26°C., 31 - 33°C., and 29 - 35°C. These figures bore no obvious relation to the original states of the melanophores, which varied widely. Several interesting features of the response were noted at this time.

(i) Although the temperature range for producing complete reversible melanophore dispersion was small, the change required to produce the full response in a single melanophore or group of melanophores was even smaller, often being less than 1°C.

(ii) The effective temperature varied from day to day for the same region of the same individual. The fish whose response occurred at 31 - 33°C. above showed complete

darkening at 25 - 27°C. when retested 24 hours later. Similar variations were often found in later experiments but rarely during a single experiment.

(iii) The threshold temperatures for different types of melanophore were often different. At least four groups of melanophores could be distinguished by their responses;

1. large melanophores forming the lateral stripe and a patch at the base of the tail fin;
2. large melanophores forming the pattern on the back of the fish;
3. small melanophores on the back;
4. small melanophores forming a patch on the tail just dorsal to that of group 1.

The similarity to the physiological groups described by Healey (1951) is marked (Figure 8). As the temperature was slowly raised dispersion occurred for each group in the order listed and on lowering the temperature recovery occurred in the reverse order. Small melanophores of the fins and ventral body surface always appeared to be thermally inert. Thus while the change of temperature required for a response by each group was small, full darkening of the fish often needed a wider range. One fish with a very gradual overall response gave the following figures:-

Group 1 : 26 - 28°C.

Group 2 : 29 - 31°C.

Figure 8. After Healey (1951).

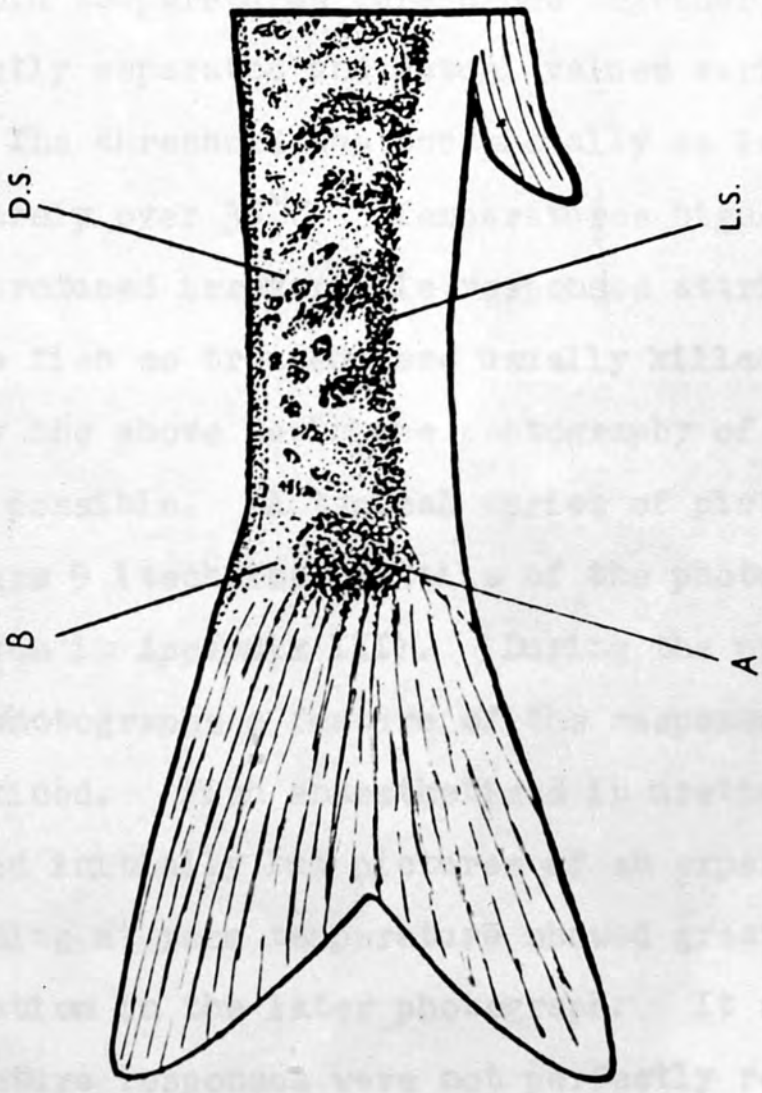


Diagram of the tail region of a minnow to show the regions observed: *A* = enlarged tail region of lateral stripe; *B* = region of small melanophores; *D.S.* = small melanophores on dorsal surface; *L.S.* = large melanophores on lateral stripe.

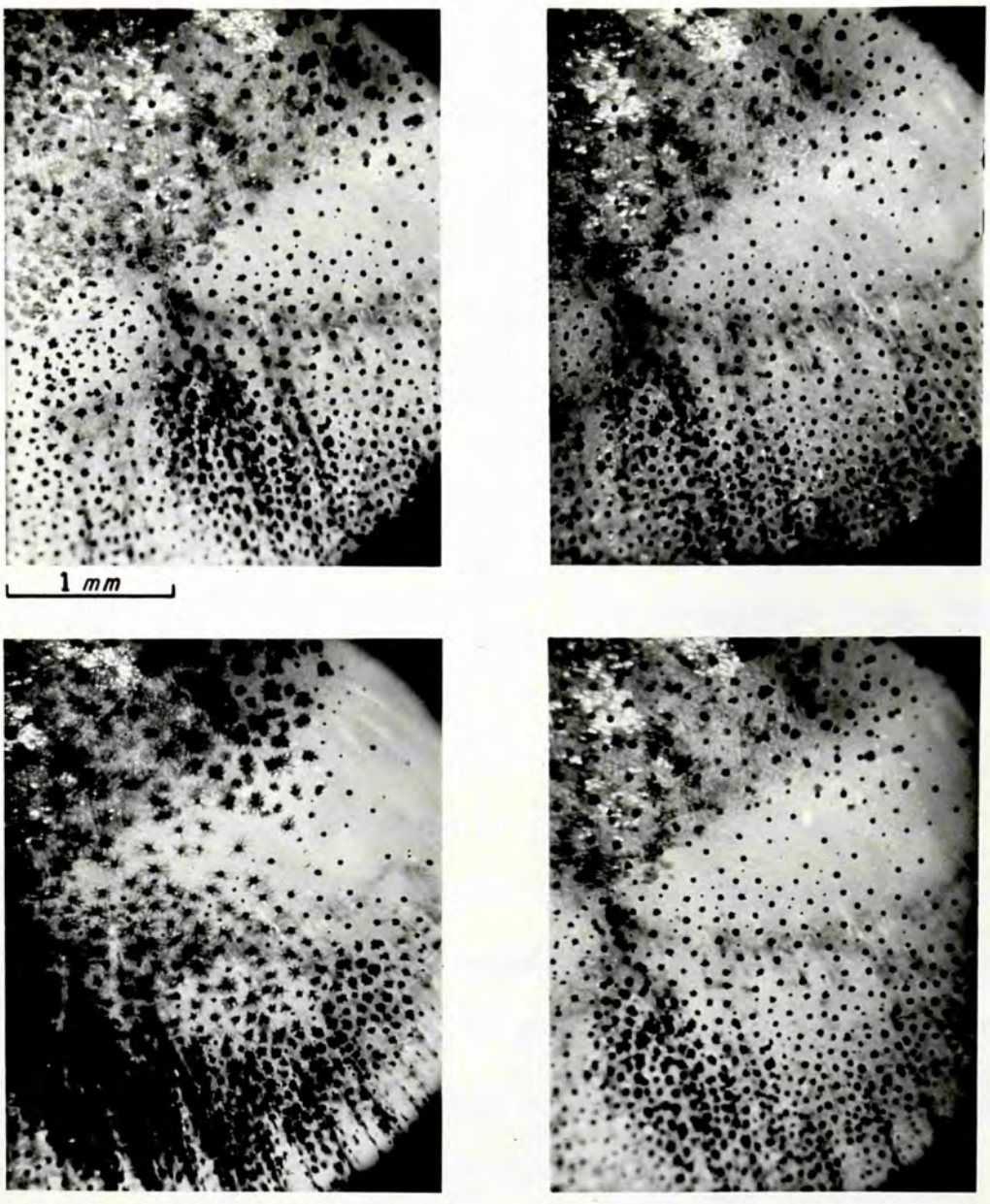
Group 3 : 32 - 35°C.

Group 4 : no response at 35°C.

As for the experiments described in Chapter II, all these effects were seen without exception in a large number of fish during later experiments. In some cases the threshold temperatures were close together, in others distinctly separated and actual values varied from day to day. The threshold was occasionally as low as 20°C. and very rarely over 35°C. Temperatures higher than 35°C. often produced irreversible responses attributed to damage and the fish so treated were usually killed.

By the above technique photography of the responses became possible. A typical series of pictures is given in Figure 9 (technical details of the photographic method are given in Appendix III). During the preparation of these photographs a feature of the response to urethane was noticed. Fish anaesthetised in urethane invariably darkened initially but pictures of an experiment starting and ending at room temperature showed greater melanophore aggregation in the later photograph. It appeared that the temperature responses were not perfectly reversible but exhibited some hysteresis. That this was not so was demonstrated by reversing the order in which the extreme temperatures were presented. Further observation and pictures of six fish, taken without thermal stimulation, showed that the fish darkened at first under urethane and

Figure 9.



The normal local temperature effect in the minnow.

Top left: a part of the tail after 45 minutes at 16°C. under urethane anaesthesia.

Top right: the same after 10 minutes at 0°C..

Bottom left: the same after 10 minutes at 35°C..

Bottom right: the same after 10 minutes at 16°C..

then slowly paled for 30 - 45 minutes. The fish used for the photographs of Figure 9 was kept under anaesthetic for 45 minutes before the experiment was begun but comparison of the first and last pictures shows a slight further aggregation. This effect is therefore not related to the temperature responses which may be assumed to be completely reversible.

b. Changes of Threshold.

The change of threshold for the same fish from day to day can be explained by the present theory with one further premise; that all the fibres of one group become blocked in turn over a fairly wide range of temperatures with the majority being inactivated at some intermediate value. The range of effective temperatures for each group may then overlap; say aggregating fibres are blocked in turn from 25 - 35°C. but mostly around 30°C., whilst dispersing fibres are only affected by temperatures above 30°C. and the majority remain active to about 35°C. Since only the blocking of active fibres is effective, the levels of activity in the two groups would affect the temperature at which the melanophore becomes unbalanced. In the anaesthetised fish there is no control over the level of nervous activity which might vary from one experiment to the next. It would therefore be of great interest to

examine the threshold temperatures of fish responding normally to backgrounds of various shades.

In a preliminary experiment two fish were kept on a black, and two on a white background for fourteen days. Each was anaesthetised in turn on a background of the same colour and tested for high temperature responses. No obvious differences could be distinguished between the thresholds of the two pairs and since it was considered that anaesthesia could upset the tonic balance (if it exists) no experiments were attempted on statistically significant numbers of fish. This experiment also indicated that the level of melanophore aggregating hormone in the blood did not normally affect the threshold. This question was re-examined later.

Microscopic studies of the melanophore responses to background colour may be made with the Constant Observation Tank apparatus designed by Healey and further developed by Gray (1955a, 1956b). A minnow, trained by previous confinement to suppress locomotory activities, sits in the corner of an aquarium with its tail projecting into a perspex observation cell, while an ingenious arrangement of slides allows black or white backgrounds to be presented to the fish. A great deal of time was spent in attempting to control the temperature within the observation cell independently of that in the main body of the tank. The final method was to plug the entrance of the cell around

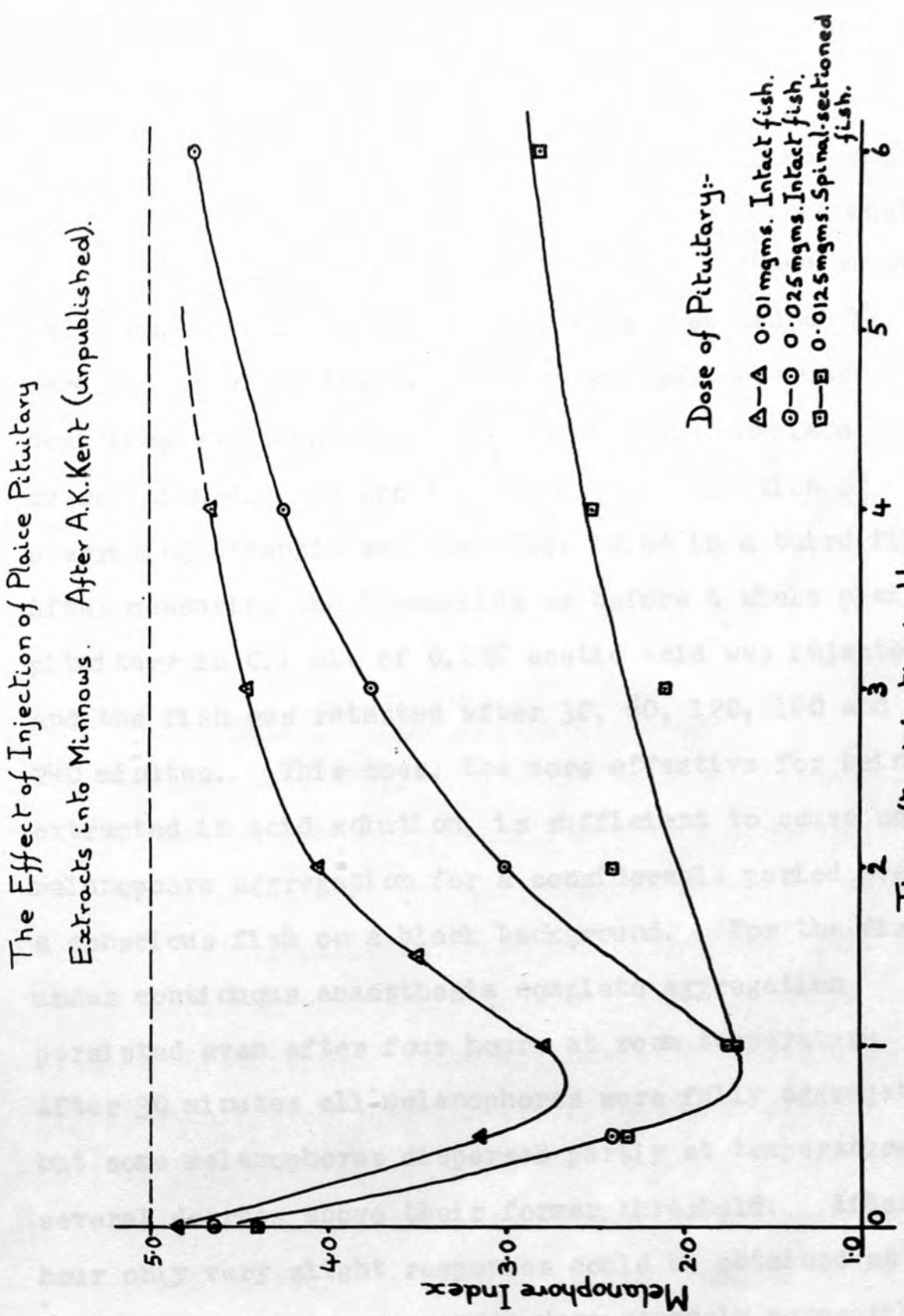
the body of the fish lightly with cotton wool and to warm the cell by a miniature immersion heater manufactured from an electric gas-lighter element. This heater was so placed in a channel beneath the cell that convection currents warmed the whole cell evenly and smooth control of temperature was obtained by a rheostat. While technically possible the technique failed on biological grounds. Such restraint upon a normally active fish is endured well after a short period of training by restriction in a tube, although the method introduces certain complications (Gray, 1956a), and the fish showed no objection to the cotton wool plugging. But in nearly every case a slight rise in temperature within the cell evoked violent struggling movements and the cotton plug was released. Following the work of Rubin (1935) and the experiments of Chapter IId, the lateral line and lateral cutaneous nerves were cut on both sides in four fish. Sensory pathways appeared not to be interrupted since these fish behaved just as badly as the others. Only a very few fish lay quiet long enough for high temperature dispersion to occur at all and controlled measurements proved to be impossible. Many attempts were made to improve the technique in view of its obvious advantages and potentialities but the project had finally to be abandoned.

Another attempt to shift the threshold temperatures

was made by injecting extracts of minnow pituitary into the experimental animals. Such extracts have a strong paling influence due to the presence of melanophore aggregating hormone. Pituitary glands dissected from freshly decapitated minnow were ground up with a miniature pestle and mortar in a small quantity of distilled water. The whole mixture, usually about 0.1 ml. in volume, was injected intraperitoneally. The effect of similar injections into an active fish on a black ground is shown in the curves of Figure 10 (after Kent, unpublished). The vertical scale represents the state of the melanophores on an arbitrary scale from 1.0 (fully aggregated) to 5.0 (fully dispersed); Hogben and Slome (1931), Healey (1951). The dose of 25 µgm. of plaice pituitary is approximately equivalent to that of one minnow pituitary (a whole plaice pituitary weighs about 500 µgm.). 5 µgm. produces a response of equal magnitude in a spinal-sectioned fish and recovery takes much longer, so that the nervous system appears to exert a strong restoring influence in the normal fish (see Chapter Vb).

A minnow was anaesthetised, the temperature thresholds for each type of melanophore carefully measured and one minnow pituitary in 0.05 ml. of water was injected. The threshold was redetermined under maintained anaesthesia after 30 minutes and 60 minutes. Although considerable paling occurred at normal temperatures the melanophores

Figure 10.


The Effect of Injection of Plaice Pituitary Extracts into Minnows. After A.K. Kent (unpublished).
Melanophore Index
5·0
4·0
3·0
2·0
1·0
0
1
2
3
4
5
6
Time after injection in Hours.
Dose of Pituitary:-
Δ—Δ 0·01 mgms. Intact fish.
○—○ 0·025 mgms. Intact fish.
□—□ 0·0125 mgms. Spinal-sectioned fish.

dispersed their pigment at exactly the same elevated temperatures as before injection. Another fish was retested after 30, 60 and 180 minutes; again there was no change in response except for one group of melanophores whose threshold rose only at 180 minutes. Since most of the effect of injection had by then worn off and anaesthesia had become difficult to control, this rise cannot be regarded as significant. In these cases nervous dominance was maintained throughout since complete aggregation did not occur. A further injection of overriding strength was therefore tried in a third fish. After measuring the thresholds as before a whole plaice pituitary in 0.1 ml. of 0.25% acetic acid was injected and the fish was retested after 30, 60, 120, 180 and 240 minutes. This does, the more effective for being extracted in acid solution, is sufficient to cause complete melanophore aggregation for a considerable period even for a conscious fish on a black background. For the fish under continuous anaesthesia complete aggregation persisted even after four hours at room temperature. After 30 minutes all melanophores were fully aggregated but some melanophores dispersed partly at temperatures several degrees above their former threshold. After one hour only very slight responses could be obtained at 35°C. and after two hours responses were scarcely perceptible at this temperature. At three hours dispersion again

occurred at 35°C. and at four hours the threshold was barely higher than originally. Thus an extremely large amount of pituitary hormone is required to raise the threshold temperatures. Since prolonged exposure of a normal fish to a white background does not affect the time course of darkening when transferred to a black background, the level of hormone in the blood of normal fish can never approach even that due to the first doses used. It must therefore be assumed that temporal changes in temperature threshold are not related to varying levels of pituitary hormone.

c. Local Heating of the Autonomic Chain.

If the temperature effect is due to differential nerve block in the skin, a similar effect may be produced by heating or cooling the motor nerves at a more central level. The sympathetic chain was chosen for testing as it is small and reasonably accessible without much vascular disturbance. The apparatus of Figure 6 was filled with Ringer solution and fitted with a finer jet, bent at right angles near the tip. The new jet limited the total flow so that the differential taps produced unstable mixtures. A smaller pair of taps with a large control disc was therefore made as shown in Figure 7b. These experiments also led to the adoption of Young's

Freshwater Teleost Ringer solution instead of that of Steinhausen, as described in Appendix IV.

The fish was anaesthetised and laid in the wax dish with a tube supplying anaesthetic orally as before. A horizontal incision about ½ - 1 cm. long was made mid-laterally in the body wall to expose the swim-bladder which was partially collapsed by puncturing. The fine jet could then be placed so that it played upon the thin renal tissue covering the sympathetic chain. If placed posteriorly in the body cavity even the renal tissue was present in negligible proportions. The dorsal mesenteries were not removed. Fluid from the jet filled the cavity and overflowed at the incision. A new dish with a central drain was constructed so that these fluids would not make their way to the regions under observation. In order further to prevent temperature changes in the area of the skin observed, a jet of water at room temperature was kept flowing over it. This also drained to the centre of the dish and prevented backflow of warm Ringer, while urethane solution from the gills prevented it from flowing forwards. Temperature changes in the skin of the fish were monitored by a second thermocouple in a hypodermic needle (see Appendix I), whose tip was placed subcutaneously.

The experiment was performed successfully on ten fish in slightly different ways. Only one fish showed no response while three showed only weak or local responses.

The other six produced dispersion at high temperatures as if the jet had been applied to the skin itself. The results were as follows:-

1 and 2. The jet was placed posteriorly in the body cavity after collapsing the posterior part of the swim-bladder. Normal responses were found in the tail with thresholds at 26 - 28°C. in each case.

3 and 4. The jet was placed anterior to vertebra 15 after collapsing the anterior part of the swim-bladder. One fish showed rather weak and patchy dispersion over the whole body but the other gave no response at all.

5 and 6. Since the sympathetic chain is closely applied to the aorta, these responses might be due to the heating of arterial blood passing to the tissues. To check this the first experiment was repeated after a second incision had been made more anteriorly to expose the dorsal aorta. Thresholds were found to be 26 - 30°C. and 30-34°C. in the two fish. Their dorsal aortae were then completely cut by a knife jab through the anterior incisions. Melanophore responses continued at the same threshold temperatures in each case until respiratory movements ceased. Aortic rupture was later checked by dissection.

7 to 10. In order to reduce the initial loss of blood due to a double incision in the body wall it was decided to cut the ventral aorta by a knife jab between the heart and the gills. The blood released flowed away and

could not possibly be involved in the transfer of heat. Also this procedure does not involve damage to the autonomic chain. The remaining four fish were set up with the Ringer jet anterior to the 15th vertebra. All showed responses anteriorly although one case was rather weak and another patchy. In all at least one reversible response was observed after opening the ventral aorta. Thus the normal responses observed in these experiments cannot be attributed to the circulation of heated blood.

A further factor considered was that of direct heat conduction through the other tissues. The speed of melanophore reaction, even without blood circulation, once the threshold temperature was reached, and the fact that the threshold temperature was independent of the rate of warming, seemed to preclude this. Also the strictly localised nature of the response when the skin of an intact fish is heated suggests that little heat conduction occurs. MacLeod, Self and Taylor (1921) found that local heating of the shaven abdomen of the rabbit by temperatures greater than 15°C. above the body temperature produced heat spread for 5 - 7 cm. into the viscera but only 2 cm. laterally. Even "relatively vascular tissues" were penetrated but two hours application were necessary to obtain a rise of 2°C. at such a distance.

In the present experiments the flow of room-temperature water over the area observed should reduce the

risk of heat spread but monitoring with the second thermocouple does not provide a sure guide. The thermal conductivity of steel is presumably much greater than that of muscle so that the bimetal junction might be cooled much more effectively by the cool jet than the tissues themselves. Experiments on three fish with intact circulation but no cooling water showed that tissue temperature can indeed rise under the conditions of these experiments. The only way to eliminate these difficulties unequivocally appeared to be by repetition on a much larger fish, where observations could be made at a considerably distance from the warm jet. Experiments were therefore continued at Plymouth on the plaice (Pleuronectes platessa L.).

This species, as described in Chapter IV, shows melanophore responses to temperature which are similar to those of the minnow. Little is known of the chromatic pathways in this species but they are probably similar to those of the minnow. Hewer (1926) states that in Limanda limanda (L.), the dab, chromatic fibres pass from the spinal cord to the sympathetic chain at the level of the sixth vertebra.

Due to lateral compression the renal tissue overlying the sympathetic chain is much thicker than in the minnow and dissection showed that it continues around the posterior wall of the body cavity. Attempts to reach the

sympathetic chain of plaice anaesthetised in MS222 produced so much loss of blood that death soon followed. Injection of desoxycorticosterone as a shock preventative helped very little. Measurements on a cadaver of the thermal conductivity of the walls of the haemal canal showed that it would not be possible to heat the sympathetic chain more posteriorly. Finally the experiment was performed on the spinal nerves just peripheral to the point where they receive the rami communicantes.

Three large plaice were used under anaesthetic and in each fish three adjacent spinal nerves were exposed for a distance of 1 - 2 cm. on the upper (right) side, just ventral to the haemal canal and about half way along the body. Three nerves were taken as a minimum since the areas served probably overlap. No melanophore reactions were elicited by exposing the nerves, nor when they were warmed to 35°C. or cooled with carefully crushed frozen Ringer solution. At the end of each experiment the nerves were severed and the ensuing dark band (first observed by Pouchet, 1876) was taken to indicate that the chromatic fibres were still physiologically active. This assumption is valid by all theories of the band formation. A similar experiment on a single flounder (Platichthys flesus (L.)) in which four consecutive spinal nerves were exposed, produced only very weak responses. Further experiments had to be abandoned for lack of material due

to gales at that time.

d. The Correlation with Respiratory Movements.

The above experiments on the sympathetic chain, although not conclusive, led to a further interesting discovery. In early attempts to achieve the minnow preparation the body wall incision was made dorso-laterally in order to obtain direct access to the sympathetic chain. This involved cutting several ribs and spinal nerves rather near their roots. Soon after this operation the dorsal fins of the fishes began to twitch violently. When this ceased, respiratory movements were suddenly discontinued together with melanophore activity in response to thermal stimulation. This phenomenon was assumed to be due to spinal shock and was completely cured by making a ventro-lateral incision in the soft belly wall and using a longer glass jet to reach the region of the sympathetic chain.

In the experiments involving disruption of the circulation it was again noted that chromatic responses persisted until the time at which respiratory movements ceased. Then, even if the melanophores were half-way in an active response or recovery, further movement of pigment halted abruptly. Again widespread nervous reaction or shock might have caused the coincidence but the correlation was later supported in a third way. Intact minnows,

responding normally to externally applied temperatures, suddenly ceased to do so if anaesthesia became so deep that respiratory movements were interrupted. Provision was made in the anaesthetic apparatus to provide tap-water (or in extreme cases dilute carbonic acid) instead of 0.25% urethane if necessary, and in such cases respiratory movements soon restarted. The interrupted melanophore response was then completed and further reactions could be obtained in the normal way.

These phenomena, first noticed when minnows were kept for long periods under urethane, were later observed in several other species (see Chapter IV). Flatfish in particular proved difficult to control under urethane; they either became active or ceased to perform respiratory movements. For this reason urethane was replaced by MS222 for the larger marine teleosts and the respiratory movements were carefully observed throughout all temperature experiments. The correlation was always upheld; a cessation of melanophore activity observed microscopically indicated that the respirator apparatus required attention. Responses in the absence of respiratory movements were never observed in any fish.

Possibly this relationship indicates that the source of tonic activity proposed for the chromatic nerve fibres is linked in some way with rhythmic activity in the respiratory centres. No other explanation can be suggested

at present. A rather tenuous connection might be suggested between this effect and that observed by Cole and Schaeffer (1936, 1937) and Cole (1937). These authors measured the rate of background adaptation of Fundulus at different temperatures. They stressed that the temperature coefficients they obtained (Arrhenius' μ was 9,700 and 10,900 in sea-water and 11,400 in fresh-water; see Chapter Ib) were strongly suggestive of respiratory dependence, since they were very close to the value for respiratory movements in the same species (μ = 8,400 in sea-water, 11,300 in fresh-water; quoted from Sizer 1935-6). Belehradek (1930) however questions the significance of temperature coefficients when used in this way.

According to Healey (1957), Springer (1928) reported that section of the optic lobes of some teleosts results in irregularities of respiratory movement. This may indicate a link between the optochromatic system and the respiratory centres, but the experimental details were said to be imprecise (Healey).

e. The Effect of Blinding.

Smith (1928) reported that following bilateral optic-nerve section, Fundulus produces very slow denervated responses to applied heat and cold (see Chapter Id). He states..."It is not necessary then to stimulate denervated

cells in order to observe pigmentary responses to temperature changes associated with the direct action of heat and cold upon melanophores. All that is necessary to ensure the occurrence of such reactions is to prevent by some suitable means the motor impulses from reaching the pigment cells. If this can be done by leaving the nervous system intact, the presence of such connections does not hinder the responses". No further references are made to this statement however, and he finally considers the constant "exposure time" necessary at all liminal temperatures to be definite evidence of reflex action.

An explanation of this effect is possible on the basis of the present theory since a complete lack of sensory input may lead to quiescence of the tonic chromatic motor centres. If this were so there would be no motor impulses and the melanophores would be free to act independently to temperature (the denervated response).

The position in the minnow may be more complex however since v.Frisch (1911c) found that the roof of the diencephalon is photosensitive and that blinded but otherwise intact minnows continue to change colour in response to changes of illumination (this has not been demonstrated in Fundulus). The independent response to light intensity by the melanophores of some minnows (Healey 1948, 1951) is insufficient to account for the whole of this effect. An interesting experiment would be to test the temperature

responses of intact minnows during prolonged periods of darkness. Obviously this could not be achieved with the jet technique due to the necessity of handling the fish, but it should have been possible with the constant observation tank technique. It was hoped to measure the light intensity at which the reactions might become affected, but the project had finally to be abandoned (see Chapter IIIb).

A repetition of the experiment as performed by Smith, with bilateral optic-nerve section was initially disallowed by the Secretary for State in the licence for experiments on living animals, but special permission was obtained to blind twelve fishes provided that they were killed within 30 hours. Section of the optic nerve was performed under urethane anaesthesia, through a small incision dorsal to the eye which was later closed by a single stitch. The fishes appeared to be little incapacitated by the procedure and swam normally on recovery. Nerve section was later confirmed by dissection in each fish.

Three fish were each tested 1 hour, 3 hours and 24 hours after the operation at 35°C. and at 0°C. One gave denervated responses at three hours but later died. The other two gave denervated responses only at 24 hours and in one these responses were very weak. A further group of three fish were allowed to recover from the operation

and were tested only at 24 hours when all three showed fairly strong innervated responses.

Finally three fish were observed under maintained anaesthesia for periods of 5 - 8 hours, then returned to water and re-examined after 24 hours. Following the operation innervated responses became sluggish and weaker and in one case rather patchy. Thresholds fluctuated and varied in sharpness. No pattern could be discerned in this activity and no reversal of response was observed. After 24 hours one fish behaved as before and then suddenly changed to the denervated response which was repeated three times before the fish was killed. The second died overnight and the melanophores of the third proved totally unresponsive to temperature changes.

The permission for three further fish was reserved in case the experiment could be repeated in the constant observation tank.

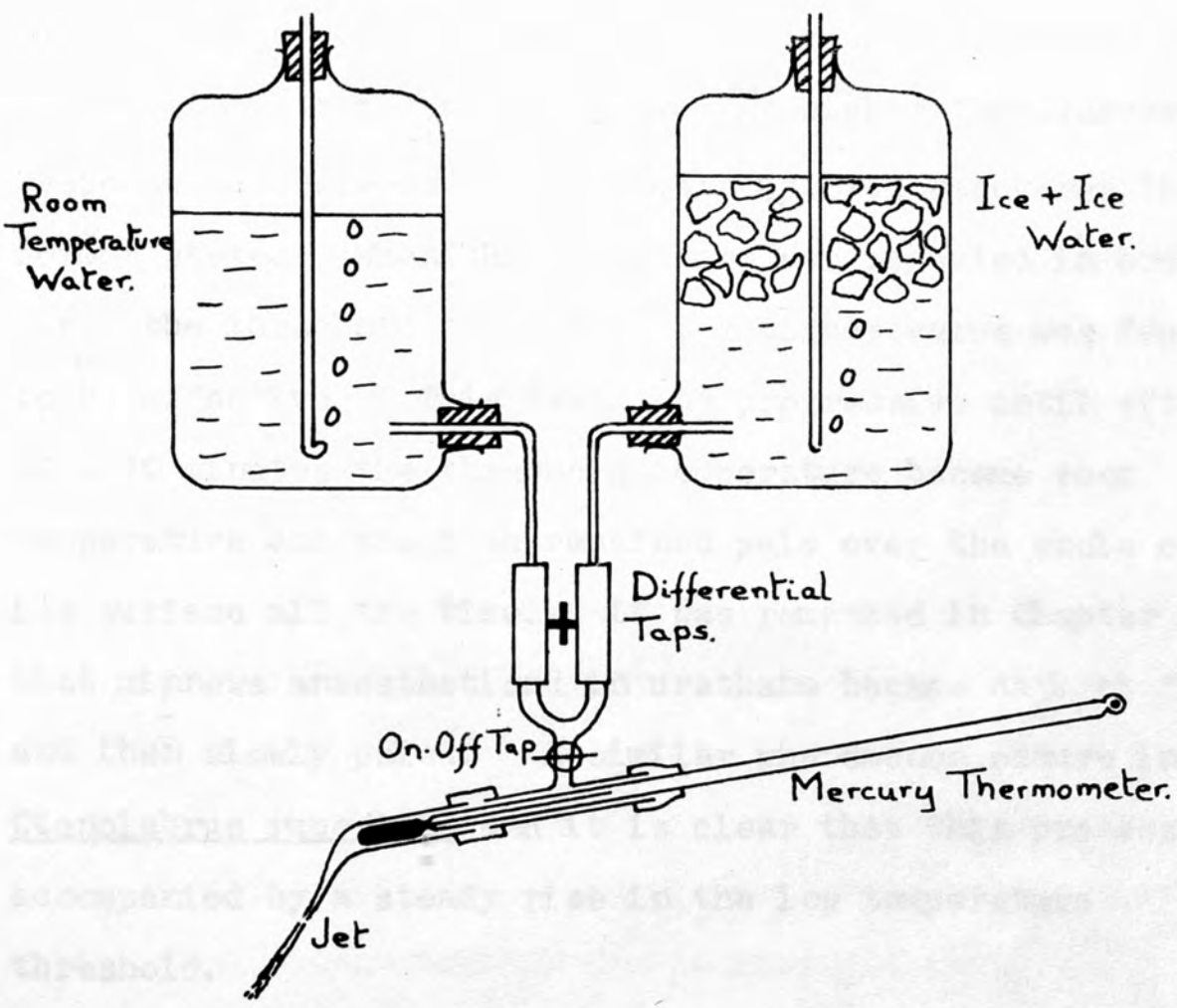
Although Smith's result cannot be fully confirmed, optic nerve section undoubtedly had some effect in Phoxinus. In most fish the response became very weak, thresholds fluctuated from hour to hour and the response was reversed in four fish out of nine. None of these effects has been observed in a very large number of intact fish. The results may indicate a weakening or fluctuation of chromatic motor tonus but several other explanations are possible.

f. The Low Temperature Effect.

The response to low temperature was examined by a technique similar to that used for the high temperature responses (Chapter IIIa). A controlled jet of cold water was obtained with the apparatus shown in Figure 11. This produced temperatures down to about 2°C. By comparison with the warm jet apparatus (Figure 6) the extreme temperature was reached rather slowly as the reservoir temperature could not greatly exceed that required at the output and thermal inertia was high. Also at low temperatures the pressure of the jet was reduced by the increased viscosity. This was corrected in use by compensatory adjustment of the total flow tap, in order to prevent changes in mechanical stimulation.

The experiments were commenced at Plymouth on Ctenolabrus rupestris (L.), a species which exhibits very clear temperature responses similar to those of Phoxinus (see Chapter IV). Early experiments with the application of pieces of frozen sea-water to the skin had produced little response at first, but as the cooled skin warmed towards room temperature after removing the ice, complete aggregation occurred. Further warming produced complete recovery of the original melanophore states. Three of the same fish were used in the present experiments. After anaesthetisation in urethane each fish was laid upon

Figure 11.



The Cold Jet Apparatus.

the wax dish and supplied orally with 0.25% solution of urethane in sea-water. The apparatus was filled with sea-water at 16°C. and melting sea-water ice.

As the temperature of the jet was reduced, complete aggregation of the melanophores was produced suddenly over a temperature range of 2 - 3°C. At higher temperatures the response was reversed and the melanophores recovered their former state. When this procedure was repeated in order to check the threshold temperature, a higher value was found to be effective. This trend was progressive until after 60 - 90 minutes the threshold temperature became room temperature and the fish remained pale over the whole of its surface all the time. It was remarked in Chapter IIIa that minnows anaesthetised in urethane became dark at first and then slowly paled. A similar phenomenon occurs in Ctenolabrus rupestris and it is clear that this process is accompanied by a steady rise in the low temperature threshold.

Each fish was later retested and the initial estimation of cold threshold temperature was performed as quickly as possible after anaesthetisation. This was rather difficult as the responses were slower than the high temperature responses and time had to be allowed at each test temperature for its effect to be determined. Values between 6°C. and 10°C. were obtained but again the threshold rose slowly to room temperature. During each

experiment the jet was removed from the body of the fish and run fully cold before being reapplied (at 2 - 3°C.). Each time this produced no response but when the jet temperature was raised slowly, complete aggregation occurred over a narrow temperature range. After a further increase, recovery occurred at the cold threshold temperature. It therefore appeared that the application of too low a temperature prevented normal responses. This agrees with the observation following the removal of frozen sea-water. Further investigation of this phenomenon would be desirable since it was also observed in several other species (Chapter IV).

In one experiment the body temperature of the fish, some distance from the jet, was monitored by a thermo-electric junction in a needle inserted sub-cutaneously. No change could be detected after prolonged application of a jet at 4°C. In some cases it was found that responses ceased when respiratory movements were suspended by too deep a state of anaesthesia, just as found for the high temperature response (Chapter IIId).

A single specimen of Crenilabrus melops (L.) was also available from the experiments of Chapter IV. Responses were again observed for the melanophores surrounding the centre of each scale. Complete aggregation was observed at temperatures below 8 - 12°C. but the threshold appeared to be rather wide and indeterminate. No progressive

shift of threshold temperature could be distinguished. Sudden application of the jet at 2 - 3°C. produced complete aggregation in contrast to Ctenolabrus rupestris. All responses were completely reversible at temperatures somewhat above the threshold (12 - 16°C.).

Two specimens of Gobius flavescens Fabricius were also tested. As shown in Chapter IV only certain of the melanophores of this species responded to the application of sea-water ice. The results were rather inconclusive. One fish showed some evidence of inhibition of response by the larger melanophores at very low temperatures, while the other suggested some rise of threshold for the smaller melanophores with time. Neither effect could be confirmed on the other specimen. Both showed that the low temperature response could occur completely and reversibly over a range of a few degrees.

Two Pleuronectes platessa (L.) (see Chapter IV) were also tested. Prolonged exposure to temperatures down to 3°C. produced no chromatophore responses in either fish, although subsequent contact with blocks of frozen sea-water produced aggregation in both cases. It is therefore assumed that 3°C. is above the threshold temperature for this species. Absence of response was noted in a third specimen but weak and irregular breathing movements caused this result to be rejected.

These experiments were later continued on minnows

which were obtained in breeding condition from Gravetie. The apparatus of Figure 11 was now filled with fresh-water. Six chromatically normal fish were anaesthetised in turn and tested with temperatures down to 3°C. and with small pieces of melting ice. In no case could any response be detected in any of the melanophores. Paling under anaesthesia proceeded at varying rates and was complete in each fish within 15 - 60 minutes. The same fish were then tested for high temperature responses. All showed the normal reaction (pigment dispersion) at temperatures varying from 28 - 35°C. although the response was weak in two specimens. The following day all were subjected to spinal section anterior to the 15th vertebra, and 48 hours after this five of the fish were again tested for low temperature responses (one died following the operation). All produced maximal melanophore dispersion at 2°C. with no clear threshold temperature and a graded response.

It was suggested that these abnormal responses might be due to chromatic changes associated with the breeding condition. The external indications of this state were lost quite quickly in the stock tanks and after three weeks the sexes were chromatically indistinguishable. Six fish from this stock were then tested but low temperature water and the application of ice produced no distinguishable responses. Two further groups of six fish each also produced no low temperature responses

although normal responses were produced by local heating.

Thus it appeared that the fish from this population produced normal responses to high temperatures except when denervated (Chapter IIe) and normal responses to low temperatures only when denervated. It seems unlikely that the breeding condition caused the discrepancy between these results and those of v.Frisch and those obtained with Welsh minnows and with minnows from Hertfordshire. It can only be suggested that the population from Gravetie differed in some way from those of the other sources used. Similar differences between other chromatic responses in different populations of Phoxinus have been found by Healey (1948, and unpublished).

g. Conclusions.

In Chapter IIf a possible explanation of the responses of melanophores to temperature changes was put forward. The results reported in this Chapter do not prove the correctness of these ideas but are nevertheless compatible with them. The discovery that both the high and the low temperature responses of innervated melanophores occur at definite threshold temperatures with apparent absence of accommodation render a sensory origin improbable. The narrow range of effective temperature changes sometimes observed compares favourably with the results of Bremer

and Titeca (1946) shown in Figure 5. The theory of tonic imbalance by thermal blocking thus receives some support. The fluctuations and broadening of the thresholds may be explained by postulating variations in the absolute and relative levels of tonicity in the two sets of fibres. This argument leads naturally to the conclusion that under urethane anaesthesia the activity of melanophore dispersing fibres is first stimulated and then slowly depressed, whilst paling fibre activity is scarcely affected. But since darkening fibres have not been conclusively demonstrated (see Chapter V) and thermal interference is not proven, these conclusions must be regarded as tentative.

The absence of the cold response in some species at very low temperatures may represent a total block of all chromatic fibres. On rewarming the paling fibres would then be released first and produce complete aggregation until the dispersing fibres were released. If this were so one would expect the melanophores to disperse their pigment at very low temperatures as an independent response. Their failure to do this suggests that at such temperatures the activity of the effector cells is also suppressed.

The failure of the constant observation tank technique was disappointing since it would have allowed the responses to be observed in unaesthetised fish under a wide range of controlled conditions. Variations of

illumination and background colour might have permitted more valid conclusions about the tonic states and removed much of the doubt about the present interpretations.

The correlation between both high and low temperature responses and respiratory movements was always very clear although totally unexpected. A direct connection between the respiratory and chromatic motor centres has been suggested. Possibly the former acts as a pacemaker for both the latter (if two exist). But if this is the case it is difficult to see why the melanophores of the quiescent fish, supposedly relieved of motor control, do not react independently to the temperatures present. The speed and certainty with which the responses are suppressed and regained almost certainly rule out any suggestion that tissue metabolism is affected by respiratory conditions. The conclusions of v.Frisch regarding Anämieaufhellung (see Chapter Ic, IIa) also discount this possibility.

The experiments on heating the sympathetic chain of Phoxinus appeared to give considerable support to the tonus interference theory. Responses were always so rapid and exhibited such clearly defined thresholds that the conduction of heat through the tissues appeared to be an impossible vector of the stimulus. Unfortunately subsequent experiments with Pleuronectes only served to confuse the situation. Experiments on a large fish with

an 'accessible' sympathetic chain are desirable, for it is possible that thermal blocking is more effective at synapses or neuro-chromal junction than in the uninterrupted fibres of the spinal nerves. An attempt was made to construct a small thermo-electric thermode which would permit extremely local heating and cooling of either the sympathetic chain or a free loop of the superficial ophthalmic nerve (see Chapter V). This proved to be beyond the technical ability of the author and had to be abandoned.

CHAPTER IV.

THE TEMPERATURE EFFECT IN SOME OTHER SPECIES.

a. Introduction.

The temperature responses of the minnow have been observed in only a few other species of teleost. v.Frisch (1912) describes similar responses, reversible on denervation, in Crenilabrus pavo and in the erythrophores of Trigla corax and T.lineata. Smith (1928)

investigated the responses of Fundulus heteroclitus while Smith and Smith (1935) obtained similar results for the erythrophores of Holocentrus ascensionalis. In all these species normal colour changes are very quick and there is adequate evidence of nervous control of the chromatophores involved.

Neill (1940) compared the colour changes of three species of teleost and defined two distinct types of colour change. Quick changes as shown by Lebistes reticulatus and Salmo salar were taken to indicate predominantly nervous control with or without humoral support, whereas the much slower changes of Anguilla anguilla suggested humoral control alone. This idea was supported by Waring (1942). An investigation of the chromatic responses of some marine teleosts at Plymouth in January 1958 led to a survey which later included some freshwater fishes. The aim was to ascertain how widespread the temperature responses are and to test their relationship to the presence of nervous control as judged by Neill's criterion.

The method adopted was very simple. Rate of change of colour was measured by reversing the backgrounds of some fully adapted fish and recording the time when they were judged to be indistinguishable from others maintained on the same background. In order to reduce the subjective influence of background colour, parts of the

larger species, especially the Heterosomata, were viewed through a hole in a screen of white paper. This macroscopic method is crude and time estimates are always less than those obtained by microscopic observation of the chromatophores, but it is quicker and sufficient for the present purpose. Since the fish need not be disturbed this method is also possible with much smaller numbers of fish. Most of the experiments were not continued for more than a few hours so that absence of response to background in some species may not exclude the presence of very slow adaptation.

Temperature tests were performed under anaesthesia. High temperatures were produced by the apparatus of Figure 6 (filled with sea-water for marine teleosts) and low temperatures by the application of ice or ice-water to the skin of the fish. Sea-water ice was carefully broken and mixed to prevent temperature variations and possible osmotic effects. Observation was always made microscopically as for the minnow and respiration was always carefully controlled. A large wax dish was prepared for some marine fish but the larger Heterosomata were mounted on a sloping dissection board.

An attempt was made to obtain results for four individuals of each species but, as no inconsistencies occurred, the results for smaller numbers have been included. Judgment of background reversal times are

rather more subjective in the case of single specimens due to the lack of comparison. Special attention was paid to those fishes which did not show quick colour changes as in some cases the presence of chromatic nerves was indicated by other means (e.g. by nerve-section).

The results are here presented in full as the chromatic responses of many species have been examined for the first time and it is felt that they may form a useful basis for future work. The notes are given in taxonomic order following nomenclature used by the British Museum (Natural History). Dr. E. Trewavas kindly advised in this matter.

b. Notes on the Species Examined.

Order Isospondyli.

Family Salmonidae.

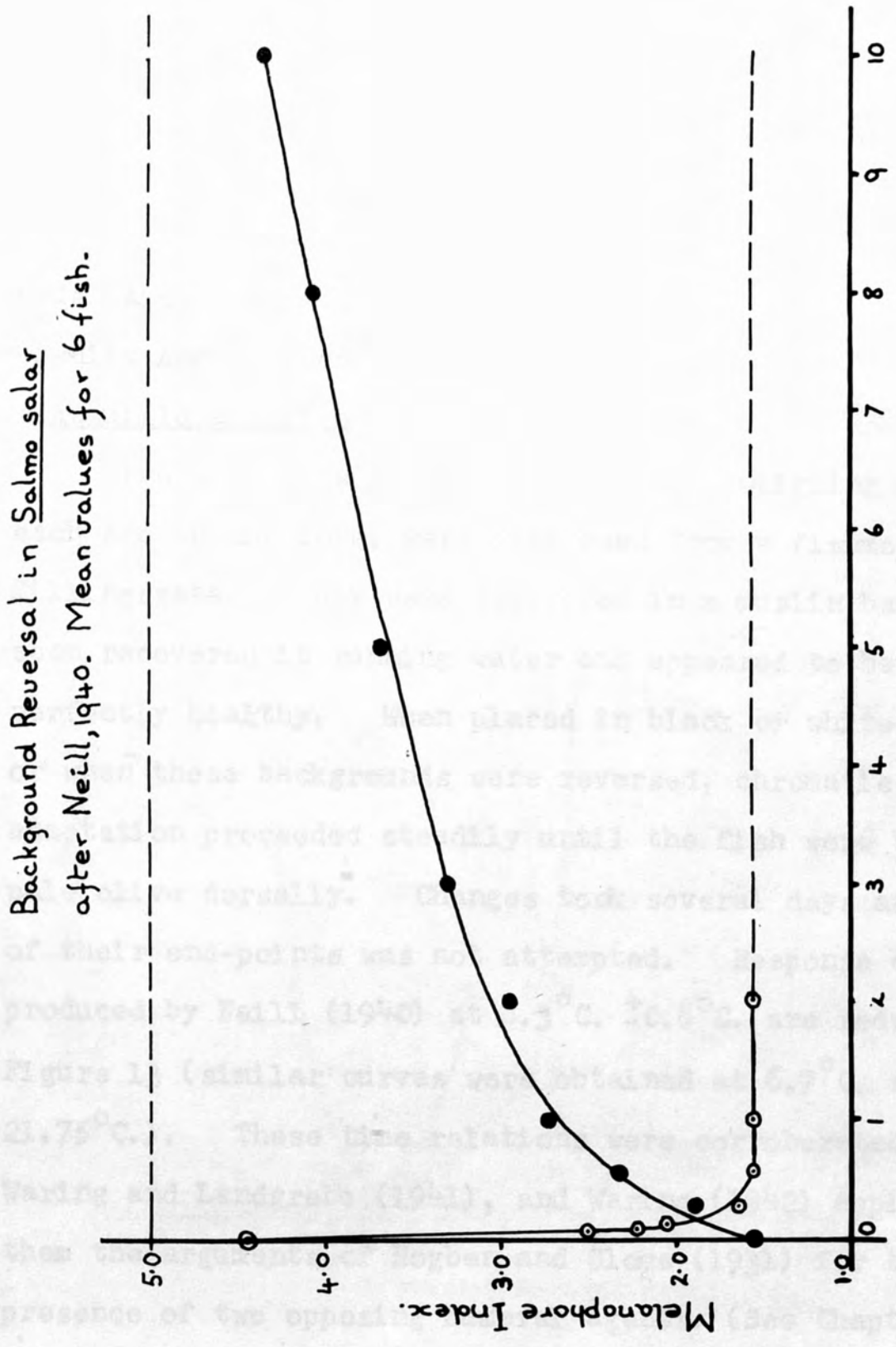
Salmo salar L. The Salmon.

Seven parr aged three to four months and about 3.7 cm. long were obtained from the Freshwater Research Laboratory of the Ministry of Agriculture, Fisheries and Food, by kind permission of Dr. V. Parry. The fish were kept at 15°C. in a constant temperature room and supplied with vigorous aeration since they are very sensitive to warmth and to low oxygen tensions.

Background adaptation was marked. After several hours on a black background the fish became generally dark with eleven black saddle-shaped 'parr' markings along the back. Fish on a white ground became very pale and the markings were almost invisible. On changing from a black to a white background paling appeared to be complete in 3 - 5 minutes, but the reverse change took 2 - 4 hours. Neill's results for fish of a similar age at 13.5°C. are shown in Figure 12 (1940). He estimated that paling took 30 minutes and darkening 10 hours. The much shorter estimations made here are probably due to the macroscopic method which cannot follow the later asymptotic stages of the responses. That one change is very much quicker than the other is confirmed and appears to be unique for all the species studied here or described in the literature. It appears certain that nervous control predominates, at least for paling. Neill also stimulated the fish electrically and obtained rapid, reversible paling responses.

The fish were successfully anaesthetised with a 1/20,000 solution of MS222 chilled to 15°C. Microscopic examination showed many small melanophores with larger ones forming the skin pattern as in Phoxinus. High temperature tests were confined to the extremity of the tail to prevent excessive heating of the body. The four fish tested showed complete, reversible, local dispersion of all melanophores at 24 - 26°C., 26 - 28°C., 24 - 28°C.

Figure 12.


Background Reversal in Salmo salar
after Neill, 1940. Mean values for 6 fish.
Melanophore Index.
5.0
4.0
3.0
2.0
1.0
0
1
2
3
4
5
6
7
8
9
10
Time in Hours.

and 24 - 28°C. respectively. Application of small pieces of ice to the skin produced almost complete melanophore aggregation which was reversible on rewarming. All the fish recovered when returned to cool water and were kept in good health for several weeks.

Order Apodes.

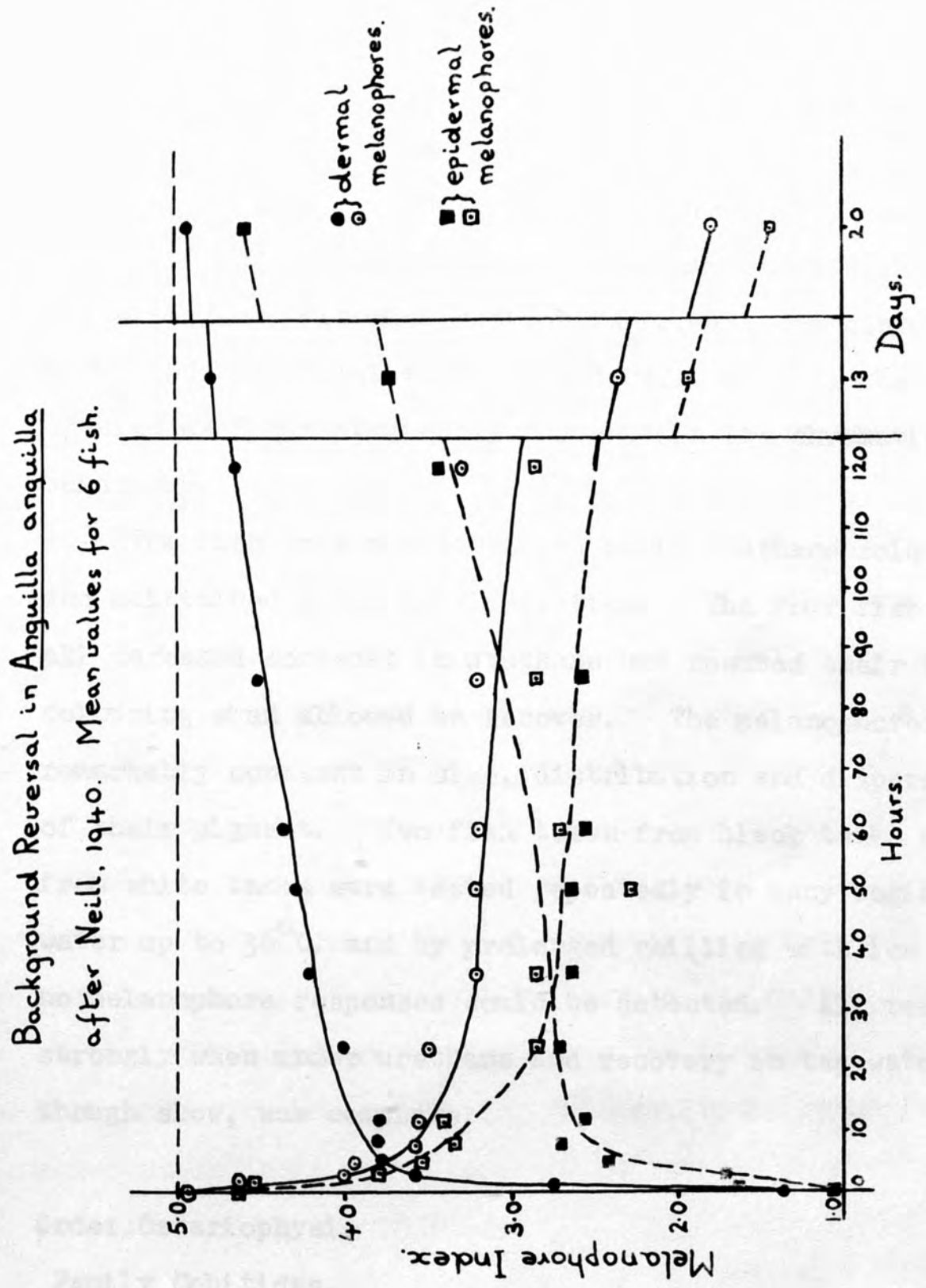
Family Anguillidae.

Anguilla anguilla (L.). The Eel.

Five specimens in the yellow phase, weighing 240 gm. each and 50 cm. long, were purchased from a fishmonger of Billingsgate. They were delivered in a muslin bag but soon recovered in running water and appeared to be perfectly healthy. When placed in black or white tanks, or when these backgrounds were reversed, chromatic adaptation proceeded steadily until the fish were black or pale olive dorsally. Changes took several days and timing of their end-points was not attempted. Response curves produced by Neill (1940) at 8.3°C. ±0.8°C. are redrawn in Figure 13 (similar curves were obtained at 6.7°C. and 21.75°C.). These time relations were corroborated by Waring and Landgrebe (1941), and Waring (1942) applied to them the arguments of Hogben and Slome (1931) for the presence of two opposing humoral agents. (See Chapter Ia).

There is nevertheless some evidence for the presence of chromatic motor nerves in this species although their

Figure 13.


Background Reversal in Anguilla anguilla
after Neill, 1940. Mean values for 6 fish.
dermal melanophores.
epidermal melanophores.
Melanophore Index.
5.0
4.0
3.0
2.0
1.0
Hours.
0
10
20
30
40
50
60
70
80
90
100
110
120
Time.
Days.
13
20

influence in the normal animal is extremely slight. The action of melanophore aggregating nerves in hypophysectomised eels was demonstrated by Waring (1940) and this was confirmed by electrical stimulation. Parker (1948) claims to have discovered dispersing fibres in North American eels by nerve section experiments. All authors agree that such control is normally completely dominated by humoral factors, and Neill regards the species as "physiologically archaic" in its chromatic behaviour.

The fish were anaesthetised in 1% urethane solution and maintained under 0.25% solution. The four fish used all darkened somewhat in urethane but resumed their former colouring when allowed to recover. The melanophores were remarkably constant in size, distribution and dispersion of their pigment. Two fish taken from black tanks and two from white tanks were tested repeatedly in many regions by water up to 36°C. and by prolonged chilling with ice but no melanophore responses could be detected. All respired strongly when under urethane and recovery in tap-water, though slow, was complete.

Order Ostariophysi.

Family Cobitidae.

Nemacheilus barbatulus (L.) The Stone Loach.

A number of specimens 6 - 7 cm. in length were caught

from a stream near Essenden, Hertfordshire. The adaptive response to black and white backgrounds was similar in extent to that of the minnow although patterning of the skin was less marked at all stages. Reversal of backgrounds led to rather variable effects. If the fish were left quietly in shallow dishes, complete adaptation took 12 hours or more, but if slightly excited by occasional movement of the dishes or stirring of the water with a glass rod, adaptation was almost complete in 30 mimutes. This was observed in each of the four specimens tested.

Each fish was then anaesthetised in 0.5% urethane and transferred to the testing table with 0.25% urethane supplied orally. The melanophores were seen to be rather small and very uniform in appearance. Slight variations in the distribution of the melanophores produced the rather indistinct skin pattern. No responses were obtained up to 35°C. or under melting ice, or after the removal of the ice. This was repeated and checked on each specimen. These results confirm earlier experiments on a single specimen obtained from the River Chess at Rickmansworth, Hertfordshire. In all cases breathing under anaesthesia was strong and subsequent recovery complete.

Family Cyprinidae.

Phoxinus phoxinus (L.). The Minnow

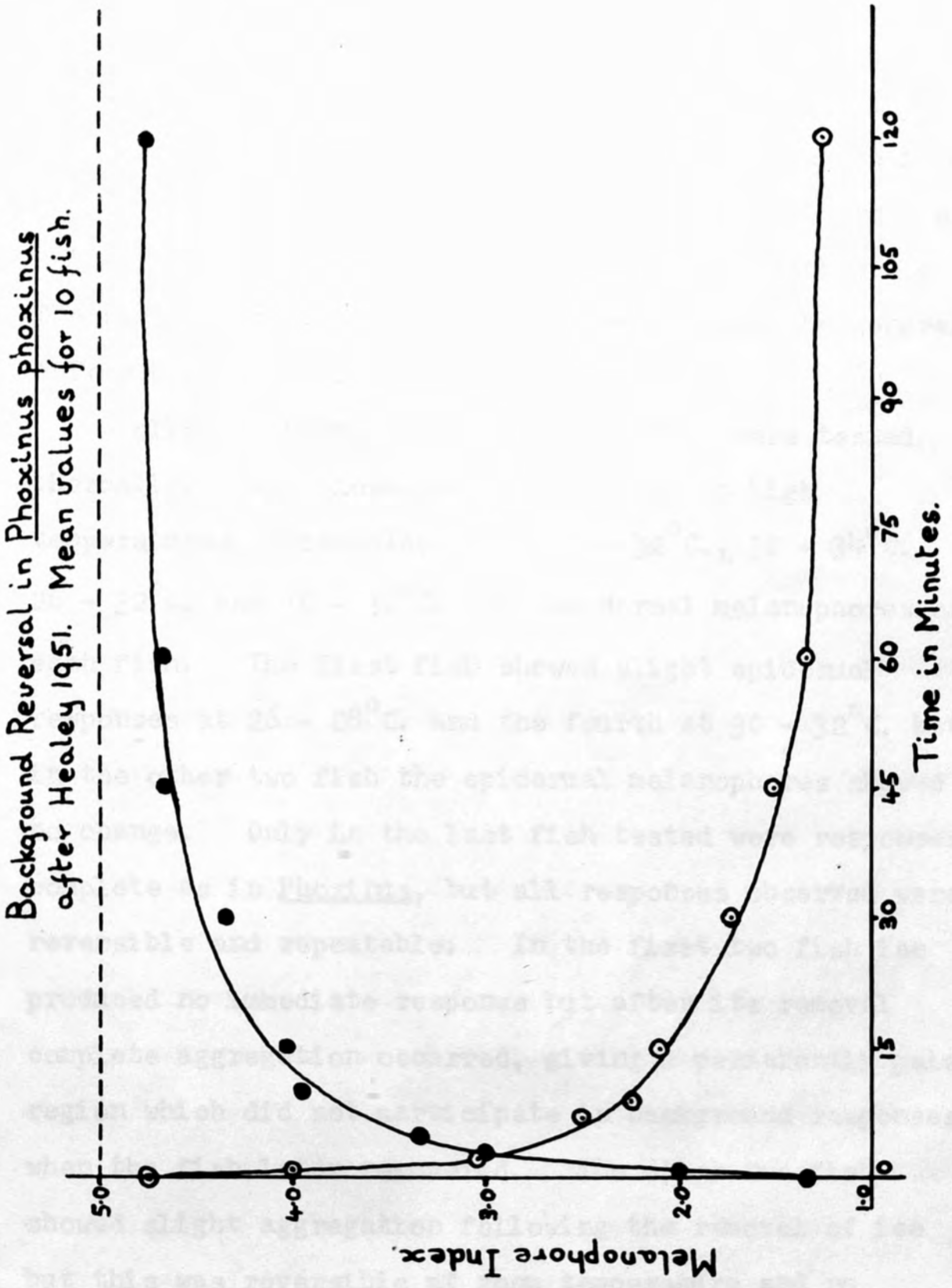
The background reversal tests as used with other species were performed on the minnow in order to compare the results of this method with those obtained microscopically by Healey (1951) and shown in Figure 14 (Hogben and Clark in an unpublished communication to Waring (1942) stated that changes in each direction take one hour). Both paling and darkening appeared to be complete in about 15 minutes although fading of the dark pattern took somewhat longer. This confirms that macroscopic comparison cannot follow the later stages of melanophore response and that the true response times are therefore underestimated by this method. The figures given for other species in this Chapter can only be taken as an approximate guide to the rate of reaction.

Rutilus rutilus (L.). The Roach.

Many fish 7 - 12 cm. in length were caught in May from the primary filter beds at the Metropolitan Water Works, Sunbury, by kind permission of Mr. Ridley, B.Sc. Adaptation to background appeared to be similar in extent to that of the minnow but was achieved rather more quickly, taking only 2 - 3 minutes in either direction.

Dr. A. K. Kent found on the same group of fish (unpublished) that an extract of roach pituitary caused melanophore aggregation in dark adapted roach and minnows. Thus as in the minnow a predominantly nervous control

Figure 14.


Background Reversal in Phoxinus phoxinus
after Healey 1951. Mean values for 10 fish.
Melanophore Index.
5·0
4·0
3·0
2·0
1·0
0
15
30
45
60
75
90
105
120
Time in Minutes.

mechanism may be supported by humoral control from the hypophysis.

Anaesthesia in 0.5% urethane followed by 0.25% solution proved satisfactory. Microscopic examination showed an even layer of dermal melanophores with rows of epidermal melanophores along the margins of each scale. The latter formed a reticulate pattern above the general colour of the skin.

Following normal procedure four fish were tested thermally. All showed some dispersion at high temperatures, thresholds being 30 - 32°C., 30 - 34°C., 26 - 32°C. and 30 - 32°C. for the dermal melanophores of each fish. The first fish showed slight epidermal responses at 26 - 28°C. and the fourth at 30 - 32°C. but in the other two fish the epidermal melanophores showed no change. Only in the last fish tested were responses complete as in Phoxinus, but all responses observed were reversible and repeatable. In the first two fish ice produced no immediate response but after its removal complete aggregation occurred, giving a permanently pale region which did not participate in background responses when the fish later recovered. The other two fish showed slight aggregation following the removal of ice but this was reversible at room temperature and no permanent damage was caused.

Further experiments on four other fish with the

graded cold jet apparatus of Figure 11 produced no response between 18°C. and 2°C. Thus the roach appears to show rather weak high temperature responses and a dubious low temperature effect. 2°C. is insufficient, but 0°C. may cause irreversible damage to the chromatic system.

Gobio gobio (L.). The Gudgeon.

Several specimens 10 cm. long were caught in the River Colne at Watford. Adaptation and response times appeared to be very similar to those of the minnow, but there was some individual variation, especially in the degree of dark adaptation attained.

Four fish were anaesthetised in turn by 0.5% urethane followed by 0.25% solution. The melanophores were rather small with short thick branches. All were of similar appearance but they were arranged more closely around the edge of each scale and in the darker parts of the skin. These pattern melanophores appeared to be under separate control from the ground-colour cells despite their similar appearance, since marked differences in pigment dispersion between the two groups were sometimes noticed.

No temperature responses of any kind could be evoked despite repeated tests up to 36°C. and under ice. All fish respired strongly and recovered completely when returned to water.

Family Ameiuridae.

Ameiurus melas (Rafinesque). One of the North American Catfish.

A number of 6 - 7 cm. fish were purchased from an aquarium dealer. The extremes of adaptation were dense black and pale olive-green. Darkening was complete in about 30 minutes. Considerable paling occurred within 1 - 1½ hours of being placed on a white background but adaptation was not complete for a much longer period. This time cannot be indicated as the later paling appeared to be influenced by complex factors such as the size of vessel used, whether or not the fish were disturbed, and even the presence of other fish in the vicinity. In 1 litre beakers some individuals never became fully pale and even in large aquaria they often darkened partly when disturbed. This species is normally sluggish by nature when given a sandy or muddy bottom but in clean glass tanks, especially white ones, they appear to be unhappy and are continuously active. This state of excitement may interfere with normal colour changes.

Parker (1934b) and Abramowitz (1935) reported that Ameiurus nebulosus (Le Seur) pales completely in 3 - 3½ hours and darkens in one hour. Parker described experiments demonstrating nervous activity and this was confirmed by electrical stimulation experiments by

Wykes (1938). Parker's experiments involving chromatic nerve section in this fish provide a great deal of the available evidence for double innervation of melanophores (discussed in Chapter V). Unfortunately the same species could not be obtained in this country.

0.5% urethane followed by 0.25% solution proved satisfactory for anaesthetisation. The majority of the melanophores were extremely small and evenly distributed in the epidermis. Some larger melanophores were observed in an iridescent dermal layer. No responses to cooling by ice or to warming to 36°C. could be obtained in any of these fish.

Order Microcyprini.

Family Poecilidae.

Lebistes reticulatus (Peters) Regan. The Guppy.

Four female fish, about 4 cm. long, were obtained from an aquarist. All were fully grown and gravid. The fish were isolated in 1 litre beakers which were transferred between a white thermostat tank and a black one. Both baths were maintained at 25 - 27°C. to which temperature the fish were accustomed. Adaptation was marked although a dark mesh pattern outlining the large scales was distinct at all times (as indicated by the specific name). During the experiments one fish gave birth to about twenty young which immediately began to

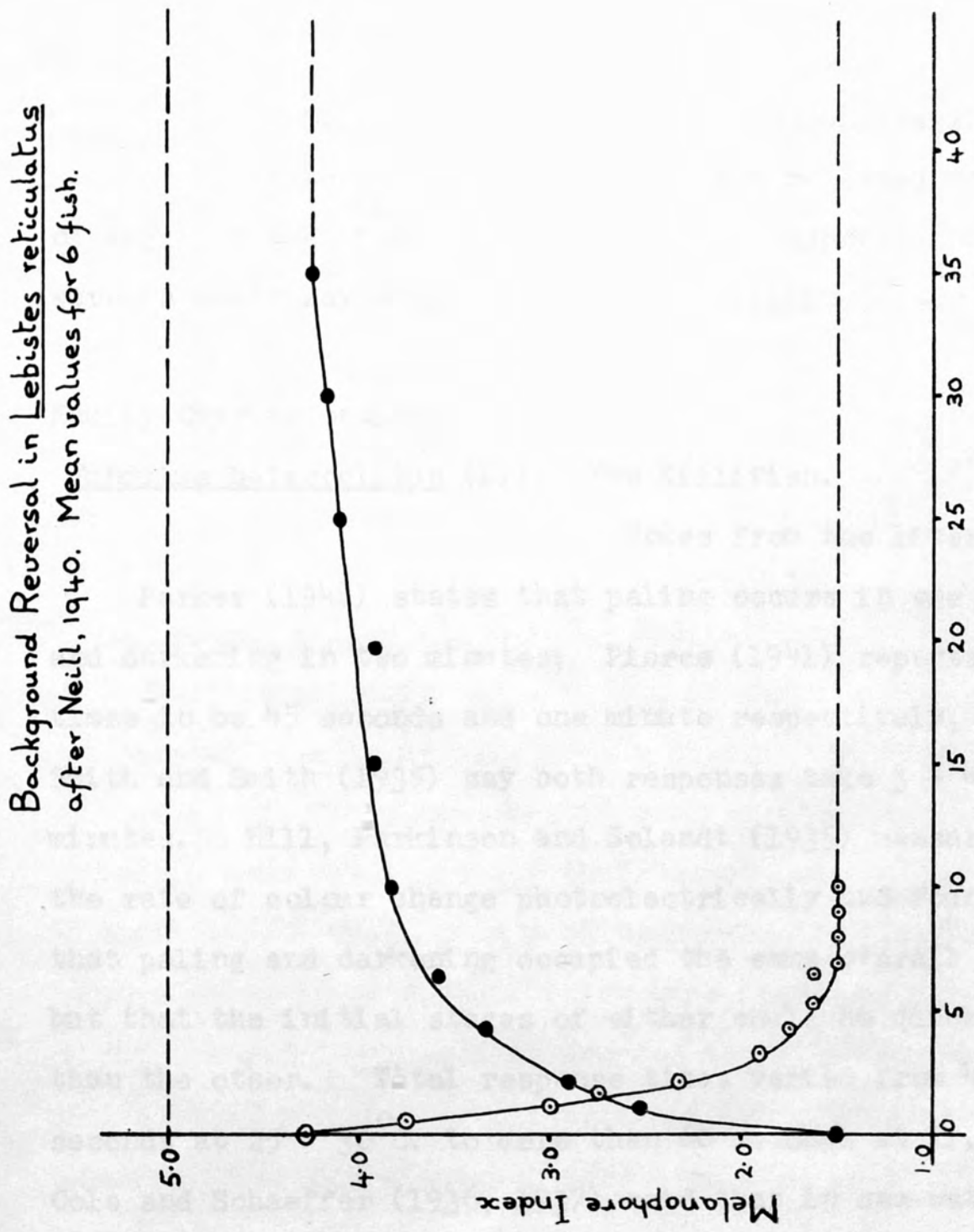
change colour in response to the background. Microscopical examination of these baby fish in a Petri dish showed their response times to be similar to those of the adults.

This is the third species studied by Neill (1940) and his results taken at 16°C. (Figure 15) show a very slight but protracted late darkening phase. Apart from this the results are confirmatory; complete paling was estimated to take 5 mimutes and darkening about 8 - 10 mimutes. Nervous control of the melanophores was established by Neill on the basis of electrical excitation.

For the temperature tests a small room was warmed to 26°C. together with the anaesthetic solutions. 1% urethane was required to produce a coma but 0.25% solution maintained this state satisfactorily. The melanophores were small in the centre of each scale with a line of larger ones peripherally. This line consisted of a single row on the sides of the fish but dorsally it was two or three cells wide. The skin and flesh were translucent, giving a strange appearance to the pale fish.

High temperatures produced almost complete pigment dispersion in all melanophores of each fish. Threshold temperatures, as expected for a tropical fish, were rather high, being 36 - 40°C., 36 - 40°C., 32 - 38°C and 36 - 40°C. respectively. These responses were local, fully

Figure 15.



Background Reversal in Lebistes reticulatus after Neill, 1940. Mean values for 6 fish.

reversible and repeatable. Small pieces of ice were laid upon the tail for a short time only, but all produced pronounced aggregation which was fully reversible at 26°C. In one case aggregation was complete for the pattern melanophores only but in another all melanophores gave the maximal response. These tests were not repeated for fear of injuring the fish. The temperature responses observed were in every way similar to those of Phoxinus.

Family Cyprinodontidae.

Fundulus heteroclitus (L.). The Killifish.

Notes from the literature.

Parker (1948) states that paling occurs in one minute and darkening in two minutes; Pierce (1941) reports these times to be 45 seconds and one minute respectively, while Smith and Smith (1935) say both responses take 3 - 4 minutes. Hill, Parkinson and Solandt (1935) measured the rate of colour change photoelectrically and found that paling and darkening occupied the same overall time but that the initial stages of either could be quicker than the other. Total response times varied from 40 seconds at 25 - 30°C. to more than 60 seconds at 11.5°C. Cole and Schaeffer (1936, 1937) said that in sea-water paling occurs 2.8 times more rapidly than darkening. In fresh-water paling was slightly slower than darkening and both reactions were much slower than in sea-water of

the same temperature. Mills (1932, vide Parker, 1948) claimed to have demonstrated the presence of both aggregating and dispersing nerve fibres in this species on the basis of nerve section experiments (see Chapter V). The killifish seems to be the antithesis of Anguilla since humoral control has been shown to be very weak (Mathews, 1933). The experiments of Smith (1928) on the temperature responses of this species have been fully described in Chapter Id.

Order Anacanthini.

Family Gadidae.

Pollachius pollachius L. The Pollack.

Four individuals, 12 - 19 cm. in length, were caught on rod and line by members of the Plymouth Angling Society. All were kept for a few days in running sea-water and appeared none the worse for their treatment. Colour changes were marked. On a white background this fish became olive-green dorsally with blue iridescent patches along the back. When dark-adapted the fish were dark brown in colour and the iridescent patches were obscured. Changes in either direction were completed in about three minutes.

Anaesthesia was obtained by a 1% solution of urethane in sea-water, followed by a similar 0.25% solution. This produced some darkening of the fish.

Respiratory movements were strong and subsequent recovery in running sea-water was complete in about 30 minutes. Dorsally the melanophores were so crowded that they were difficult to distinguish individually in the dark fish. There appeared to be at least two types present, small 'chunky' dermal melanophores and large 'straggly' epidermal ones with few branches. Laterally over a silvery reflective layer were found large, well spaced melanophores. Patches of erythrophores were also observed and other types of melanophore may well be present.

No chromatophore responses could be obtained in any region by warming to 35°C. or by chilling with sea-water ice.

Experiments were also attempted with the related species Gadus luscus L., the Pouting or Bib. This fish showed rapid colour changes in the tanks of the Public Aquarium but freshly caught specimens proved extremely difficult to keep alive. A few survived for a few days but none of them withstood the handling necessary for the background reversal experiments.

Onos mustelus (L.). The five-bearded Rockling.

Five specimens, 10 - 13 cm. in length were provided at Plymouth. When placed individually in large jars upon black or white backgrounds no adaptation could be

detected after several hours, and slight individual variations of colour were maintained. At a later date microscopic observations by Dr. Kent revealed very slight changes in two specimens after several days on different backgrounds. On black the melanophore index was 5.0 (fully dispersed) and on white a patchy variation between 4.0 and 5.0.

Coma was slowly induced in a 1% solution of urethane in sea-water and maintained by a 0.25% solution. The melanophores were seen to form a very dense layer of pigment in the dermis but occasional 'straggly' melanophores were found in the epidermis. No change in either microscopic or macroscopic appearance could be detected at temperatures up to 34°C. or when chilled with ice.

One fish, on recovery in sea-water, became a pale mottled red colour for several hours although the other fishes were unaffected. Microscopical examination of this fish showed that all the melanophores were almost completely aggregated, revealing a light brown ground colour with some erythrophores. Temperature tests were repeated on the fish in this state but no responses could be obtained. Later it was found that the condition can be produced by injections of pituitary extract (from Gadus luscus) and Dr. Kent (unpublished) has confirmed that the species is very sensitive to acid

extracts of pituitary from Pleuronectes platessa. Thus the melanophores are physiologically active and the fish appears to possess a suitable activating agent although this is hardly used for background adaptation.

Later one pale fish was spinal sectioned at an anterior level but no further darkening ensued. Post mortem examination showed complete section between the 2nd and 3rd vertebrae. It seems fairly certain that chromatic nerves do not exist in this species.

Order Beri comorphi.

Family Holocentridae.

Holocentrus ascensionalis (Osbeck). The Squirrel Fish.

Notes from the literature.

Smith and Smith (1935) report that this species effects colour changes mainly by the action of erythrophores. On a black background the fish becomes bright red and on a white background very pale. Changes are extremely rapid, paling being complete in five seconds while darkening takes ten seconds. Parker (1948) reports these times as 4.5 - 8.5 seconds and 16.5 - 22.0 seconds respectively. This evidence of nervous control was supported by the earlier workers by nerve section experiments, since denervated areas became dark red. Peripheral section in the skin was repaired by regeneration in 10 - 15 days. After autonomic chain section normal function was restored

remarkably quickly and it was believed that an alternative pathway became established since regeneration did not occur at this site. The authors maintain that the chromatic tracts follow the same paths as the melanophore motor fibres in Phoxinus (Figure 1a).

A jet of sea-water at 35°C. produced strong local reddening of the intact fish but denervated regions were paled. Conversely a cold jet (temperature not stated) paled normal fish but reddened denervated ones. Xanthophore responses were said to be similar but more sluggish. Thus the temperature response was shown to be exactly the same as for the melanophores of Fundulus. The authors also state that similar responses could not be obtained from the erythrophores of Phoxinus or of Trigla spp. This can be confirmed for Phoxinus and Giersberg (1930) states that the erythrophores and xanthophores of this fish are subject only to humoral control. v.Frisch (1912) however maintained that temperature responses are shown by the innervated erythrophores of Trigla lineata and T. corax.

Order Percomorphi.

Family Sparidae.

Pagellus centrodontus (de la Roche). The Common Sea-bream.

A single 17 cm. specimen was obtained at Plymouth. On a white background it became light brown with a faint

striped pattern dorsally, shading laterally to a silver belly. In a black tank the fish became almost black dorsally with no pattern visible, while the sides assumed a graded dark brown colour. Changes between extremes appeared to be accomplished in less than three minutes in either direction.

Anaesthetisation in 1% urethane solution in sea-water was slow and difficult to control when later maintained under 0.25% solution. At least two types of melanophore were present; 'straggly' ones with a few thin branches and 'chunky' ones with shorter, thicker branches. All the melanophores were fairly large and spaced well apart so that observation was easy. High temperature responses were shown by all melanophores between 22 - 28°C. and sea-water ice produced aggregation. When respiratory movements stopped under urethane no further responses were obtained. The fish died some time later and no more specimens could be obtained due to the January gales.

Family Centrarchidae.

Eupomotis gibbosus L. The North American Sunfish.

Two specimens (one male and one female) were obtained from an aquarium dealer and kept as pets for some years. At the time of testing each was about 9 cm. in length. On a white background the fish exhibited a light brown reticular pattern, shaded dorsoventrally, with a number of

rather darker vertical stripes on the back. On a black background this pattern was almost completely obscured by a uniform black colour dorsally shading to a silver belly. Reversal was accomplished with great rapidity; the changes appeared to be complete in 3 - 4 minutes but their major part was accomplished in 30 seconds.

Both fish were extremely resistant to urethane, requiring more than 20 minutes in a 1% solution to become quiescent. When placed on the wax dish with an oral supply of 0.25% urethane both lay quietly and breathed strongly. The reticular pattern was seen to be formed by a group of melanophores at the centre of each scale. The edges of the large scales were free of melanophores and a few scales (randomly sited) were completely free from melanophores. Beneath the scales there was a uniform layer of densely packed iridescent granules which did not appear to be confined to iridophore cells. The whole pattern was thus the reverse of that observed in Crenilabrus melops (see below).

No chromatic responses could be obtained at temperatures up to 35°C., or under ice, or after removing the ice. The tests were thoroughly repeated and confirmed. Both fish recovered completely on return to their tanks.

Family Labridae.

Labrus bergylta, Ascanius. The Ballan Wrasse.

Two immature specimens 7 cm. and 10 cm. in length were obtained at Plymouth. Both were uniformly dark green in colour dorsally and background adaptation was slight. Changes appeared to occur for about 15 minutes but accurate estimates were impossible due to the weakness of the response. Urethane anaesthesia was used as for other small fishes. The melanophores appeared to be of one type, uniformly scattered but at varying depths in the skin. The chromatophores of this species were described by Pouchet (1876). No responses to temperature could be obtained up to 35°C. or under sea-water ice. Later both fish were successfully spinal-sectioned at an anterior level. This produced immediate darkening in both cases but neither fish survived more than a few hours. It is probable that some degree of nervous control exists in this species despite the barely perceptible background adaptation. Experiments on adult fish which possess different colouring and pattern might be interesting.

Crenilabrus melops. (L.). The Corkwing Wrasse.

Four small specimens were obtained at Plymouth, two 11 cm. long and two 7 cm. long. On a white background these fish showed a delicate light-green and sandy-brown pattern which was almost obscured by black when dark-adapted. The marked adaptive states could be reversed

in 5 minutes. The fish were anaesthetised as before in 1% and 0.25% urethane. Each scale had at its centre a patch of iridophores and the surrounding melanophores, which may obscure them when dispersed, were easily observed. Other melanophores at deeper levels were difficult to distinguish in outline.

All superficial melanophores showed complete dispersion in each fish at high temperatures, the measured thresholds being 20 - 26°C., 18 - 22°C., 18 - 26°C. and 22 - 26°C. respectively. The responses were strictly local, reversible and fully repeatable. The deeper melanophores appeared to be unresponsive. Responses to low temperatures are described in Chapter IIIf.

Crenilabrus pavo, Brunn. Notes from the literature.

v.Frisch (1912) stated that temperature responses shown by this Mediterranean species were similar to those of the minnow. The direction of the reactions was reversed when the chromatic motor tracts were interrupted.

Ctenolabrus rupestris (L.). The Rock Wrasse or Gold Sinny.

Four specimens, 5 - 10 cm. in length, were obtained at Plymouth. On a white background all became a delicate pearly-pink colour but on a black background there was considerable individual variation in the colour

attained. Each fish darkened to a greater or lesser extent and in three specimens a number of broad vertical bars of darker colour became visible. These changes, while clear to the eye, did not involve darkening beyond a 'khaki' colour. Microscopical examination under urethane anaesthesia showed that the melanophores were evenly but sparingly distributed above a pink reflecting layer and were more numberous in the darker individuals. Erythrophores outnumbered melanophores by about two to one. Changes in colour were completed in about three minutes in either direction.

Temperature responses were observed in all melanophores but not in the erythrophores. High temperature responses occurred at 22 - 28°C., 18 - 22°C., 20 - 26°C. and 18 - 24°C. Again it was found that no responses could be obtained during suspension of respiratory movements although normal responses returned on resuscitation. Application of sea-water ice for a few minutes produced an unusual response; the branches of each melanophore maintained their former extent although some pigment appeared to be withdrawn evenly in each cell. Some seconds after the removal of the ice, however, complete melanophore aggregation occurred. This was reversible on further approaching room temperature. The sequence was observed in each of the four fish tested. The phenomenon was further investigated

as described in Chapter IIIf and two possible explanations are put forward in Chapter IIIg.

Family Gobiidae.

Gobius flavescens Fabricius (=G. ruthensparri Euphrasen).

The Spotted Goby.

Six specimens, each 5 cm. long, were obtained at Plymouth. Unlike most members of the family this species swims actively in the water and seldom rests on the bottom. Colour reversal is also quicker than in most gobies. White-adaptation occurred in 2 - 3 minutes and dark-adaptation in 3 - 4 minutes with slight individual variation. Changes were marked in extent by almost complete fading or intensification of the complex but very attractive pattern. Microscopical examination under urethane showed that at least four types of melanophore were present:-

1. large epidermal cells on the dark saddle-markings and around the edge of each scale;
2. small epidermal melanophores ventrally and laterally;
3. large epidermal melanophores generally distributed on the back; and
4. very large dermal cells on the sides and back.

Under urethane the fish darkened considerably, mainly by complete dispersion of type (1) and these melanophores remained unresponsive to temperature changes. On raising

the temperature the other groups dispersed their pigment in turn until complete darkening occurred at 24°C. All recovered in turn by 17°C. and the response was completely reversible. Ice produced considerable aggregation in types (3) and (4) only. The small melanophores were unaffected but a pale region was easily distinguishable macroscopically. The effect was slowly reversed on being allowed to return to room temperature. All four fish tested showed remarkable conformity of response, even to the temperatures involved, and all recovered completely when replaced in sea-water.

Gobius minutus Pallas. The Common Goby.

Six fish, 5 - 6 cm. in length, were obtained at Plymouth. Only very slight, quick colour changes could be distinguished and no further adaptation occurred after prolonged exposure to either background. Dr. Kent (unpublished) in April of the following year reported that distinct changes were visible microscopically, taking 20 minutes to darken and 45 - 60 minutes to pale. Examination under urethane anaesthesia (1% and 0.25%) showed the melanophores to be very variable in size and degree of dispersion. No temperature responses could be observed in any melanophores up to 30°C. or under sea-water ice. The fish withstood this treatment well and recovered very quickly in sea-water.

Later two fish were spinal-sectioned anteriorly and showed extreme darkening which persisted for several days on a white background, in marked contrast to unoperated fish on a black background. Post mortem dissection confirmed complete section of the spinal cord between the 2nd and 3rd vertebrae in each case. It thus seems likely that melanophore motor-nerves are present but the lack of marked background adaptation at that time is puzzling.

Family Callionymidae.

Callionymus lyra L. The Dragonet.

Twelve specimens were made available at Plymouth; six were immature fish 6 - 9 cm. in length, two were adult females 18 cm. long and four were adult males 22 cm. long. Although random fluctuations in intensity of pattern occurred, no responses to background colour could be distinguished in any of these fish. Spinal section at an anterior level in two of the smaller specimens produced no darkening.

This species was investigated by Pouchet (1876) who figures dark and light-adapted specimens and also dark patches produced by section of each branch of the trigeminal nerve. The absence of adaptation in the Plymouth fishes was later confirmed by Dr. Kent (unpublished) who also reported no response to peripheral nerve section or to large doses of pituitary extracts

from other teleosts. Perhaps marked geographical differences exist within the species and a wider survey of the chromatic responses would be interesting.

All six specimens were examined for temperature responses under urethane. Temperatures up to 36°C. and cooling by sea-water ice produced no effect in any melanophores. Respiratory movements were strong and all the fish recovered quickly in sea-water.

Family Blennidae.

Blennius gattorugine L. The Tompot Blenny.

A single 12 cm. specimen obtained at Plymouth, showed a very clearly defined pattern of large brown markings. On black backgrounds the whole fish darkened considerably and the pattern was somewhat obscured but on a white background the pattern became more distinct, revealing light-brown patches on a very pale ground colour. Changes in either direction (judged without a control) appeared to take about five minutes. This was later confirmed with three other specimens by Dr. Kent.

Microscopical examination under urethane revealed large distinct melanophores, well spaced apart in the pale regions but densely packed in the darker markings. Local heating of the skin produced complete dispersion in both regions over the range 16 - 22°C. This was reversible and repeatable. Application of ice caused

some aggregation but many melanophores became completely aggregated only after the ice was removed. On further warming towards room temperature all melanophores recovered their former condition. The fish recovered normally in sea-water.

Blennius pholis L. The Shanny.

Five specimens, 5 - 12 cm. in length, were obtained at Plymouth. Background adaptation was the most spectacular in extent found in any species of teleost (this had earlier been observed in 'tame' specimens kept at Aberystwyth). On black backgrounds they became intensely black and on white a very pale green colour with a faint brownish pattern (due to free pigment in the skin) and iridescent spots. In a poor light these fish were extremely difficult to see in thick glass aquaria painted on the outside, where the glass added a greenish tinge to the paint. The tests on all species however were performed with backgrounds presented inside the aquaria, except for clear beakers used to hold smaller specimens. These beakers were placed in coloured trays with a few inches of water to prevent reflection from below. Colour changes of the Shanny appeared to be complete in about 5 minutes in either direction although there was some individual variation. Marked colour changes were also produced by 'psychic' conditions when frightened, handled or fed.

Microscopical examination under urethane anaesthesia showed a wide variety of melanophores in a range of dispersive states. Classification of these cells would appear to require detailed examination of their structure, position and responses. They were densely packed in most parts of the skin but spaced further apart in small patches. Dr. Kent found that section of the pectoral fin rays caused dispersion in the denervated band.

Temperature responses were slow but distinct. Total dispersion in each case occurred at 24°C. but required 2 - 3 minutes to be effected. Threshold measurements were therefore made by small adjustments of the jet temperature with long pauses between. The values obtained were 16 - 24°C., 17 - 24°C., 22 - 24°C. and 17 - 24°C. for the four fish tested. In no other species were slow responses of this type seen, the response usually being practically instantaneous. The widely spread low 'threshold' temperatures for this species and B.gattorugine were also unusual. The application of ice had variable effects. Complete aggregation occurred in one fish, partial aggregation was followed by further (reversible) aggregation after removal of the ice in two others and a complete lack of any response was found in the fourth.

This species can be recommended for further study for many reasons. It proved very easy to control under urethane anaesthesia and recovery was always complete.

Order Scleroparei.

Family Triglidae.

Trigla lucerna L. The Saphirine Gurnard.

Three specimens 27 - 30 cm. in length, were obtained from the trawler at Plymouth. The general colour was red and small erythrophores were found to be the predominant chromatophore, with sparsely scattered large melanophores. Colour changes between red and pink in response to black and white backgrounds appeared to be extremely quick so that comparison of dark and light-adapted fish on the same background was difficult. The responses did not however involve complete aggregation or dispersion of the erythrophores. Microscopical observation of the erythrophores was found to be greatly assisted by a green filter (kindly loaned by Dr. Alexandrovitch) placed in front of the illuminating lamp. MS222 was used as an anaesthetic and breathing was strong. In none of the three fish could any response be seen to temperatures up to 32°C. or to cooling by sea-water ice. All the fish recovered quickly on return to sea-water.

Trigla cuculus L. The Red Gurnard.

A single 33 cm. specimen was caught with the T.lucerna above. Rather a deeper red in colour, its colour changes and skin appearance were very similar to the other species. Again neither erythrophores nor melanophores showed any

temperature responses. The fish recovered fully in sea-water.

(Trigla corax Bonap. (=T.lucerna L.) The Saphirine Gurnard.
(
(Trigla lineata Gmelin. The Streaked Gurnard. Notes from the literature.

v.Frisch (1912) stated that in both these species the erythrophores are innervated in a similar manner to the melanophores of Phoxinus and show similar temperature responses. In denervated regions the response was reversed. This is in conflict with the results obtained at Plymouth. Smith and Smith (1935) however maintain that the erythrophores of Trigla spp. (sic), Scorpaena ustulata and Phoxinus phoxinus show no temperature responses. The same authors (1934) stated that extracts of the pituitary gland of Trigla spp. caused pronounced paling when injected into Scorpaena ustulata.

Family Cottidae.

Cottus bubalis Euphrasen. The Long-spined Bullhead.

Four specimens, 6 - 10 cm. in length, were obtained at Plymouth. Coloration was generally dark with a number of irregular pale bands dorsally on the body and with white underparts. This disruptive effect was increased by an irregular outline and mottled fin patterns. Colour changes appeared to be complex. The limited paler areas

showed quick responses to background colour with a slower final phase of adaptation. Thus on a white background the skin became very pale in 1 - 2 minutes but after an hour had become remarkably white. Darkening appeared to be similar though the dark hue of the rest of the skin was never equalled. Thus the skin pattern was intensified on a pale background but was still distinct on a dark one. The dark skin of the head became distinctly paler after 1 - 2 hours on a white background but redarkened after a variable interval. Redarkening also occurred if the fish were disturbed and paling did not then recur during prolonged exposure to a white background. Further experiments on this region are described in Chapter V. The dark regions of the body were never seen to pale appreciably.

Anaesthetisation with 1% urethane followed by 0.25% solution was successful (the latter was supplied through a rubber tube to suit the large mouth) and the fish remained upright on the wax dish. The dark parts of the body were seen to contain very closely packed melanophores while the pale areas had only sparse melanophores with many erythrophores. Despite extensive experiments involving quick and slow temperature changes between 35°C. and chilling with sea-water ice in both the dark and pale regions of the body (not on the head) no thermal responses of any kind were observed. All the fish endured the

experiments well and recovered normally in sea-water.

Cottus gobio L. The Freshwater Bullhead or Miller's Thumb.

Six 10 cm. fish were netted from the River Chess near Rickmansworth, Hertfordshire. Skin patterns in these fish were not so marked as in C.bubalis but a similar disruptive arrangement of irregular dark and light areas was present. The fish were extremely difficult to detect in their normal environment of pebbly streams. The extent and speed of colour change varied considerably between individuals. Some darkened and paled over the whole body while in others the darker regions of the skin appeared more or less incapable of paling, as observed for C.bubalis. Thus on a white background some individuals became generally pale while others became strongly patterned. Times for adaptation in either direction appeared to be about the same for each individual but varied between 10 and 30 minutes for different fish. Similar results had been obtained at earlier dates on several specimens caught at Hardwick Park, Derbyshire. Miller and Kennedy (1946) reported that marked colour changes could be observed in the freshwater sculpin (Cottus cognatus) of North America. No details were given except that complete paling of the pattern could be accomplished in 2 - 3½ minutes on a white background.

Microscopic examination after anaesthetisation by 0.5% and 0.25% urethane showed a greater density of melanophores in the darker regions of the skin, and that these melanophores were generally more dispersed than in the paler areas. In some individuals there was apparently some free melanin beneath the chromatophores of the dark regions.

No temperature responses were observed in any of the melanophores during repeated tests between 0°C. and 36°C. All the fish respired strongly and recovered completely when returned to their tank.

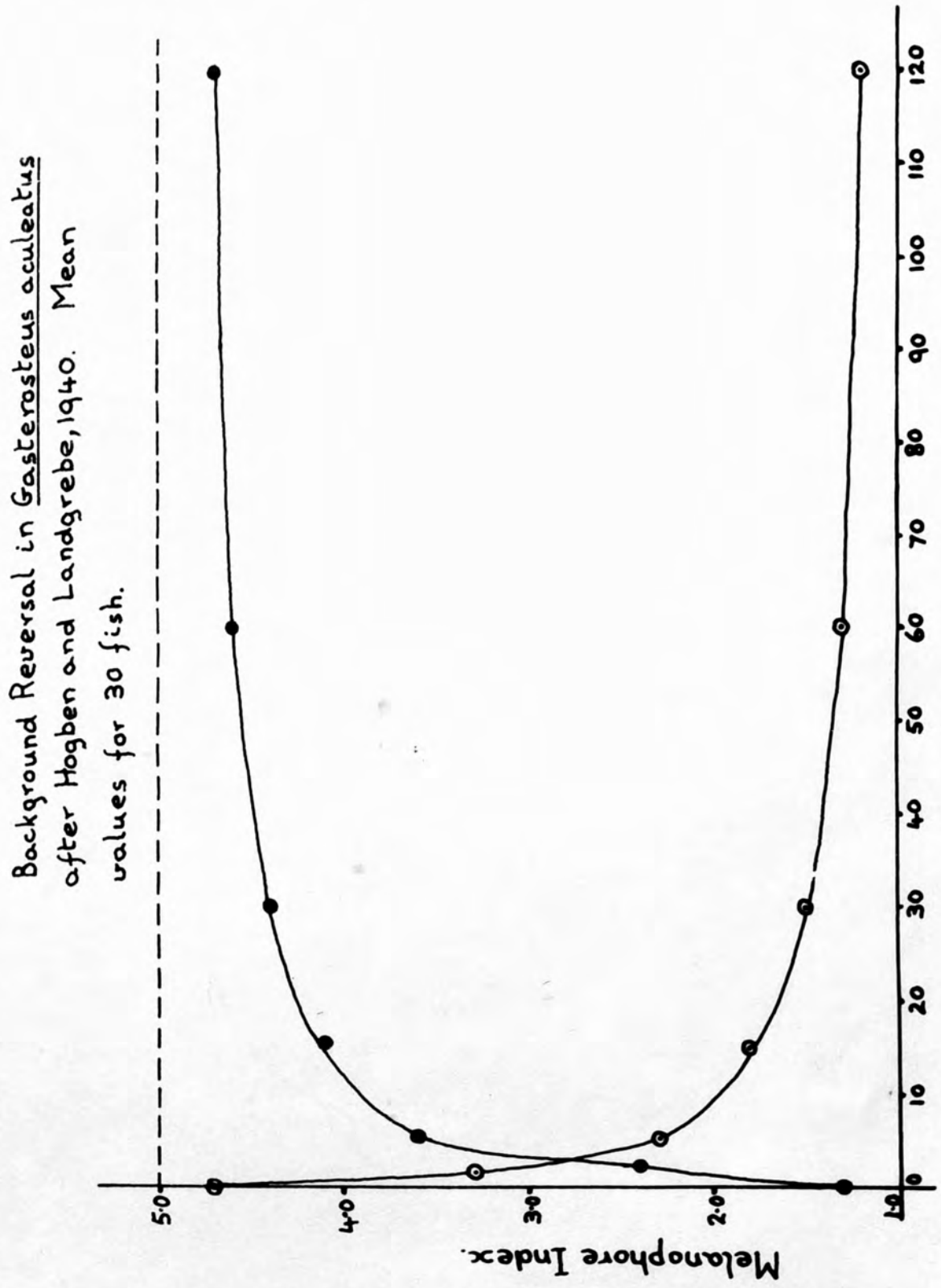
Family Gasterosteidae.

Gasterosteus aculeatus L. The Three Spined Stickleback.

Five specimens, 3.5 - 5.0 cm. in length, were obtained from a dealer in a consignment of minnows from Gravetie, Surrey. The extent of background adaptation appeared to be similar to that of Phoxinus but there were no pattern markings at any stage. Times for background reversal varied somewhat between individuals but were generally similar to those of Phoxinus. This is supported by microscopical observations by Hogben and Landgrebe (1940), Figure 16, and by Griffiths (1948), Figure 17. Hogben and Landgrebe stated that both nervous and humoral control of the melanophores were present.

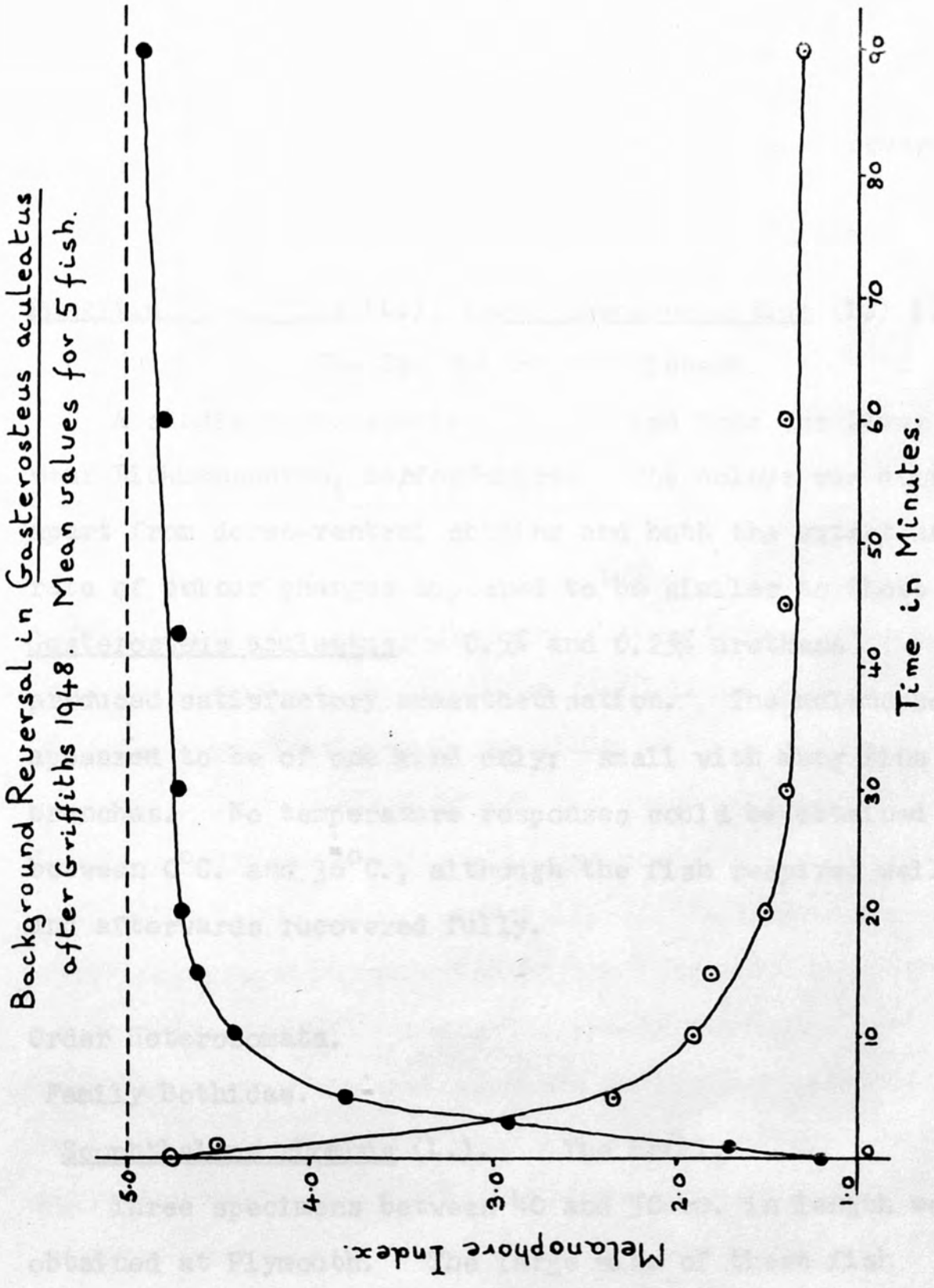
For anaesthetisation 1% urethane was required, followed

Figure 16.



Background Reversal in Gasterosteus aculeatus after Hogben and Landgrebe, 1940. Mean values for 30 fish.

Figure 17.


Background Reversal in Gasterosteus aculeatus
after Griffiths 1948. Mean values for 5 fish.
Melanophore Index.
5·0
4·0
3·0
2·0
1·0
0
10
20
30
40
50
60
70
80
90
Time in Minutes.

by 0.25% as usual. The melanophores were extremely small and became rather dispersed in urethane. No temperature responses could be evoked between 0°C. and 35°C. Respiratory movements were strong and the fish recovered normally in tap-water.

Pungitius pungitius (L.). (=Pygosteus pungitius (L.)).

The Ten Spined Stickleback.

A single 5 cm. specimen was netted from the River Chess, near Rickmansworth, Herfordshire. The colour was uniform apart from dorso-ventral shading and both the extent and rate of colour changes appeared to be similar to those of Gasterosteus aculeatus. 0.5% and 0.25% urethane produced satisfactory anaesthetisation. The melanophores appeared to be of one kind only; small with many fine branches. No temperature responses could be obtained between 0°C. and 36°C., although the fish respired well and afterwards recovered fully.

Order Heterosomata.

Family Bothidae.

Scophthalmus rhombus (L.). The Brill.

Three specimens between 40 and 50 cm. in length were obtained at Plymouth. The large size of these fish rendered background reversal tests difficult. They were eventually placed in large trays of sea-water which were

lined with black or white paper. Reversal appeared to be complete in 30 minutes in either direction. This figure must be treated with caution since the fish, although quiet, were cramped and also because adaptation was not marked. Macroscopic comparison proved difficult without controls (only two trays were available). Schaeffer (1921) reported that colour changes in Rhombus maximus (=Scophthalmus maximus) were very marked but not so rapid as in the Pleuronectidae. Pouchet (1876) first demonstrated the presence of chromatic nerves in Rhombus maximus.

Each fish was set up in turn under urethane anaesthesia. Melanophores of two distinct types were seen to form the blotchy pattern. In the first fish all melanophores dispersed completely and reversibly in regions heated to 20 - 24°C. No further effect was produced up to 30°C. Local cooling by sea-water ice produced incomplete but reversible aggregation, especially of the larger melanophores, although a few of the large cells appeared to be refractory. Towards the end of the experiment respiratory movements stopped and no further responses could be obtained until resuscitation restored the opercular movements. The fish recovered completely in running sea-water.

The second fish stopped respiratory movements before temperature tests could be applied and although revived

artificially the fish had to be returned to the stock tank where it later died. The third fish also stopped respiratory movements and could not be resuscitated. These experiments were performed before MS222 was adopted as an anaesthetic for flatfish and no further specimens could be obtained due to gales.

Family Pleuronectidae.

Pleuronectes platessa L. The Plaice.

Several specimens were available at Plymouth and four fish about 30 cm. long were chosen for testing. These were placed in black and white dishes of sea-water and marked adaptation was observed. On reversal of backgrounds in either direction the fish rapidly became blotchy and even reversal was complete in 3 minutes. Further changes were slow and could not be detected after 2 hours. Some individual variation was noted in the adaptive states attained but the containers were not really suitable for such large fish. Schaeffer (1921) also reported extremely rapid but rather slight responses in this species.

Four fish were anaesthetised in 1% urethane and laid upon a sloping board with 0.25% urethane supplied orally. All the melanophores were similar in appearance and evenly distributed. Complete local dispersion was obtained between 22 - 26°C., 22 - 26°C and 18 - 24°C. in three fish. In the fourth complete dispersion was

present at 28°C. but the resting state at 16°C. was one of considerable dispersion so that the first response was difficult to judge. In each fish recovery was complete at the lower temperature. Almost complete reversible aggregation was produced by chilling with frozen sea-water in the last two fish; this experiment was not performed on the first two. Some difficulty was experienced in controlling respiratory movements under anaesthetic but all recovered when returned to sea-water tanks.

The first two fish were later subjected to spinal nerve section in the manner of Pouchet (1876) on Scophthalmus maximus. A longitudinal incision was made close to the lateral line about midway along the body and a small knife was then used to cut the spinal nerves either dorsally or ventrally for 2 - 3 cm. (the section of each nerve was indicated by a twitch of the associated skeletal muscles). The wound was then sutured and the fish allowed to recover. In each case a dark band formed by total melanophore dispersion appeared after a few minutes, running dorsally or ventrally over the segments concerned. These bands remained distinct for several days especially if the fish were placed upon a white background. This confirmed the nerve section experiments performed by Schaeffer (1921) on this species. Twenty-four hours after the operation the band melanophores were retested for temperature responses. No effect could be obtained in

either case up to 34°C. or by chilling with frozen sea-water, although melanophores immediately outside the band gave normal responses. These results appear to give clear evidence of nervous control in this species and indicate that as in Phoxinus chromatic nerves are involved in the temperature response. Erythrophores of the red spots appeared to be completely inactive in both background and temperature responses.

Limanda limanda (L.). The Dab.

A single 7 cm. specimen was available at Plymouth. Background adaptation was clearly distinguishable macroscopically but estimates of the times involved were difficult since the small size of the fish rendered it almost transparent and the background colour could be seen through it. The following year Dr. Kent (unpublished) estimated from six 20 cm. specimens that reversal in either direction takes about one hour. Spinal nerve section in these fish produced segmental dark bands lasting for about three days. A detailed examination of the colour changes of this species has been described by Hewer (1926) but no times for background reversal were given. Outflow of chromatic nerve-fibres from the spinal cord was found to occur at the level of the sixth vertebra.

Temperature testing under urethane anaesthesia produced total, reversible melanophore dispersion between

22 - 28°C. Chilling by sea-water ice produced almost complete aggregation, reversible at room temperature. The fish recovered completely when returned to its aquarium.

Platichthys flesus (L.). The Flounder.

A single 24 cm. specimen was caught on rod and line by the Plymouth Angling Society and kept for several days in a tank of running sea-water. Background adaptation and reversal appeared to be very similar to those of Pleuronectes platessa. On a white background paling was uneven, producing a blotchy appearance, as also observed in Pleuronectes.

Anaesthetisation was effected by immersion in a 1/12,500 (w/v) solution of MS222 and this same solution was supplied continuously during experiments. Several types of chromatophore were seen to be present (Hewer, 1931). Certain large melanophores and others overlain by white chromatophores showed no temperature responses, nor did the erythrophores. The rather smaller melanophores contributing to the ground colour of the pattern dispersed somewhat when warmed to 35°C. and aggregated completely when chilled. Both these responses were reversible and repeatable. The fish behaved very well under anaesthesia and recovered completely afterwards.

Microstomus kitt (Walbaum). The Merry Sole.

Four 28 cm. specimens were obtained at Plymouth.

These fish refused to lie quietly in the black and white trays used for observing colour changes in other flatfish. Adaptation to white sinks and the almost black stock-tanks appeared to be extremely small in extent, the marbled pattern changing little if at all in appearance. Each fish was anaesthetised in turn in urethane. The melanophores were seen to be all rather small with a very large number of fine radial branches. No responses to temperature could be evoked despite repeated tests between 16 - 32°C. and chilling with frozen sea-water. Respiratory movements were strong and this species proved to be the most easily controlled of all flatfish tested under urethane. Some spinal nerves were cut in each fish as for Pleuronectes, and all four fish recovered from the operation normally. No darkening of the denervated region was observed although the fish were examined frequently for over a week.

Family Soleidae.

Solea solea (L.). The Common Sole.

Four specimens between 34 and 40 cm. in length were available at Plymouth. In black and white trays the fish lay quietly but showed no discernible adaptation for a considerable time. Each was anaesthetised in 1/12,500 MS222 solution which produced some darkening. No responses could be evoked in any fish by temperatures up

to 32°C. or by chilling under ice. Control of respiration proved to be very easy and all the fish recovered well. In two of the specimens spinal nerves were cut ventrally but no darkening of the skin was produced.

Buglossidium luteum (Risso). The Solenette.

Several specimens supplied at Plymouth ranged in length from 5 - 10 cm. (fully grown). No colour changes in response to black or white backgrounds could be observed even after some days. Anaesthesia was produced by 0.5% and 0.25% urethane solutions, which appeared to be quite satisfactory for this species. No temperature responses could be evoked from the melanophores or erythrophores. One fish was successfully spinal sectioned but no darkening was produced and the fish was killed after several days. Post mortem dissection showed that complete section occurred at the level of the 8th vertebra, possibly too far posteriorly. Spinal nerve section in one case produced darkening of the whole body posterior to the point of section. This was traced in subsequent dissection to damage to the aorta. In another case ventral nerve section produced a ventral dark band which persisted for only three hours. No circulatory damage could be found in this specimen. The case for chromatic control must therefore remain undecided at present. The identification of this species was later confirmed by

Dr. D. W. Tucker of the British Museum (Natural History).

c. Discussion.

The results described in this Chapter are summarised in Table I. Thirty-one species have been newly investigated and experiments on two more are repeated. Information is now available on thirty-seven species of twenty-one families and nine orders. Nervous control of chromatophores appears to be present in thirty-one of these species and may be present in others which did not show quick background adaptation. Temperature responses similar to those of the minnow were found in nineteen species (although V.Frisch's observations on Trigla corax could not be substantiated) of twelve families and seven orders, each of which shows strong evidence for nervous control of colour change. In no case was the temperature response observed in a fish with sluggish colour change. Further, no response to extreme temperatures other than that seen in intact minnows was ever observed for any single melanophore. The phenomenon first described by v.Frisch was either present or absent and no variations were discovered.

There appears to be little taxonomic significance in the distribution of these results. Six species of three families of the Order Scleroparei which were investigated

here all showed evidence of nervous control with no temperature response, but two species of Trigla (one of which was repeated here) were stated by v.Frisch to show normal temperature responses. A more complete survey of the Triglidae would be interesting. The families Cyprinidae, Labridae, Gobiidae and Pleuronectidae contain some members with, and other members without the response. The families Gadidae and Pleuronectidae also contain members with clear evidence of nervous control and one member in which this could not be demonstrated.

No intraspecific discrepancies were observed except for slight variations in the low temperature response where temperatures could not be accurately controlled, for example in Rutilus rutilus, Lebistes reticulatus and Blennius pholis. On the other hand observations by Dr. Kent and myself do not accord with those of Pouchet (1876) on the adaptation of Callionymus lyra.

In general these results confirm the dependence of the temperature response on the presence of chromatic nerves, but indicate that the phenomenon is not a necessary corollary of nervous control. The absence of any other temperature response and the lack of taxonomic significance appear to weigh against a theory involving the evolution of complex thermosensory pathways. The absence of response in fish in which chromatic nerve-fibres were probably present but normally ineffective may also favour the

peripheral interference theory.

The dependence of the response upon normal respiratory rhythm has also been confirmed by accident in Pagellus centrodontus, Ctenolabrus rupestris, Scophthalmus rhombus and Pleuronectes platessa. In all cases the responses reappeared when the fish was revived to the point where respiratory movements were resumed.

The one new effect noted was the occurrence or completion of the low temperature response only after the removal of chilling ice, regardless of the duration of its application. This was observed in some or all specimens of Rutilus rutilus, Ctenolabrus rupestris, Blennius gattorugine and Blennius pholis. Except for two specimens pf Rutilus rutilus this reaction was reversed on allowing the tissues to warm further towards room temperature, and was then repeatable. Two possible explanations of these effects are given in Chapter IIIg.

TABLE I

Classification of Species	Chromatic Innervation	Temperature Responses
Order Isospondyli, Family Salmonidae, *Salmo salar* L.	Present. Confirms Neill (1940).	Present.
Order Apodes, Family Anguillidae, *Anguilla anguilla* (L.).	Absent or ineffective. Confirms Neill (1940), etc.	Absent.
Order Ostariophysi, Family Cobitidae, *Nemacheilus barbatulus* (L.).	Present.	Absent.
Family Cyprinidae, *Phoxinus phoxinus* (L.).	Present. Confirms v. Frisch (1911a), etc.	Present. Confirms v. Frisch (1911b), etc.
Rutilus rutilus (L.).	Present.	Present.
Gobio gobio (L.).	Present.	Absent.
Family Ameiuridae, *Ameiurus melas* (Rafinesque).	Present. Confirms Parker (1934b) etc. on *A. nebulosus*.	Absent.
Order Microcyprinidae, Family Poecilidae, *Lebistes reticulatus* (Peters).	Present. Confirms Neill (1940).	Present.

TABLE I (Continued)

Classification of Species	Chromatic Innervation	Temperature Responses
Family Cyprinodontidae, *Fundulus heteroclitus* (L.).	Present (Parker 1948, etc.)	Present (Smith 1928)
Order Anacanthini, Family Gadidae, *Pollachius pollachius* L.	Present.	Absent.
Onos mustelus (L.).	Absent or ineffective.	Absent.
Order Beric omorphi, Family Holocentridae, *Holocentrus ascensionalis* (Osbeck).	Present (Smith and Smith 1935).	Present (Smith and Smith 1935).
Order Percomorphi, Family Sparidae, *Pagellus centrodontus* (de la Roche)	Present.	Present.
Family Centrarchide, *Eupomotis gibbosus* L.	Present.	Absent.
Family Labridae, *Labrus bergylta* Ascanius.	Present but weak.	Absent.
Crenilabrus melops (L.).	Present.	Present.
Crenilabrus pavo Brunn.	Present (v.Frisch, 1912).	Present (v.Frisch, 1912).

TABLE I (Continued)

Classification of Species	Chromatic Innervation	Temperature Responses
Ctenolabrus rupestris (L.).	Present.	Present.
Family Gobiidae, Gobius flavescens Fabricius.	Present.	Present.
Gobius minutus Pallas.	Present but weak.	Absent.
Family Callionymidae, Callionymus lyra L.	Absent. Contradicts Pouchet (1876)	Absent.
Family Blennidae, Blennius gattorugine L.	Present.	Present.
Blennius pholis L.	Present.	Present.
Order Scleroparei, Family Triglidae, Trigla lucerna L.	Present. Confirms v. Frisch (1912) etc.	Absent. Contradicts v. Frisch (1912).
Trigla cuculus L.	Present.	Absent.
Trigla lineata Gmelin.	Present (v. Frisch, 1912).	Present (v. Frisch, 1912).
Family Cottidae, Cottus bubalis Euphrasen.	Present.	Absent

TABLE I (Continued)

Classification of Species	Chromatic Innervation	Temperature Responses
Order Scleroparei (contd.)		
Cottus gobio L.	Present.	Absent.
Family Gasterosteidae, *Gasterosteus aculeatus* L.	Present. Confirms Hogben and Landgrebe (1940), etc.	Absent.
Pungitius pungitius (L.).	Present.	Absent.
Order Heterosomata, Family Bothidae, *Scophthalmus rhombus* (L.).	Present.	Present.
Family Pleuronectidae, *Pleuronectes platessa* L.	Present. Confirms Schaeffer (1921)	Present.
Limanda limanda (L.).	Present. Confirms Hewer (1926).	Present.
Platichthys flesus (L.).	Present.	Present.
Microstomus kitt (Walbaum).	Absent or inactive.	Absent.
Family Soleidae, *Solea solea* (L.)	Absent or inactive.	Absent.
Buglossidium luteum (Risso).	Absent or inactive.	Absent.

CHAPTER V.

THE ELECTRICAL STIMULATION OF CHROMATIC NERVE FIBRES.

a. Introduction.

The responses of the melanophores of some teleost fishes to extreme temperatures may now be assumed to depend in some way upon the nervous system. A theoretical mechanism has been advanced and the experimental results obtained appear to be consistent with this theory. Certain assumptions have however been made concerning the nature of the nervous control of melanophores, and these will now be examined.

It is probably safe to assume that the melanophores are tonic effectors. All that is known of the autonomic system, of other autonomic effector organs and of the structure and responses of melanophores makes any alternative suggestion very improbable. But whether there is a single tonic motor-supply or a double, opposed and reciprocally-balanced control is by no means certain. By the arguments of Chapter IIf a theory of peripheral interference by extreme temperatures cannot be supported on the basis of a single innervation. On the other hand if double innervation can be demonstrated the theory provides a feasible explanation of the results obtained so far. Furthermore independent stimulation of the two systems would allow the blocking temperatures of each to be

measured directly and provide a conclusive test of the peripheral interference theory. The following investigation was undertaken with this aim.

b. The History of the Double Innervation Theory.

Both Pouchet (1876) and v.Frisch (1911a) found that melanophores dispersed their pigment when newly denervated but aggregated it when the intact motor nerves were electrically stimulated. At that time they concluded that nerve fibre activity caused paling while its interruption allowed complete relaxation of the effector in a state of paralysis. This view has been widely held and the existence of these paling fibres has never been questioned.

Giersberg (1930) and Parker (1932) revived the idea (originally attributed to Bert, 1875, on the chameleon) that melanophores might be subject to double nervous control. Parker obtained evidence that the dispersion following nerve section was not a passive process but an active one, which, he proposed, was due to repeated discharges from the cut ends of darkening fibres. Between 1932 and 1942 Parker published a series of papers (summarised in 1948) describing experiments on denervated tail-bands in Fundulus and Ameiurus. Two of these experiments appear to be critical and at present afford no more satisfactory explanation than that advanced by Parker.

(1) Wyman (1924) observed that a small cut near the base of the caudal fin of Fundulus produced a sharply defined dark band running peripherally to the edge of the fin. This was attributed to the section of chromatic nerve fibres running parallel to each other through the fin and serving the fin melanophores. Within this denervated band the blood could be observed to be flowing normally since the vessels anastomosed across the fin rays; so circulatory disturbance was ruled out. If the fish were then placed on a white background the dark band faded in two to three days by recession of its margins. Parker suggested that this effect was due to diffusion into the band of chromo-neural transmitter substances from adjacent active paling fibres.

Parker (1934b) produced such a band and when it had paled completely, made a second, smaller cut within the denervated area. This produced a new, smaller band in exactly the same way. Parker argued that if darkening were due to relaxation of paling tonus, resection within the paralysed region should have no effect; therefore the second response demonstrated that an active process was involved. Later (1936, 1937) Parker demonstrated that in both Fundulus and Ameiurus the second cut also produced antidromic responses anteriorly as far as the first cut. The absence of antidromic activity from the first cut was attributed to dominant control by paling

fibres which were still active in the region concerned. In 1941 Parker obtained responses from three and even four consecutive cuts, each within the region denervated by the last.

Parker (1948) stated that similar results had been obtained by Matsushita (1938) on Parasilurus, by Tomita (1938, 1940) on Pterophyllum, by Fries (1942, 1943) on Gobius and two species of Fundulus, and by Parker (1937) on Holocentrus. Osborn (1938) on Ameiurus, Vilter (1938, 1939) on Gobius and Wykes (1938) on Ameiurus were all listed as having failed to obtain responses to second cuts. Parker attributed this to their experimental method or to working at too low a temperature.

Adelman and Butcher (1937) performed similar nerve-section and electrical stimulation experiments on Fundulus and were unable to support the presence of double innervation. (2) Parker (1934a) applied a small cold block at 0°C. to -10°C. to a new caudal band, about halfway along its length. He observed that distal to this point the band quickly faded, whereas proximally it was unaffected. Recutting beyond the cold block again produced darkening. Parker argued that each cut produced a succession of impulses within the darkening nerve fibres which could not pass the local cold region, while the fibres beyond the block were still potentially active.

These experiments cannot be repeated in the minnow

as the caudal fin melanophores of this fish are too scarce to form a well defined band. Gray (1955a, 1956b) developed a technique for producing a similar band, without vascular disturbance, on the tail near the base of the caudal fin. There is insufficient room to perform a second cut at this site as Parker did, so Gray performed three rather different experiments on the minnow. In each case the first cut was effected by spinal section anterior to the 15th vertebra, so that the whole surface of the fish was chromatically denervated. After eight days on a white background, when all the fish had become uniformly pale, second cuts were made as follows:-

(1) A second spinal section behind the first but still anterior to vertebra 15. No redarkening occurred.

(2) Section of the sympathetic chain anterior to vertebra 15. No redarkening occurred.

(3) Peripheral section at the base of the tail. A tail band of dispersed melanophores was formed, but the intensity and duration were much reduced compared with the response of the normal fish.

All three experiments were then repeated by Gray on spinal-sectioned fish which, after eight days in a white tank, were transferred to a black tank for a few hours to become partially black-adapted. Experiments (1) and (2) now showed some dispersion following the second cuts, and in (3) the response was much stronger. Gray therefore concluded that

the dispersion due to a second cut could be obscured or suppressed by concentrations of melanophore aggregating hormone in the blood on a white background.

Parker's interpretations of these effects have been much criticised (Waring, 1942; Young, 1950) on the grounds that repetitive discharges from the cut ends of nerve fibres would not be expected to continue for two or three days or longer, as required by this theory. Parker however cited Adrian (1930), Fessard (1936), Hoagland (1933c), and O'Shaughnessy and Slome (1935) for evidence of similar activity in nerve fibres of higher vertebrates. The phenomenon described by Adrian in sensory fibres of mammals does show that repetitive discharges may be due to permanent local depolarisation at cut ends of fibres and may last for some time, but the conditions cited by this author do not seem to be applicable to severed motor fibres in teleosts. Hoagland stated that similar responses in the lateral line nerve of Ameiurus lasted for only 15 minutes.

Umrath and Walcher (1950), working on Macropodus, have produced the only alternative theory to date. According to Gray (1955a), they assumed the presence of double innervation, but suggested that the original dispersion on cutting was due to the sudden loss of paling tonus. Repaling they attributed to the development of injury potentials from the cut ends of the paling fibres and

they also postulated that the cold block of Parker's experiment actively stimulated these paling fibres. This theory is rather unsatisfactory and was strongly criticised by Gray on the grounds that repaling does not occur on a black background and so cannot be an active process due to paling fibre stimulation. Gray therefore supported Parker's ideas.

Thus at present there seems to be little justification for rejecting Parker's theory. On this basis his results appear to suggest but not directly to prove the presence of double innervation.

Further evidence of double innervation has been produced by electrical stimulation experiments. Parker and Rosenblueth (1941) employed a square-wave stimulator connected to bipolar electrodes on the skin of Ameiurus. They reported that 8 volt pulses, 4 - 8 msec. long and delivered at a rate of 15 - 25 pulses per second produced paling after 15 - 25 mimutes. 6 - 8 volt pulses, 300 - 500 msec. long at 1 - 2 pulses per second produced darkening after 10 mimutes. These results seem to be rather unsatisfactory for three reasons.

(1) Both stimuli had to be applied for long periods before the response could be seen. It must be admitted that normal colour changes in this fish are rather sluggish (Chapter IV), but comparison with the response times obtained with Phoxinus does not suggest that direct

stimulation was being produced.

(2) For darkening, the mark/space ratios quoted vary between 3/7 and infinity. Thus the stimuli might be compared with a continuous direct current which could have the effect of blocking paling fibres. The effect of continuous current does not appear to have been investigated by Parker and Rosenblueth.

(3) Despite the heavy duty cycle of direct current pulses, no attempt was made to use non-polarisable electrodes. The actual stimulating currents would thus be complex and their effects difficult to assess theoretically. Nevertheless, the possibility that melanophore-dispersing fibres were actually stimulated cannot be ruled out.

Giersberg (1930), working on Phoxinus, discovered that the effects of electrical stimulation were reversed after the injection of a mixture of ergotamine and acetylcholine. He argued that ergotamine depressed the adrenergic paling-fibre system while acetylcholine sensitised a system of cholinergic darkening fibres, so that electrical stimulation then produced darkening. V.Gelei (1942) combined this technique with nerve section and claimed to have traced the pathways of the darkening fibres (Chapter Ia, Figure 1b). These experiments are repeated and criticised in Chapter VI.

The histological pictures produced by Ballowitz (1893) show a large number of small nerve endings associated with

each melanophore and derived from more than one nerve fibre. There is no indication that the different fibres have different physiological properties but the preparations do allow this possibility. According to Parker (1948), Eberth and Bunge produced similar preparations (1893-5). Pictures of the skin of Phoxinus by Whitear (1952), and slides produced by similar methods by Gray (unpublished), do not show such a rich profusion of nerve endings around the melanophores. However Parker (1933) suggested that chemical transmitters (neurohumours), liberated by chromatophore nerve-fibres, may diffuse over quite large distances in the skin. If this is so, either single or double innervation could be mediated by nerve endings lying randomly in the skin in some species. The asymmetrical responses described by Gray (1955b) are still possible in this model, as responses to concentration gradients of diffusing neurohumours.

Finally an argument may be advanced on the basis of the curves of Figure 10. Kent (1960) has shown that injections of a given amount of teleost pituitary extract has a stronger and more lasting blanching effect on spinal-sectioned minnows than on intact fish. The obvious inference is that chromatic nervous activity opposes the effect, but it is difficult to see how the known paling fibres could do this. Thus one might assume that on a black background there are darkening fibres which antagonise

the influence of the injected paling principle. But an alternative possibility is that denervation sensitises the melanophores to humoral influences. The paling fibres are probably adrenergic, and increased sensitivity to adrenalin after denervation has been demonstrated for teleost melanophores by Smith (1941); it was already well known for other autonomic effectors. It is not clear however whether this change is extended to other agents which produce the same response. There is no evidence for a structural affinity between adrenalin and the active principle of teleost hypophysis extracts, and the latter is probably not sympathomimetic in other respects. Spinal section may also be responsible for decreasing the activity of tissues responsible for extracting the hormone from the blood and inactivating or excreting it.

The literature on this subject thus appears to leave the case for double innervation unproven. Experiments have been reported whose explanation is made easier by postulating a double system, but little direct evidence of its existence has been obtained. Some part of this work was therefore devoted to a further investigation of the problem.

c. Selective Stimulation.

Electrical stimulation of any chromatic nerves in the

minnow produces paling. Spatial discrimination of different effects in different nerve trunks appears to be impossible and if darkening fibres exist, their effect is always masked. Possibly the paling fibres are more sensitive to stimulation or are more vigorous in their effect. A method of selective stimulation was therefore investigated in an attempt to resolve the motor influences.

Lapicque (1908a) devised an electrical stimulator, driven by a mechanical fly-wheel and capable of producing pulses of various 'shapes'. By using currents of rising intensity he was able to study the rate of nerve accommodation and discovered that it was greater in fibres of higher excitability and lower threshold. By carefully adjusting the rate of increase of stimulating current, he was able to stimulate selectively either a frog or a slower toad sciatic nerve, when both were mounted on the same pair of electrodes. Gastrocnemius muscle twitch was used as an indication of excitation. A further stimulator using two capacitors (Lapicque, 1908b; Lapicque and Lapicque, 1908) provided stimulating pulses whose temporal pattern imitated that of a nerve impulse and allowed more sensitive discrimination.

Fabre (1927b) plotted maximum intensity threshold curves for saw-tooth waves (rising linearly and falling abruptly to zero) of different durations, obtained from an electronic time-base circuit (Fabre, 1927a). For

very short durations the threshold curve approximated to that produced by square waves. As the pulses were made longer, the curve stopped falling towards the rheobase and rose instead to approach asymptotically a straight line which passed through zero. The slope of this line was termed the 'limiting slope', as pulses of lower slope failed to stimulate the nerve however far the voltage rose. On a frog gastrocnemius-sciatic preparation the stimulation curve reached a minimum at about 7 msec. and approached the limiting slope at about 30 msec. The limiting slope was believed to be a fundamental parameter of the nerve as a measure of its rate of accommodation. A further stimulator (Fabre, 1931a) depended on variation of the aperture of a photoelectric system and allowed faster rise-times to be investigated. Between 1927 and 1934 Fabre published a number of papers in which he developed theories of excitation based upon these results. Much of this work led to the design of theoretical excitation constants which were severely criticised by Rushton (1935). Monnier (see Fabre, 1934) suggested that Fabre's results were due to break-excitation rather than to the ascending current slope. But a series of papers by Blair (1932a and b), Hill (1936a and b) and Rashevsky (1948) on the theory of excitation processes, were able to uphold Fabre's curves on mathematical grounds. The action of linearly rising currents has also been

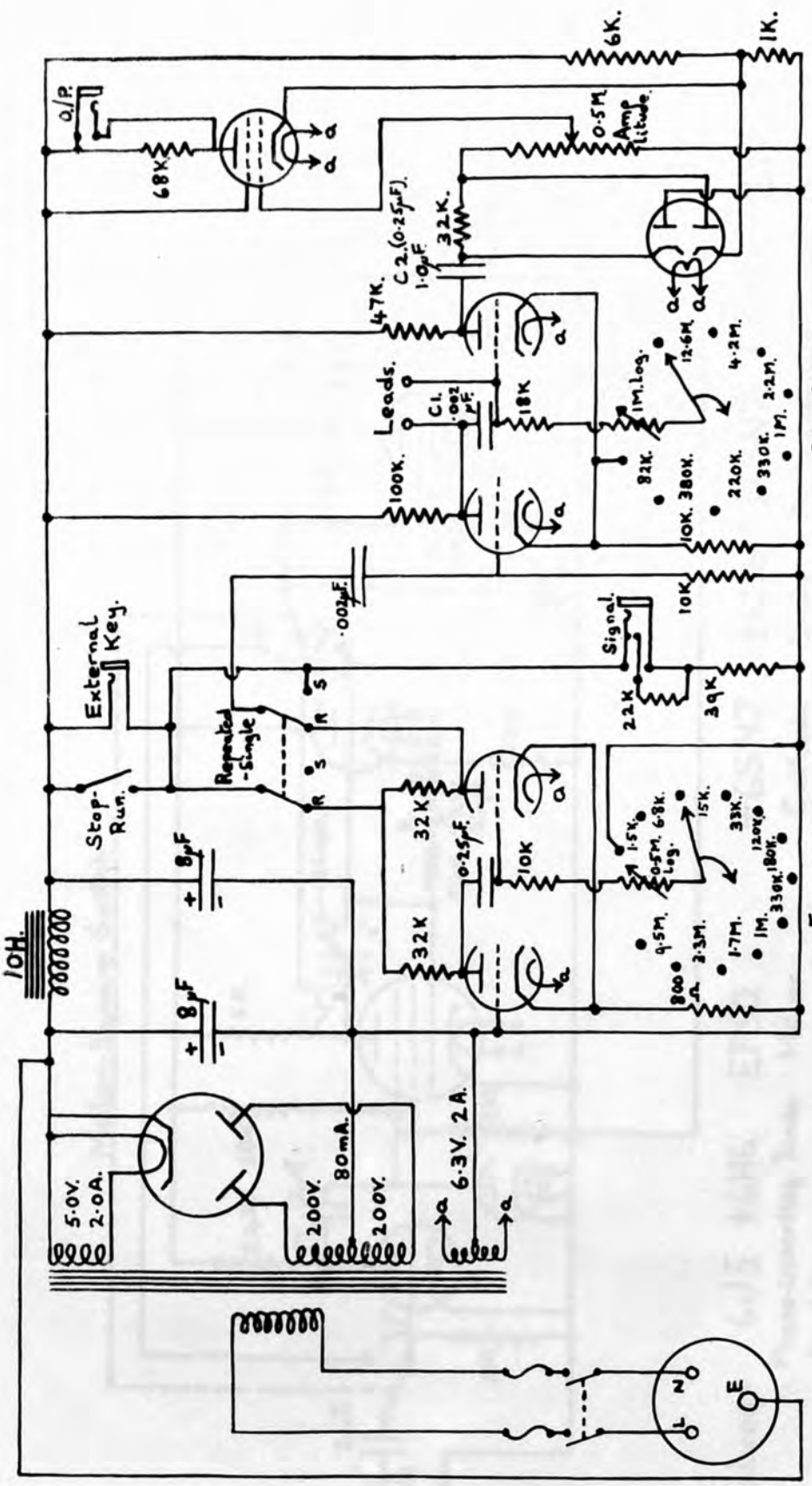
investigated recently at one or more nodes of Ranvier by Frankenheuser (1953) with confirmatory results. Whatever the precise mechanism involved, it had been possible, by controlling the rate of rise of stimulating current, to increase the threshold of quick, sensitive fibres above that of slower, less sensitive ones. It was decided to attempt this technique on the chromatic nerves of the minnow and a stimulator was developed for the purpose.

d. The Flickertron Stimulator.

A square-wave generator available at the commencement of this work, had been built to a design published by Bernstein (1950). This was used to gate the present stimulator after slight modification as shown in Figure 18. Two leads were attached to C_1 (0.002 μF) so that external capacitances could be connected in parallel, to increase the pulse-length if required. The time-constant of the output circuit was increased accordingly by changing C_2 from 0.25 μF to 1.0 μF.

The final circuit of the Flickertron pulse-shaping is shown in Figure 19. The square-wave generator fed into a phase-inverting amplifier which gated a Miller integrator producing a saw-tooth pulse (for this reason saw-tooth pulses are sometimes referred to as Miller pulses in this thesis). A Gibbs and Rushton cathode

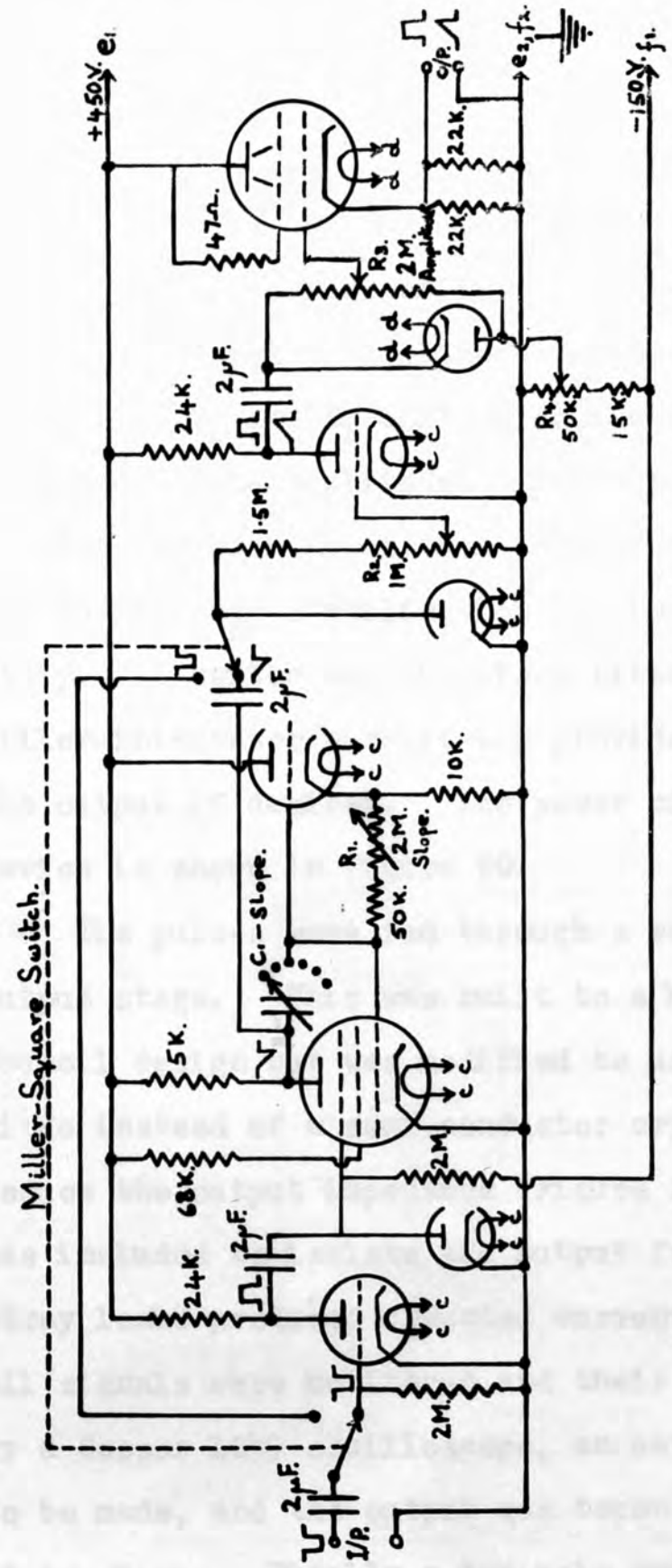
Figure 18.



Valves:- 6Z5.GT. 6SN7.GT. 6SN7.GT. 6H6.GT. 6V6.GT.

Square-Wave Generator as modified after Bernstein, L., 1950.
(high impedance output form)

Figure 19.



Values:- 6J5. ½6H6. EF50. ½6SN7. ½6H6. ½6SN7. EA50. 6V6.

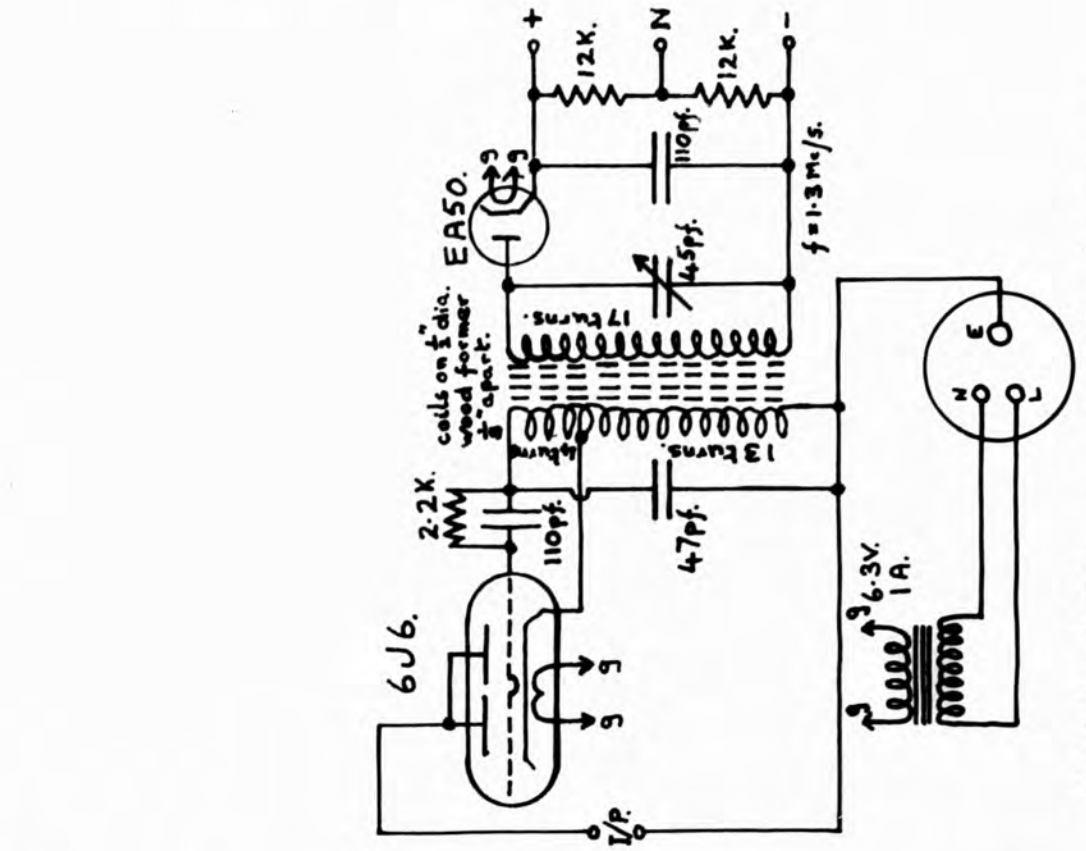
Phase-inverting Diode Miller Cathode Diode Phase-inverting Diode Cathode
Amplifier. Limiter. Integrator. Follower. Clamp. Amplifier. Clamp. Follower.

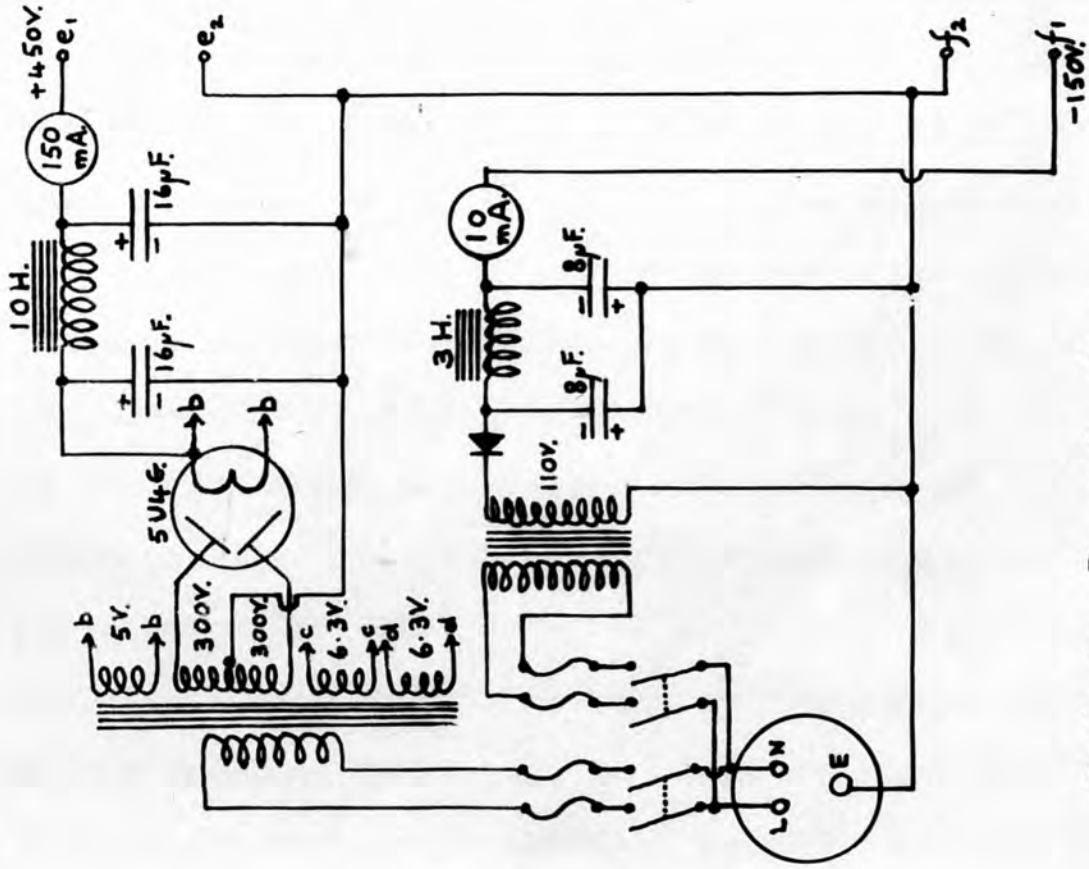
The Flickertron Pulse Shaping Unit.

follower was used in the control-grid circuit to allow low slopes to be generated. A very similar arrangement was later found to be used in a stimulator designed by Dr. Malcolm of the Medical Research Council Unit, Mill Hill (private communication). The signal was then inverted and fed to a cathode-follower biased just to cut-off. C_1 and R_1 controlled the slope, R_4 the cut-off bias and R_3 the amplitude. The output impedence was found to be much lower than that of the Bernstein circuit and the voltage stabilisation was improved proportionately. A switch was therefore fitted to by-pass the Miller-integrator circuit and provide square waves at the output if desired. The power circuit for the device is shown in Figure 20.

The pulses were fed through a radio-frequency output stage. This was built to a Medical Research Council design but was modified to use a thermionic diode instead of a semi-conductor crystal in order to reduce the output impedance (Figure 20). This unit was included to isolate the output from earth, in case stray leaks produced unwanted currents. However, as all signals were monitored and their voltages measured by a Cossor 1049 oscilloscope, an earth connection had to be made, and the output was taken in balance about it as shown. Finally a two-pole double-throw switch allowed the phase of the pulses to be reversed at will.

Figure 20.





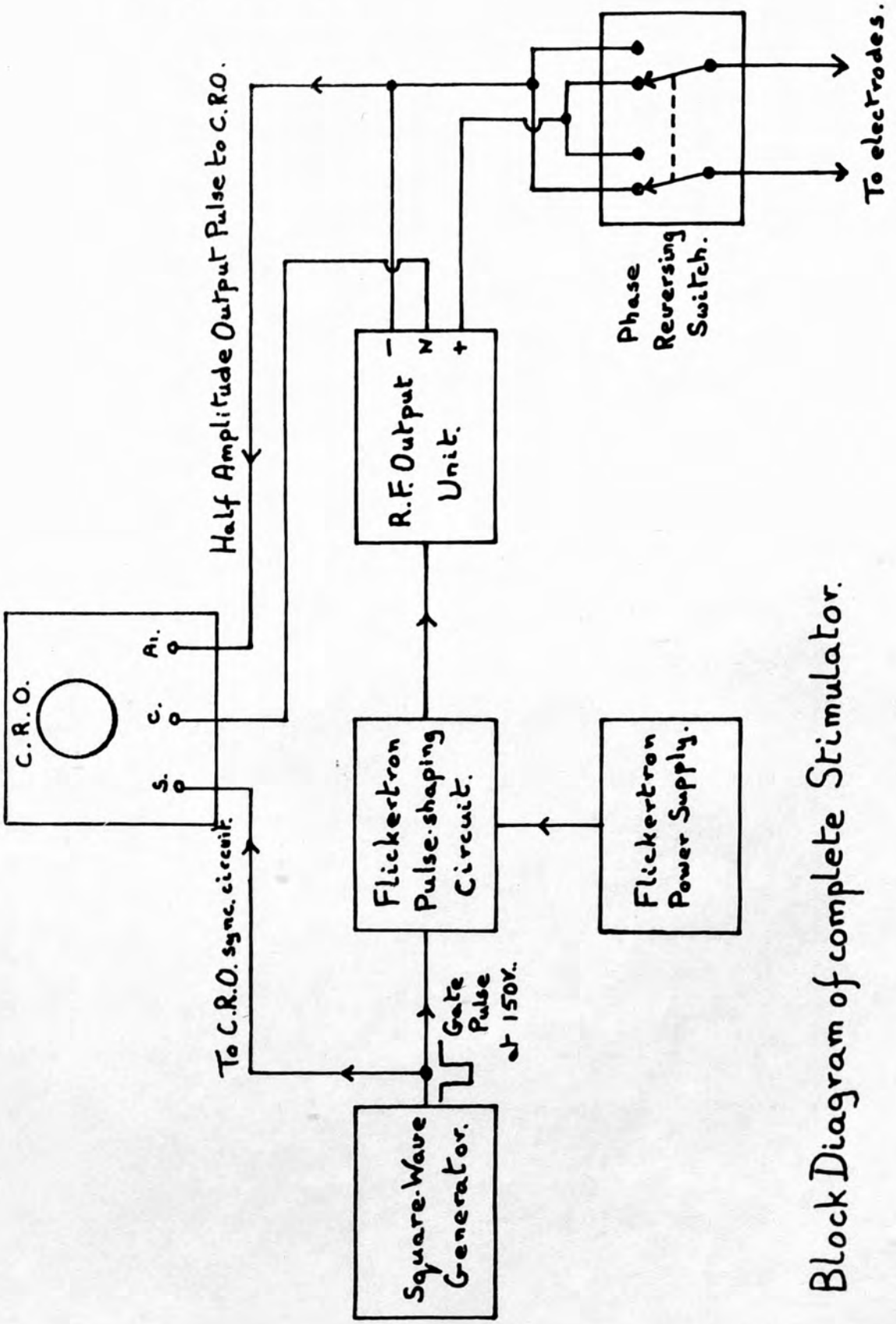
Flickertron Power Supplies.

A plan of the layout of the complete equipment is shown in Figure 21.

The electrodes used were silver/silver-chloride, sealed in glass tubes with mercury to contact amalgamated copper wires from the stimulator.

Preliminary tests were made with this circuit on sciatic-gastrocnemius preparations of frog (Rana temporaria) and toad (Bufo vulgaris). Isolated preparations were attached to an unloaded lever with which the twitch-threshold for single shocks was estimated. The nerve was laid upon electrodes 1 cm. apart with the cathode nearer the muscle (descending current). Eight frog preparations were tested and typical strength-duration curves are shown in Figure 22. For square waves the curve was of normal shape, approaching the rheobase asymptotically. For saw-tooth pulses, where the voltage measured was the peak value, the curve followed closely that described by Fabre (1927b), with the exception of rather lower thresholds, probably due to the use of non-polarisable electrodes. The limiting slope was found to vary somewhat between different preparations and with time. As shown in Figure 23, when a preparation was left quiescent but moistened with Ringer for some hours, the rheobase rose and the saw-tooth pulse curve assumed a humped shape. This was observed in all the five preparations which were left to stand

Figure 21.



Block Diagram of complete Stimulator.

Figure 22.

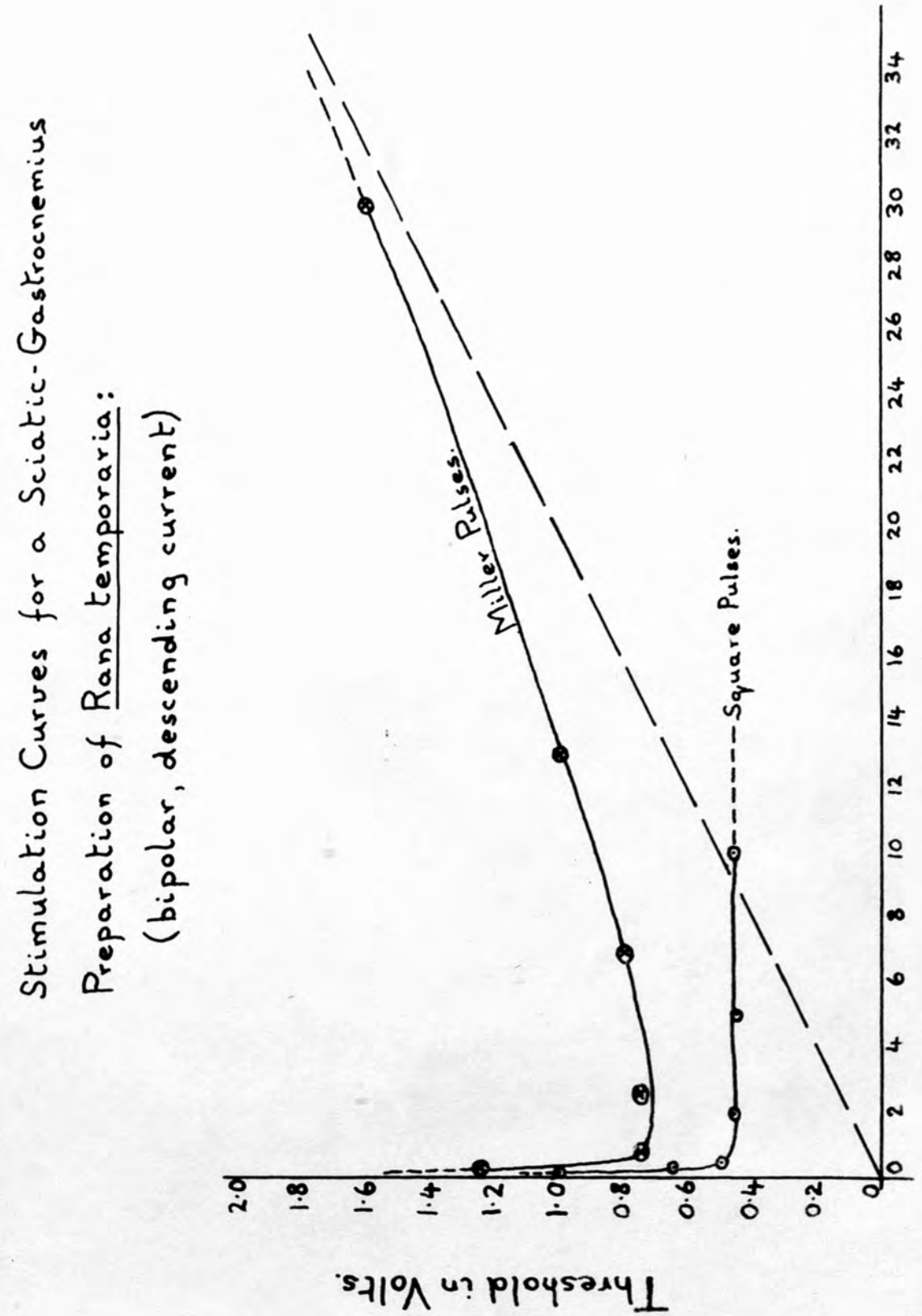
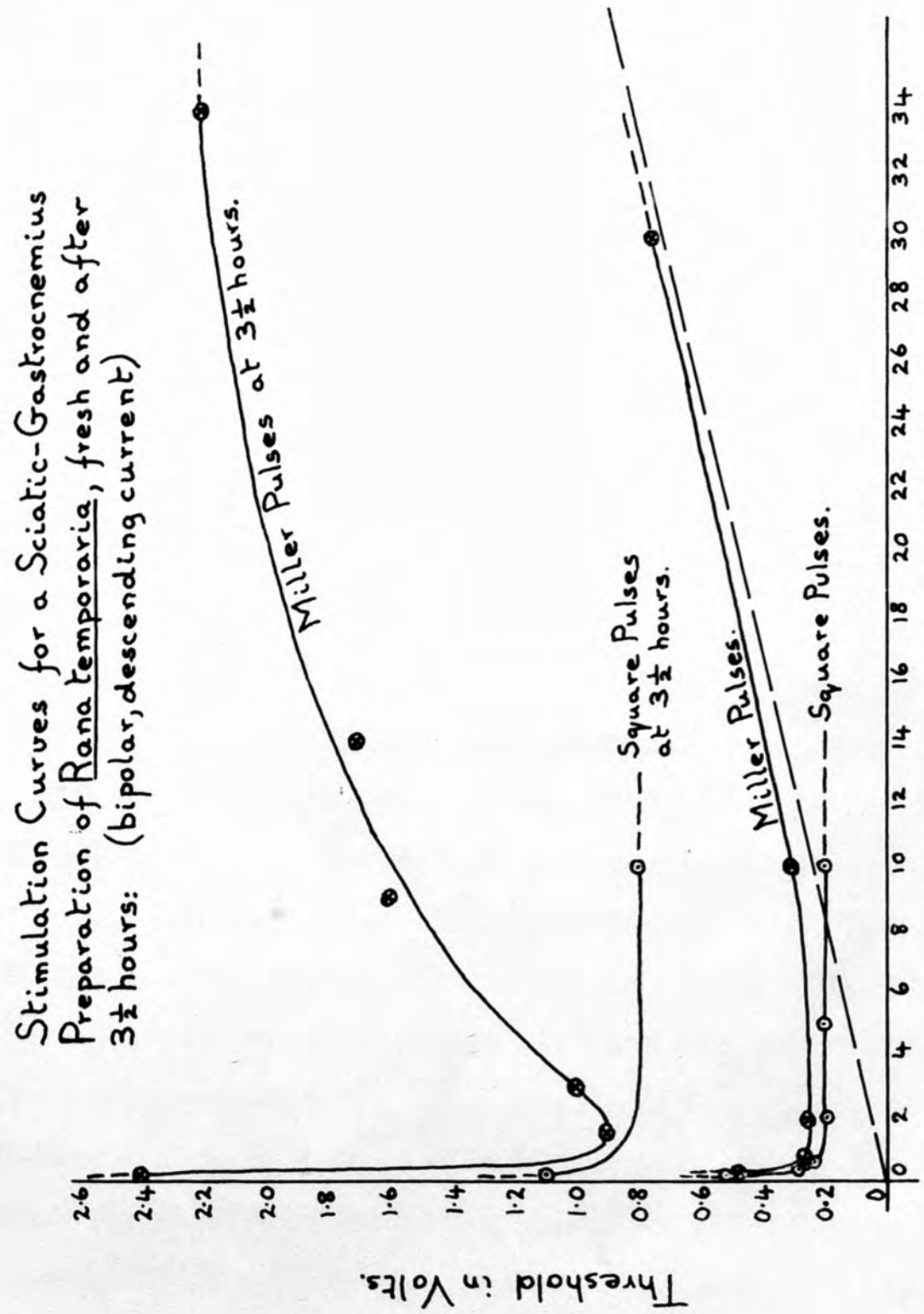

Stimulation Curves for a Sciatic-Gastrocnemius Preparation of Rana temporaria: (bipolar, descending current)
Threshold in Volts.
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Miller Pulses.
Square Pulses.
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Pulse Duration in Milliseconds.

Figure 23.


Stimulation Curves for a Sciatic-Gastrocnemius Preparation of Rana temporaria, fresh and after 3½ hours: (bipolar, descending current)
Miller Pulses at 3½ hours.
Square Pulses at 3½ hours.
Miller Pulses.
Square Pulses.
Threshold in Volts.
Pulse Duration in Milliseconds.
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34

before retesting. A description of such changes cannot be found in the literature. Fabre (1931b) reported a great increase in the limiting slope during ageing but he does not appear to have described the shape of the later curve.

Five similar preparations from the toad were tested. Threshold curves for square-waves were similar to those of the frog but a rise in threshold for 'Miller pulses' only occurred when very long pulses were used (more than 50 msec.), and the limiting slope was much lower. Changes in the shape of the curves of ageing preparations occurred as for the frog.

In another experiment two preparations, one from a frog and one from a toad, were attached to separate levers and both nerves were placed over the same electrodes. The thresholds for both nerves were raised, presumably by mutual shunting, but differential stimulation was easily accomplished. Square pulses, below threshold for the toad nerve, produced strong contractions of the frog muscle, while the opposite result was obtained by Miller pulses of longer than 40 msec. duration. Lapicque (1908a) stated that the threshold curves crossed at 30 - 40 msec., so the results obtained here confirm his original findings very closely.

Further experiments were performed on long sciatic-nerve preparations of the frog. Nerve impulses, about

4 cm. from the stimulating electrodes, were examined by platinum electrodes feeding an Ediswan preamplifier and oscilloscope. For short supraliminal square waves the α, β and γ elevations of the composite pulse could be readily identified. By using Miller pulses of gradually decreasing slope the α and β elevations appeared to be successively eliminated. Selective stimulation could not be proven since the lower slopes produced sequential stimulation and the oscillogram became much changed in appearance. Similarly Fabre described stepped contractions of muscle when the motor nerve was stimulated by long saw-tooth pulses of low slope. A separate, synchronous off-discharge, due to break excitation could be seen when the pulse duration and slope were increased sufficiently. This contradicts Monnier's criticism that Fabre's results were due to break excitation.

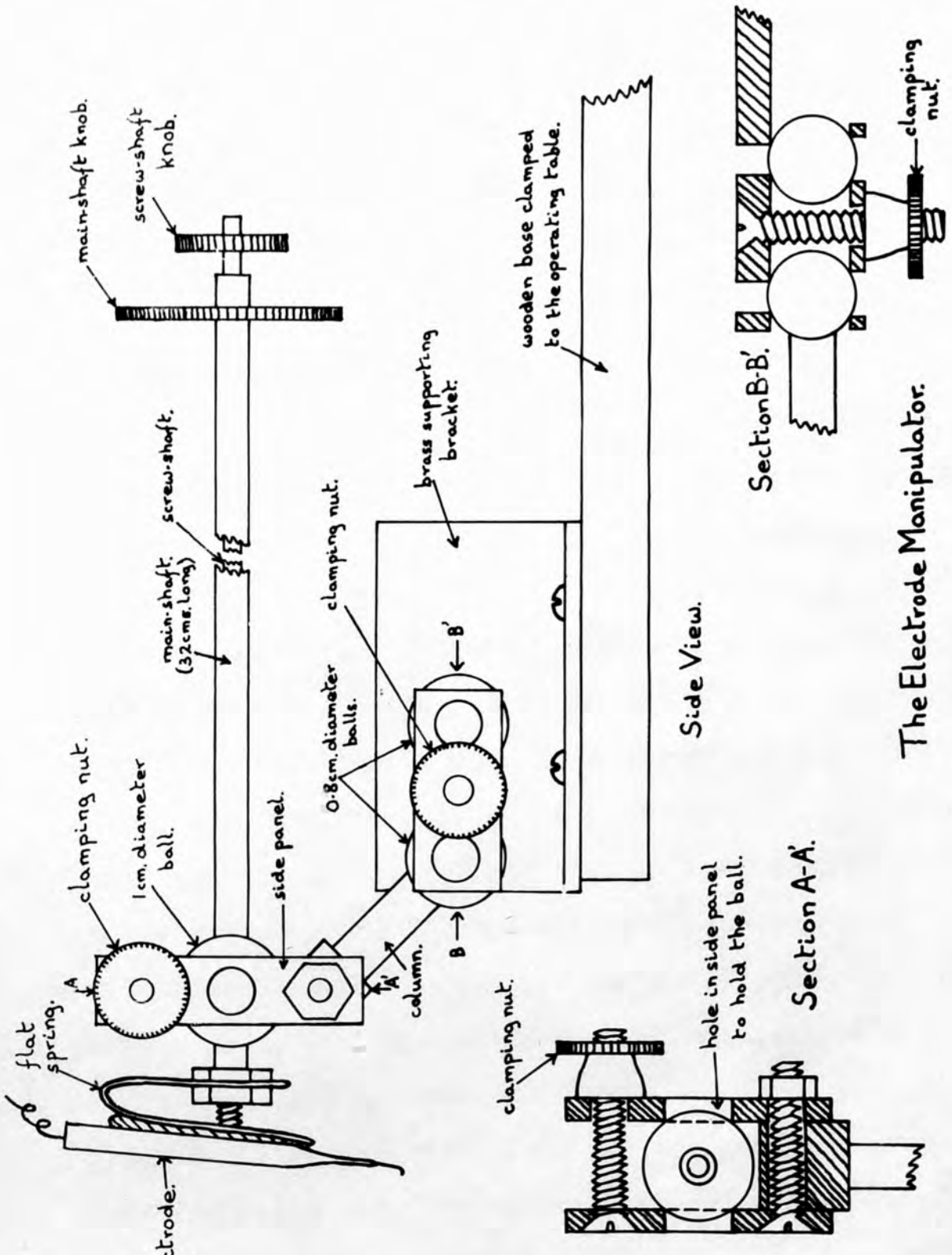
e. <u>A Teleost Preparation: Experimental Method</u>.

Several experiments were made with decapitated and anaesthetised minnows in an attempt to stimulate chromatic fibres in the skin, spinal cord or sympathetic chain. Several types of Ringer, Ringer-agar, and wick electrodes were employed in order not to damage the delicate sympathetic chain. Failure to measure

thresholds accurately and repeatably was attributed to insufficient localisation of the electrode and to excessive shunting and capacitative effects within the body of the fish. The requirement was a section of chromatic nerve which could be locally isolated for stimulation. Only the superficial ophthalmic nerve within the orbit appeared to be suitable for this purpose. Dissection showed that when the eye was removed a short loop of this nerve could be freed from the connective tissues and picked up on a small hook.

Suitable electrodes were constructed of 40 S.W.G. (= 0.122 mm. diameter) silver wire, sealed with 'Araldite' into small glass tubes and slightly bent near the end. They were lightly chlorided under controlled conditions. A prototype manipulator was made from Meccano and finally constructed in the form shown in Figure 24. The electrode assembly was held by Chatterton's compound onto a small U-shaped spring, $\frac{1}{8}$" wide, which was in turn attached to the short arm of a hollow first-order lever. The fulcrum of the lever consisted of a sphere clamped between two drilled and bevelled plates. Displacement of a knob on the long shaft produced movements in two dimensions of arc with an amplitude reduction of 21:1, while rotation of this knob turned the whole structure about its longitudinal axis. Rotation of a second knob, on an

Figure 24.


main-shaft knob.
screw-shaft knob.
wooden base clamped to the operating table.
brass supporting bracket.
screw-shaft.
main-shaft. (32cms. long)
clamping nut.
B'
0.8cm. diameter balls.
clamping nut.
1cm. diameter ball.
side panel.
A
A'
column.
B
flat spring.
electrode.
Side View.
Section B-B'.
clamping nut.
The Electrode Manipulator.
clamping nut.
hole in side panel to hold the ball.
Section A-A'.

inner, concentric shaft, terminating in a short length of studding, controlled flexure of the spring and provided movement in the third dimension. Since all movements executed by the electrode tip were along arcs, slight compensatory movements in the other directions were required in order to follow a straight line. Nevertheless after a little practice with a binocular microscope a sufficient degree of accuracy was achieved. The whole device was controlled with one hand.

The electrode could be held rigidly in any position by tightening the clamping nut. For coarse adjustment the device was mounted on a short arm terminating in a second ball-joint, which could also be clamped rigidly. Finally the base of the manipulator was clamped to the operating table with a small G-clamp.

The experimental procedure was as follows. A fish, deeply anaesthetised in 0.5% urethane, was laid upon the wax operating dish with an oral supply of 0.25% urethane and a large silver/silver-chloride electrode beneath its belly. Rolls of Ringer-soaked filter paper were placed to support the body at the right angle and to prevent it from slipping about. Under a dissecting microscope a careful incision was made round the eye, the extrinsic muscles and the optic nerve were cut in turn, and the eye was removed. The exposed orbit was washed frequently with Ringer.

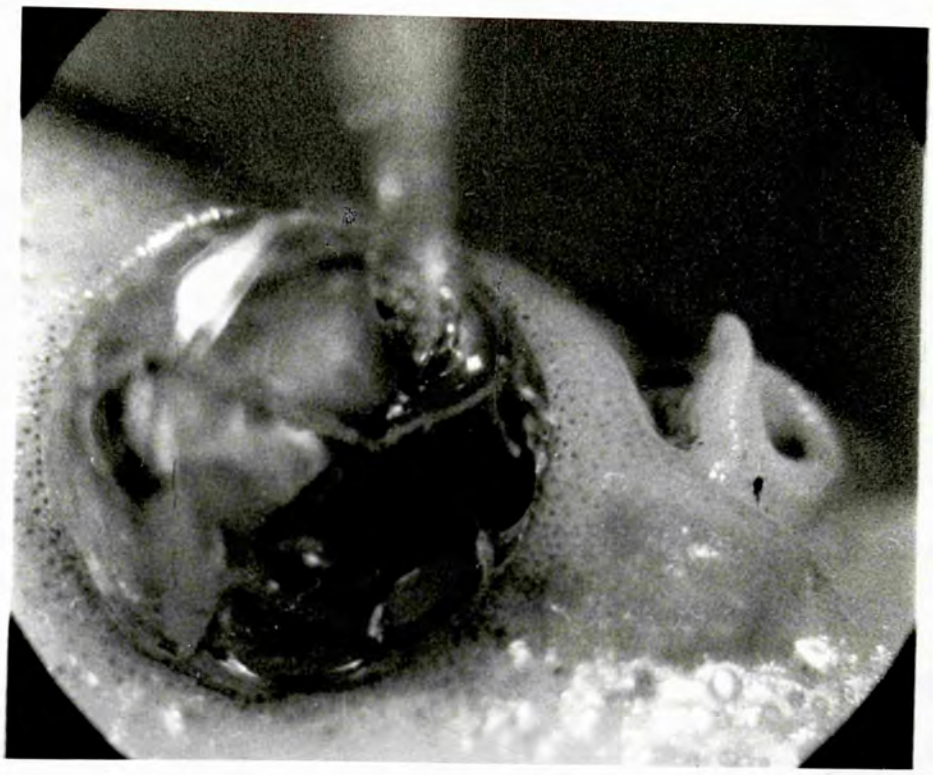
With fine hooks the superficial ophthalmic nerve was freed from the connective tissues surrounding it, but was not cut or damaged. Care was taken not to cut the blood-vessel which lay alongside this nerve, for although Smith (1931b) had demonstrated the presence of a collateral blood supply to the region served, the release of blood reduced visibility for some time. The smaller electrode was then positioned somewhere in the orbit, manipulated to pick up the loop of nerve and clamped. Finally the orbit was drained with a pipette and refilled with clean liquid paraffin. A photograph of such a minnow is shown in Figure 25.

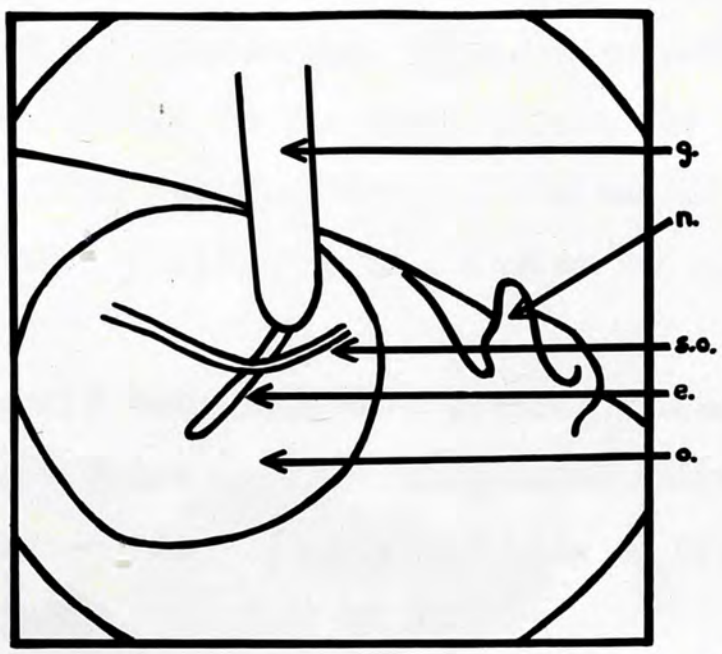
In other experiments the same nerve was cut without being stimulated electrically. In these cases a small dorsal incision was made, 3 - 4 mm. in length. When the eye was gently depressed, the superficial ophthalmic nerve could be located and severed. A single stitch of fine nylon through the epidermis sutured the wound satisfactorily, and the fish recovered completely. This method caused far less disturbance than that of Smith (1931b), whereby either the whole eye or "a triangular section of the orbit" was removed.

f. Results.

These techniques were first employed at Plymouth on Cottus bubalis which has a large orbit. The species

Figure 25.





A photograph of a superficial ophthalmic nerve preparation of a minnow. The apparent tension of the nerve is an illusion caused by refraction at the surface of the liquid paraffin.

e: Silver-silver chloride electrode (40 S.W.

g: Glass electrode mounting.

n: Right nostril of the minnow.

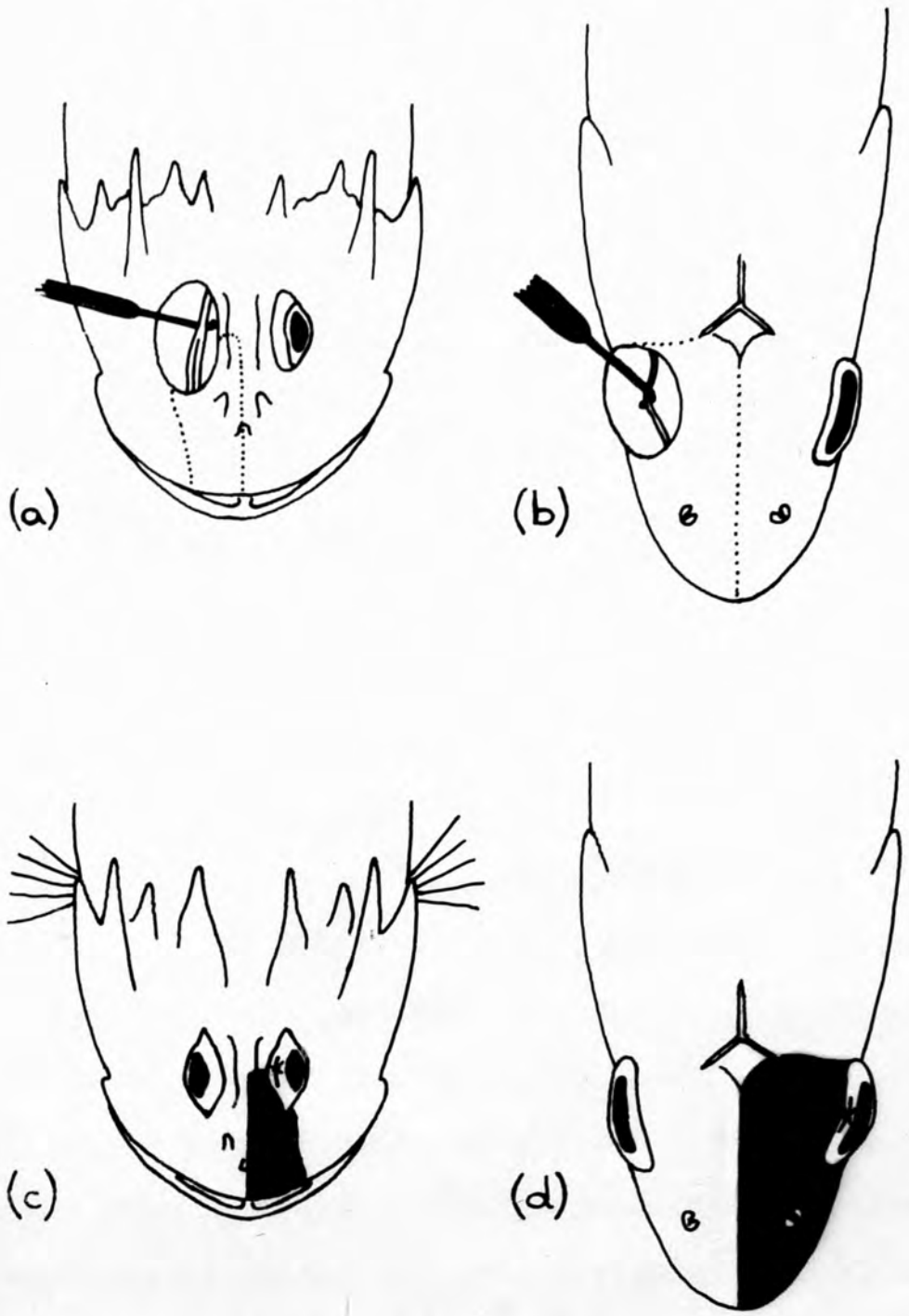
o: Orbit filled with liquid paraffin.

s.o: Superficial ophthalmic nerve.

stood up well to operative techniques and was thought to possess chromatic nerves (see Chapter IV). Figure 26c shows the effect of section of the superficial ophthalmic nerve. This was performed on the left side in two fishes and produced maximal darkening in the area shown. As stated in Chapter IV, the head of this species darkens after a time even on white backgrounds, and the patch was thus soon obscured. The fish were therefore kept in black tanks and periodically placed in white ones to produce temporary paling of the regions not affected by the operation. In this way the denervated patches were observed for five days in one fish. A very striking feature of the patch was the sharpness of its boundaries, thus demonstrating the precise area served by the nerve beyond the point of section. Since the fish were maintained in black tanks, marginal paling due to invading neurohumors did not occur.

Eventually both fish were reanaesthetised in turn and the right sides used for electrical stimulation as shown in Figure 26a. The dotted line of this diagram shows the region blanched by electrical stimulation. Again the boundary was very sharp, matching exactly the area darkened by nerve section. Thus it may be assumed that melanophore aggregation was a direct result of nerve stimulation and was not due to more

Figure 26.



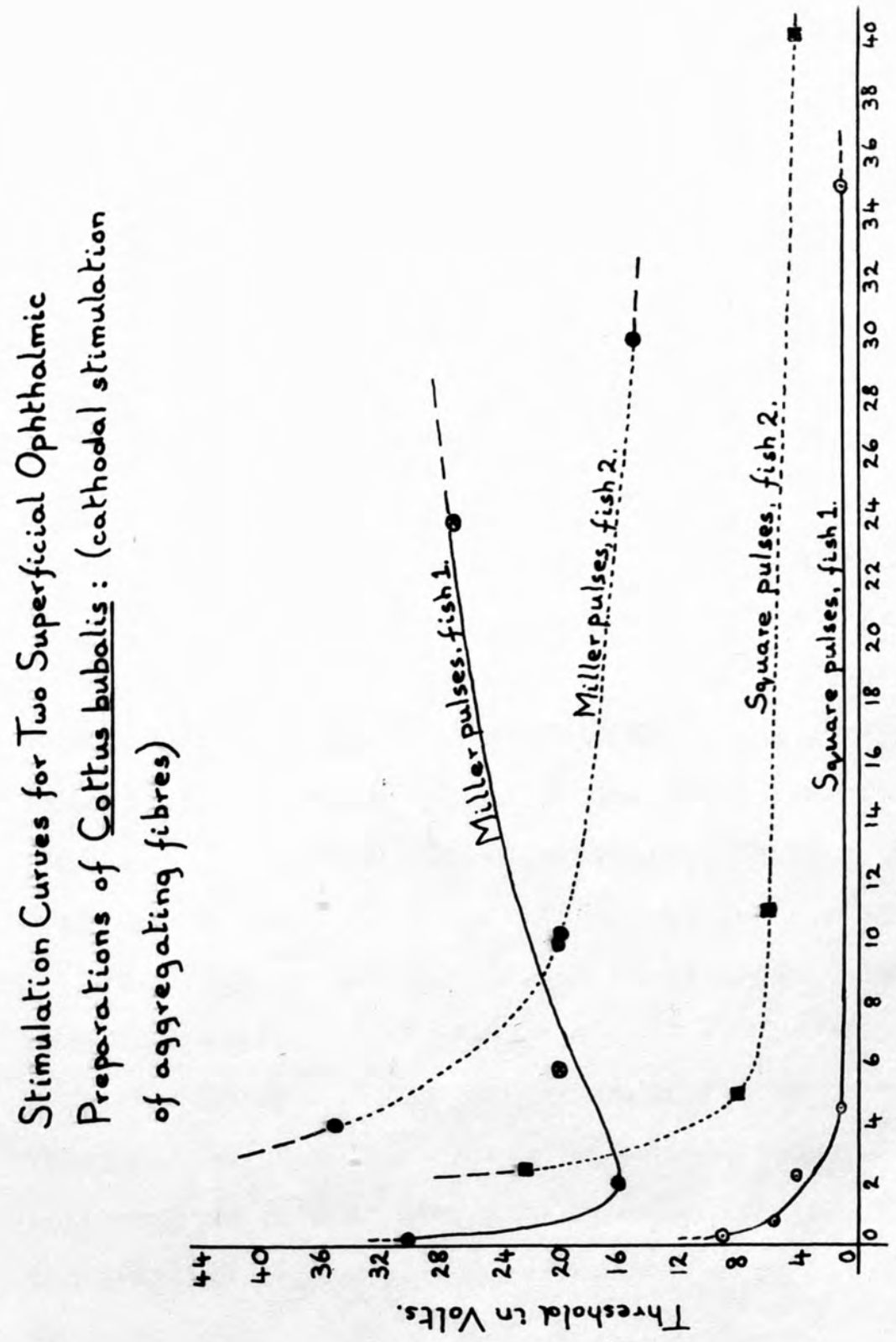
(a) Cottus and (b) Phoxinus superficial ophthalmic preparations. Dots outline the area of aggregation.
(c) and (d). The same showing areas of dispersion following section of the superficial ophthalmic nerve.

general stimulation.

Strength-duration curves for these two preparations are shown in Figure 27. In Fish 1 the curves are more or less as expected except that the Miller curve is slightly humped as in the ageing preparations of the frog sciatic-nerve. In Fish 2 the central end of the nerve was cut and the end wrapped in filter paper which had been soaked in 1% procaine solution in Ringer, to prevent injury discharges. This was done in an attempt to eliminate afferent impulses and to restrict stimulation to the motor fibres. The much higher threshold values indicated that some damage had been produced and the technique was discontinued. No rise in Miller threshold for the longer pulses was detected, although very long pulses were not tried; a toad sciatic-nerve might have produced curves of similar shape over the same range. In every case electrical stimulation produced quick and complete melanophore aggregation within the affected area, and there were no signs of the stimulation of darkening fibres. Cessation of stimulation was followed by slow recovery, taking 2 - 3 minutes, to an intermediate state similar to that over the rest of the head.

Similar experiments on the minnow are illustrated in Figure 26b and d. The left superficial ophthalmic nerve was cut in five fishes which were then returned to a white background. Complete dispersion was observed

Figure 27.


Stimulation Curves for Two Superficial Ophthalmic Preparations of Cottus bubalis: (cathodal stimulation of aggregating fibres)
Miller pulses, fish 1.
Miller pulses, fish 2.
Square pulses, fish 2.
Square pulses, fish 1.
Threshold in Volts.
Pulse Duration in Milliseconds.
(frequency 2 per second)
0
4
8
12
16
20
24
28
32
36
40
44
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40

in each fish in the area shown in Figure 26d. This confirms the results of v.Frisch (1911a) and of Smith (1931b). After 7 - 9 hours each patch had faded somewhat and the margins were less distinct. After 24 hours the patches had either disappeared or were barely distinguishable. In all cases dispersion did not extend below the level of the upper jaw. The lack of marginal fading, as observed in tail-band cuts (Gray, 1955a; Parker 1934a) was also observed for denervated cephalic areas of Ameiurus by Parker (1941).

In a further experiment three fish were submitted to anterior spinal cord section and returned to a white tank, in one case for three weeks and in the other two for eight days. At the end of these times the effects of denervation had disappeared and the fish were uniformly pale. In each fish the superficial ophthalmic nerve was then cut on the left side. The newly denervated patches of these fish showed only slight darkening and rather irregular edges. When the fish were returned to a white tank, the patches faded completely in 1 - 1½ hours. These effects were similar to those obtained by Gray for tail-band section after spinal section (Chapter Vb), and confirm that darkening after denervation is not a passive process.

Electrical stimulation of the superficial ophthalmic nerve of the minnow produced complete aggregation within

the area shown in Figure 26b. This matches exactly the area darkened by denervation. The first paling-threshold curve obtained from one of these preparations, by cathodal square pulses, is shown in Figure 28. It is of perfectly normal form. Ten preparations were made from intact fish and threshold determinations were made on each. Four different types of pulse were investigated; square and saw-tooth (Miller), both anodal and cathodal. The first measurement made on each preparation was checked at frequent intervals and when it began to rise no further measurements were made. In only one case was it possible to obtain complete curves for all four types of pulse, and these are shown in Figure 29. Similar curves were found in all the other preparations. In two preparations the effect of pulse-repetition rate was investigated for 5.0 msec. and 0.5 msec. square pulses, between 1/2 sec. 100/sec. No changes in threshold or response rate could be detected. The rate of 2/sec. was therefore made standard so that longer pulses could be delivered without change in frequency.

These curves present several interesting features. There were no discontinuities which might suggest the presence of a second, opposing system of nerve fibres, and melanophore dispersion was never observed in response to electrical stimulation. No rise in threshold for Miller pulses was observed, even when pulses several hundred

Figure 28.

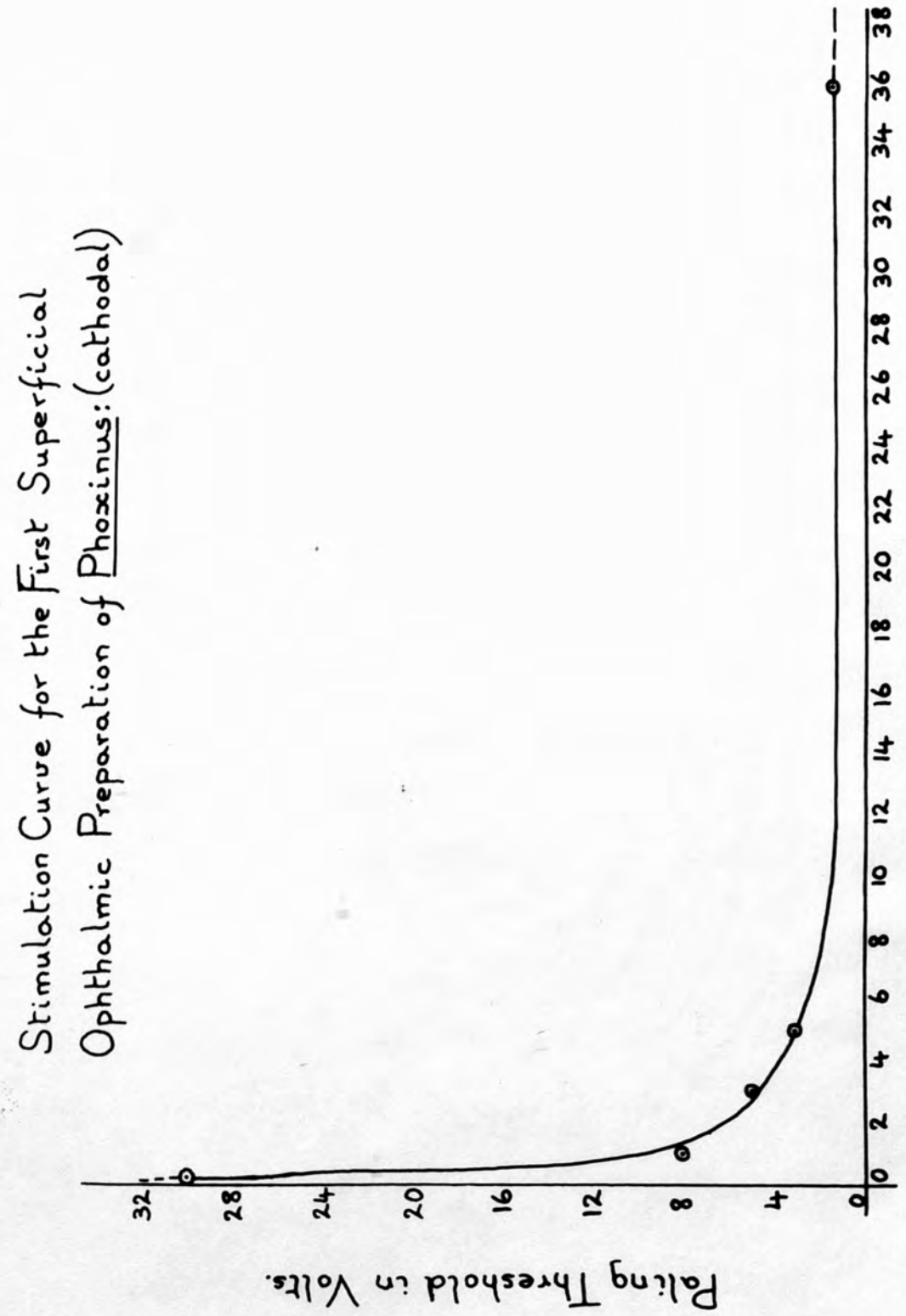
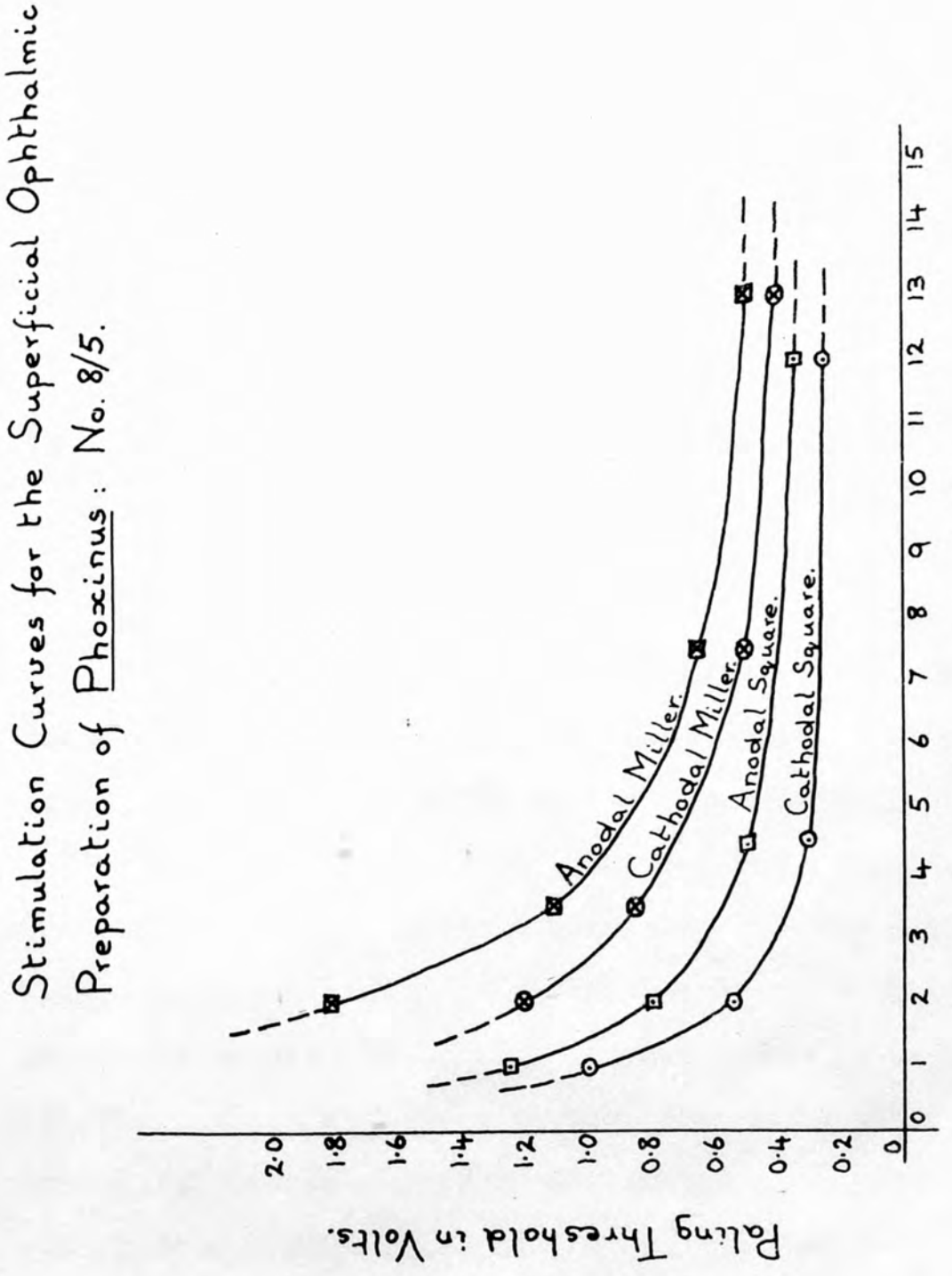

Stimulation Curve for the First Superficial
Ophthalmic Preparation of Phoxinus: (cathodal)
Firing Threshold in Volts.
32
28
24
20
16
12
8
4
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
Square Pulse Duration in Milliseconds.
(frequency 2 per second)

Figure 29.


Stimulation Curves for the Superficial Ophthalmic
Preparation of Phoxinus: No. 8/5.
Rolling Threshold in Volts.
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Pulse Duration in Milliseconds.
(frequency 2/second)
Anodal Miller.
Cathodal Miller.
Anodal Square.
Cathodal Square.

milliseconds in duration were used. This is in contrast to one of the Cottus bubalis preparations.

Most surprising of all was the effectiveness of anodal unipolar stimulation. Stimulation by direct current pulses is expected to occur only at the cathode, but a large 'indifferent' electrode placed below the belly of the fish could only cause general excitation. Yet the melanophore response to anodal stimulation was just as strictly limited to the area shown in Figure 26b as for cathodal stimulation. In preparation 8/5 cathodal stimulation gave a slightly lower threshold than anodal pulses (Figure 29) but in some preparations this relation was reversed (e.g. 1/5, Figure 30). Further, in ageing preparations, when the threshold began to rise, the cathodal threshold was several times observed to rise to ten times its former value without any change in the anodal curve. The reverse condition was never encountered. This suggested a possible explanation for the anodal effect. Figure 31 shows a diagram of the nerve and smaller electrode within the orbit. During cathodal stimulation (i) the current must pass through the nerve which is then stimulated in the region of the cathode. Anodal stimulation merely reverses the current flow (ii). Since the nerve is surrounded by insulating paraffin, the current must leave the nerve at the two points where it rejoins the body, and these regions will constitute 'effective

Figure 30.

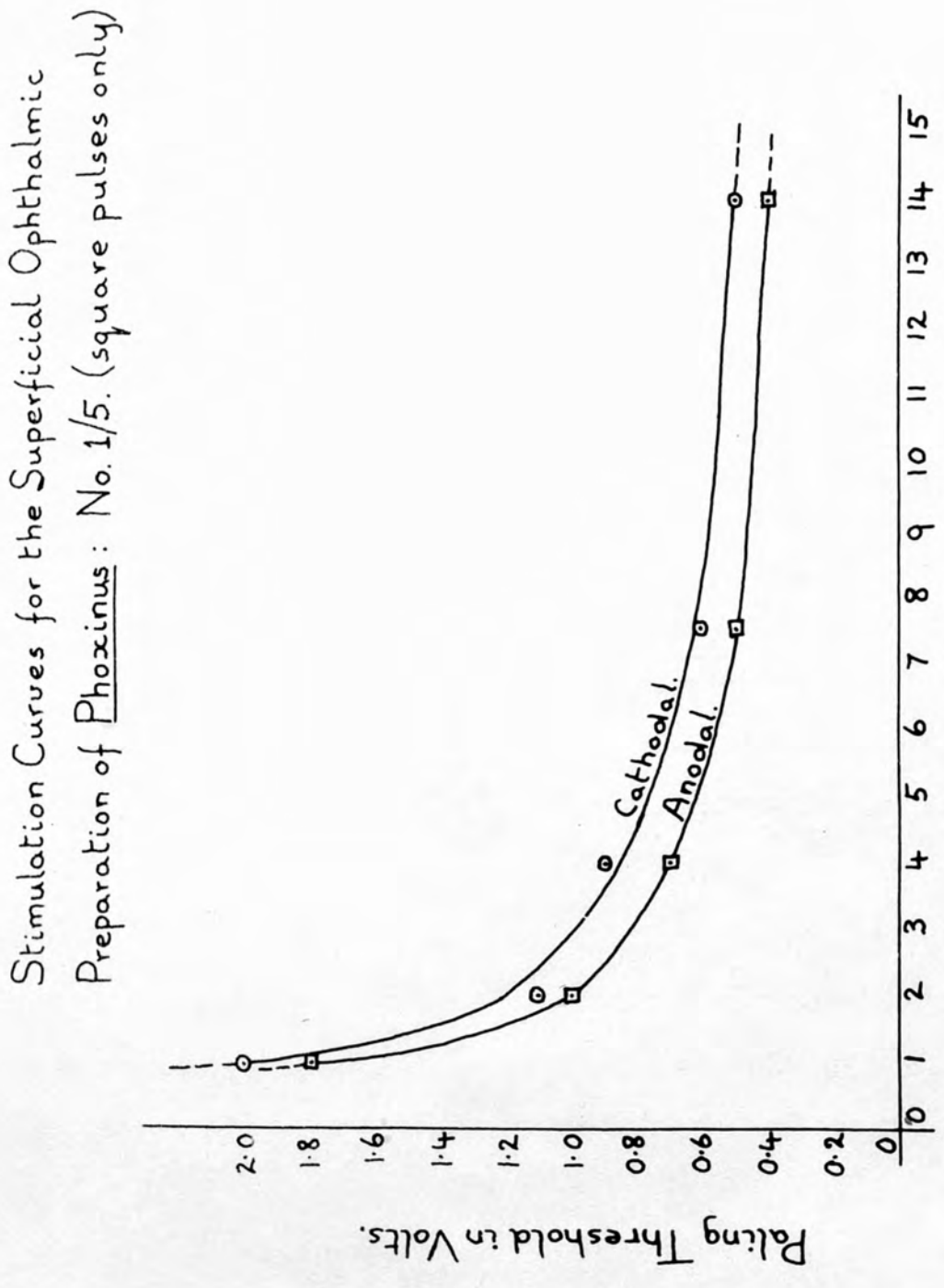
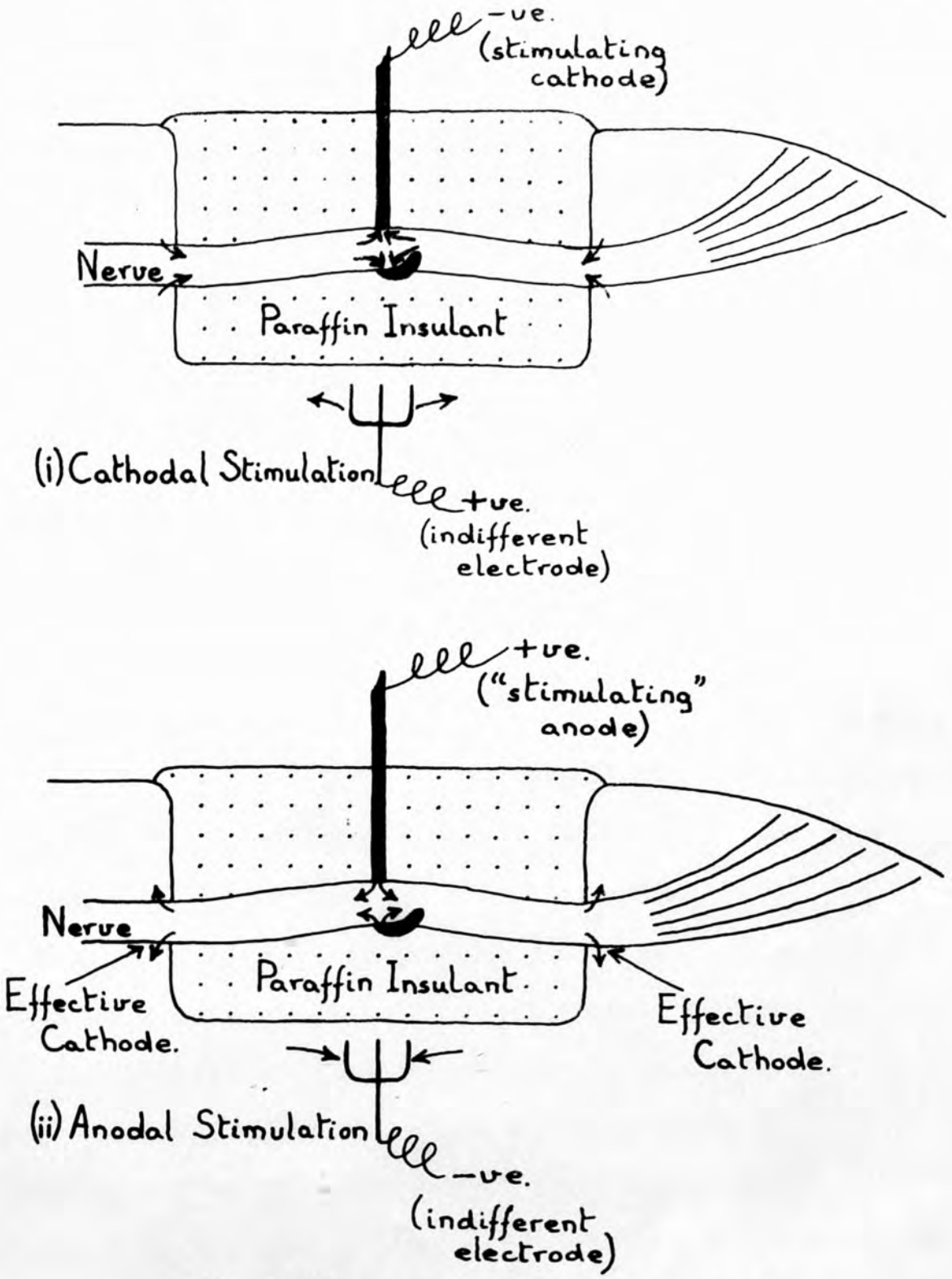

Stimulation Curves for the Superficial Ophthalmic
Preparation of Phoxinus: No. 1/5. (square pulses only)
Firing Threshold in Volts.
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Cathodal.
Anodal.
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Pulse Duration in Milliseconds.
(frequency 2 per second)

Figure 31.



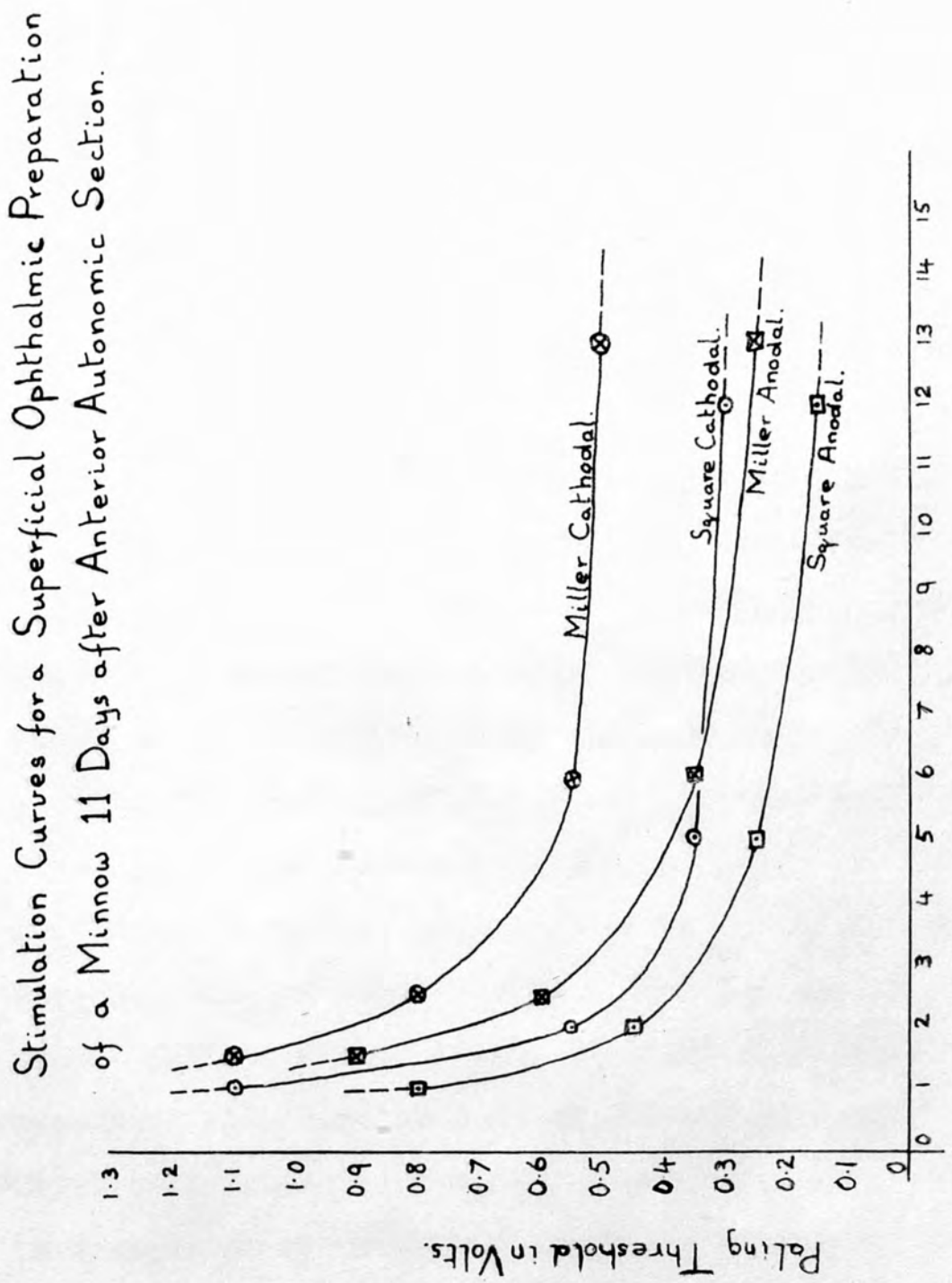
Block Diagrams of the Superficial Ophthalmic Preparation.
(arrows show direction of current flow)

cathodes'. Excitation will then occur, at suitable threshold levels, in a ring around the nerve at each of these sites.

As the preparation ages, it may be supposed that anoxia, or other conditions of depression, will occur first at points farthest from the body - in the region of the smaller electrode. Thus cathodal threshold will rise due to reduced excitability, while for anodal stimulation the 'effective cathodes' will remain excitable since they are within the body where the blood-circulation is intact. Slight differences in the relative thresholds for anodal and cathodal pulses in different preparations are probably related to slight differences in electrode contact and in the way in which the nerve re-enters the connective tissues. The anodal and cathodal curves, therefore, probably relate to very similar effects.

A further preparation was made from a fish which had been on a white background for 11 days, following section of the sympathetic chain anterior to vertebra 15. All four threshold curves were plotted as shown in Figure 32. They appear to be no different from those of the intact fish, thus suggesting that degeneration, at least of the paling fibres, had not occurred in this region. Similar readings were made on a fish which had been subjected to anterior spinal section 13 days previously, but a complete set of curves could not be obtained. These results

Figure 32.


Stimulation Curves for a Superficial Ophthalmic Preparation
of a Minnow 11 Days after Anterior Autonomic Section.
Miller Cathodal.
Square Cathodal.
Miller Anodal.
Square Anodal.
Pulse Threshold in Volts.
Pulse Duration in Milliseconds.
(frequency 2 per second)
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

support the arguments of Chapter IIf regarding v.Frisch's theory that reversal of temperature response after denervation might be due to motor-nerve degeneration.

g. Conclusions.

It is clear that stimulation by differential accommodation failed to demonstrate the existence of double innervation for minnow melanophores. But it also produced no evidence to the contrary, since the Miller-pulse thresholds did not rise asymptotically to a limiting slope. If this had occurred, distortion or discontinuity of the curves might have been expected if double innervation were present. The absence of such features from curves which included pulses of very long duration, might have given grounds for arguing against double innervation. In the circumstances no inference of any kind can be made. No reason can be suggested, at present, to explain why the threshold failed to rise with longer pulses.

A further method of differential stimulation which has been used with some success on nerve-muscle preparations might be tried here. A continuous direct current has been shown to block highly excitable fibres at lower levels than slower, less excitable fibres (Wood, 1958). This is another form of differential accommodation, but does not depend on time-relations. Rosenblueth (1941a and b) has

shown that in mammalian motor-nerve fibres the effects of direct currents are not nearly so simple as is generally thought. Spatial and temporal variations, and changes of current intensity and electrode size, enable a wide variety of effects to be obtained. These are often in the opposite direction to those expected, for example anodal facilitation and cathodal depression.

The same preparation and the same stimulator could be used. By adjustment of R_3 (Figure 19) a positive D.C. component may be produced at the output and the stimulating pulses will be superimposed on this. By taking the cathode loads to the negative line (with suitable increase in resistance) either positive or negative D.C. voltages could be produced. Unfortunately this technique could not be investigated in the time available.

Reference to other points arising from these experiments will be made in later Chapters.

CHAPTER VI.

THE EFFECT OF ERGOTAMINE ON CHROMATIC CONTROL.

a. Introduction.

Giersberg (1930) repeated some of the nerve-stimulation

experiments of v.Frisch but was unsatisfied with the idea of a single, melanophore-aggregating, innervation. He argued that in such a system the state of melanophore dispersion must be passive in order to explain the quick adaptation to a black background. Many experiments, notably those of Spaeth (1916a and b) on isolated scale melanophores, had rendered this unlikely. (This is a point which is still not settled. Parker (1940, 1948) collected considerable evidence that melanophores are quiescent at any equilibrium state of pigment dispersion and are only active when the pigment gramules are migrating. This is also supported by the work of Kinosita (1953) but it cannot be said to be proven conclusively.) Giersberg therefore attempted to find melanophore-dispersing fibres by a method employing selective blocking and stimulating drugs.

As v.Frisch's paling fibres were probably adrenergic, Giersberg suggested that the darkening fibres may be cholinergic. In order to block the adrenergic system he injected minnows with either nicotine or ergotamine. Fish treated with nicotine became completely dark, presumably due to loss of aggregating tonus, and were unsuitable for the investigation of dispersing activity. With ergotamine, however, the fish darkened for about an hour and then paled, regardless of background colour. This was attributed to an initial loss of aggregating

tonus followed by a direct action of the drug on the effector cells, although Spaeth and Barbour (1917) had shown that ergotamine phosphate had little effect on the melanophores of isolated Fundulus scales. Stimulation of the chromatic nerves of fish in this condition was without effect. If such fish were injected with acetycholine nerve stimulation produced reversible melanophore dispersion. The inference was that acetylcholine increased the excitability of cholinergic nerves and that the presence of a parasympathetic type of innervation was thus demonstrated.

V.Gelei (1942) used this method, together with nerve-section techniques, to map the course of dispersing fibres, as v.Frisch had earlier done for aggregating fibres. He placed a single stimulating electrode in the medulla oblongata and an indifferent electrode beneath the body of the fish. Section of the autonomic chain and aorta, by a single knife-thrust (as used by v.Frisch), prevented the darkening action from appearing more posteriorly, while responses anterior to the cut remained normal. Since the level at which the section was made had no effect upon this result, v.Gelei argued that the dispersing fibres must emerge from the spinal cord in the first or second spinal nerves and then pass posteriorly in the autonomic chain. This course is shown in Figure 1b.

Unfortunately v.Gelei did not attempt either (1) to

control the experiment by repetition without ergot treatment, or (2) to try the effect of stimulation at other levels in the nervous system. Because the paths described do not conform with the results of Healey (1948, 1954) and Gray (1955a), this evidence for double innervation was reinvestigated.

b. Stimulation Experiments without Ergotamine Treatment.

Three normal minnows were anaesthetised in turn, and in each a small part of the medulla and anterior end of the spinal cord was exposed, as for spinal section. A fine electrode, made from lightly chlorided 40 S.W.G. (= 0.122 mm. diameter) silver wire, was then inserted into the nerve mass, and a larger silver/silver-chloride electrode was placed beneath the belly of the fish. Stimulation with square negative pulses 2 msec. in duration and delivered at a rate of 10/sec. from the Flickertron stimulator (see Chapter Vd), produced complete paling over the whole surface of the fish. At a level of 2.5 V. the response was complete, while partial paling occurred down to 2.0 V. The aggregation of melanin was rapid and completely reversible when stimulation was interrupted. Successive cuts were then made in the autonomic chain, first posteriorly in the haemal canal and later in the body cavity anterior to vertebra 15. These were performed by

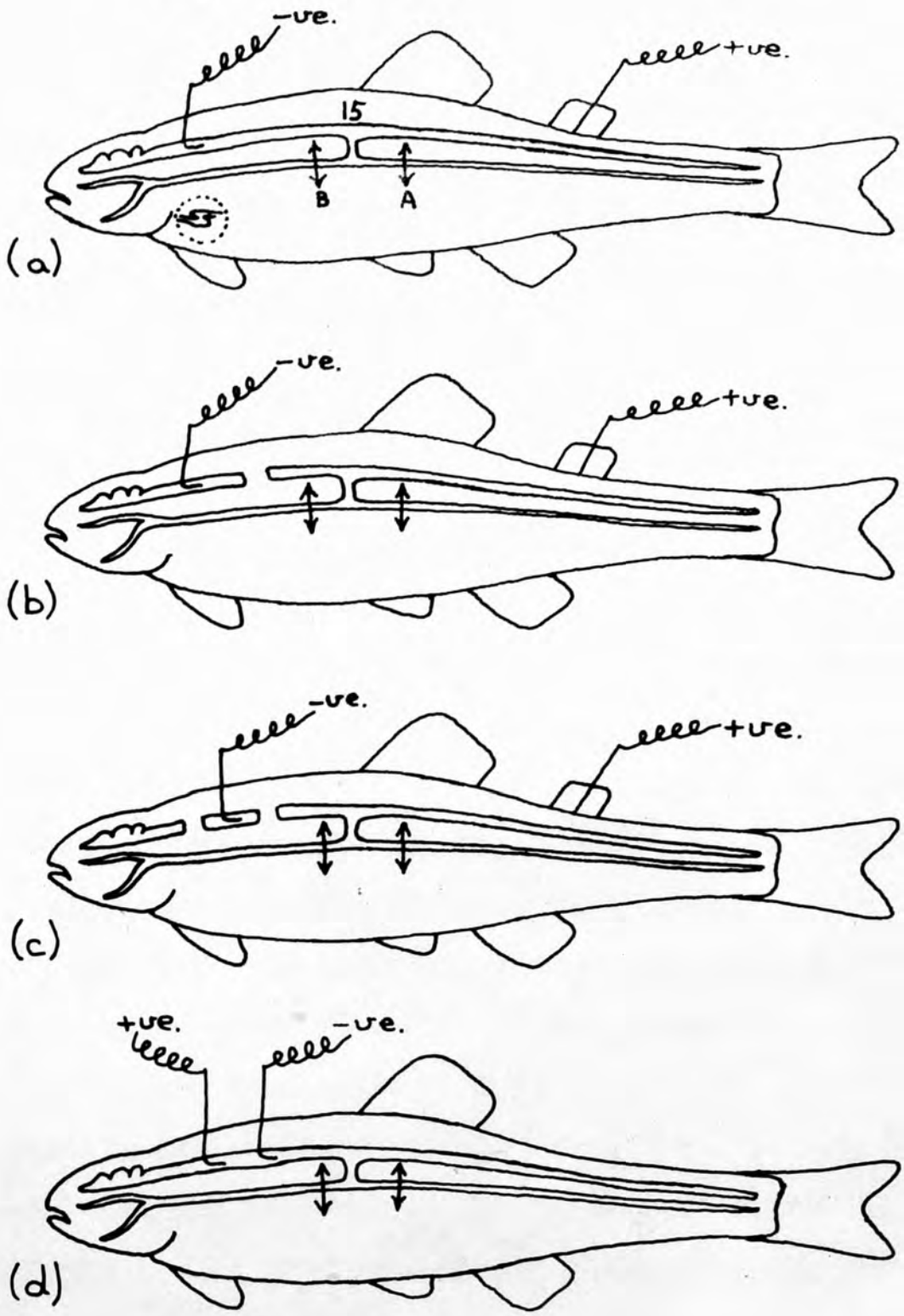
a knife jab through the body wall, at the levels A and B in Figure 33a. Each cut in turn prevented the paling from appearing more posteriorly.

In the case of cuts posterior to vertebra 15, this result is to be expected from the pathways described by v.Frisch; but on this basis paling should only occur behind a cut made anterior to vertebra 15. This was not so. While a cut at an anterior level caused complete darkening anteriorly, this region was readily blanched by electrical stimulation of the medulla. No other region was affected. The responses were always observed microscopically in order to be quite sure of this.

Thus v.Gelei's experiment, repeated without ergot treatment, produced exactly the same results, except that darkening was replaced by paling. v.Frisch's paling-fibre pathways have been confirmed by a great deal of experimental work, so it must be concluded that some alternative pathway was involved. Tests involving the section of various spinal and cranial nerves in the region of the stimulating electrode produced no change in the responses and led to the following experiments.

Three fish were subjected to spinal section anterior to vertebra 15, so that v.Frisch's fibres were occluded. After a week on a white background each fish was used for the above experiment. Stimulation was performed in the spinal cord anterior to the point of section as shown in

Figure 33.



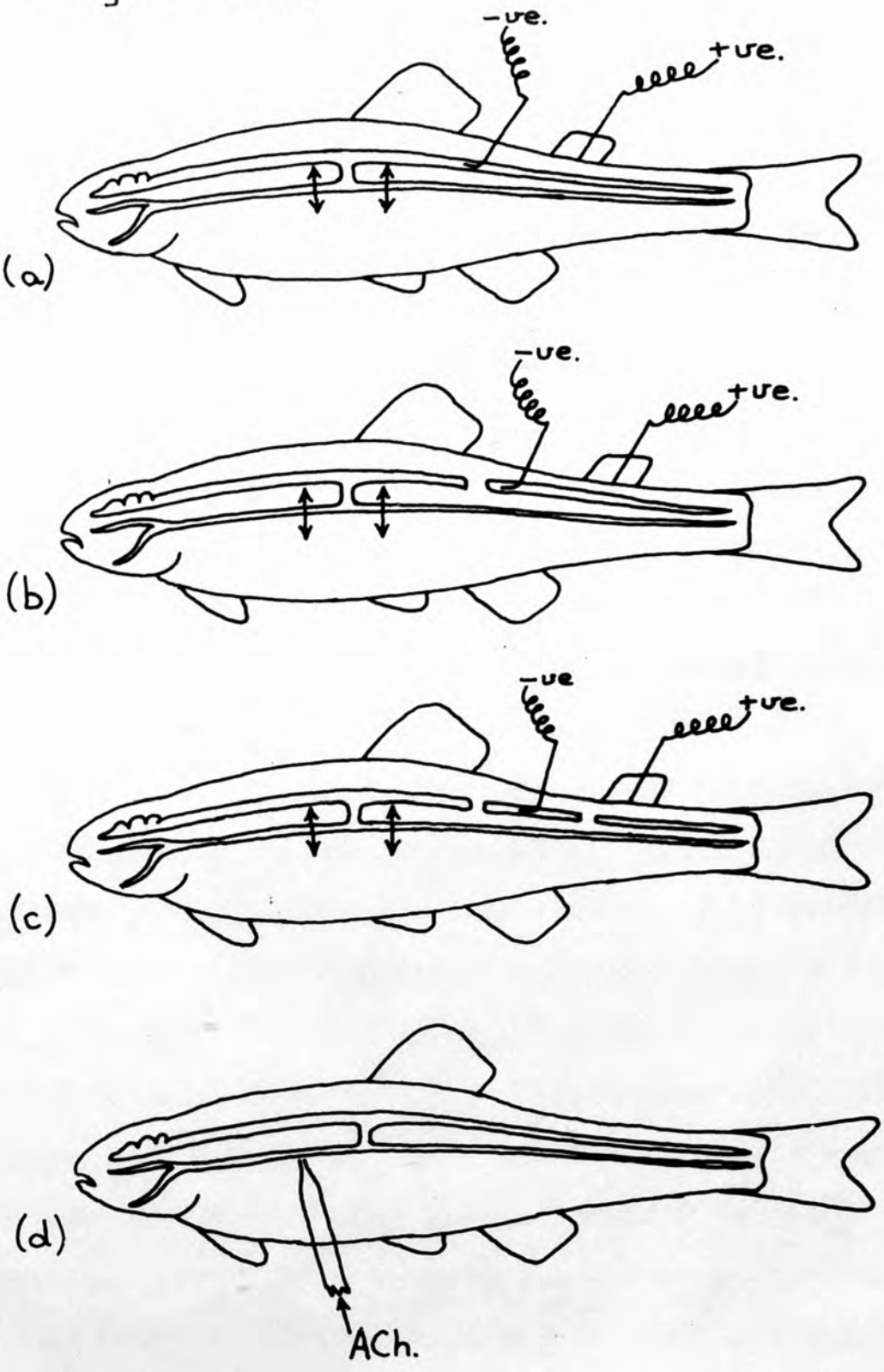
Some experiments involving stimulation of the Central Nervous System in Phoxinus.

Figure 33b. The results were exactly as before; paling did not occur posterior to any autonomic-chain section.

Three more fish were subjected to double spinal section. Two pieces of spinal cord, each occupying 2 - 3 vertebrae and both anterior to vertebra 15, were removed in order to isolate a piece of spinal cord 6 - 7 vertebrae long. The wounds were sutured and the fish returned to a white tank for 24 hours. At the end of this time each fish was reanaesthetised and stimulated as before, but with the local electrode placed in the isolated section of spinal cord, as shown in Figure 33c. The results were exactly as before; cuts in the autonomic chain both posterior to vertebra 15, and later between vertebra 15 and the electrode level, both prevented the paling response from appearing posteriorly. In this case excitation could not pass by either v.Frisch's pathways or by v.Gelei's more anterior pathways.

In the next experiment two fish were stimulated after placing the electrode in the spinal cord posterior to vertebra 15, in a region generally accepted to be chromatically inactive (Figure 34a). Paling occurred, as before, over the whole body. Cuts in the autonomic chain were made anterior to vertebra 15 and paling then occurred only posteriorly to the section. A further cut posterior to vertebra 15, but anterior to the electrode, further restricted the paling response to the posterior region.

Figure 34.



Further experiments on stimulation of the Central Nervous System of <u>Phoxinus</u>.

In the fifth experiment spinal section was performed in three fish just posterior to vertebra 15. After 24 hours each was stimulated by an electrode placed behind the section (Figure 34b). The paling response was again restricted by cuts in the autonomic chain (both anterior and posterior to vertebra 15) to the region posterior to the more posterior cut. In this case, as in the last, the cuts resulted in complete loss of circulation in the region where responses were observed, since the aorta was severed with the chain. This tends to discount any suggestion that the response was mediated by blood-borne substances.

Double posterior spinal section was performed upon three further fish, which were later stimulated by an electrode placed within the isolated piece of spinal cord (Figure 34c). The 'islands' prepared in this way were only 4 complete vertebrae long, due to the small size of the spinal cord posteriorly. Nevertheless paling was obtained exactly as before. Again cuts in the autonomic chain prevented the influence from passing forwards.

In order to be quite certain that these effects were independent of blood-borne factors, three normal fish were stimulated by an electrode placed in the anterior end of the spinal cord. After normal paling responses had been obtained, the heart of each fish was

completely removed and stimulation repeated (Figure 33a). Responses were exactly as before and cuts in the autonomic chain both posterior and anterior to vertebra 15 prevented the influence from passing further back.

Several points of general observance may now be noted.

(1) Care was taken throughout to ensure that stimulation was restricted to nervous tissues in the region of the electrode. General stimulation could be evoked at much higher voltage levels, causing violent muscular contractions and general paling of the skin. This response was not restricted in extent by section of the autonomic chain, whereas the responses described above were always sharply discontinuous at such a point. In spinal section experiments, muscle twitch first appeared only in segments served by the stimulated section of the spinal cord; this was only a narrow band in the case of double spinal sections. Thus it may be assumed that the isolated sections of cord remained active and that stimulation did not spread across the gaps where nervous tissue had been removed. In all these experiments the gaps in the spinal cord were carefully dried and filled with liquid paraffin before stimulation, in order to hinder such spread of excitation.

(2) As a further check on the stimulating process the active electrode was removed from the spinal cord in many

of the above experiments and laid upon connective tissues or muscles adjacent to the nervous tissue. The responses always disappeared until the electrode was replaced in the spinal cord. In another fish two small electrodes were inserted into the spinal cord as shown in Figure 33d. Normal responses were obtained by stimulation between one of these and the large indifferent electrode usually used beneath the body of the fish. Then both small electrodes were used for bipolar stimulation. This restricted the current flow within the body but the responses occurred at the same voltage levels as before. When the electrodes were applied to nearby muscles, no paling occurred. The electrodes were then replaced and the autonomic chain was cut anterior to vertebra 15. Paling was again elicited only anterior to the cut.

(3) Reversal of polarity of the stimulating pulses was tried in many of these experiments. In all cases the large 'indifferent' cathode produced wide-spread muscle twitches, presumably by direct stimulation in the flanks. Paling, if any, was always restricted to the region of the cathode and there was no evidence of nerve stimulation at the local anode. These findings support the conclusions of Chapter Vf regarding successful anodal stimulation in the superficial ophthalmic nerve preparations.

(4) In many preparations the fish stopped breathing at some stage, due to depth of anaesthesia, to nerve shock

when electrodes were removed and reimplanted, causing excessive damage to the spinal cord, to section of the aorta or to heart extirpation. In all cases normal responses were obtained for a considerable time, usually until 'Anämieaufhellung' set in, thus showing that the melanophore nerves were still potentially active. This was to be expected, but it helps to confirm the conclusions of Chapter IIId and Chapter IV concerning the dependence of the normal temperature responses on respiratory rhythm.

(5) In all cases, section of the autonomic chain produced rapid darkening in the regions denervated with respect to v.Frisch's pathways (Figure 1a), although this was less apparent in fish which had been subjected to anterior spinal section. A cut anterior to vertebra 15 produced complete darkening anteriorly, whereas a cut posterior to vertebra 15 gave darkening posteriorly. As both cuts were produced (in varying order) in each preparation, every fish eventually became dark at both ends with a paler band centrally. This darkening did not affect the paling responses, but only the resting states. Stimulation produced melanophore aggregation over the whole body as far as the autonomic cut nearest to the active electrode, whether this region was technically denervated or not.

(6) All fish which had received operations were examined after death to establish the effectiveness of spinal section and the vertebral levels at which this had been performed. The length of isolated sections of spinal cord, in the cases of double section, was established at this stage. Section of the autonomic chain was considered to be demonstrated during the experiment by the consequent darkening and interruption of paling responses. In any case it would be difficult to establish complete section by dissection, due to the very fine structure of the chain.

The conclusion which is drawn from these experiments is that stimulation at these voltage levels within the spinal cord does not directly excite any known chromatic tracts. Since separate pathways, running in all the directions necessary to support these results, are unlikely to exist, it must be assumed that the known tracts are stimulated indirectly. The hypothesis put forward is that local stimulation of any region of the spinal cord leads to a high level of activity in the adjacent rami communicantes. This could result in general excitation of several ganglia of the sympathetic chain. In view of the largely unspecific nature of activity within the autonomic nervous system, it is possible that chromatic tracts present in each ganglion would then be excited.

According to Nicol (1952) and Young (1931), Wernóe (1926) obtained evidence that the chromatic fibres, after

emerging from the spinal cord (at the 15th vertebra in Phoxinus) run for long distances in the autonomic chain and give off collateral branches within each ganglion. He found that excitation of fibres within any ganglion was transmitted both orthodromically and antidromically to cause colour changes along the whole length of the fish. This mechanism could provide a complete explanation of the effects observed here. Chromatic responses resulting from the excitation of any part of the spinal cord can therefore provide no evidence of an outflow of chromatic motor-fibres in that region.

If this hypothesis is correct, the general excitatory process within each ganglion may be supposed to depend on the rate of stimulation. At high pulse-rates the chemical transmitters involved might accumulate, whilst at low pulse-rates enzymatic activity may limit the level achieved and result only in specific excitation across direct synaptic junctions. The latter could not cause chromatic responses at the anterior and posterior levels used in the above experiments.

Three normal fish were therefore anaesthetised in turn and the stimulating electrode was placed in the anterior end of their spinal cords. Square negative pulses were applied at the standard rate of 10/sec., as in all earlier experiments, and paling was observed in each case. When the rate was immediately reduced to 1/sec.,

partial or complete recovery occurred. After an interval to allow complete recovery, pulses were applied at the rate of 1/sec.; the responses were absent or were represented by only a very slow and very slight melanophore aggregation. The same pulses at the rate of 1/2 sec. rarely produced any observable response. Above 10 pulses/sec. the response was quick and complete, and no further changes could be detected above 50 pulses/sec. Variation of the duration and voltage of these pulses was without effect at any repetition rate, provided that they were initially supra-liminal.

Thus the response was found to vary markedly with the frequency of stimulation. In view of the independence of the superficial ophthalmic preparation to an even wider range of pulse-frequencies, the result of the above experiments strongly suggests that the chromatic fibres were being stimulated indirectly. The proposed hypothesis is therefore supported.

The stimulation used by v.Gelei consisted of repeated spikes from a shocking-coil which was self-driven by a make-and-break in the primary circuit. No information is available regarding the frequency at which these shocks were delivered. A standard laboratory induction coil manufactured by Messrs. C. F. Palmer & Co. was tested oscillographically to determine the frequency at which shocks were produced. While adjustment of the

make-and-break contacts appeared to change the buzzing note considerably, the frequency only varied between 38 and 42 spikes per second. By making the armature strike the core of the coil forcibly during each cycle, the rate could be increased to just 50 spikes per second, but no decrease in rate below 38 per second could be achieved. It therefore seems probable that v.Gelei used stimulation rates of this order and that his results are perfectly compatible with those obtained here. The induction-coil was used to stimulate several of the above preparations, after each had been tested with the electronic stimulator, and the responses were found to be no different.

If the above assumptions are correct, it should be possible to obtain the same result by the artificial addition of acetylcholine to the ganglia, for even the adrenergic tracts (sympathetic or thoraco-lumbar outflow) possess cholinergic synapses within the ganglia of higher animals. Two fish were therefore anaesthetised and the autonomic chain was carefully exposed within the body cavity, anterior to vertebra 15, as for the sympathectomy operation. Instead of being severed, the nerve-cords were bathed in a 1% solution of acetylcholine chloride in Young's Ringer (as in Figure 34d). This solution was removed and replaced several times to ensure that it was not diluted by body fluids or by the Ringer introduced during the exposure operation. No

melanophore responses were observed in any part of either fish. The 1% solution was replaced by 10% solution which was replaced twice. Again no responses were obtained. This result either indicates that the hypothesis is incorrect, or else that the nerve-cords were surrounded by a relatively impermeable barrier of connective tissue. The latter could not be teased open, due to the small size of the nerve connectives and ganglia. A low rate of diffusion may be ineffective as acetylcholinesterase would be expected to break down the drug rapidly and so prevent accumulation within the ganglia.

A further corollary of the hypothesis of general excitation is that responses should not be confined to the melanophores. Two other sympathetic effectors which can be observed easily are the heart and the iris. Counts of the heart-rate (the pulse is clearly visible in the body wall near the heart) and observations of the iris, in several of the foregoing experiments, showed no detectable responses. Research on these matters in the literature revealed that cardiac-accelerator fibres are absent in teleosts (Kisch, 1948; Nicol, 1952), and that pupillary adaptation in teleosts is only shown by the Apodes, the Heterosomata and by Uranoscopus (Walls, 1942). No vasomotor responses could be observed in the blood vessels of the fins and

no other simple tests could be devised.

A further point against the hypothesis is that general excitation within the sympathetic nervous system would be expected to release quantities of adrenalin into the blood-stream. This cannot account for the responses obtained in the above preparations and the quick rate of recovery of the melanophores, on cessation of stimulation, suggested that it did not occur. However, no alternative proposal can be suggested at present.

c. The Effect of Injections of Ergotamine.

Wyman (1924) injected Fundulus intraperitoneally with 0.5 ml. of ergotamine phosphate solution which was "adjusted to the strength of fluid-extract ergot U.S.P." The melanophores showed a quick maximum aggregation followed by a slower dispersion to an intermediate condition which lasted indefinitely. Smith (1931a) also obtained an intermediate condition by injecting Fundulus with 0.3 - 0.5 ml. of 10% solution. But this author found that the ability to adapt completely to a white background was unaffected for up to 1½ hours after injection, after which the intermediate condition prevailed regardless of the background colour. These responses do not agree with those obtained by Giersberg and v.Gelei on Phoxinus (Chapter VIa). V.Gelei injected

minnows intramuscularly with 0.08 - 0.15 ml. of a Sandoz ergotamine preparation called Gynergen. The Sandoz preparation available in Great Britain was named Femergin, but was stated by the manufacturers to be identical in constitution. The preparation contained 0.05 mgm. of ergotamine tartrate in 0.1 ml. of placebo.

Two fish on a white background and two on a black background were each injected intraperitoneally with this dose of ergot and were replaced in their dishes. After 15 minutes the pale fish had darkened very slightly and the dark fish were unchanged. Within an hour the fish on the black background had paled until they were indistinguishable from those on the white background. The latter had shown no further change, so that all four fish finally became pale, but not maximally so. None of the fish showed the initial state of complete darkening for 1 - 2 hours, reported by Giersberg and v.Gelei, although the final pale condition reported by these workers was confirmed.

To check this result, a further sixteen fish were taken, eight from a white ground and eight from a black ground. Each was given 0.1 ml. of undiluted Femergin intraperitoneally and replaced. All became almost fully pale within one hour, with no initial darkening. The recovery time for regaining complete background adaptation varied individually from 24 to 48 hours.

Since the earlier workers on Phoxinus had injected intramuscularly, this method was also tested. Eight fish on a white background were injected in the muscles of the back with 0.1 ml. of Femergin. Again only very slight darkening, mainly by a faint flecking of the skin, was observed. This appeared within 15 minutes and no further responses occurred. A further eight fish were injected from a black background and all became almost completely pale within 5 minutes. Thus the responses were rather more rapid but otherwise were indistinguishable from those following intraperitoneal injection. Since it was both slow and difficult to inject 0.1 ml. into the muscles of such a small animal, intraperitoneal injection was always used in later experiments.

Altogether in these and subsequent experiments 109 normal fish were injected with doses of Femergin and the responses were always observed closely. In no case was there a period of marked darkening. Regardless of background colour the fish all came gradually to an almost fully pale colour which persisted for many hours. The discrepancy between these results and those obtained by v.Gelei can only be attributed to some differences in the minnows used. The results obtained by Wyman and Smith on Fundulus are different from both these.

d. Stimulation Experiments after Ergotamine Treatment.

Most of the experiments of section b of this Chapter were repeated on minnows which had been injected with ergotamine solution and had paled on a black background.

Three fish were injected with 0.1 ml. of Femergin and when pale were anaesthetised and stimulated by negative pulses from an electrode in the anterior end of the spinal cord (as in Figure 33a). Melanophore dispersion occurred over the whole surface of each fish. The response varied somewhat between different groups of melanophores; each showed a clear dispersion of melanin but in none was the response maximal, as when stimulated to aggregate. Nevertheless the skin of the fish darkened considerably when viewed macroscopically. The response was rather slower than the blanching without ergot and recovery after stimulation was slower (3 - 5 minutes), though complete.

The speed and extent of the response was again dependent upon the rate at which stimulating pulses were delivered. One pulse per two seconds had barely any effect; 5 pulses per second gave rather greater responses and 10 - 50 pulses per second produced the greatest and most rapid effects. Pulses of 2 - 3 V. were completely effective, as for stimulating aggregation. At the level required to produce wide-spread muscle-twitch (8 - 12 V.) the dispersive response gave way to one of

complete aggregation.

In two fish in which the autonomic chain was cut by a knife-thrust anterior to vertebra 15 the subsequent dispersion produced by electrical stimulation only occurred anterior to the point of section. The heart was then removed from each fish without affecting the responses.

Smaller doses of Femergin were prepared by diluting the preparation with Young's Ringer solution. Two fish were given 0.1 ml. of x2 dilution, two received 0.1 ml. of x^4 dilution and two others had 0.1 ml. of x^8 dilution each. All paled normally on a black background. One fish was stimulated as before, 3 hours after the x^8 dilution dose, and two others 1 hour and 4 hours after x^4 dilution dose. No dispersion could be elicited in any of these. Another fish was stimulated one hour after x2 dilution. Slight dispersion was produced by pulses similar to those used before. A cut in the autonomic chain anterior to the 15th vertebra prevented the excitation from passing further back, and the response was unchanged after removal of the heart. The earlier dose of 0.1 ml. of undiluted Femergin therefore appeared to be ideal for eliciting the response and was made the standard dose thereafter.

Three fish were then subjected to double anterior spinal section, to leave an isolated, but otherwise intact, section of spinal cord occupying 6 - 7 vertebrae. All

darkened normally on a white background. After 24 hours each was injected with 0.1 ml. of Femergin and after one hour was anaesthetised and stimulated by an electrode placed in the isolated piece of spinal cord (as in Figure 33c). In each case dispersion occurred when 2 msec. pulses were delivered at levels of 2 - 8 V. and at a rate of 50/sec. Autonomic-chain section behind the isolated piece of spinal cord but anterior to vertebra 15, restricted the response sharply to the anterior region.

Three more fish were given a spinal section posterior to vertebra 15. Chromatic responses to background colour appeared to be unaffected. After 24 hours each was injected with 0.1 ml. of Femergin, and, when pale, anaesthetised and stimulated by an electrode in the spinal cord posterior to the point of section (as in Figure 34b). Darkening of the whole body was produced in each case. Section of the autonomic chain and aorta by a knife jab anterior to vertebra 15 restricted the effect to the posterior region.

A single intact fish was injected with ergot. When pale it was anaesthetised and the autonomic chain was exposed within the body cavity. Applications of 1% and 10% solutions of acetylcholine chloride in Ringer (Figure 34d) were without effect on the melanophores.

On four of the above fishes the electrode was removed from the spinal cord during the experiment and

positioned so that it touched the skin of the fish. Stimulation as before produced darkening of a small area, 2 - 3 mm. in radius, around the point of electrode contact. These responses were slowly reversible. This showed that stimulation within the spinal cord, leading to darkening of the whole body, must have involved dissemination of the excitation by means other than current spread. In non-ergotised fish similar effects may be obtained, but the localised response is always an aggregation of melanin.

Finally, an intact fish was injected with 0.1 ml. of Femergin and, when pale, was prepared for stimulation of the superficial ophthalmic nerve (see Chapter Ve). A single small electrode was used with a large indifferent electrode beneath the body of the fish. Either positive or negative pulses were effective in producing melanophore dispersion within the area served by this nerve (Figure 26b), but no response was elicited elsewhere on the body. The threshold was found to be rather variable, and accurate measurements were difficult but responses were obtained from 4 V. with 5 msec. and 10 msec. pulses. On cessation of stimulation the melanophores recovered their almost fully aggregated state in 30 - 90 seconds. These responses were repeatedly obtained for some time.

e. Conclusions.

The same precautions were exercised with the experiments on ergotised fish (section d) as on non-ergotised fish (section b). In particular, care was taken to establish that no general excitation occurred but that stimulation was restricted to the nerve-trunks in the region of the local electrode. The experiments were conducted in exactly the same way and produced the same results, except for aggregation in one case and dispersion in the other.

The experimental results of v.Gelei were therefore confirmed, although it was not found necessary to administer acetylcholine after ergotamine in order to elicit a darkening response. But the experiments without ergot and with stimulation in various parts of the spinal cord show that v.Gelei's arguments for the course of the dispersing fibres are erroneous. Both paling and darkening could be elicited from any part of the spinal cord by the same levels of stimulation. The hypothesis of indirect stimulation within the ganglia of the autonomic chain therefore appears to be the only satisfactory explanation for these phenomena.

The superficial ophthalmic nerve experiment could not be repeated due to shortage of time, but the single result indicated that the direct stimulation of a motor nerve (without the possible presence of neurone switching-

circuits in intermediate ganglia) also produces dispersion when ergotamine is present. The low threshold recorded suggested that, if melanophore-dispersing fibres do exist, their excitability is little different from that of the aggregating fibres. Thus it is possible that during the electrical stimulation of nerves in non-ergotised fish the paling fibres are not preferentially stimulated but are dominant in their influence on the melanophores. If this is the case, differential stimulation by saw-tooth pulses would not be expected to succeed (see Chapter V).

Nevertheless it is impossible to state at this stage whether these experiments truly indicate the presence of melanophore-dispersing nerve fibres. Gray (1955a) suggested (1) that stimulation within the spinal cord may result in a release of adrenalin into the blood-stream, the effect of which may be reversed in the presence of ergotamine and would be restricted by section of the aorta; or (2) that ergotamine may reverse the response of effector cells to stimulation from adrenergic (paling) fibres. The first of these suggestions has already been ruled out, together with all other possible humoral influences. The second possibility will be examined more closely in Chapter VII.

CHAPTER VII.

FURTHER EXPERIMENTS WITH ERGOTAMINE.

a. Introduction.

As already stated, adrenalin is a powerful blanching agent for teleosts. If the substance is injected into whole fish, or if isolated pieces of skin are immersed in a solution containing it, complete melanophore aggregation ensues rapidly. Barbour and Spaeth (1917) found that the melanophores of isolated scales of Fundulus showed a sensible response to a solution containing only 1 part of adrenalin in 5×10^7 parts of Ringer solution. They also discovered (Barbour and Spaeth, 1917) that following exposure to a solution of ergotamine, the melanophores of these preparations became dispersed, instead of aggregated, in response to adrenalin. It is also interesting to note that Barbour (1936) reported that the sympathomimetic action of deuterium oxide on melanophores was also reversed by ergot. Gray (1955a) and Healey (1957) reported that Verne and Vilter (1935), Vialli (1927) and Ciabatti (1929) obtained similar reversals to adrenalin in Carassius, Gambusia and Scardinius. Gray (1955a) therefore suggested that in v.Gelei's experiments ergotamine merely reversed the response of melanophores to the transmitter-substances released when adrenergic (paling) fibres were stimulated.

Barger and Dale, in 1910, first distinguished between

sympathin, found in perfusates after sympathetic stimulation, and epinephrine (adrenalin), the secretion of the adrenal medulla. They suggested that adrenalin was a methylated form of sympathin and this has since been confirmed (cited in Goodman and Gilman, 1956). Describing the effect of each substance on blood-pressure, they stated that the presence of ergotoxin "... leaves the amino-base with a remnant of pressor action, while that of the methylamino base (adrenine) is replaced by depressor action". On the other hand, the motor response of the retractor-penis muscle of the dog to both adrenalin and sympathetic stimulation is gradually suppressed but is never reversed by ergotoxine. In other cases both responses may be reversed but to different extents. "... certain normally motor effects of adrenine are reversed by smaller doses of ergotoxine than are needed for the reversal of the corresponding motor effects of stimulating sympathetic nerves". Goodman and Gilman (1956) state that this has now been confirmed and is generally accepted.

In the circumstances, it seemed advisable to examine the responses of minnow melanophores to each of these substances, both before and after treatment with ergot. Wyman (1924) was unable to support Spaeth and Barbour's result by the injection of intact Fundulus. Giersberg also remarked that minnows injected with 0.1 ml. of 0.05%

ergotamine solution, and used for nerve stimulation experiments, produced maximal aggregation when injected with adrenalin. It was therefore decided to use both intact minnows and isolated skin preparations in these investigations.

b. Tests by Injection.

Twelve normal minnows were allowed to adapt fully to black backgrounds. Then eight were injected with 0.1 ml. of Femergin (0.05 mgm. of ergotamine tartrate). After one hour all eight were almost fully pale (see Chapter VIc). Four of these fish and the four untreated fish were then each injected intraperitoneally with 0.1 ml. of a solution containing 1 part in 5,000 of Adrenalina B.P., (= adrenalin = adrenaline = adrenine = epinephrine = methylamino-ethanol-catechol), manufactured by Messrs. British Drug Houses. Of the fish which had received adrenalin only, two paled maximally in 30 minutes and the other two were pale except for a few flecks of colour dorsally. After an hour all four were beginning to darken somewhat and after two hours they had recovered completely to the dark-adapted state on the black background. No difference was detectable by the unaided eye at any time between the fish which had received ergotamine only and those which had received both

ergotamine and adrenalin. All stayed almost fully pale on a black background.

In another experiment eight normal fish were allowed to adapt to a black background and then four were given 0.1 ml. of Femergin each. After an hour all eight were injected with Ringer solution saturated with adrenalin. The non-ergotised fish all paled maximally within 15 minutes, but after an hour were noticeably darker again. Recovery was not quite complete after 2½ hours. The fish treated with ergot became fully pale 15 - 30 minutes after injection with adrenalin. After one hour each fish was darkening slightly and after 2½ hours had regained the typical colour of an ergot-treated fish. This condition persisted for many hours as usual. The response to adrenalin was only slight, since the fish were already almost fully pale on a black background, but it can be stated emphatically that at no time was there the slightest indication of melanophore dispersion in any of these fish.

The same experiment was repeated, using l-noradrenalin (= norepinephrine = sympathin = levartenol = amino-ethanol-catechol) bitartrate, manufactured by Messrs. L. Light and Co. Four black-adapted fish were each injected intra-peritoneally with 0.1 ml. of a solution containing 1 part in 5,000 of noradrenalin. All paled completely, except for slight flecks dorsally, within 10 minutes. The

flecks were rather more pronounced after an hour but the fish remained otherwise pale. All redarkened almost completely within 2½ hours from the time of injection.

To test the effect of ergot on this dose, twelve black-adapted fish were divided in to three groups of four fish each. Eight fish were given 0.1 ml. of Femergin intraperitoneally and allowed to pale for one hour. Then four of these fish and the four untreated fish were each given 0.1 ml. of 1 part in 5,000 l-noradrenalin solution. The fish with noradrenalin only rapidly became completely pale and were clearly distinguishable from those which had received ergotamine only. Two fish showed flecks dorsally but after an hour one of these had become evenly and maximally pale. All four were much darker after two hours and at three hours had recovered almost completely. The four fish which received both ergotamine and noradrenalin were at all times indistinguishable from those which had been given ergotamine only. They showed neither a darkening response nor a further paling. All recovered from the ergotamine treatment only after several hours.

These experiments on intact fish indicate that the administration of 0.05 mgm. of ergotamine tartrate may suppress melanophore reaction to 0.02 mgm. of either adrenalin or noradrenalin. Adrenalin at least, is still effective in massive doses (about 0.1 mgm.). No other

doses were tested, but the effects of the above doses, when administered alone, suggest that they were neither abnormally high nor critically low. No evidence was obtained for a reversal of aggregating effects, and the results of Wyman (1924) on injected Fundulus and of Giersberg (1930) on injected minnows are therefore confirmed.

c. Tests on Isolated Skin Preparations.

Spaeth and Barbour (1917) exposed scales, bearing melanophores, to solutions of 1 part of adrenalin in 10,000 of Ringer. The normal aggregating response was "temporarily reversed" by prior immersion in a solution containing 1 part in 3,000 of ergotamine phosphate for 30 minutes, while exposure for an hour to ergot solution caused "prolonged reversal". If allowance is made for the dilution of an injected dose within the body fluids of the fish, these concentrations are much higher than those encountered by the melanophores in the injection experiments.

Pieces of minnow skin were prepared as described in Chapter IIe. This method succeeds in isolating the melanophores from the complex influences of the fish body, and is the nearest approximation which can be made to the isolated scale preparations of Fundulus, as used by Spaeth et al. The response of these preparations to

high concentrations of adrenalin has already been used as a test for responsiveness at the end of each experiment in Chapter IIe.

A normal fish was anaesthetised in 0.5% urethane, a strip of skin was removed from its flank and the fish was decapitated. The skin was divided into four pieces, each of which was transferred to a small dish of Young's Ringer. Microscopical observation showed that for the first 15 minutes the melanophores were unaffected by this solution (see Appendix IV). The Ringer solution was then carefully replaced by a similar solution containing 1 part in 10,000 of adrenalin. All the melanophores aggregated completely in 7 - 10 minutes, the response being quickest at the periphery of each preparation and slowest at the centre. The adrenalin solution was removed at this stage and replaced by fresh Ringer which was changed frequently. After a further 45 minutes the melanophores of the thinnest piece of skin started to redisperse their pigment and in a further 15 minutes they had regained their original state. The melanophores of the other three pieces, with rather more body tissue attached, showed no signs of recovery up to 1½ hours after removal of the adrenalin solution. After a further 12 hours in plain Ringer solution all melanophores were found to be fully aggregated. Whereas Spaeth's preparations showed reversible and repeatable responses, the melanophores

were in much more intimate contact with the surrounding solutions than were those of the minnow preparations. The epidermis may be regarded as fairly impermeable and the sub-epidermal tissue which accompanied these preparations must have presented a barrier to diffusion. In view of the extreme sensitivity of the melanophores to adrenalin, it is hardly surprising that leaching of the drug to an ineffective level did not always occur. The lack of reversibility and the slowness of the responses, while unfortunate in permitting each experiment to be performed only once, are therefore not regarded as invalidating the preparation or its responses.

A second fish was anaesthetised and a strip of skin was removed from each flank. Each strip was divided into four pieces and all were put in Ringer. The melanophores showed no changes after 10 minutes. Four pieces were then transferred to a dish of Femergin (1 part of ergotamine in 2,000 parts of placebo). In each of these the melanophores aggregated completely in 30 minutes while those left in Ringer showed no change. All were then washed in plain Ringer and placed in separate dishes containing a solution of 1 part adrenalin in 10,000 parts of Ringer. The non-ergotised preparations showed complete melanophore aggregation within 5 minutes. They were then transferred to plain Ringer where some recovery was apparent after an hour. The ergot-treated preparations

showed no further response up to 3 hours. The experiment was repeated on eight pieces of skin from another fish with exactly the same result.

The ergotamine phosphate used by Spaeth and Barbour was dissolved in an ionically balanced solution whose composition was similar to that of Young's Ringer (see Appendix IV). In the above experiments ergotamine tartrate was already dissolved in a placebo consisting of sodium chloride and tartaric acid (see Appendix IV). Spaeth (1913) reported that excess sodium ions caused strong melanophore dispersion, so that ergotamine must have overcome this response in the above experiments. It is possible, however, that the ergotamine and placebo had damaged the melanophores in the aggregated condition so that no further responses could be expected. To test this, 1 part of crystallised ergotamine tartrate (manufactured by Messrs. Burrough's Wellcome) was added to 2,000 parts of Young's Ringer solution. This formed a light flocculent precipitate which dissolved on the addition of a small amount of tartaric acid. A similar quantity of tartaric acid was added to some fresh Ringer for use as a control medium.

Eight pieces of skin were obtained from a normal fish as before. Four were placed in ergotamine-Ringer solution and four in the control-Ringer solution. All melanophores in ergot became completely aggregated, while those in the control solution assumed a wide range of

conditions. Upon being transferred to a solution of 1 part of adrenalin in 10,000 parts of Ringer the ergot-treated melanophores showed no change while the control melanophores aggregated completely and rapidly. In a further experiment on another fish the melanophores were not completely aggregated by ergot and, when placed in adrenalin, a further aggregation was observed. The control pieces, as before, showed aggregation only in adrenalin.

This experiment was then repeated with l-noradrenalin solution. Eight pieces of skin were removed from a fresh fish; four were placed in ergot (Ringer + ergotamine tartrate (1:2,000) + tartaric acid), and four in control solution (Ringer + tartaric acid). After an hour the ergot-treated melanophores showed considerable, but not maximal, aggregation while the controls were unchanged. All melanophores in both series of preparations aggregated completely when placed in l-noradrenalin solution (1 part in 10,000 of Ringer). Similar results were obtained in an experiment on a second fish, after ergot and control-solution treatment for 30 minutes.

It was still possible that the treatment of pieces of skin in solutions of ergotamine was not equivalent to the effect of injecting the drug into intact fish. Two fish were therefore injected, each with 0.1 ml. of undiluted Femergin (as for the nerve stimulation

experiments), whereupon they paled on a black background. After one hour the fish were anaesthetised and four pieces of skin from each were removed to adrenalin solution (1 part in 10,000 of Ringer). In one case aggregation was already almost complete and no further change occurred. In the other case complete aggregation of all melanophores occurred in 20 minutes.

Finally two normal fish were injected with 0.1 ml. of Femergin each and pieces of skin were removed after 30 minutes in one case and an hour in the other. Pieces of skin were also prepared from a third, untreated, fish. Neither of the ergot-treated series of preparations showed complete aggregation, while the untreated pieces were almost completely dispersed. When transferred to a solution of l-noradrenalin (1 part in 5,000 of Ringer), all aggregated completely and rapidly.

In these last experiments the melanophores received exactly the same treatment as in the nerve-stimulation experiments but no reversal of response to either adrenalin or l-noradrenalin could be demonstrated. In many cases there still remained an aggregating response. These results indicate that the dispersion which occurs in ergot-treated fish, when chromatic nerve-tracts are stimulated electrically, is not due to a reversal of response to these particular transmitter substances. It might therefore be assumed to be a response to the

excitation of dispersing fibres when the adrenergic sensitivity is reduced as originally suggested by Giersberg.

Nevertheless it is still possible that the aggregating nerve-fibre endings in the skin of the minnow produce neither adrenalin nor noradrenalin. Several other sympathomimetic substances with related molecular structure are now known. Their natural occurrence is doubtful, but one of them may occur in the minnow. In view of the differential and varying effects of ergot on adrenalin and noradrenalin (Barger and Dale, 1910; Goodman and Gilman, 1956), the substance produced in the minnow may be more susceptible to reversal of its influence than either of these. Unless the chemical mediator (or neurohumor) liberated by melanophore-aggregating nerve-fibres can be identified, the experiments with ergotamine cannot conclusively establish the presence of double innervation.

d. Nerve Section after Ergotamine.

Since the chromatic response to nerve stimulation is reversed by treatment with ergotamine, it would be of interest to investigate the effect of this drug on other chromatic responses. The local darkening of newly denervated areas was considered by Parker to demonstrate the presence of dispersing fibres (see Chapter Va) and ergot, unlike many other adrenergic blocking agents, does

not result in complete melanophore dispersion. The two influences were therefore applied together.

Three normal minnows were allowed to adapt to a black background. Then each was injected intraperitoneally with 0.1 ml. of Femergin. After 20 minutes, 40 minutes and 1 hour, respectively, each fish was anaesthetised and subjected to both caudal-band section (after Gray, 1955a) and unilateral superficial-ophthalmic-nerve section (see Chapter Ve). The wounds were sutured and the fish allowed to recover on a black background. No sign of any responses could be detected, either microscopically or by the naked eye, on the head of any fish, up to 2 hours after nerve section. On the tails of two of the fish there was a suggestion of very slightly greater aggregation within the denervated area. This was very indefinite because of the pale condition of the rest of the skin.

In order to check on this response, the experiment should be repeated using a second drug to oppose the direct action of the ergot, and so bias the melanophores in the direction of greater dispersion. This principle would have to be quite independent of the nervous system in its action. Melanophore-dispersing hormone prepared from mammalian pituitary glands would appear to be suitable, but no active preparation was available at the time.

The experiment is worth reporting for the lack of dispersion alone. If ergot merely reduces adrenergic sensitivity, as Giersberg supposed, then stimulation of dispersing fibres by cutting them, as Parker supposed, would be expected to be all the more effective. The direct (aggregating) effect of ergot on the melanophores could hardly be held to prevent them from dispersing, since they accomplish this readily when stimulated electrically. On the other hand if, as has been widely held, the initial dispersion after denervation is due to loss of aggregating tonus, a further loss after the fibres have been blocked by ergot would have no effect. In this case the dispersion elicited by electrical stimulation is difficult to explain. If ergot reverses the influence of adrenergic paling-fibres, as Gray suggested, a further loss of tonus due to nerve section should result in further aggregation as a direct response to ergotamine. This was not observed on the head but may have occurred on the tail. These results therefore suggest that either Giersberg or Parker was incorrect in his assumptions, but the position requires much more detailed investigation and this, unfortunately, could not be attempted in the time available.

e. Temperature Responses after Ergotamine.

The theory of the local temperature effect put forward in Chapter IIf supposes that double innervation does exist in the minnow. An investigation of this response after ergot treatment was therefore made.

Smith (1931a) found that in Fundulus treated with ergotamine (as described in Chapter VIc) the melanophores dispersed their pigment at low temperatures and aggregated it as high ones. He assumed that the response was equivalent to that of denervated melanophores and represented a direct response to temperature on the part of the effector cells after the thermal reflex had been blocked.

Four fish were in turn allowed to adapt to a black background and injected intraperitoneally with 0.1 ml. of Femergin. When paling had progressed to the normal ergotised condition, each fish was anaesthetised in urethane and subjected to temperature tests as performed in Chapter IV. Three fish became a little darker when comatose, but much less so than normal fish, and one remained pale. In every case cooling by melting ice produced considerable local darkening, although melanophore dispersion never became complete. This response was carefully checked and repeated several times on each fish. Recovery of the original condition always occurred at room temperature. When the temperature of a

jet of water was raised slowly, two fish showed no response (one was very pale), and the other two showed distinct local paling at 35°C. This was reversible and repeated several times. Each fish maintained a steady respiratory rhythm throughout the experiment and afterwards recovered completely.

Thus it seems that, as Smith found in Fundulus, the temperature effect becomes reversed (or perhaps the denervated response is produced) after administration of ergotamine.

The results of this Chapter will be discussed in Chapter VIIIg.

CHAPTER VIII.

EXPERIMENTS WITH SOME OTHER DRUGS.

a. Rogitine.

Goodman and Gilman (1956) stated that the specificity of ergot is low, although the effectiveness of adrenergic blockade is high. Effects upon the central nervous system were said to be complex. It therefore seemed advisable to repeat some of the foregoing experiments with an

alternative sympathicolytic agent.

Rogitine was claimed to be one of the most potent and most selective of adrenergic blocking agents available. Produced by Messrs. CIBA Laboratories Ltd., it is also known as Rogetine, Regetine, and C-7337. Its rigorous chemical name is 2(N-p-tolyl-N-(m-hydroxyphenyl)-aminomethyl)-imidazoline, and it is supplied in ampoules containing a solution of its methane sulphonate. The manufacturers claimed that "...in comparatively small doses, Rogitine blocks the peripheral effect associated with stimulation of the sympathetic nerves" and that it "...does not antagonise acetylcholine or atropine". This has been confirmed quantitatively by Trapold, Warren and Woodbury (1950), but these authors also stated that the rise in blood pressure in response to epinephrine (in dogs) was reversed by doses of Rogitine between 0.8 and 1.0 mgm./kgm. Side effects were said to be few and general toxicity to be very low.

This drug therefore appeared to be a suitable sympathicolytic agent for comparison with ergotamine.

In the absence of any previous records of the effects of Rogitine on colour change in teleosts, a fairly extensive series of tests was performed. The drug was available in ampoules containing 5 mgm. of the methane sulphonate in 1.0 ml. of placebo. One white-adapted minnow was injected intraperitoneally with 0.1 ml. of this

preparation, a dose of 0.5 mgm. or about 140 mgm./kgm. Complete darkening occurred within 8 minutes and the fish was dead after 17 minutes. The contents of the ampoule were then diluted with an equal amount of Ringer and six fish were each given 0.1 ml. of this solution. All darkened maximally but three died after an hour; the other three recovered completely. This dose (0.25 mgm., or 70 mgm./kgm.) appeared to be about the acute lethal dose for 50% of the recipients. Messrs. CIBA stated that toxicity occurs in mammals at a dose of 75 mgm./kgm. Subsequently one part of placebo was diluted with three parts of Ringer and 0.1 ml. of this solution was used as the standard dose (0.125 mgm. or 37.5 mgm./kgm.) No ill effects were observed in the ensuing tests.

(1) Three fish were maintained on a black background for a considerable period so that a low level of hypophyseal melanophore-aggregating hormone should be present in the blood-stream. They were then placed on a white background until just white-adapted and each was injected with a standard dose of Rogitine. All darkened completely within 15 minutes. After 6 hours each fish was distinctly paler, and after 12 hours all were almost completely white-adapted again.

(2) Three more fish which had been long black-adapted were each injected with a dose of Rogitine into which had been ground one acetone-dried minnow pituitary gland (containing

a melanophore-aggregating principle). The fish were replaced on a black background. All paled almost completely at first, as if given pituitary extract alone, and slowly recovered their fully dark condition in about 6 hours.

(3) The same experiment was then repeated on three fish newly adapted to a white background. All darkened somewhat following the injection, but then paled completely from 30 to 60 minutes after injection. Each fish then darkened to an intermediate condition after 3 hours and paled again up to 18 hours.

(4) Three further fish which were each injected on a black background with one minnow pituitary extracted in 0.1 ml. of Ringer, paled maximally in about 45 minutes and darkened again in 3 - 5 hours (see Figure 10). From this it appears that Rogitine acts more quickly than injected melanophore-aggregating hormone, and that its effect lasts longer, but that the hormone is dominant for some time at the levels administered.

(5) Three fish were allowed to adapt to a white background in a good light for nine days, by which time the concentration of aggregating hormone in the blood should have reached equilibrium at its normal high level (Healey, 1951). When injected with a dose of Rogitine all three fish darkened completely within 30 minutes and paled slowly over some hours. A normal level of hormone does

not therefore appear to affect the responses to this dose of Rogitine.

(6) Four more long white-adapted fish were injected with 0.1 ml. of 1 part Rogitine in 9 parts of Ringer, a dose of 0.05 mgm. All darkened normally at first but complete recovery occurred in 3 - 6 hours in different individuals. The larger dose (0.125 mgm.) was therefore used subsequently.

A controlled dose of injected minnow pituitary hormone thus appears to be suitable for opposing the dispersing action of Rogitine (whatever the mechanism of this action), to permit investigation of nervous dispersing activity under the influence of this drug.

b. <u>The Effect of Rogitine on some other Responses</u>.

A fish was injected with 0.1 ml. of Rogitine (diluted x4) containing one minnow pituitary gland. After an hour, when the paling phase was maximal, the animal was anaesthetised and prepared for electrical stimulation of the superficial ophthalmic nerve, as in Chapter Ve. Pulses from 2 to 20 msec. in duration were applied at frequencies from one per second to 100 per second in each of three electrode configurations; unipolar cathodal, unipolar anodal, and bipolar. In every case the voltage was raised to the level which caused violent muscle twitch

over the whole body (about 12 - 20 V.), but no melanophore responses could be evoked. Two hours after injection the melanophores were beginning to disperse their pigment somewhat as the effect of the aggregating hormone decreased, but neither aggregation nor dispersion occurred upon electrical stimulation. In case the melanophore nerves had been irreversibly damaged by the injection, the fish was allowed to recover. After 24 hours it was again anaesthetised and the superficial ophthalmic nerve in the other orbit was stimulated. Normal aggregating responses occurred as in Chapter Vf. The fish, now bilaterally blinded, was decapitated under anaesthetic.

This experiment was carefully repeated on two further fish and exactly the same results were obtained. Thus Rogitine appeared to abolish nervous influences completely but temporarily, while leaving the melanophores free to respond to humoral factors if these were present in large quantities.

Four fish were then injected with Rogitine on a white background. After 30 minutes, when each was completely dark, it was anaesthetised and tested for temperature responses, as in Chapter IV. No responses could be obtained by local heating up to 36°C., nor under melting ice, nor after the removal of ice. Each test was repeated both quickly and slowly, and prolonged exposure to the extreme temperatures was used, but no

changes occurred in the melanophores. All the fish breathed regularly and recovery from both anaesthetic and Rogitine appeared to be complete.

In case Rogitine exerted too powerful a dispersing influence, the experiment was repeated with four fish which each received Rogitine and one minnow pituitary gland. Each was anaesthetised in the pale phase and tested as the effect of the hormone gradually diminished. In every case marked but not complete dispersion occurred when heated to 36°C. and also under melting ice. These responses were always reversible and were repeatedly evoked in each fish for a considerable period. In one fish respiratory movements ceased during exposure to water at 35°C. and no recovery occurred at 20°C. When the fish was resuscitated by dilution of the anaesthetic supply, melanophore recovery took place and both high and low temperature responses were again obtained. All the fish recovered normally in freshwater.

Finally the response of melanophores to both Rogitine and adrenalin was tested. Three fish were injected with Rogitine on a white background and when fully dark were transferred to a black background. Three further fish were injected with a similar quantity of Ringer and placed upon a black background. Thirty minutes after the first injection all six fish were each given 0.1 ml. of adrenalin (1 part to 5,000 of Ringer). All paled equally and at similar rates. The course of recovery varied somewhat

between individuals but no distinction could be made between fish of the two groups. Thus Rogitine appeared to have no effect upon chromatic responses to injected adrenalin at the doses used.

c. Atropine.

This drug was selected as a readily available cholinergic depressant. Goodman and Gilman (1956) stated that clinical doses of atropine in mammals blocked the influence of post-ganglionic cholinergic fibres, leaving the adrenergic system intact and dominant. Barbour and Spaeth (1917) found that concentrations between 1 in 1,000 and 1 in 100,000 produced dispersion in isolated scale melanophores of Fundulus. Wyman (1924) confirmed this by injecting Fundulus with 0.5 ml. of 0.5% solution of atropine sulphate, a dose of 2.5 mgm. per fish. This produced complete dispersion in 4 minutes. Smith (1931a) injected Fundulus with 0.3 - 0.5 ml. of 0.013 - 0.11% concentrations of atropine sulphate. The results were said to be uniform regardless of the dosage. On a white background the fish became intermediate in colour within 5 - 15 minutes; on a black background no change occurred. Similar results were obtained by the same author on Phoxinus (1931b). When transferred from one background to the other, atropinised Fundulus showed adaptive

responses between the two states described. The complete response, therefore, had the opposite effect to that described for Fundulus treated with ergotamine (see Chapter VIc). At higher doses Smith was able to confirm Wyman's results, but none of the fish survived. Smith argued that the lethal dose caused complete darkening by general paralysis, while smaller doses had a more specific effect.

Smith also tested the effect of local temperature changes on atropinised Fundulus. After doses sufficient to produce partial darkening on a white background, the melanophores produced complete aggregation at low temperatures and complete dispersion at high temperatures. The actual temperatures employed were not defined, but Smith concluded that normal responses were not affected by atropine treatment.

For the present work atropine was available as the sulphate. One fish was injected intraperitoneally with 0.1 ml. of a solution containing 1 part of the salt in 200 of Ringer, a dose of 0.5 mgm. for a 3.5 gm. fish. On a white background maximum melanophore dispersion occurred and the fish died after 20 minutes. Three fish were then given 0.1 ml. of 1 part in 1,000 solution on a white background and all darkened completely in 15 minutes. Recovery of the white-adapted condition was not quite complete after 7 hours. This dose (0.1 mgm.) was made

the standard for subsequent tests.

Three fish were given a dose of atropine sulphate with one minnow pituitary each, upon a black background. Paling occurred in 30 - 45 minutes, although it was incomplete in one fish. Redarkening took place gradually over the next 3 - 5 hours. When three other fish were similarly tested upon a white background slight dispersion occurred initially but the fish paled again after 20 - 45 minutes; darkening occurred again about 4 hours after injection but this did not become complete and the fish became maximally pale again at 16 hours. Thus the response to atropine appeared to be similar to that produced by Rogitine. This was surprising since the two drugs are supposed to produce different effects in the autonomic nervous system. The results of Smith cannot therefore be confirmed in the minnow. Relatively small doses produced responses which were only given by lethal amounts in Fundulus.

d. The Effect of Atropine on some other Responses.

Three normal fish were each injected with atropine and one minnow pituitary gland and were left on a black background until darkening commenced. Then each was anaesthetised and prepared for electrical stimulation of the superficial ophthalmic nerve. Unipolar stimulation

produced slow melanophore aggregation which was quickly reversible and repeatable. Thresholds appeared to be unaffected by the drug and bipolar stimulation was also effective in producing aggregation. The slowness of the response did not therefore seem to be caused by a decreased excitability of the nerve-fibres but by some antagonism of their influence.

Four minnows were given standard doses of atropine upon a white background. After 30 minutes, when fully dark, each was anaesthetised and tested for local temperature responses. No changes could be produced by heating to 36°C., by cooling under melting ice, or after the removal of ice. All fish produced regular respiratory movements and recovered quickly in an aquarium. Four further fish were injected with atropine together with one minnow pituitary gland each. When paling was complete on a black background the fish were anaesthetised in turn and examined as before. The melanophores were now largely in the aggregated condition but complete dispersion was produced both under melting ice and at about 35°C. in every fish. The responses were all reversible at room temperature and were all carefully checked. These results are at variance with those of Smith on Fundulus and resemble instead those obtained with minnows treated with Rogitine. Respiratory movements ceased for a time in one fish while the melanophores were at 35°C. No recovery

occurred at 16°C. until the opercular movement was restored. A similar incident occurred with one of the fish treated with Rogitine (Chapter VIIIb) and was frequently observed in normal minnows (Chapter IIId).

e. Acetylcholine.

This substance has often been supposed to act as the chemical mediator for the hypothetical melanophore dispersing fibres. When used to influence teleost melanophores it has generally produced little effect. Barbour and Spaeth (1917) reported that the isolated-scale melanophores of Fundulus were unaffected by concentrations as high as 1 part in 1,000. Slight responses in either direction were described for various other species by Parker (1948). It is generally believed that cholinesterases in the tissues break down the substance too quickly for marked responses to occur. More reliable results have been obtained by injecting Ameiurus with eserine (Parker, 1948) so as to antagonise acetylcholinesterase and enhance the effect of natural or injected acetylcholine. This procedure produced strong darkening with both lethal and sub-lethal doses. Giersberg (1930) and v.Gelei (1942) believed that both acetylcholine and ergotamine were necessary in order to produce dispersive responses to electrical stimulation.

It was felt that the experiments reported here would be incomplete without a brief investigation of the effect of acetylcholine in the minnows used. The absence of response when the sympathetic chain was bathed in solutions of the drug was described in Chapter VIb and d.

Acetylcholine chloride, produced by Messrs. Roche and Co., was dissolved in Ringer solution at various concentrations and was prepared afresh for each experiment. A minnow was injected intraperitoneally with 0.1 ml. of 10% solution on a white background. It became comatose very quickly and showed considerable darkening in patches. It died after 15 minutes. Four more fish were each given 0.1 ml. of 1.0% solution on a white background. Two darkened and died within 50 minutes, a third became somewhat darkened in 20 minutes but later recovered, and the fourth appeared to be unaffected chromatically, although it was rather unexcitable for some time.

Four further fish were given 0.1 ml. of 0.25% solution on a white background. No chromatic responses or harmful effects appeared to be induced. Finally four fish were given this dose upon a black background, again without effect. V.Gelei stated that the doses he administered varied from 0.02 to 0.04 ml. of 0.5 - 1.0% solution. These figures give extreme values equivalent to 0.1 ml. of 0.1 - 0.4% solution. The doses used above were therefore comparable to these.

Four fish on a white background and four more on a black background were each injected with 0.1 ml. of Femergin (a standard dose of ergotamine). After 45 minutes, when all were almost fully pale, each was further injected with 0.1 ml. of 0.25% acetylcholine chloride. No changes were detectable up to 2½ hours.

Thus lethal doses of acetylcholine may produce uneven darkening. This does not seem to be a very satisfactory response since it could be due to complex side-effects. These simple experiments produced no evidence that the drug affects the chromatic responses of the minnow.

f. Urethane and Acetylcholinesterase.

After the experimental work for this thesis had been completed, it was found that the urethane grouping has been reported to be antagonistic to acetylcholinesterase (this is discussed by Wright, 1952). The possibility arose that urethane itself acted as an anticholinesterase. This was disturbing in view of its wide use as an anaesthetic in experiments on double innervation. Also it provides a ready explanation, in terms of cholinergic darkening fibres, for the darkening observed on anaesthetisation (but not for the subsequent paling - see Chapter IIIa).

At this time Dr. J. C. McAlpine, of the Institute of Laryngology and Otology, London, was making a comparative

survey of cholinesterase activity in different animals. He kindly agreed to examine the effects of urethane in the minnow. The following experiments were performed by Dr. McAlpine with assistance from the author.

Small blocks of muscle were cut from the flanks of several normal minnows immediately after they had been decapitated. Each specimen was rapidly frozen in a jet of carbon dioxide and placed in a cryostat at -20°C. Cold microtome frozen sections, 10μ thick, were cut and mounted on glass slides. These sections were then treated by a thio-acetic acid method (McAlpine, unpublished) in order to demonstrate acetylcholinesterase activity in the motor-end-plates of the skeletal muscles. No unspecific cholinesterases were found. Further sections were incubated for both 10 minutes and 20 minutes in 2M, M, M/2 and M/10 solutions of urethane (ethyl carbamate). No inhibition was found at M/10, and only slight inhibition occurred in M/2 and Molar concentrations. In the 2M solution completely negative results were obtained in every case (indicating complete suppression of enzyme activity) although the controls (without urethane) showed normal activity. Thus it was concluded that urethane does antagonise acetylcholinesterase but only in concentrations greater than half-molar, which represents a concentration of 4.45% w/v. Similar results were obtained with sections of rat-tongue which was used

as standard material for the method.

As an anaesthetic, urethane was generally used in concentrations of only 0.5% and 0.25% (Chapter Ie), but it is possible that the gills of the fish actively absorbed the salt and that higher concentrations could have been attained in the body fluids. One fish was therefore anaesthetised by the normal method and was maintained under 0.25% urethane for one hour. Then this fish and an untreated animal were decapitated and sections of their muscles were prepared as before. Sections from both fish showed equal acetylcholinesterase activity by the thio-acetic acid method. When incubated in the presence of eserine, at a concentration of 1×10^{-5}., both showed complete inhibition of enzyme activity.

Further sections from these two fish were treated by an alternative method due to Gomori (1952). This consisted of two separate tests, (1) by acetylthiocholine iodide (ATCI) to demonstrate specific acetylcholinesterase, and (2) by butylthiocholine iodide (BTCI) for unspecific cholinesterases. All were incubated for one hour at 40°C. Again no distinction could be drawn between the anaesthetised and unaesthetised fish from the ATCI sections. The BTCI test failed to demonstrate the presence of any unspecific enzymes and ATCI in the presence of eserine produced completely negative results. This concludes the work of Dr. McAlpine for this thesis.

It can be stated therefore that true acetylcholinesterase is found in the muscle-end-plates of the minnow and that its activity is not suppressed either by normal anaesthesia in urethane or by direct exposure to urethane in concentrations less than about 4.45%. This does not preclude effects on centres within the central nervous system which might cause the responses of the normal anaesthetised fish.

No activity could be seen to be associated with the muscle melanophores or with the melanophores of the skin, although transverse sections were probably far from ideal for observing this. In connection with some other work at an earlier date, the author had treated whole pieces of minnow skin (removed as in Chapter IIe) by an acetylcholinesterase method due to Holmstedt (1957a and b). By presenting various combinations of substrates and inhibitors, this method produced eight specimens showing complete inhibition and all combinations of specific and unspecific enzyme activity. Two minnows were allowed to adapt completely on a white background so that melanin would not obscure enzyme activity at the periphery of the melanophores. They were then decapitated and skin preparations were made from each as quickly as possible to prevent melanophore dispersion following denervation. Examination of the slides showed no activity around any of the melanophores in any of the

preparations. It was later learned that much longer incubation times are possible with this method and that very fine nerve-endings are sometimes only visible after extended treatment. Perhaps a more complete series of histochemical tests along these lines might demonstrate chromatic cholinergic endings, but negative results would not disprove double innervation.

g. Conclusions.

Nicol (1952) stated that the use of stimulating and depressing drugs on the autonomic nervous system of teleosts has produced largely inconsistent results. This is borne out by the experiments of this and the preceding chapters. The results are briefly summarised in Table II and previously reported results for Fundulus are given in Table III.

In Fundulus some case may be argued for double innervation, as was done by Smith (1931a), since the effects of ergot and atropine are complementary. However, ergot should suppress the aggregating activity, whereas the treated fish were unable to darken completely. Conversely, atropine, if it had any effect, might have suppressed dispersing activity, but the fish were unable to pale completely. The reversal of temperature responses by ergot, but not by atropine, was interpreted by Smith as

showing that the nerve fibres of the thermal reflex were adrenergic. These results do not support the theory of the thermal mechanism put forward in this thesis (Chapter IIf).

The results obtained with the minnow bear little similarity to the above and throw little light on chromatic control in this species. The effects of injections of ergot differed from those in Fundulus and from those reported in Phoxinus by other workers. The reversal of response to electrical stimulation and, to some extent, to thermal stimulation are confirmed. Rogitine should have produced similar responses, judging by its effects in mammals, but this was not so. The complete suppression of electrical responses suggests that only an adrenergic system is present. In this case ergot must have caused reversal of the response as suggested by Gray (1955a). These conclusions are placed in doubt because atropine produced very similar responses, with the exception of the electrical one, although it is a cholinergic suppressor. The thermal responses obtained with both these drugs are completely inexplicable at present, especially since the dependence on respiratory rhythm was upheld in both cases, at least for the high-temperature response.

The results of the experiments of Chapter VII indicate fairly conclusively that ergotamine does not reverse the

responses of melanophores to either adrenalin or l-noradrenalin. However, the possibility that adrenergic chromatic nerves in the minnow may produce a slightly different mediator, whose influence is more readily reversed by ergotamine, cannot be ruled out. It therefore seems that the use of drugs by analogy with their effects in mammals is not permissible. Until a more complete knowledge of the pharmacology of teleosts is available, such methods cannot yield conclusive evidence about the nature of autonomic control in these animals.

TABLE II. THE RESPONSES OF PHOXINUS AFTER VARIOUS DRUGS.

	Normal	Ergotamine	Rogitine	Atropine
Injection on white or black background.	-	Almost complete paling (1).	Complete darkening.	Complete darkening.
Electrical stimulation of nerves.	Paling (2).	Darkening (3).	No response.	Paling.
Local low temperature.	Paling.	Darkening.	Darkening.	Darkening.
Local high temperature.	Darkening.	Paling.	Darkening.	Darkening.

(1) Giersberg (1930) and v.Gelei (1942) report an initial darkening phase.

(2) Confirms v.Frisch (1911a).

(3) Confirms Giersberg (1930) and v.Gelei (1942).

TABLE III. REPORTED RESPONSES OF FUNDULUS AFTER DRUGS.

	Normal	Ergotamine	Atropine
Injection on a white background.	-	No effect (1).	Complete darkening (2) Intermediate state (1)
Injection on a black background.	-	Intermediate condition (1).	No effect (1, 2).
Electrical stimulation of nerves.	Paling (2).	-	-
Local low temperature.	Paling (3).	Darkening (1).	Paling (1).
Local high temperature.	Darkening (3).	Paling (1).	Darkening (1).

(1) Smith (1931a). (2) Wyman (1924). (3) Smith (1928).

CHAPTER IX.

GENERAL CONCLUSIONS.

The conclusions drawn in Chapters II and III were that the mediation of thermal responses, either by sensory (reflex) control or by independent melanophore activity, were rendered less likely than before, and that some support was given for the theory of selective nerve block leading to tonic imbalance. The results of experiments on the temperature effects were found to be compatible with this theory, although in some cases it was necessary to make further assumptions regarding the nature of tonic nervous control. All these assumptions appear to be physiologically reasonable, but as no success was achieved with attempts to vary the tonus under controlled conditions, the arguments involved must be regarded as purely hypothetical. There appeared to be little virtue in continuing this type of experiment on such a doubtful theoretical basis. In this respect Chapter IV merely serves to confirm the observations on *Phoxinus*.

On the other hand, the major assumption of the theory was that the melanophores were controlled by two sets of opposing nerve-fibres. The available evidence for this was discussed in Chapter V, where it was considered that none of the arguments advanced hitherto was conclusive. More definite information was required, which would allow

a more direct experimental approach to the temperature problems. However, the attempt to achieve differential stimulation by electrical means failed to contribute anything of value in this respect. The threshold curves are believed to be the first obtained for a nerve-melanophore preparation but they throw no light on the possible presence of opposing (dispersing) nerve fibres.

Attention was therefore turned to the use of selective drugs in association with electrical and thermal stimulation, and the results are described in Chapters VI, VII and VIII. The work of v.Gelei (1942) was in general confirmed, but subsequent experiments showed that his arguments regarding the pathways of dispersing fibres could not be supported. No drug other than ergotamine produced comparable results and the electrical properties of the 'dispersing fibres' were very similar to those of the aggregating fibres in the non-ergotised fish. However, the experiments of Chapter VII showed that reversal of the effector response in the presence of ergot must be ruled out, unless the melanophore-aggregating fibres of the minnow produce a sympathomimetic mediator other than adrenalin or l-noradrenalin. This chance appears to be small but it nevertheless leaves some doubt about the evidence for double innervation obtained in Chapter VI.

If melanophore-dispersing fibres do exist, their

pathways may now be indicated. It was argued that the experimental results of Chapter VI can only be interpreted on the assumption that the technique causes general excitation within the autonomic ganglia and does not indicate a chromatic outflow at any particular level of the spinal cord. Healey (1954) and Gray (1955a) showed that section of the autonomic chain causes complete denervation of the region which is peripheral to the section according to Figure 1a. No site could be found to cause differential elimination of darkening or paling fibres. This was not in accord with the conclusions of v.Gelei. However, now that v.Gelei's fibre paths can be discounted, the anomaly disappears. It must therefore be assumed that if darkening fibres exist, they run along exactly the same pathway as the paling fibres, that is, as shown in Figure 1a.

This conclusion accords well with Parker's view that complete dispersion after peripheral nerve section is not due to loss of aggregating tonus but to the active stimulation of dispersing fibres, an opinion supported for Phoxinus by Gray. The plan advanced by Wernøe (see Nicol, 1952; Young, 1931) of long chromatic fibres in the autonomic chain with segmental collaterals to the spinal nerves is also supported by these arguments. The similarity of darkening responses after ergot treatment and the paling without ergot suggest that if two systems

exist they both follow this arrangement.

The original intention, to apply the results of differential stimulation to the theory of differential nerve block, could not be carried out since the conclusions in both fields were fraught with conjecture. It would be interesting to investigate whether the effect of electrical stimulation in the spinal cord is prevented by local high skin temperatures in both the ergotised and untreated fish. Although the electrical threshold levels were similar in each case, the temperatures required for the suppression of each response might be expected to differ if all the foregoing hypotheses are correct. Unfortunately time did not permit this experiment to be attempted. Even if the expected result were obtained, it alone could not be accepted as conclusive proof of all the arguments leading to it.

The mechanisms behind these phenomena must therefore remain as hypotheses for the present time. When only descriptive features of the phenomena are considered, rather more positive conclusions may be drawn for this work. At the risk of undue repetition, these are listed in full as follows:-

A. Temperature Effects.

(1) The thermal responses are not peculiar to Phoxinus, but are widespread among teleosts in which chromatic nervous control is active. Many other fish, and all

those in which chromatic nerves are absent or of doubtful occurrence, showed no such responses. The responses appeared to be little affected by anaesthesia with urethane or MS222.

(2) The high-temperature response occurs sharply at certain threshold temperatures which differ for different types of melanophore in the same region of the same fish. This may indicate a separate innervation of each type of melanophore, or a graded sensitivity to the concentration of diffusing neurohumors in the skin. The threshold for each type of melanophore varies between individual fishes and for the same individual from day to day but remains fairly constant during one experiment.

(3) The low-temperature response occurs sharply at certain threshold temperatures in some species but this was not checked on Phoxinus. The threshold temperature for the melanophores of a single fish rises steadily throughout each experiment (under urethane anaesthesia) in some species.

(4) Both responses are dependent upon normal melanophore innervation but do not appear to be mediated by sensory receptors and reflex arcs. In particular the lateralis system and lateral cutaneous nerves play no part in the responses.

(5) The high-temperature response may be elicited by heating the sympathetic chain in Phoxinus but not by

heating spinal nerves in Pleuronectes. The possibility of heat conduction through the tissues of Phoxinus cannot be ruled out.

(6) Both responses are dependent upon normal respiratory movements in several of the species investigated and may be so in all.

(7) The responses of denervated melanophores show no discontinuity and are not dependent upon respiratory rhythm. They are probably direct responses by the independent melanophores to temperature.

B. Nervous Control of Melanophores.

(1) Electrical threshold curves were obtained for the stimulation, by square waves, of melanophore-aggregating fibres in a Phoxinus preparation.

(2) Similar curves for saw-tooth pulses produced no rise in threshold with increased pulse duration and no discontinuities which could have indicated the presence of dispersing fibres.

(3) Both sets of curves showed complete independence of pulse-repetition rate over a wide range.

(4) The results of v.Gelei on the electrical stimulation of the spinal cord of ergotamine-treated minnows were confirmed but it was found that melanophore-dispersing activity could pass forwards and backwards from a stimulating electrode at any level of the spinal cord.

(5) Identical results were obtained for the melanophore-aggregating activity in non-ergotised fish.

(6) Both responses were slow and incomplete at pulse repetition rates below 10/sec.

(7) All electrical responses were independent of respiratory rhythm.

(8) The presence of ergotamine does not reverse the responses of melanophores to proprietary preparations of adrenalin and l-noradrenalin in the minnow.

(9) The effects of some drugs other than ergotamine on the responses of minnow melanophores to background colour, to local temperature variations and to electrical stimulation are not consistent with the effects of these drugs in mammals, the effects reported in other species of teleost, or the effects of ergot in the minnow.

Finally, it is sincerely hoped that further work will be undertaken in an attempt to solve the many questions which have been posed by this work and to confirm or to refute the theories which have been put forward.

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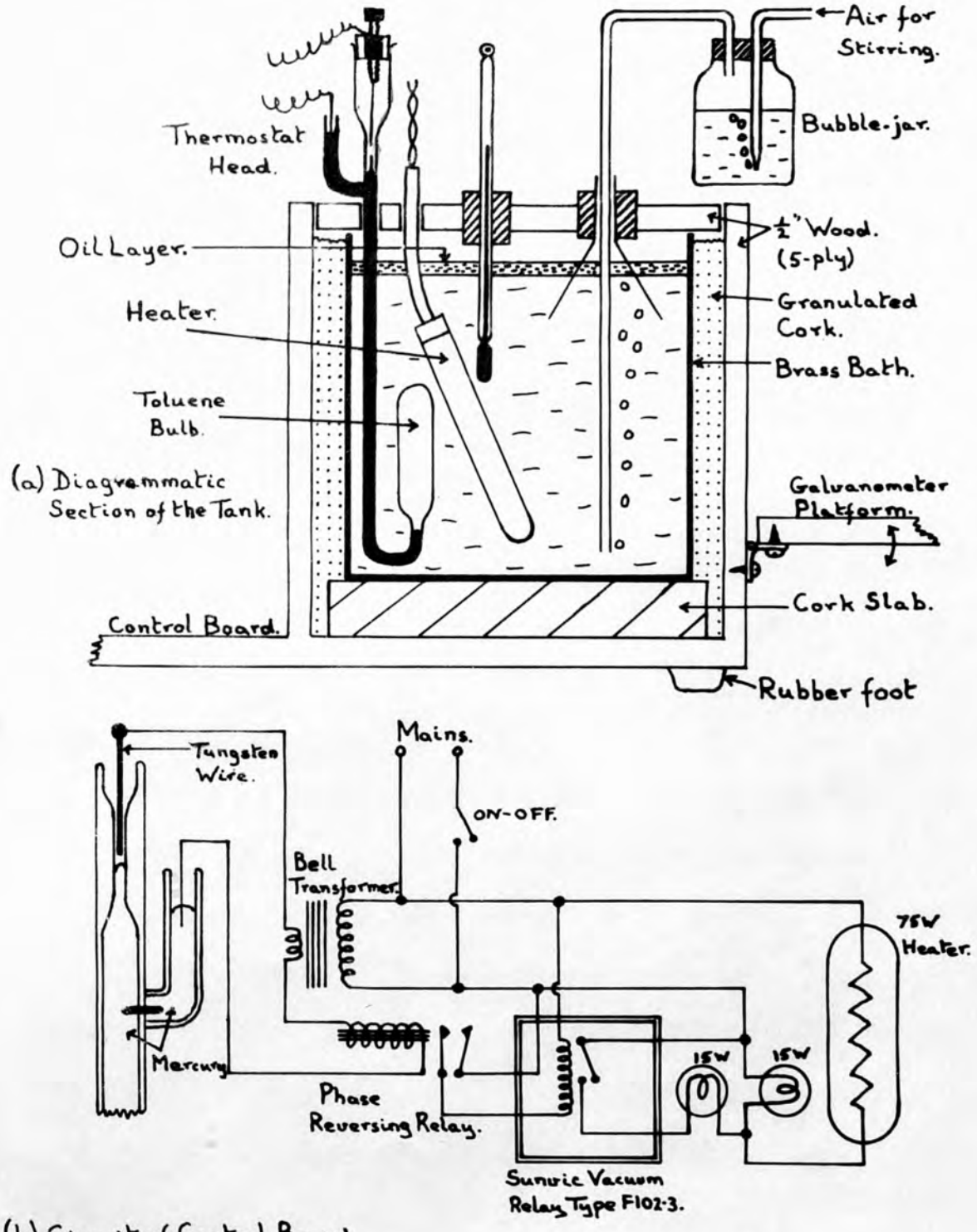
APPENDIX I. THERMOMETRY.

The basis for all temperature measurements made in this work was a Baird and Tatlock mercury-in-glass thermometer with a Certificate of Examination from the National Physical Laboratory. The scale range was -5°C. to +50°C. divided to 0.1°C. and the stated accuracy was within 0.03°C. over the range used.

With this sub-standard thermometer six ordinary laboratory mercury-in-glass thermometers were carefully checked by slow cooling and heating over the whole range in a water bath and calibration-correction curves were produced for each. Only one showed an error at any point greater than 0.1°C. The speed of action of each, as compared with the sub-standard thermometer, was noted and each thermometer was fitted with an identifying tag.

For measurement of temperature in confined spaces or where accurate localisation was required, a thermo-electric thermometer was constructed. The reference temperature selected was 40°C. so that an electrical control system could be used, and an accurate thermostat bath was constructed for this purpose as shown in Figure 35. A cylindrical brass vessel, 16 cm. in diameter and 16 cm. deep, was placed in a square wooden box and thermally insulated from it by a cork slab and granulated cork. A close-fitting wooden lid was drilled

Figure 35.



(a) Diagrammatic Section of the Tank.

(b) Circuit of Control Board.

The Thermocouple Reference Bath.

so that all the fittings were held snugly. The bath was filled with water, covered with oil to prevent evaporation and stirred by a stream of air-bubbles which were collected at the surface by an inverted glass funnel. The air supply for this was passed through a bubble-jar to wet the air and to allow the flow-rate to be judged visually (Figure 35a).

The control circuit operated from a toluene-mercury thermostat via a phase-reversing relay, a Sunvic vacuum relay and a 70 watt aquarium immersion heater. Due to thermal delay in the toluene bath, heating and cooling were made to be very slow by the arrangement shown in Figure 35b, which worked very well. One 15 watt light bulb in series with the heater reduced the power so that an equilibrium temperature of 35-38°C. was produced in the bath. A second bulb switched in parallel with the first allowed the temperature to rise slowly. In this way a temperature differential of less than $\pm$0.05°C. was produced. The thermostat was left running continuously and required no attention.

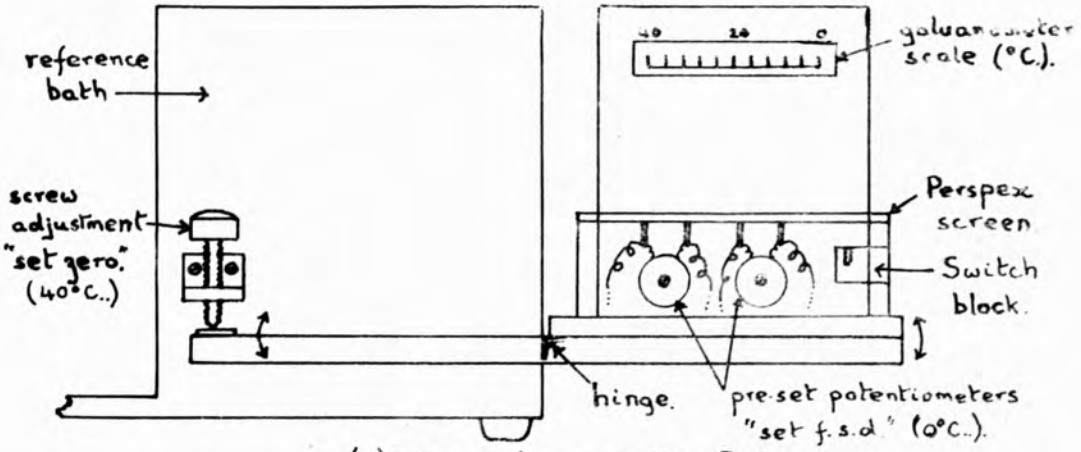
The galvanometer used with the thermocouples was a Tinsley moving mirror type with 8Ω D.C. impedance and 33μA. full scale deflection. Attempts to centre this instrument by the means provided prove inconvenient but the suspension was found to be very sensitive to lateral tilting. The meter was therefore mounted on a platform

hinged to the side of the thermostat (Figure 36a) and zero adjustments were easily carried out by means of a screw bearing on a lever attached to this platform. Full scale deflection at 0°C. was arranged by means of an 8Ω. preset potentiometer in shunt with the meter. Two such circuits were made and the meter switched to either by dipping its amalgamated leads in pools of mercury in a perspex block. The whole of the wiring was enclosed in a perspex box covering the galvanometer terminals to prevent air currents from causing stray thermoelectric potentials at other bimetal junctions in the circuit (Figure 36a and c).

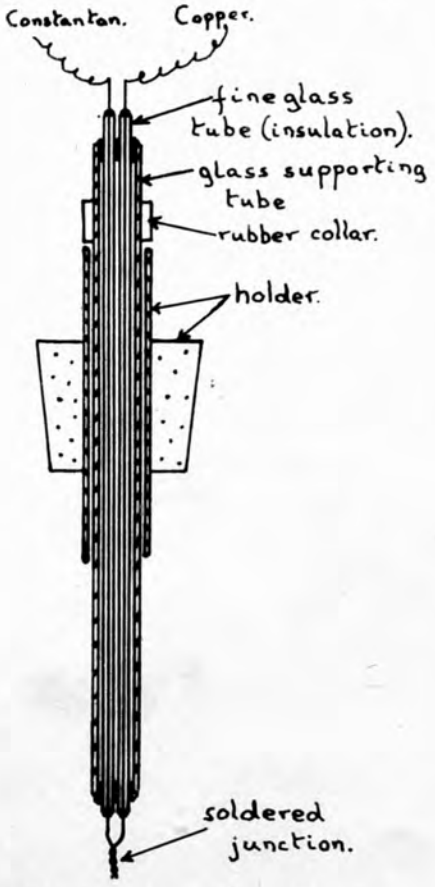
The couples used were mainly copper/constantan/copper and the construction of the reference couple is shown in Figure 36b. With hypodermic needle couples the circuit consisted of constantan/steel/constantan, the reference junction being a steel knitting needle with a cooling vane near the top and connections of piano wire. At Plymouth a second galvanometer, giving 1 μA/°C. was sometimes used as a second channel.

For normal purposes the scale deflection was taken to be linear between 0°C. and 40°C. The theoretical error for copper/constantan/copper is shown in Figure 37, and the actual calibration of the thermoelectric thermometer against the N.P.L. sub-standard is shown in Figure 38. The ends of the scale were adjusted for each pair of couples and

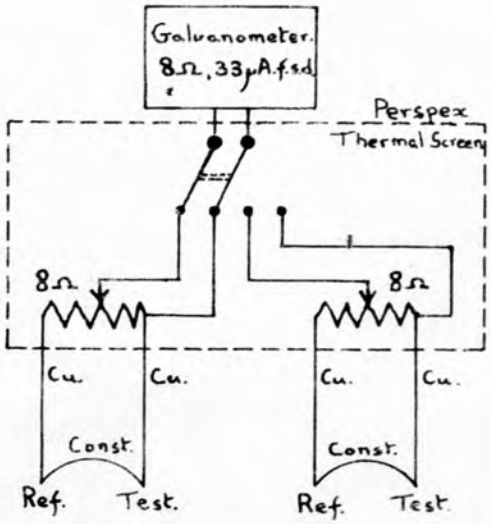
Figure 36.



(a) The Galvanometer Balance.



(b) A Reference Junction.



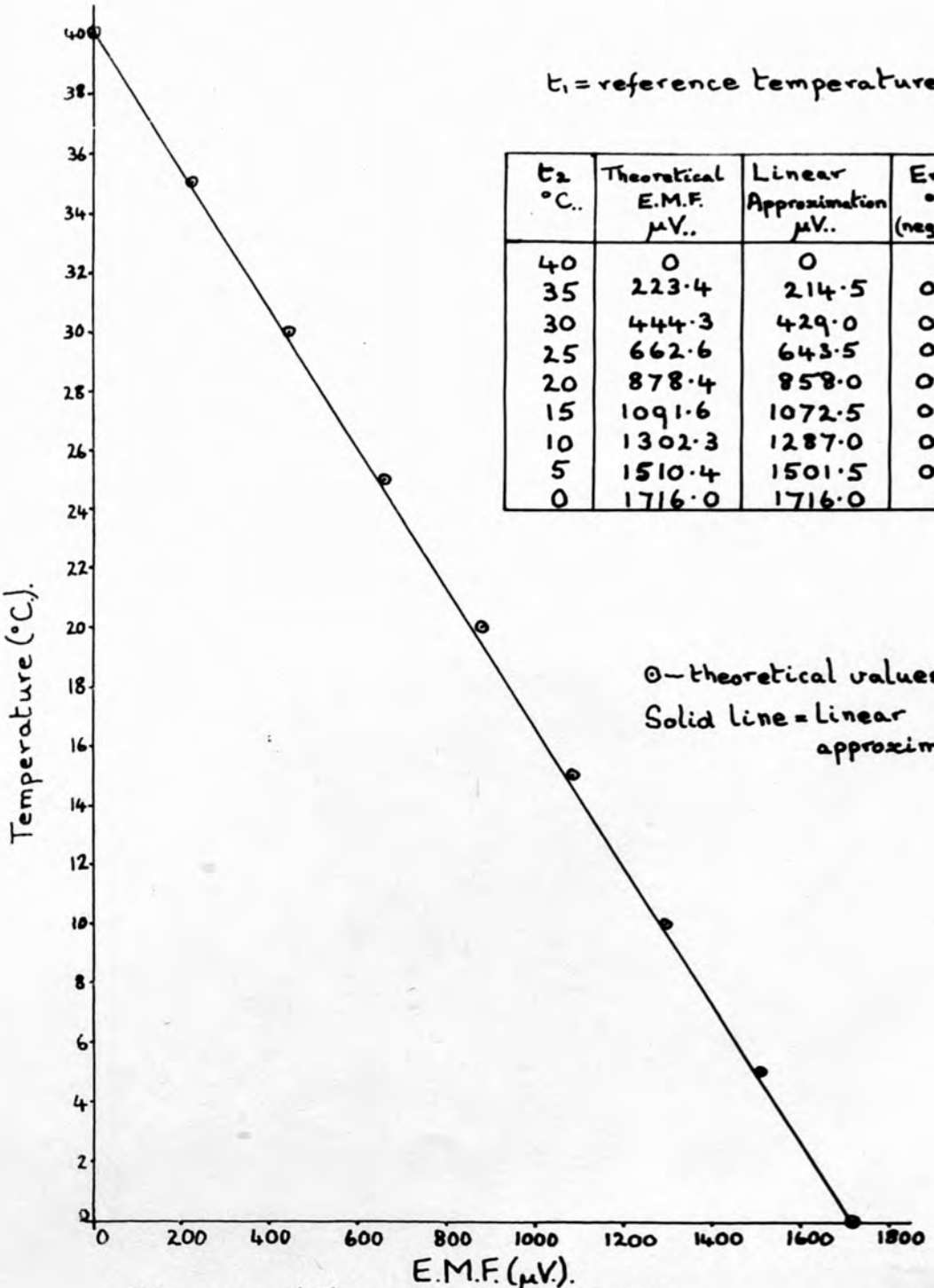
(c) Circuit of the Thermometer.

The Thermoelectric Thermometer.

Figure 37.

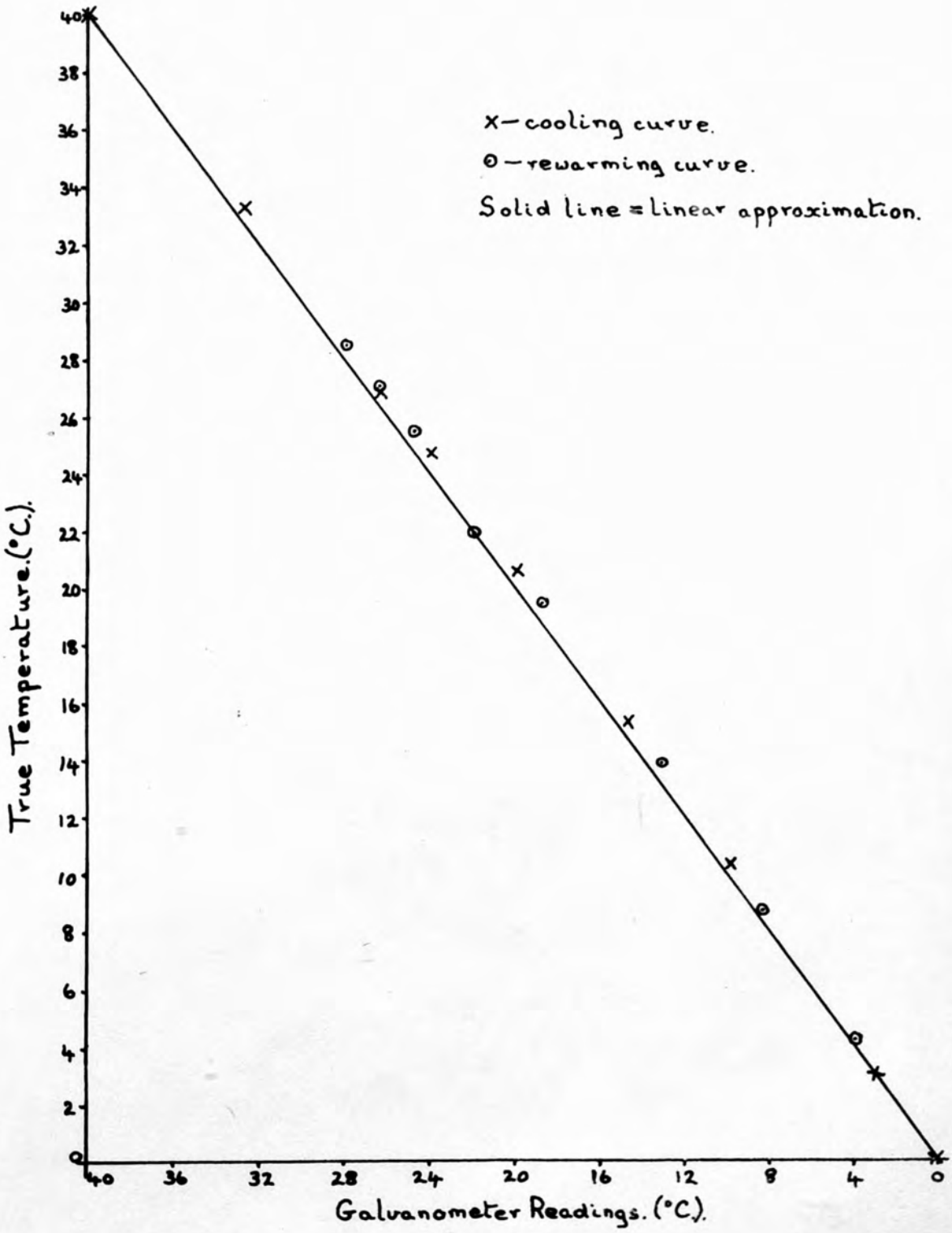
t_1 = reference temperature = 40°C..

t_2 °C..	Theoretical E.M.F. μV..	Linear Approximation μV..	Error °C.. (negative)
40	0	0	0
35	223·4	214·5	0·20
30	444·3	429·0	0·34
25	662·6	643·5	0·42
20	878·4	858·0	0·46
15	1091·6	1072·5	0·44
10	1302·3	1287·0	0·35
5	1510·4	1501·5	0·20
0	1716·0	1716·0	0



Theoretical Error of the Thermoelectric Thermometer using a Linear Scale.

Figure 38.



Calibration Curve of Thermoelectric Thermometer.

checked before each experiment but rarely needed readjustment.

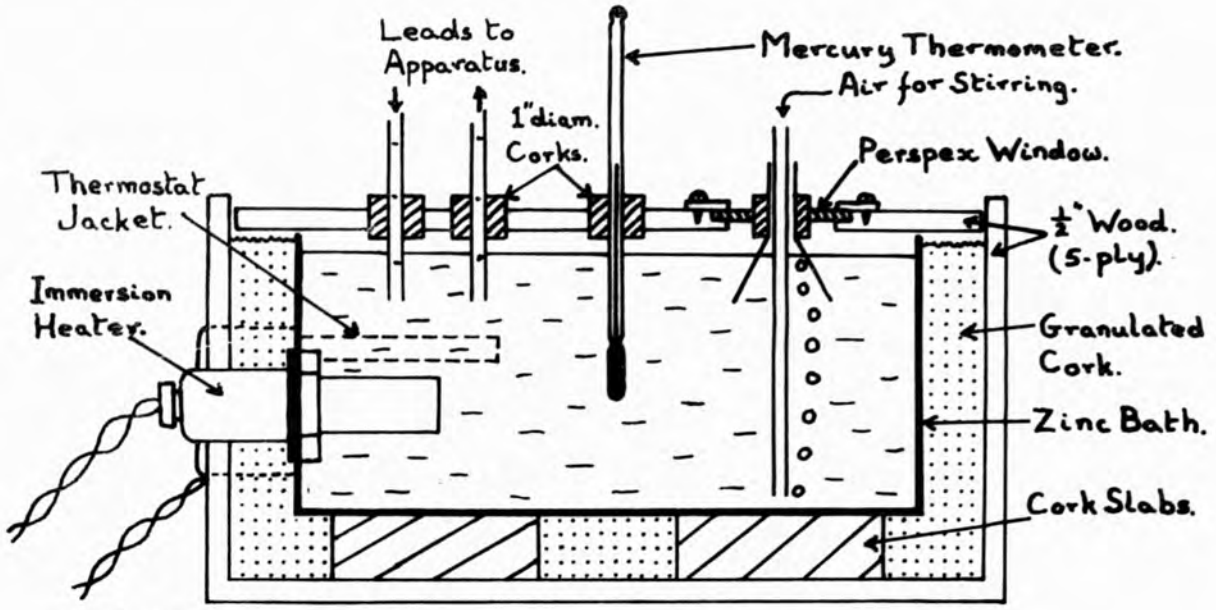
APPENDIX II. TEMPERATURE SOURCES.

It was decided to build two accurate thermostat baths to act as temperature sources from which all heating apparatus could be run, and all such apparatus was designed accordingly. This obviated the need for a separate temperature control system in each apparatus.

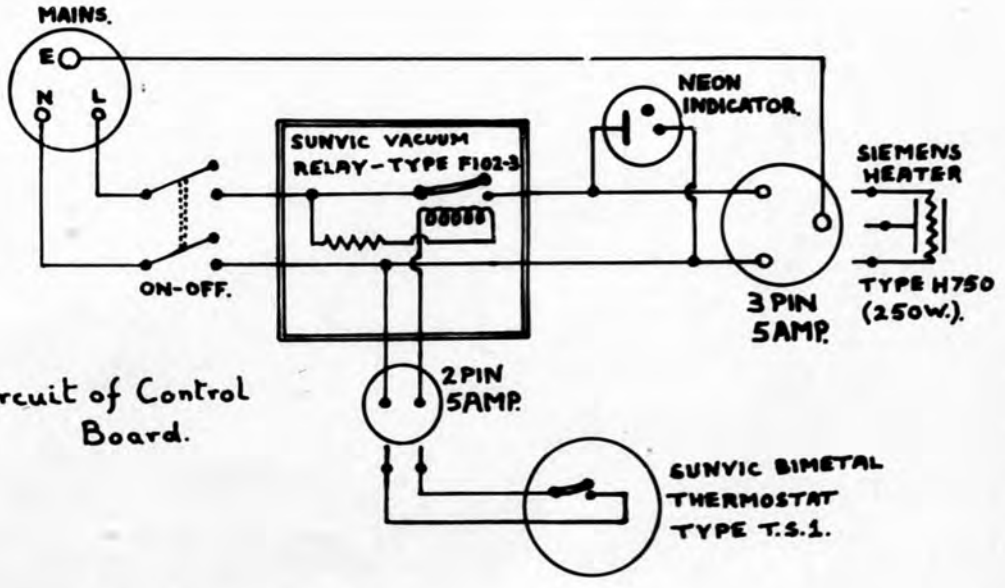
The construction of each of these thermostats is shown in Figure 39a. A zinc bath, holding about 16 litres of water, was supported on cork slabs in a wooden box and the gap of about 3.5 cm. was filled with granulated cork for thermal insulation. Heating by a blade-type immersion heater was controlled by a Sunvic bimetal thermostat and vacuum relay. The heater and thermostat casings projected a little from the wooden box for convenience and the control circuit with an indicator lamp (Figure 39b) was mounted on a separate board. Careful adjustment of the thermostat enabled a differential of $\pm 0.1^{\circ}C.$ to be obtained.

The bath was stirred by air-bubbles, collected at the water surface by an inverted funnel which was held in a perspex window so that the rate of bubbling could be seen and adjusted. Corks in the wooden lid held glass tubes through which water was drawn or returned. Leads to the apparatus were thick-wall polyvinyl chloride

Figure 39.



(a) Diagrammatic Section of the tank. (×¼).



(b) Circuit of Control Board.

The Thermostat Tanks.

(P.V.C.) tubing which gave low thermal losses.

The water was circulated by air-lift pumps to a small reservoir on the outside of the thermostat from which it siphoned back into the tank (Figure 6, Chapter III). In the temperature-controlled microscope stage the high thermal inertia of the apparatus rendered continuous circulation unnecessary. In such cases where thewater was not returned to the tank the external reservoir acted as a level indicator to prevent the heater and thermostat elements from being uncovered. For low temperature work the insulated tanks, without the electrical system, acted as excellent ice-boxes.

APPENDIX III. PHOTOGRAPHY.

(a) The melanophore photographs of Figure 9, Chapter III, were taken through a monocular microscope with a Cooke, Troughton and Sims quarter-plate eyepiece camera. The experimental technique was normal. The fish was anaesthetised in 0.25% urethane solution throughout and jets of water of various temperatures were played on the skin a short distance from the field of view. Immediately before each exposure the jet was turned off to prevent refractive distortion.

Illumination was by a 500 watt projector lamp mounted in a housing on an adjustable, counterbalanced arm with two 10 cm. diameter lenses giving a focal length of 18 cm. Focussing was carried out with incident illumination from a microscope lamp and the larger lamp was only switched on immediately before the exposure. Thus the effects of illumination and unwanted heating were reduced to a minimum.

The optical system consisted of a 25 mm. objective with a x15 eyepiece. Exposure at full aperture at 1/25 second was used to minimise blurring due to respiratory movements of the fish. The emulsion used was the fastest then obtainable (Kodak P 2000) and the plates were developed as advised by the makers for the fastest

possible emulsion speed.

(b) The photograph of the superficial ophthalmic preparation, Figure 26 of Chapter V, was taken with a Kodak Retinette 35 mm. camera mounted on one eye-piece of a Beck binocular dissecting microscope. The other eyepiece was used as a viewfinder and for focussing (camera focus set to infinity). A $1\frac{1}{2}$ inch objective was used with x10 eyepieces. Illumination from an electronic flash-tube working at 100 Joules was used. The camera aperture was f3.5 and the film (H.P.3.) was developed normally in fine grain developer (I.D.11.).

APPENDIX IV. RINGER SOLUTIONS.

At first the Ringer solution produced by Steinhausen (1928) was used for operations on minnows. In the experiments of Chapter III which involved prolonged exposure of the sympathetic chain, the preparation became quiescent after a short time. It was noticed that Steinhausen's formula differed markedly from that of Young (1933) especially in the amount of sodium required (see Table IV, a and b).

In order to determine the better of these solutions, 0.5 ml. of minnow body fluid was obtained by lightly centrifuging pieces of several freshly decapitated fish. After dilution this fluid was very kindly analysed by Miss Bond of the Physiology Department, Bedford College, in a flame spectrometer. This indicated an original sodium content of 2.4 gm./l. Young's Ringer for fresh-water teleosts was then used in all subsequent experiments and operations with complete success. Both the sympathetic chain and the superficial ophthalmic nerve were exposed to it for long periods with little change in activity or response. The same solution was found to be suitable for the isolated skin preparations used in the experiments described in Chapter IIe and Chapter VIIc. The formula originally used by Spaeth

and Barbour (1917) is similar to that of Young except for the amount of potassium used (see Table IVc). The Sandoz placebo mentioned in Chapter VIIc is given in Table IVd.

For experiments on marine teleosts the standard solution available at the Plymouth laboratory was used. This was Young's formula for marine teleosts (Table IVe).

TABLE IV.

Component	(a) Steinhausen's freshwater teleost Ringer.	(b) Young's freshwater teleost Ringer.	(c) Spaeth's balanced melanophore solution.	(d) Sandoz placebo for ergotamine tartrate.	(e) Young's marine teleost Ringer.
NaCl.	8.45 gm.	5.50 gm.	4.78 gm.	2.50 gm.	13.45 gm.
KCl.	0.13 gm.	0.14 gm.	1.01 gm.	-	0.60 gm.
$CaCl_2$.	0.26 gm.	0.11 gm.	0.26 gm.	-	0.25 gm.
Other.	$NaHCO_3$. 1.30 gm.	-	-	Tartaric acid 0.25 gm.	$MgCl_2$. 0.35 gm.
Water.	ad.1 litre.	ad 1 litre.	ad 1 litre.	ad 1 litre.	ad 1 litre.
Total Na^+.	3.68 gm.	2.16 gm.	1.88 gm.	0.98 gm.	5.29 gm.

APPENDIX V. A BIBLIOGRAPHY OF THE EFFECTS OF TEMPERATURE UPON THE CHROMATIC RESPONSES OF ANIMALS OTHER THAN TELEOST FISHES.

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Pye, J.D. 1960. J.Laryng., 74, 718-29.

A theory of echo-location by bats.

Pye, J.D. 1961. Endeavour, 20, 78, 101-11.

Echo-location by bats.

Ormerod, F.C. and Pye, J.D. 1961. Acta Oto-laryng., Stockh., in the press.

Echo-location in bats.

(An unpublished abstract of the text of this paper is given here).

A THEORY OF ECHOLOCATION BY BATS

BY

J. D. PYE (B.Sc.)

(London)

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A THEORY OF ECHOLOCATION BY BATS*

By J. D. PYE (London)

TOWARDS the end of the eighteenth-century, Lazzaro Spallanzani (1794) demonstrated that the location of obstacles and prey by insectivorous bats depends on hearing rather than on their inadequate vision. Captive bats avoided fine wires in complete darkness or when their eyes were destroyed. Blinded bats were released and on subsequent recapture were found to have caught and eaten just as many insects as normal animals. Yet when their ears were blocked, seeing bats were reluctant to fly and blundered clumsily into quite large objects. Many critical experiments confirmed the importance of hearing but the mechanism remained a mystery. Although similar results were obtained by several of Spallanzani's friends they were discredited by the influential Cuvier and the matter was almost forgotten.

In 1920, Hartridge observed bats in flight and suggested that they might produce high frequency calls, inaudible to man, and navigate by means of echoes. He pointed out the advantages of high frequencies for producing echoes from small objects. Since 1938, Griffin, Galambos, Dijkgraaf, Möhres and others have amply demonstrated the truth of this theory and a comprehensive review of these researches has been published by Griffin (1958). Their importance is such that a new type of sense at a distance must now be accepted, an active sense in which the larynx and ears take over the rôle of the eyes for orientation.

The echolocation cries of bats fall in the frequency range of 20–120 kilocycles per second, and are produced as discrete pulses of high intensity. The larynx is highly specialized for the production of these sounds (Elias, 1907; Möhres, 1953). In general there is heavy ossification and fusion of the cartilages to form a rigid framework and the cricothyroid muscles are capable of applying great tension to the short, light vocal cords. Other modifications, such as the resonating chambers of the Rhinolophidae, may be present.

The ear is specialized in several ways. The pinnae are very large, especially in the genus *Plecotus*, with various accessory structures (Figs. 1, 2 and 3); the tensor tympani and stapedius muscles are very well developed; the cochlea may be relatively enormous as in the Rhinolophidae, and

* The research reported in this paper has been sponsored in part by the Air Force Office of Scientific Research, of the Air Research and Development Command, United States Air Force, through its European Office.

in general the basilar membrane is very narrow with a strong ligamentum spirale and two laminae spirales suggesting high tension (Fig. 4). Anatomical descriptions of the larynx and ears of bats are given by Griffin (1958), Möhres (1953) and Reysenbach de Haan (1958). Galambos (1941, 1942) obtained cochlear microphonic potentials from Vespertilionid bats up to at least 98 kc/s. whereas in the guinea pig the response disappeared at about 40 kc/s.

With this apparatus the bat is able to avoid wires less than 0.5 mm. in

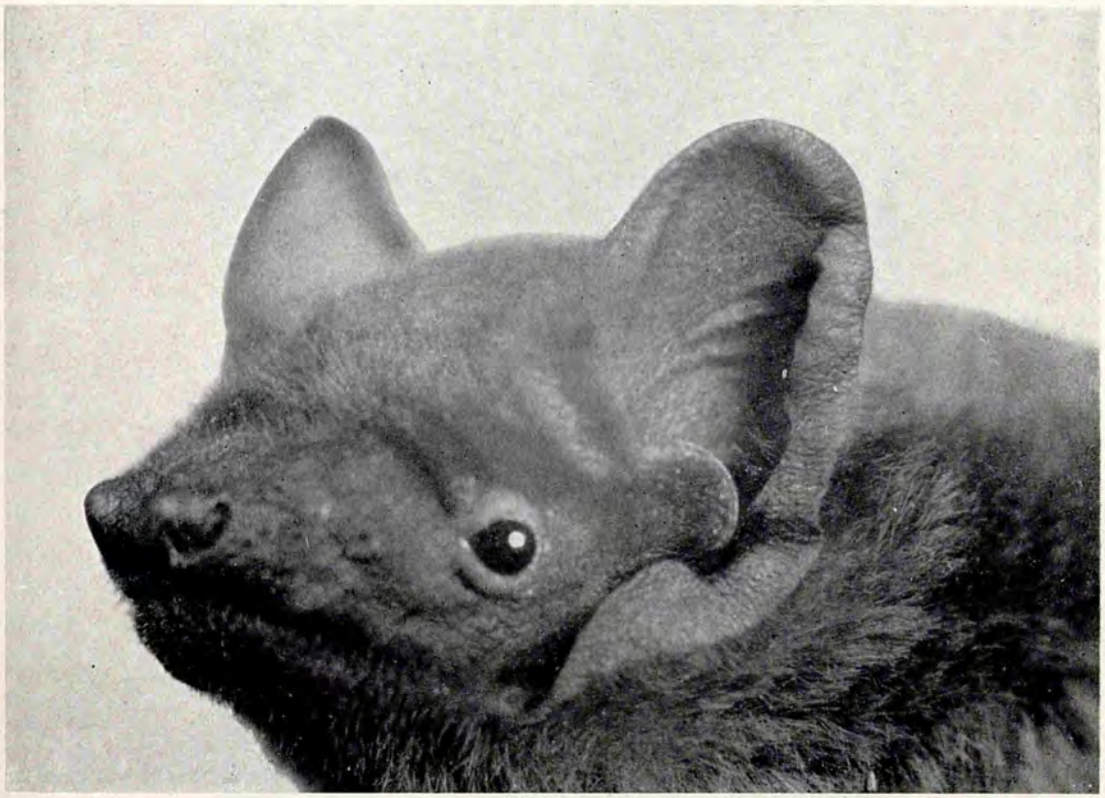

FIG. 1.

Nyctalus noctula (Vespertilionidae), The Noctule. The tragus is fairly large and the nostrils open forwards. (×4)

diameter and to catch mosquitoes at rates up to 10 per minute. Furthermore Griffin has shown that the performance is little affected by high levels of masking noise. *Plecotus rafinesqui* continues to detect the presence of wires 0.4 mm. thick in a uniform field of white noise covering the frequency range of their calls and calculated to be 45 db. above the echo intensity. Hunting is not disturbed by the calls of other bats, by multiple echoes from vegetation or rain, or by the very strong specular reflections from walls or water surfaces. The whole system is apparently highly developed and possesses great sensitivity and precision.

The theories which have been advanced to explain how bats obtain this accurate information appear to be inadequate expecially for short range

operation. Since such ideas are still largely speculative, there appears to be some excuse for offering at this stage an alternative general theory which although untested provides a simple explanation of many features of this phenomenon.

The Beat Note Theory

The ear in man is principally a warning and communication instrument, and ideally should be free from distortion in order to give a true

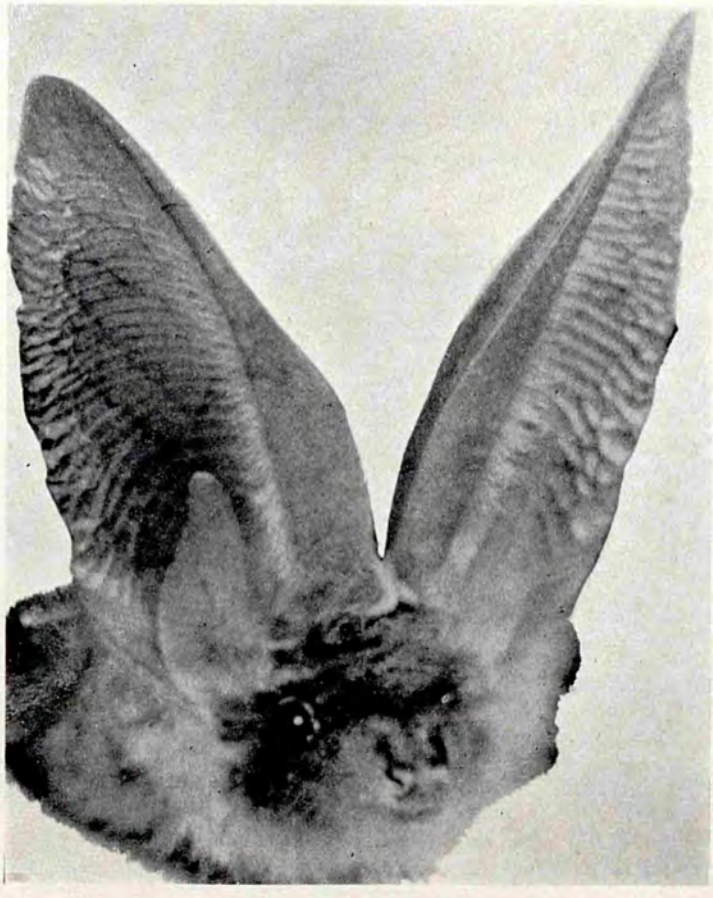

FIG. 2.

Plecotus auritus (Vespertilionidae), the European Long-eared Bat. The pinnae and tragi are enormously developed. Sound is emitted through the nostrils which open upwards. (×2·5)

appreciation of incident sounds. This does not necessarily apply to an animal which uses it ears for entirely different purposes. In the case of the bat it appears that distortion introduced by non-linearity might be of great value. A non-linear device supplied with energy of two frequencies constructs a range of overtones, summation tones and difference tones in addition to the two fundamentals. A bat's pulse and its echo could be treated in this way if heard simultaneously, that is if an object is close enough for overlap to occur at the ear. Since the call and echo will seldom be separated by more than one octave, the first order difference tone will

nearly always be of lower frequency than either of them, and will be audible as a beat note. Due to the nature of the pulse produced, this beat note has some interesting properties which may provide sufficient information to satisfy the bat's navigational requirements. The other difference tones and beats between different echoes will be of very much lower amplitude, while the harmonics and summation tones will be of very high frequency and can probably be ignored in this argument.

This theory is thus based upon a consideration of the frequency

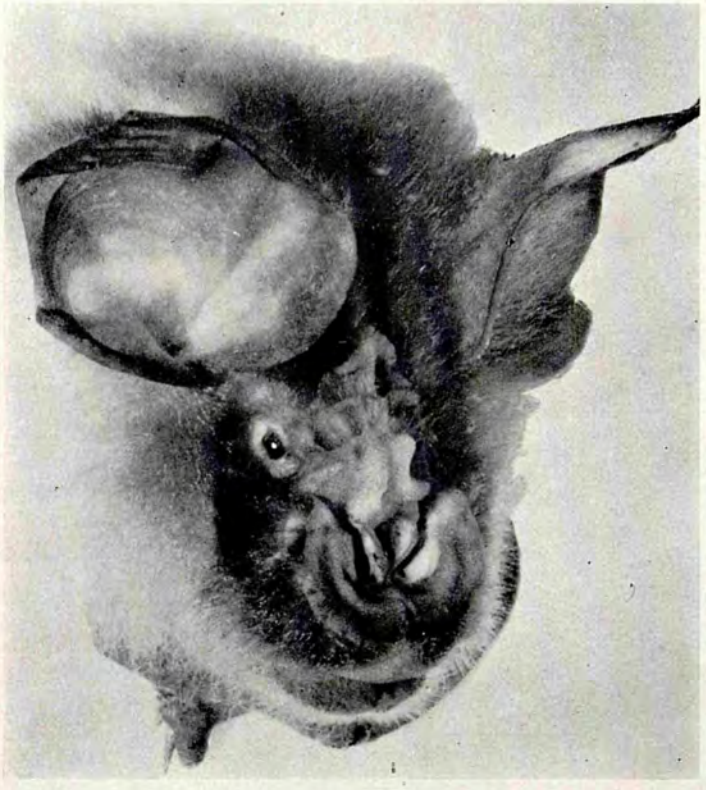

FIG. 3.

Rhinolophus ferrum-equinum (Rhinolophidae), the Greater Horseshoe Bat, from a museum specimen. There is no tragus but a large antitragus which has here been pulled outwards to reveal the inferior meatal opening. A pathway from this runs anteroventrally to a groove in the nose-leaf, which acts as a directional emitter. (×3)

structure of the bat pulses which appear to be of two types characteristic of the two families most thoroughly studied, the Vespertilionidae and the Rhinolophidae. At present it seems that most other Microchiroptera resemble either one or other of these or a combination of both, so the application of the theory will be considered for these two families only.

Vespertilionidae

These are the common insectivorous bats of temperate North America and Europe. Analyses by Griffin *et al.*, have shown the pulses to be 1–4

m.sec. in duration and of pure tone whose frequency falls steadily throughout. The frequency sweep often covers nearly an octave but seldom more. Pulse repetition rate varies from less than 10/sec. at rest to over 100/sec. when investigating obstacles or prey.

By analogy with early radar systems Hartridge (1945) supposed that the bat measures the echo delay and so deduces the distance of an object. He further suggested that the sensitivity of the ear is protected during

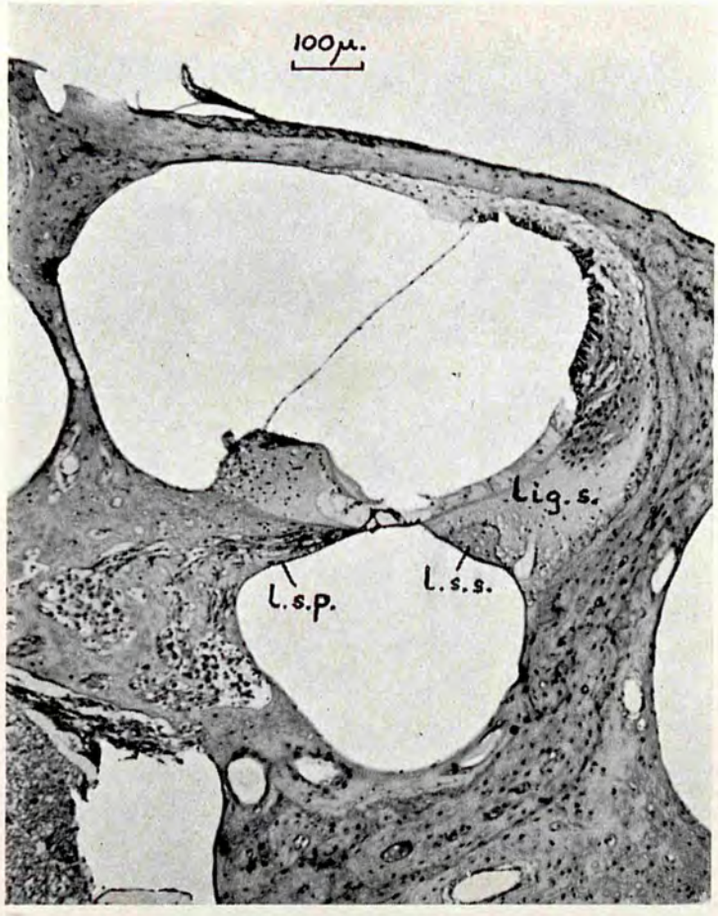

FIG. 4.

A section of the second turn of the cochlea of *Rhinolophus ferrum-equinum* (prepared by Mrs. P. N. Edwards). l.s.p. lamina spiralis primaria. l.s.s. lamina spiralis secundaria. lig.s. ligamentum spirale.

production of the very loud pulse by action of the intra-aural reflex. The transmit-receive switch of pulse radar has a similar function in protecting the receiver. Some of the many drawbacks to this theory have been discussed by Griffin (1958).

Following a short loud sound the human ear experiences a temporary rise in threshold which suppresses the reception of short range echoes. Griffin and Grinnell (1958) and Grinnell and Griffin (1959) have shown by central recording that the bat's ear recovers remarkably quickly, but the

effect must be present at short range where the echo delay is very small. At even shorter ranges the echo will overlap with the call and will tend to be masked. Griffin has suggested that frequency modulation may alleviate this since call and echo will be at different frequencies at any instant, but this separation decreases with range and for the capture of insect prey the bats must be able to work down to extremely close range. While it cannot be denied that bats must hear discrete echoes returned from 1–2 m. (Griffin and Grinnell, 1956; Grinnell and Griffin, 1958), these may only serve as an early warning system of low accuracy. At shorter ranges the

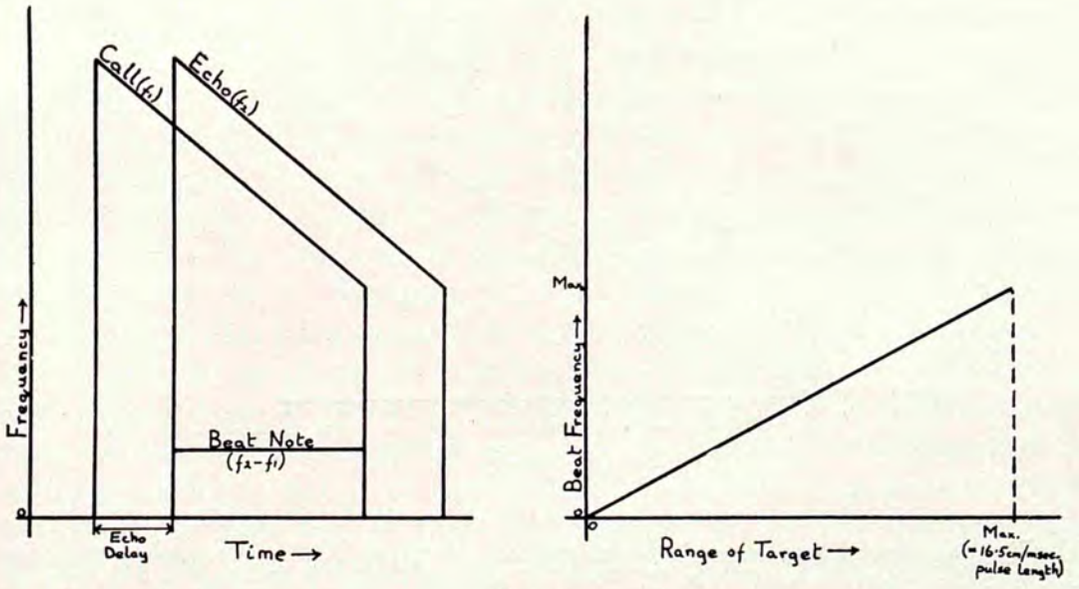

FIG. 5.

The production of beat-notes by frequency modulated pulses. The beat frequency is proportional to the range of the target ($f \propto r$). Provided the original pulse does not cover more than one octave, pulse and beat frequencies cannot overlap.

problems of reception and interpretation are greatly increased. Furthermore the simultaneous discrimination of multiple echoes by timing alone would seem extremely difficult.

The proposed action of the middle-ear muscles demands a fantastic speed of operation. Latent periods of this reflex in laboratory animals and man as measured by many workers (vide Wever and Lawrence, 1954) give values ranging from 10 to 290 m.sec. Even the shortest of these periods covers two complete call-echo cycles at the higher pulse repetition rates used by bats. The muscle relaxation rate must also be incredibly high to restore full sensitivity for short range echoes.

By listening not to the echo, but to beats between it and the call, the bat could very easily obtain accurate information about objects at close range. In the "ideal" case of linear frequency modulation as shown in Fig. 5, the beat note is of a constant frequency proportional to the range of the

target. A non-linear fall in frequency results in a beat note which is not constant but is nevertheless perfectly characteristic of the echo delay. Multiple echoes will produce beat notes of different frequencies which could be easily distinguished. Despite the smallness of the inter-aural distance there will be an appreciable difference in the beat note frequency at the two ears for objects not in the median plane. Thus provided that distortion occurs somewhere in the ear, the determination of both range and direction only requires a frequency analysis of each signal and a comparison between them. The cochleae and central auditory apparatus could accomplish this

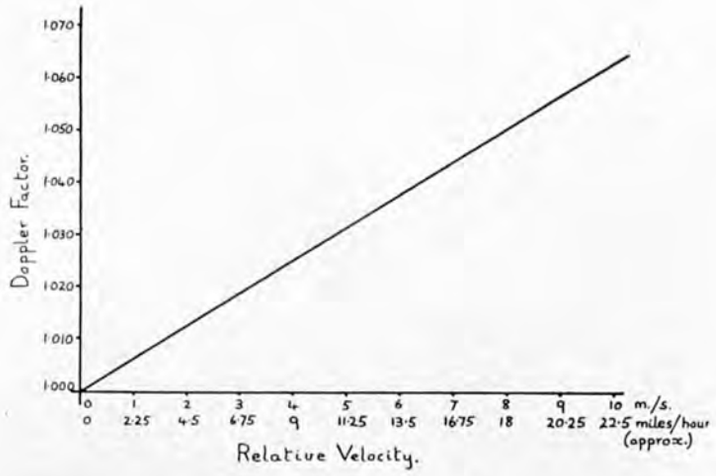

FIG. 6.

The Doppler effect for echoes at different relative velocities. The beat frequency is proportional to the relative velocity ($f \propto \frac{dr}{dt}$). Thus 100 kc/s. at 10 m/s. is returned at 106·25 kc/s., producing a beat-note of 6·25 kc/s.

and no further mechanisms need be postulated. Finally since the beat notes occupy an entirely different frequency band from the original pulse, the effects of masking will be much reduced.

Bats of this family do not move the pinnae independently but turn the whole head to examine their surroundings. By turning until a given small object is in the median plane the two beat notes are brought to the same frequency and exactly into phase. The proposed mechanism is essentially binaural and it is interesting to note that a Vespertilionid bat with one ear plugged is almost completely disorientated. A bat with both ears plugged is scarcely more helpless.

Rhinolophidae

This family comprises the Horseshoe bats of Europe and Asia. Möhres (1953) has shown that the pulses of these animals are 40–100 m.sec. in duration (much longer than those of Vespertilionids) and are of very constant, very high frequency, 85–100 kc/s. The mouth is not used for

sound emission and the sound is beamed to some extent by interference at the nostrils which are half a wave-length apart. The horseshoe structure or nose-leaf (Fig. 3) is capable of directing the beam in various directions. The pinnae are freely and independently movable. Pulse repetition rates never exceed 10/sec.

The long pulse length has led Möhres to reject timing theories and he supposes that the bat observes the loudness of the echoes. Beam movements would then give him the direction, and searching movements of the ears could give the range by triangulation. Such a mechanism would be highly susceptible to masking and depends on almost complete acoustic separation of ear and nasopharynx. In view of the small size of the head and the very high intensities produced, this seems unlikely.

The beat note theory depends on changes in frequency brought about by the Doppler shift. The beat frequency in this case is proportional to the rate of change of range, or velocity of the target relative to the bat. Since the received echo experiences a double shift which is proportional to the original frequency, the system is capable of high sensitivity at the frequencies used (Fig. 6). Location may be achieved either by movement of the beam and ears for greatest beat note intensity, or by normal binaural location involving phase and timing. The former is more likely since Rhinolophid bats, unlike the Vespertilionids, are not disorientated by plugging one ear only, although with both ears plugged disorientation is complete (Möhres, 1953). Little difference in beat frequency at the two ears would be expected so that this observation accords well with the theory. As the beat frequency will seldom rise above 6 kc/s. masking by the call will be slight.

This mechanism will work well for a bat in flight since it depends upon movement, but the stationary bat often searches around actively and appears to appreciate its surroundings. Two mechanisms may operate under these conditions. High frequency tape-recordings have recently been made by Griffin from specimens of *Rhinolophus ferrum-equinum* sent from Britain. A copy kindly presented by Professor Griffin shows that in the last 2 m.sec. or so of each pulse the frequency falls in the same way as in Vespertilionid pulses. This may give information at close range either in flight or at rest. On the other hand Möhres describes searching movements of the pinnae superimposed upon which are vibrations at a frequency of about 50/sec. Personal observation shows that these high speed movements only occur during ultrasound production. Such movements of the pinnae, acting as echo reflectors for the meatus, may introduce an artificial velocity factor and cause a varying Doppler shift. Since the tip moves faster than lower parts of the pinna a range of velocities will be represented at any instant. The result will be a range of beat notes of which the 100 c/s. component will appear to predominate due to synchronism with the ear movements. Directional information may be conveyed by this means since the

ears of these bats present a variable angle to each other. Otherwise location may be effected by the methods discussed above.

Dijkgraaf (1946) and Möhres (1953) have estimated that the maximum range of accurate echolocation by Vespertilionids does not exceed 50 cm., but Möhres states that Rhinolophids are sensitive to a range of 6.4 m. Although the latter may be assisted by beaming of the transmitted sound, it seems significant that these are the approximate maximum ranges at which pulse-echo overlap occurs in each type (50 cm. for a 3 m.sec. pulse and 6.4 m. for a 38.4 m.sec. pulse).

The Possible Source of Non-linearity

The ideal type of distortion for the reception of beat notes is a simple rectification of the signal, since if one note is of very high intensity the beat note amplitude then approaches that of the lower intensity signal (the echo). There is some evidence that this would be expected in the bat's ear under normal conditions. Distortion has been shown to occur in the cochlear microphonic potentials of laboratory animals at sound levels of 1–10 dynes/cm^2 (Wever and Lawrence, 1954). Griffin (1958) has shown that the sound intensity 10 cm. in front of a Vespertilionid bat may exceed 60 dynes/cm.^{2} and sometimes reaches 170 dynes/cm.^{2}. Möhres states that even higher intensities appear to be produced by Rhinolophids but this may be due to the beaming mechanism. Thus a small proportion of the emitted energy is sufficient to cause non-linearity and this may easily reach the ear by stray scattering from the mouth.

The source and nature of this distortion have been the subjects of some controversy. Békésy (1934) supposed that overtones are produced in the inner ear whilst difference tones are generated in the middle ear by a non-fatiguing process. Newman, Stevens and Davis (1937) measured the overtone content of cochlear microphonic potentials in the cat and guinea pig. Their results show that even harmonics, indicative of rectification and responsive to middle-ear muscle action, occur at lower distortion levels, but at higher sound intensities the odd harmonics predominate and are attributed to symmetrical limiting at the cochlear level.

It is tempting to suggest that rectification is performed by the middle-ear muscles of bats since they are so well developed. The increased pulse repetition rates of Vespertilionids investigating an object may not only satisfy an increased need for information but also cause a smoother tetanic contraction of the middle-ear muscles. However, Wever and Lawrence (1954) reject the middle ear as the source of non-linearity and attribute it wholly to the cochlear. Cochlear distortion in the bat could be induced by sound transmitted through the tissues, whereas distortion in the middle ear demands a signal entering the meatus by air conduction. The morphology of some bats appears to be ideal for providing an air conduc-

tion pathway to the outer ear and suggests that the tissue conduction signal is not used in these species.

Plecotus (Fig. 2) is known to hunt among bushes and hedgerows, and may pick non-flying prey from the foliage. Its slow, hovering flight is ideal for such a habit, and its echolocation mechanisms must be extremely accurate. This bat would not appear to need so great a range as species which hunt in the open. Short range together with the enormous pinnae would allow economy of transmitted energy. In fact Griffin has found that the American *Plecotus rafinesqui* produces rather short pulses at an intensity 30–35 db. less than that of most other Vespertilionids. In this case some mechanism might be expected to increase the energy fed to the ear as an overloading and comparison signal. With the possible exception of *Barbastella* which has similar form, *Plecotus* is the only Vespertilionid genus which flies with the mouth shut and emits sound through the nostrils. The nostrils do not open forward as in other bats but directly upward under the pinnae which are held forward in flight. Whilst this arrangement may be a mechanism for directing the outgoing sound by reflection from the pinnae, it does seem to represent a method for ensuring that the ear receives signals at sufficient intensity to cause the required distortion.

Rhinolophus emits sound through the nostrils and the nose-leaf arrangement to obtain directionality. This would appear to reduce scattering of sound to the ears. Furthermore a direct signal received by the vibrating pinnae would experience the same Doppler shift as the echoes in the stationary bat and no beats would be produced. The second opening of the meatus described by Möhres (1953) at the cartilaginous non-moving base of the pinna leads forwards down a groove in the face which enters the nose-leaf (Fig. 3). Above the nose-leaf and at the end of this pathway are the hitherto unexplained structures of the lance and shield. It is possible that these may sufficiently distort the sound field to deflect some energy along this route. The presence of this arrangement certainly does not suggest a very high attenuation between transmitter and receiver, but may be ideal for the theory proposed here.

Conclusions

This theory shows that it is possible to obtain a considerable amount of information from a frequency analysis of beats between a bat's call and its echo. Whether the emitted frequency is constant or changing, any object within range will appear to act as a source of different sounds whose nature is determined by the instantaneous or changing position of the object relative to the bat. Once the code is understood much information can be obtained in a very simple manner. Since no new physiological principles are required, the process of evolution of such systems could

be regarded merely as the development of the basic mechanism. Pumphrey (1948) argued that hearing, whatever it may have become in man, developed as a sense of "touch at a distance" whose primary function is the location of sound sources. The bat has gained independence by radiating energy itself to include passive objects. If it listens to beat notes it has changed the information code but increased enormously its powers of discrimination.

Experiments will be made to test this theory with bats, but it has already been shown to be applicable to the human ear. A simple model generating bat-like sounds and receiving the echoes through non-linear amplifiers presents the listener with audible beat notes. The information available is readily interpreted by the ear when the machine is operated in any of the three modes described above or in a combination of them. This device may possibly form the basis for a useful aid to the blind since it has many advantages over previous ultrasonic aids. A description of the model and further consideration of beat note echolocation is to be published elsewhere.

It is perhaps unfortunate that the same arguments cannot be applied to other animals known to echolocate, namely the birds *Steatornis* and *Collocalia*, the fruit bats *Roussettus* and the Cetacea. The complex nature of the sounds of these animals must involve other mechanisms. The simple but beautifully controlled frequency structure of the pulses of insectivorous bats is very striking. The theory proposed above attempts to explain this feature and show how it may account for much of the proficiency displayed by these animals.

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ECHOLOCATION BY BATS

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An important part of man's technological progress has involved the development of a variety of devices to aid and supplement his own sensory apparatus. Many of these have acquired great practical importance in helping him to find his way about, especially under difficult conditions. Thus telescopes, night-glasses, television cameras, and infra-red 'sniperscopes' increase the definition, sensitivity, and spectral response of the eyes, while microphones and hydrophones aid the ears. All these examples are 'passive' in that they rely on signals from external sources, but some other instruments show no such dependence: they radiate energy themselves and create their own signals by interaction with the surroundings. This 'active' group includes radar, asdic (or sonar), search-lights, and even the humble hand-torch. Techniques of this type are achieving increased prominence in many fields, and are often used with advantage to augment the passive ones.

It should hardly be surprising, therefore, to find that a similar development has occurred within the sensory apparatus of some animals. Specialization of the sense organs alone is insufficient to allow the full exploitation of certain habitats, and many examples are now known of

active sensory systems that produce their own exploratory energy. Some deep-sea fishes may use their luminescent organs to see their way about. Other fish such as the Gymnotids and Mormyrids, both fresh-water forms, have developed electrical methods of orientation with remarkably high sensitivity (1, 2); pulses of current are produced by the electric organs, and specialized receptors appear to detect impedance changes that distort the field around the body. The blind cave-fish (Anoptichthys) uses the lateral-line organs to detect reflections of pressure waves from its own swimming movements. This is really a semi-active mechanism, as the waves are not produced deliberately, but several of the higher vertebrates do produce sounds specially for their acoustic reflections. At least two genera of birds, Steatornis (3), the Venezuelan oil-bird, and Collocalia (4, 5), the 'birds nest soup' swiftlet, and also fruit bats of the genus Rousettus (6-9), can fly safely in complete darkness, guiding themselves by listening to the echoes of clicking noises made with the tongue. Some porpoises and dolphins make noises that are almost certainly used for underwater navigation, and the faculty may well be widespread among the Cetacea (10, 11). But true echolocation at present appears to be most highly developed in bats of the sub-order Microchiroptera, most of which are insectivorous and nocturnal.

This was the first example of echolocation to be discovered. In 1794, L. Spallanzani established that bats depend almost completely on hearing for orientation and hardly at all on sight.(12). A series of elegant, if cruel, experiments showed that bats with their ears plugged were helpless, but blinded bats flew normally and were able to catch their prey when released. H. Hartridge suggested in 1920 (13) that an echolocation mechanism using very high-frequency sounds might explain this paradox, and these sounds were detected by G.W. Pierce and D.R. Griffin in 1938 (14).

It has now been established beyond all doubt that this is the means by which bats navigate and find their food, and an account of the experiments performed by many workers over the last twenty years has been published by Griffin (15). It has been shown that small bats can fly at speed through barriers of vertical wires only 0.4 mm. in diameter and spaced only one wing span apart. They achieve this with a collision rate far below that expected from random movement, and the collisions that do occur are often only slight ones, such as a light brush with the wing tip. When feeding, the bats can catch small insects, such as mosquitoes, at the rate of one every ten seconds for up to 30 minutes (16). Each capture is accompanied by intensified emission of sound by the bat; only with

very noisy prey does the bat appear to listen passively to the insect's wing-beats. The complete manoeuvre may be completed in less than half a second, so that the information must be made available to the animal extremely quickly.

Photographs taken by the American workers have shown that the bat pursues its prey with great accuracy at close range. If slight errors are made, and the insect cannot be seized with the mouth, it may be scooped up by the tip of a wing (17). All this can be accomplished in complete darkness or when the eyes are covered, but when the ears are carefully plugged, even a seeing bat in a good light is completely disorientated and blunders into large objects.

The bat is therefore a highly successful self-directed missile that relies on its targets for fuel. But the analogy is not complete, for bats show an extraordinary resistance to jamming. Hunting often occurs with apparent success in highly compromising situations, such as in heavy rain, low over the surface of water, or among foliage of trees and hedges. In certain parts of the world, enormous numbers of bats emerge together each night from their roosting places in caves: the background noise and multiplicity of echoes in these conditions would appear to be extremely confusing, yet accidents rarely happen and the interception of prey is continued.

Some species have been forced to fly in an artificial sound-field of "white" noise whose frequency band covered

that used by the bats. Wires of 0.5 mm. diameter were avoided almost as well as in the quiet, and well above the expectation for random flight, even when the noise intensity was some 30,000 times greater than the calculated intensity of the echoes (15, 18). This performance shows an extremely effective rejection of unwanted signals that at first sight appears difficult to explain. However, recent investigations into the 'cocktail party' effect, in which single conversations can be distinguished from a background of noise and chatter, suggest that two ears may be better than one. A model employing two microphones and a non-linear method of correlation has already shown considerable improvement over the theoretical limit for linear devices (19). Development of this decision theory may help in understanding the bat's ability to deal with similar problems.

So far only two families of bats have been investigated very thoroughly, and they appear to have rather different methods of acoustic orientation. Details of the Vespertilionidae are known from the work of R. Galambos, D.R. Griffin, A.D. Grinnell, A. Novick, and others in North America, and of S. Dijkgraaf in Europe, while the European and African Rhinolophidae (horseshoe bats) have been the special study of F.P. Möhres and E. Kulzer. Only these two types can be described here, but other families which have been examined are often similar to these,

or are to various extents intermediate in character (20-22).

The orientation sounds used by all Microchiroptera are produced vocally in the larynx. The specialized structure of this organ was described in detail by M.H.A. Robin (23) and by H. Elias (24), although the functional significance of their findings was not apparent at the time. Ossification and fusion of the cartilages has occurred, and the intrinsic muscles are very well developed, especially the cricothyroids, which apply tension to the two pairs of vibrating membranes. The membranes themselves are short and extremely light, permitting the production of very high frequencies. In the Rhinolophidae there are three resonating chambers opening from the trachea, and the glottis is ring-shaped and can be raised to fit into a cartilaginous ring around the internal nares. The Vespertilionidae, with few exceptions, produce their sounds through the mouth and over a fairly wide angle, while in the Rhinolophidae emission occurs through the nostrils and is beamed forwards. This is brought about by the nose-leaf structure, on the snout (figure 1), which acts as a horn and reflector, and by the nostrils, which are a half-wavelength apart, and cause interference laterally. The nose-leaf is capable of some movement, to direct the beam in different directions, and of changes of shape that may alter the pattern of emission to some extent (25).

Both families emit the sounds in discrete pulses that may attain high energy-levels. Recordings made 10 cm. from a Vespertilionid show peaks reaching 60-170 dynes/cm^2, roughly equivalent to the level close to a pneumatic road-drill and rather more than an untrained person can produce by shouting. Rhinolophids may produce even higher intensities. The pulses of the Vespertilionidae are variable in duration, according to circumstances, but are generally very short. The typical pulse of a bat flying indoors lasts only 1-4 msec. (figure 2, a and b); some species may produce longer pulses in the open air and shorten them when obstacles or prey are approached. The pulse-repetition rate is variable, and can change from less than 10 per second, when cruising, to over 100 per second when an object is being investigated. The most characteristic feature is that the frequency of the sound falls steadily by nearly an octave throughout each pulse (figure 2c). The frequency at the start of each pulse varies with the species, between 30 and 120 kc/sec; shorter, 'close range' pulses begin at lower frequencies than the longer ones.

In quiet conditions it is sometimes possible to hear a series of faint clicks as a Vespertilionid bat flies about. Each click occurs at the beginning of a pulse, and by listening to these sounds Dijkgraaf was able to determine the rate of pulse production during various

manoeuvres (26). At the higher repetition rates, the clicks follow each other so rapidly that they form a distinct buzzing noise whose pitch is easily determined. Each click consists of a few waves of sound at about 10 kc/sec., and its intensity is very much lower than that of the subsequent high-frequency sound. Also it is more pronounced in sick, sleepy, or very young bats, and less in those that show the greatest skill in avoiding obstacles. This component therefore appears to play little part in echolocation, but it may be a useful guide to the way the larynx makes the pulses. If the crico-thyroid muscles are paralysed by cutting their motor nerves, the bat produces pulses whose frequency remains constant at about 10 kc/sec. (15). This may represent the frequency of the chords at their resting tension and subsequent changes in frequency may be in some way produced by muscular action.

The pulses of Rhinolophids are much more constant in form (25). They last for 40-100 msec. and are of very constant frequency, between 85 and 100 kc/sec. according to the species, although a fall in frequency may be observed during the last few milliseconds (figure 3). Pulse production is co-ordinated with breathing, which is itself synchronized with wing movements in the flying animal. The pulse-repetition rate therefore seldom exceeds five or six per second in flight, and is not so

variable with behaviour as in the Vespertilionids. The pulses of both these families show a very low harmonic content, although other bats may produce marked second and third harmonic components (20).

The ears of bats are highly modified for the reception of high-frequency signals. The external ear is always large - its length may exceed that of the body (figure 4) - but its structure varies considerably. The Vespertilionidae have a fairly simple immovable lobe, with a well-developed tragus looking like a second, smaller ear in front of it. Several suggestions have been made concerning the function of this 'earlet', but so far none has been substantiated. The shape of the tragus usually resembles that of the pinna (figures 4-6), which suggests that it plays some part in determining the acoustic properties of the ear.

The Rhinolophidae have no tragus, but another lobe, the antitragus, is developed as a fold in front of the pinna and forms a funnel around the ear canal (figure 1). The two lobes do not meet at their bases on the inner side: there is thus a second opening to the meatus which faces forward and downward along the snout. The ears of these bats are freely and independently mobile and, when the animal is awake, are kept in constant motion by complex musculature on the head. They appear to be actively searching for the sources of sounds or echoes, whereas the

Vespertilionidae can only turn the whole head. F.P. Möhres (25) has shown that, in addition to these searching movements, the ears are also vibrated forwards and backwards at rates up to 50 times per second, the tip of the ear often moving through an arc of 8-10 mm. This unusual action only occurs while the bat is emitting exploratory pulses, and it may be responsible for a low-pitched buzzing sound that is produced at this time. It seems to be an important part of the echolocation mechanism, since, if the ears are rendered immobile by denervating the appropriate muscles, the bat is disorientated in flight. Later the animal learns to compensate by performing rapid movements of the whole head, and the ability to orientate is regained to some extent (28).

The muscles of the middle ear are very well developed. These are the tensor tympani and stapedius, responsible for tensioning the chain of ossicles that conducts sound from the ear-drum to the inner ear. The bulla is composed of very thin bone and is restricted in extent to the external side of the cochlea (figure 7a). The cochlea is very large and occupies much of the posterior part of the skull, especially in the Rhinolophidae, where the two receptor organs almost meet in the mid-line (figure 7, a and b). The basilar membrane is very narrow

and appears to be under tension, since its supporting structures are very well developed (figure 8). On the inside are a thick spiral lamina and a very high limbus; to the outside the spiral ligament is extremely large and is further strengthened by a second spiral lamina of bone. Many features of this receptor are not yet understood, but they represent an extreme form of the pattern usually associated with hearing in the high-frequency ranges (29). This conclusion is supported by a certain amount of experimental evidence. Cochlear microphonic potentials have been obtained from Vespertilionids as far as 98 kc/sec, the limits of the apparatus used (30). In other experiments bats were trained to expect food when sounds of various pitch were heard, and responses were obtained up to 200 kc/sec. (31). By comparison, hearing in man seldom extends above 17-20 kc/sec.

Although the total size of the brain may be extremely small, the auditory regions of the bat's brain are extraordinarily well developed (15). The visual centres, by contrast, are greatly reduced. Grinnell and Griffin (32) have recorded changes in electrical potential at several levels in response to sound stimuli but especially at the posterior colliculus. They found that the greatest responses are evoked by short pulses of sound and that pairs of pulses can be resolved when separated by as little as 1-5 msec. Inhibitory interaction between

signals from the two ears was observed when the sound was projected from certain directions. There was little evidence of noise rejection at this level, but sharply tuned responses were obtained to short tones as high as 150 kc/s.

These high frequencies are an important feature of echolocation, as they increase the resolution that can be achieved. In general, waves are only reflected specularly by objects whose dimensions are very much greater than one wavelength. As size decreases the received energy becomes reflected more widely, until objects which are very small compared with the wavelength scatter incident energy equally in all directions. Wavelength is inversely proportional to frequency, and the sounds used by bats, in the range 25-120 kc/sec., have wavelengths of 15-3 mm. They are thus able to produce strong echoes from quite small objects, but the successful detection of targets smaller than this shows that to some extent useful echoes may be obtained from scattered signals.

The range at which detection may occur has been estimated by several methods. Using the increase of pulse-repetition rate of Vespertilionids as a criterion of detection, Griffin (15) has estimated that barriers of wire may be detected indoors at about 2 m., although no avoiding action was taken until the bats were much closer.

The same bats in the open will dive 5-6 m. to investigate small objects thrown into the air. However, by a series of training experiments, Dijkgraaf and Möhres estimated that occuracy of discrimination only extends to a range of about 50 cm. (31, 25). Rhinolophid bats, on the other hand, appear to perceive their surroundings accurately to a range of about 6 m. although resolution probably decreases with distance.

A further difference between the two families is seen when the ears are temporarily incapacitated. All bats are completely disorientated if the external ears are carefully plugged on both sides, but, with one ear free, a Rhinolophid bat is able to fly perfectly well and to avoid obstacles with ease. In strange contrast, the Vespertilionid bat deafened only on one side is loth to fly, and when forced to do so it blunders about as clumsily as when totally deafened.

To summarize, the Vespertilionidae produce many short, frequency-modulated pulses through a wide angle; their ears are not mobile, and both must be functioning. The Rhinolophidae make fewer, longer pulses of very constant frequency, and concentrate the sound into a beam with which they scan their surroundings. The ears move independently in a complex manner, orientation may be achieved monaurally, and they can probably probe accurately to greater ranges.

How do these mechanisms operate? What information do the bats obtain about nearby objects? Are there two or more distinct methods, or are they variations of a single one? These fundamental questions cannot yet be answered, but several theories have been proposed in attempts to explain the mechanism behind the observed phenomena. The first, that of Hartridge, resembled early radar methods in many ways (33). He supposed that the bat can estimate the range of its targets by measuring the time delay before an emitted sound returns as an echo. Normal binaural location of the direction from which the echo comes will then give precise information about the relative position of the object concerned. The very short pulses of the Vespertilionidae were considered to be suitable for such a mechanism, as they contain sharp peaks of energy which could be used to mark short intervals of time; the form of Rhinolophid pulses was not then known.

Under close examination, several points of difficulty arise regarding this early theory (34). The time delays involved are very short, and their measurement must be extremely accurate to explain the skill observed. A central mechanism capable of this accuracy must be envisaged. It is known that the time of arrival of a single sound at the two ears can be compared with great accuracy, but the timing of short intervals by the same

ear demands a different type of central analyser. Yet it would seem that range is one of the most important pieces of information, and echo delay its most direct means of measurement.

Again, it is difficult to understand how the bat can hear very faint echoes so soon after it has made such a loud noise itself. It is unlikely that much attenuation can be effected within or around the head of such a small animal, and the mammalian ear generally experiences a rise in threshold following a very loud sound. Hartridge suggested that contraction of the middle-ear muscles by the intra-aural reflex may damp the ear during pulse production and then restore sensitivity for echo reception. But the fact that very high speeds of reflex action and muscle relaxation would be needed make such an idea untenable. Direct motor control of these muscles, synchronized with that of the larynx might achieve the required result, but there is no evidence or precedent for such an arrangement.

Furthermore, at the very close ranges involved in the interception of insects, the echo front must return before production of the pulse is completed. This overlap would be expected to mask the fainter echo-signal. Griffin has suggested that frequency modulation of the pulses may overcome this difficulty, since the echo delay will ensure that pulse and echo are never

heard at the same frequency at the same time, but the difference must be small at very close ranges. The problem could also be reduced if the bat employs the principle of the recently developed chirp-radar (35). This system uses frequency-modulated pulses similar to those of the Vespertilionidae, and the receiver incorporates a delay network whose effect varies with frequency. If the pulse front is delayed more than subsequent parts, the emergent pulse is compressed in duration, and overlapping echoes may be separated. Is it physiologically possible for the bat's ear to contain such a frequency-sensitive delay network?

The ability to cope with multiple echoes demands that the timing device, whatever its mode of operation, should be able to handle a series of intervals simultaneously and to discriminate between them. The extent to which some bats can deal with such situations would place very great, though not necessarily insuperable, demands on such a system.

Möhres has rejected the idea of delay-time measurements for the Rhinolophids because of the long duration and low repetition-rates of the sounds made by these bats. There are no sharp energy-peaks in these pulses, so that only the relatively infrequent pulse-fronts and trailing edges could be used for timing, and chirp-radar principles cannot apply. Instead, Möhres

suggests that the bats observe the loudness of echoes while scanning with their beam of ultrasound and with their presumably highly directional ears. The bearing of echo targets is then easily obtained from proprioceptive information and the range could be obtained by a process of triangulation, although the base-lines involved are very small. This method could operate monaurally, and therefore satisfies the observations on this point.

The problem of masking is here even greater than for Vespertilionids. The sound does not change frequency, and the duration of each pulse will ensure that overlap of pulse and echo occurs up to a range of several metres. It is hard to believe that the bat could hear any but the loudest of echoes, and minute observation of their intensities would be very difficult.

A general theory applicable to nearly all Microchiroptera has recently been advanced by the author (34) in an attempt to overcome some of these problems. It is rather striking that the maximum ranges at which each type is thought to locate objects accurately are those at which their respective pulses and echoes just overlap. Possibly the overlap, which would seem to mask at least part of each echo, may be a necessary part of the detection mechanism. It is suggested that under these conditions the bat may not attend to the echo itself;

instead, he listens to beats between the echo and the original call. If beats occur at a frequency greater than the lower limit of hearing, they could be heard as a separate note, a beat-note.

For the frequency-modulated pulses of the Vespertilionids, the frequency of the beat-note will be characteristic of the echo delay and therefore of the range of the target (figure 9). Frequency analysis by the cochlea could thus provide an accurate method of measuring the very short time-intervals involved. Since the frequency of the pulse seldom changes by more than an octave (a factor of two), the beat frequencies will nearly always be below those of the pulse and echo, so that problems of masking will be much reduced. Multiple targets may be distinguished, as each echo produces a separate beat-note and the cochlea is known to be able to discriminate between simultaneous notes of different frequencies. A comparison of beat-notes produced in each ear could also give directional information.

The same idea has been put forward independently by L. Kay (36). He further suggests that an extension of range could be achieved if the bat repeats each pulse to itself after a controlled delay and so produces beats with later echoes. Without a provision of this kind, one must assume that the bats that detect wires at distances greater than half the pulse-length in air must be using another mechanism. But detection of an object does not

necessarily indicate that the bat possesses exact knowledge of its position. Possibly little information is gained from the discrete echoes heard at first, and accuracy is obtained only when the range is reduced and overlap occurs. The agility of bats, which can turn in 30 cm. or less, makes it very difficult to judge how much information they have when 2 m. from an obstacle. Nevertheless the initial directional perception of insects appears to be very good (15).

The beat theory applied to Rhinolophid pulses provides a sensitive method for the detection of Doppler shifts (figure 10). The bat flying towards an obstacle could measure the approach velocity, as this is proportional to the beat frequency. But, probably more important, it provides a method of preventing masking. At the relatively high speed of 10 m/sec., a pulse of 100.00 kc/sec. (as used by Rhinolophus hipposideros) will return as an echo of 106.25 kc/sec. and so produce a beat-note of 6.25 kc/sec. This is probably the maximum value that will be encountered, and the original pulse will hardly interfere with its detection. At the same time the mechanism is extremely sensitive to the very low relative velocities. Location of the target may then be achieved by observing the loudness, not of the echo as Möhres suggests, but of beat-notes from it and the call.

For this method to work, the ears of the Rhinolophid must be highly directional, and here the very rapid vibration of the pinnae may help. If each pinna acts as a reflector for collecting echoes, its own movement will introduce a range of Doppler shifts which will, at any instant, be proportional to the velocity of each part of the collecting surface. Thus the ear vibrating at 50 c/sec. will produce a range of beat-notes (figure 11). The 100 c/sec. component of this mixture will predominate, as it will synchronize with the ear movements. The effect will be greatest for an echo returning in the direction of ear movement and will decrease to either side. Even the stationary bat can detect stationary objects by this means, as the velocity component is supplied 'artificially'. Of course, if the original pulse from the nostrils were heard after reflection from the moving pinna, it would experience similar Doppler shifts to those of the echo and no particular beat-note component would predominate. It is therefore suggested that the second opening to the ear canal, at the non-moving base of the pinna, may act as a pathway for the 'direct' signal. As may be seen in figure 1, the ventral aperture faces along the snout towards a deep groove in the periphery of the nose-leaf 'transmitter'.

One of the methods of detecting beat-notes is to add the two original signals together and then to subject them

to non-linear distortion. The first action occurs within the meatus, and there is evidence that the second may occur within the ear. Studies of the cochlear microphonic potentials of cats and guinea-pigs have shown (37) that strongly non-linear characteristics appear when the ear is overloaded at intensities of 1-10 dynes/cm^2. In view of the very much higher intensities produced by bats, it would be surprising if stray sounds from the mouth or nostrils did not reach these levels at the ear. Instead of this signal being an embarrassment by masking echoes, it may be an essential part of the mechanism. The site of distortion within the ear is the source of some controversy, but there is some evidence that the type of distortion best suited to beat-note detection is caused by contraction of the middle-ear muscles (38). This may explain their well-developed condition in the bat.

Normally beat-notes are not very loud to the human ear, as the degree of distortion present is low, but under optimum conditions, that may well occur in the bat's ear, the beat-note amplitude can approach that of the smaller signal, in this case the echo. In order to see how well the human ear can discriminate the position of objects, a model has been built as shown in figure 12. Distortion is provided by a detector in conjunction with artificially generated bat-like sounds, and the operator is easily able to detect objects in any of the three modes proposed above.

Another model using the same principle but different in detail has been constructed by Kay, and similar results have been obtained.

The beat-note theory supports rather than invalidates the conclusion already reached by other workers. It attempts only to suggest a method whereby some of the expected results may be obtained and many of the drawbacks avoided. By proposing a common physiological basis for the different systems observed it allows a wide range of intermediate conditions and facilitates speculation about the evolution of these systems. It throws no light, however, on the signal-to-noise problem, nor can it be applied to the echolocation systems of other animals.

Whether the bats do in fact use one of the many methods suggested so far, or whether they have other more subtle means at their disposal, can only be decided by further investigation. This promises to be very exciting, and a better understanding of the bat's speed, accuracy, and resistance to jamming may well be of interest in fields far removed from the study of small mammals.

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FIGURE 1 - The head of Rhinolophus ferrum-equinum (Rhinolophidae), the greater horseshoe bat (x3). The nostrils are set close together at the centre of the complex nose-leaf that gives this animal its name. The antitragus is here seen edge-on across the base of the ear.

FIGURE 2 - (a) and (b) Cathode-ray oscillograph traces of pulses produced by Nyctalus noctula (Vespertilionidae) indoors. The horizontal bars of the time marking signal are each 0.1 msec. in duration. (c) The end of a similar pulse expanded to show the continuous fall in frequency. Each horizontal bar represents 0.1 msec.

FIGURE 3 - Part of an oscillograph record of a pulse from Rhinolophus ferrum-equinum. The time bars are 0.1 msec. long, and a total of 54 msec. has been removed from the two gaps in the trace. Frequency is very

constant but falls in the last 1.5 msec. The trace reads from left to right.

FIGURE 4 - The head of Plecotus auritus (Vespertilionidae), the long-eared bat (x3). The external ears are enormously developed and are held forwards in flight. The tragus is long and pointed, and the snout has no appendages.

FIGURE 5 - The head of Myotis myotis (Vespertilionidae), the mouse-eared bat (x2.7). The ears are long but are relatively much smaller than in Plecotus.

FIGURE 6 - The head of Nyctalus noctula (Vespertilionidae), the noctule (x2.5). The ears, here seen in a relaxed position, are rounded with an almost reniform tragus.

FIGURE 7 - (a) Ventral view of the skull of Rhinolophus ferrum-equinum (x2.6). The large proportions of the cochlea are apparent, but the bullae which enclose the middle ear are restricted to rings of thin bone on the outer side of each cochlea. (b) An X-ray photograph of the same skull to show the full extent of the cochlea.

FIGURE 8 - A photomicrograph of a section of one turn of the cochlea of Rhinolophus ferrum-equinum. b.m.= basilar membrane; Lim.=limbus; P.S.L.=primary spiral lamina;

S.Lig.=spiral ligament; S.S.L.=secondary spiral lamina.

FIGURE 9 - A diagram of the possible construction of a beat-note from a Vespertilionid pulse and its echo. The beat frequency (f) is directly proportional to the range (r) of the target.

FIGURE 10 - A diagram showing the production of a beat-note from a Rhinolophid pulse. The beat frequency is proportional to the rate of change of range (dr/dt), or the relative velocity of bat and target.

FIGURE 11 - A diagram of the beat-notes which could be produced by ear vibration in a Rhinolophid. The component whose frequency is twice that of the ear movements (or higher even multiples) will remain coherent and so will predominate. The beat-frequency and Doppler-shift scales are here exaggerated for clarity.

FIGURE 12 - A block diagram of the apparatus used to test the ability of the human ear to detect objects by beat-note echolocation. The generator produces different types of bat-like sounds, and beat-notes produced with the echoes are heard through headphones. The 'vibrating ear' reflectors for each microphone have been omitted from the diagram for simplicity.

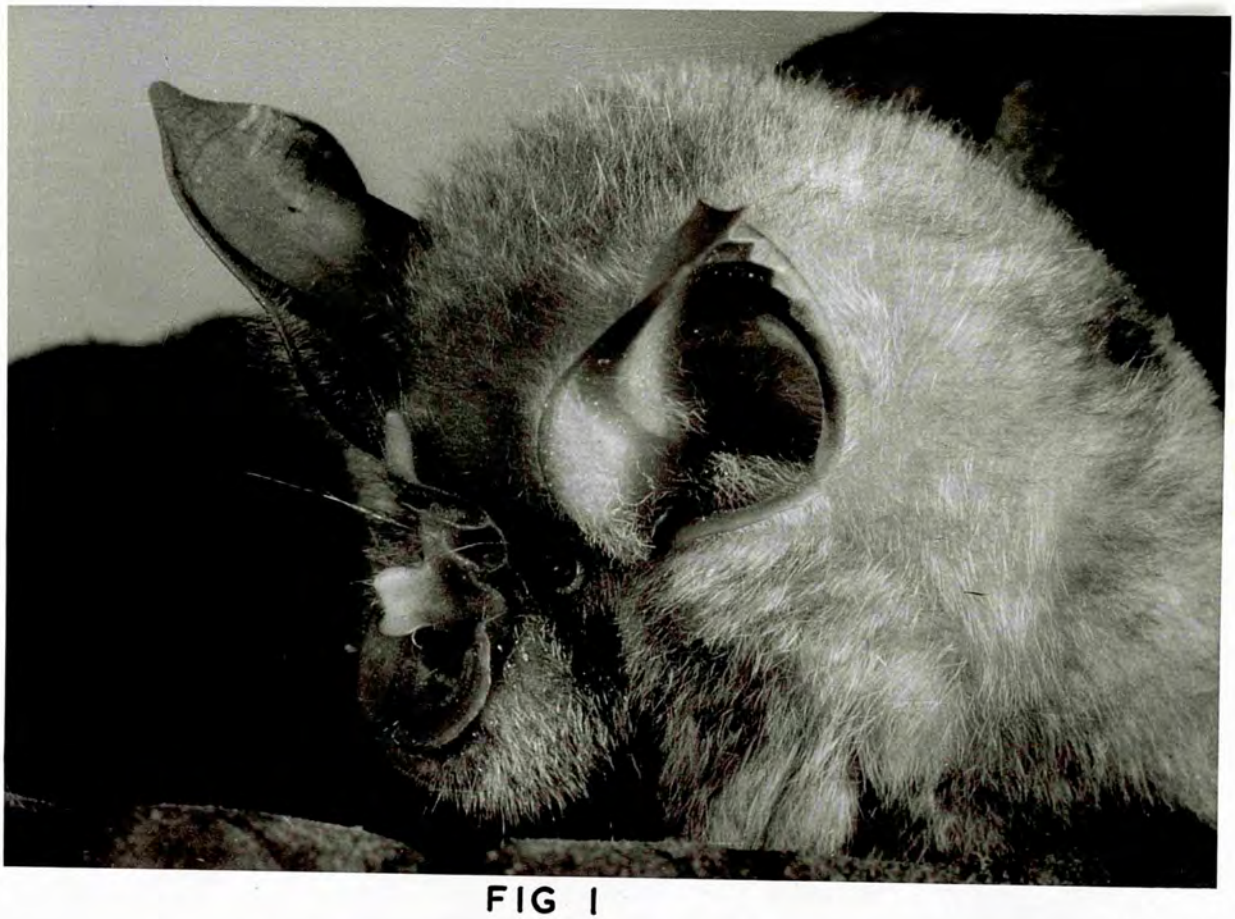

FIG 1

FIG 4

FIG 5

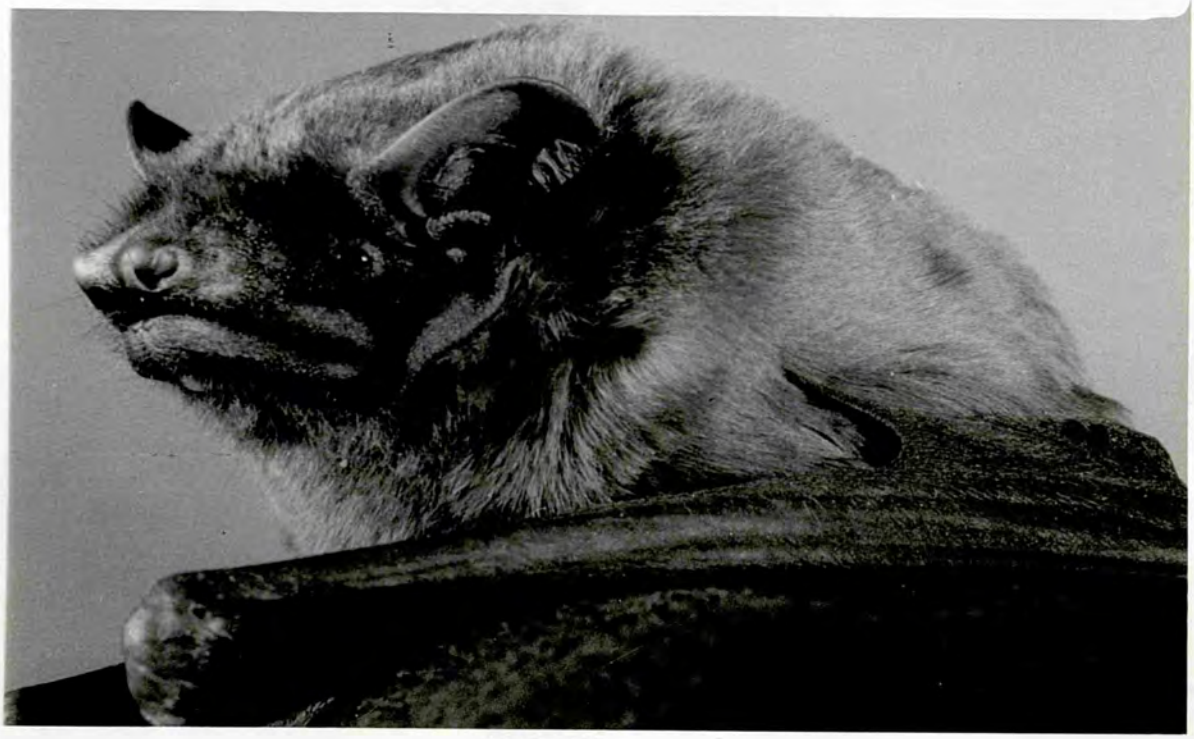

FIG 6

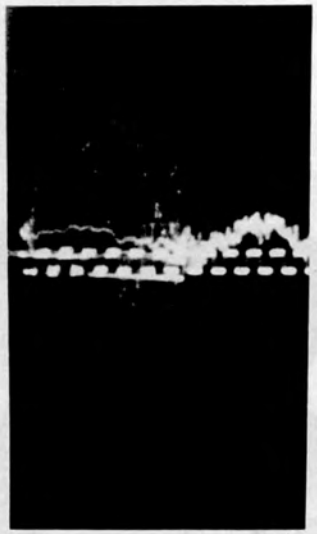

FIG 2a

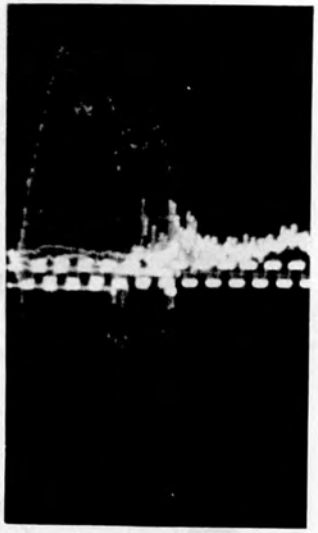

FIG 2b

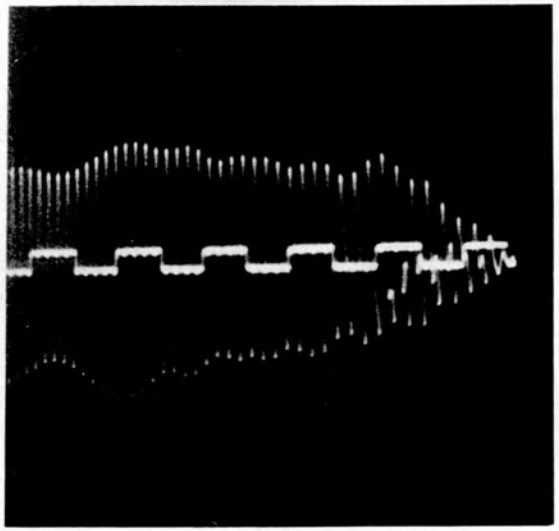

FIG 2c

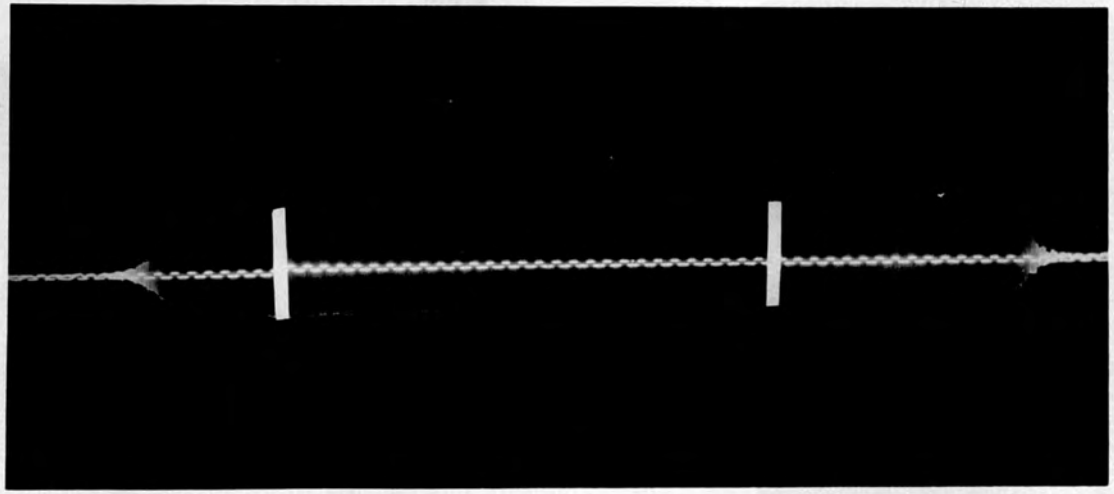

FIG 3

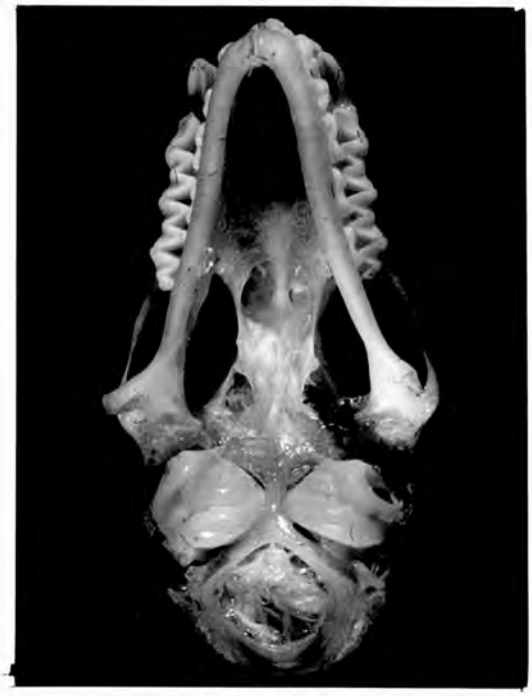

FIG 7a

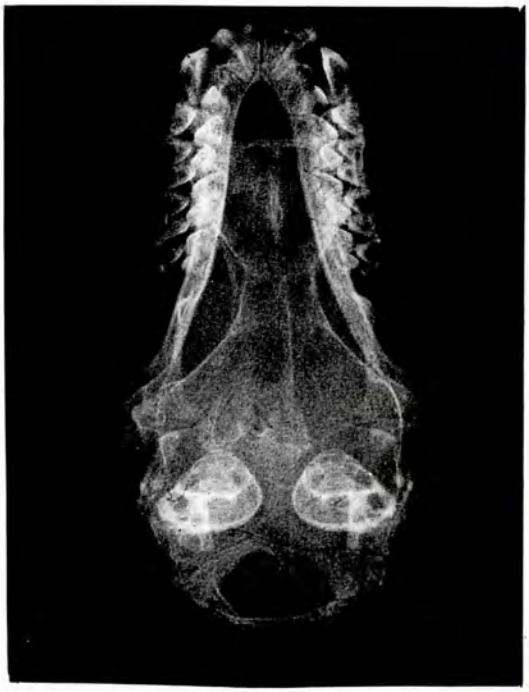

FIG 7b

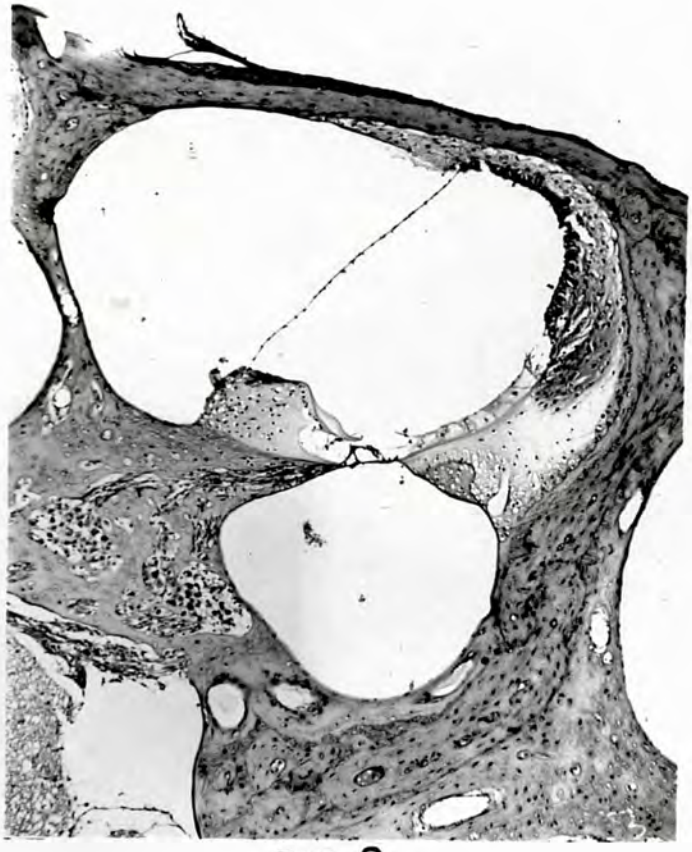

FIG 8

VESPERTILIONID - TYPE PULSES — frequency modulated

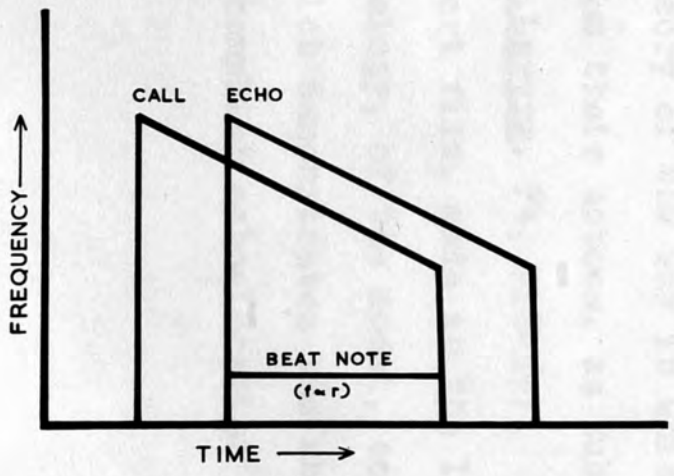

FIG 9

RHINOLOPHID-TYPE PULSES — constant frequency

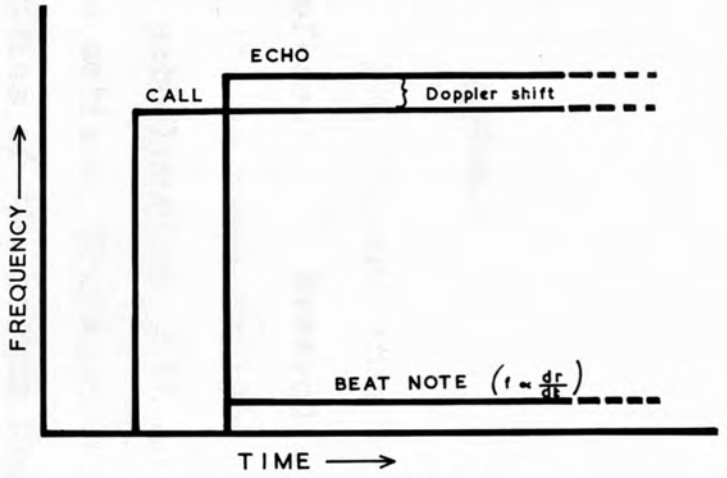

FIG 10

The stationary Rhinolophus with vibrating Pinnae

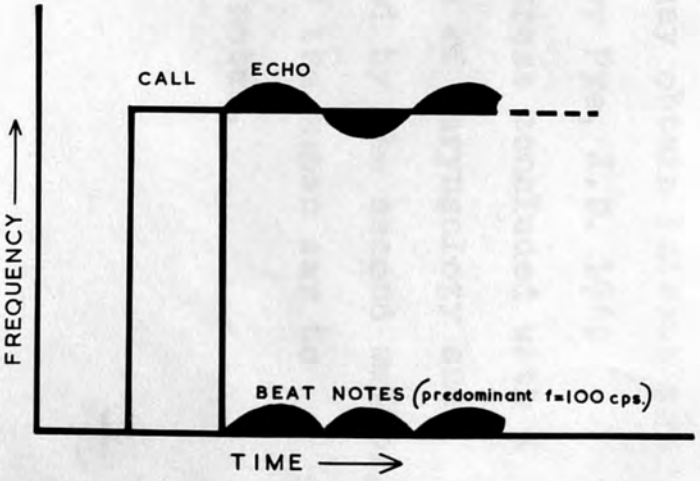

FIG 11

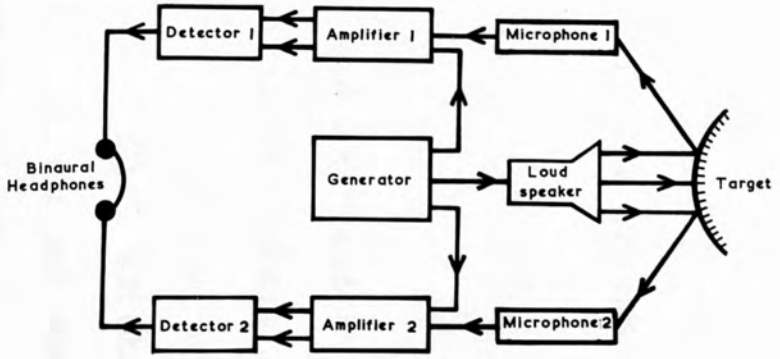

FIG 12

Ormerod, F.C. and Pye, J.D. 1961. Acta Otolaryng., Stockh. (in the press).

Unpublished abstract.

This paper forms thetext of an address read by Professor F.C. Ormerod at the Collagium of Otolaryngologists at Padua in 1960. After introducing the subject of echo-location with an account of the skill which bats can achieve, Professor Ormerod described his own anatomical studies of the larynx and cochlea of these animals. Special account was taken of the sounds produced for orientation and the nature of the echoes which must be detected. The second half of the paper introduced a new theory of the way in which bats may obtain information from their echoes, as published by Pye, J.D. 1960 (J.Laryng. 74, 718-29). The address concluded with a short film, made at the Institute of Laryngology and Otology, of the model, constructed by the second author, which demonstrates the ability of the human ear to accomplish echo-location by beat-notes.