J. Exp. Biol. (1964), 41, 525–534
With 4 text-figures
Printed in Great Britain

NERVOUS CONTROL OF CHROMATOPHORES IN TELEOST FISHES

I. ELECTRICAL STIMULATION IN THE MINNOW (*PHOXINUS PHOXINUS* (L.))

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(*Received* 13 *January* 1964)

INTRODUCTION

It has been firmly established that in many teleosts the sympathetic nervous system contains cells whose activity causes aggregation of the pigment within the melanophores and therefore paling of the skin. In the minnow these *W* (whitening) fibres were shown by von Frisch (1911) to follow the pathways shown in Fig. 1A; that is they pass from a centre in the hind-brain via the spinal cord to the level of the 15th vertebra, through rami to the sympathetic chain and then anteriorly and posteriorly to emerge through all the spinal and the trigeminal (fifth cranial) nerves to the skin. Nerve section at any level in these tracts first produces intense pigment dispersion in the paralysed melanophores, later followed by slower adaptive responses under humoral influences. Electrical stimulation of the intact tracts causes rapid melanophore aggregation, as do adrenalin and other sympathomimetic agents.

Parker (1948) claimed to have demonstrated the presence of a system of opposing, melanophore-dispersing or *B* (blackening) fibres in *Fundulus* and *Ameiurus*. His arguments are still controversial and at best provide only indirect evidence of double innervation, but two experiments seem to be critical. These are, first, that a second nerve-section within a previously paralysed region produces redispersion both orthodromically and antidromically (1934*a*, 1936, 1937); and, secondly, that the application of a small cold block just peripheral to a fresh nerve-section limits the spread of the response and prevents dispersion from appearing distally (1934*b*). From these results Parker argued that the initial dispersion is an active process produced by repetitive discharges in *B* fibres and originating from a permanently depolarized region at the site of the nerve-section. This view has met with considerable opposition from a number of sources, although no satisfactory alternative explanation has been advanced.

Parker & Rosenblueth (1941) claimed to have stimulated *B* fibres by electrical means in *Ameiurus*. They applied rectangular pulses, 6–8 V. in amplitude and 300–500 msec. in duration, at a rate of 1–2 pulses/sec. to the skin through bipolar electrodes and obtained local darkening in about 10 min. Pulses of 8 V., 4–8 msec. in duration and delivered at 15–25 pulses/sec., produced local paling in 15–25 min. These experiments may be criticized on three points: first, the mark/space ratio was very high for the pulse trains used to obtain dispersion and the d.c. component may have blocked normal

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activity in the *W* fibres for part of the time; secondly, the authors state that 'no attempt was made to use non-polarizable electrodes' so that under a heavy duty cycle the actual stimulating conditions are difficult to interpret and may have been complex; finally, the response times were extremely long compared with those normally encountered in chromatic stimulation. It does not seem wise therefore to draw dogmatic conclusions about double innervation from these results without further investigation of the processes involved.

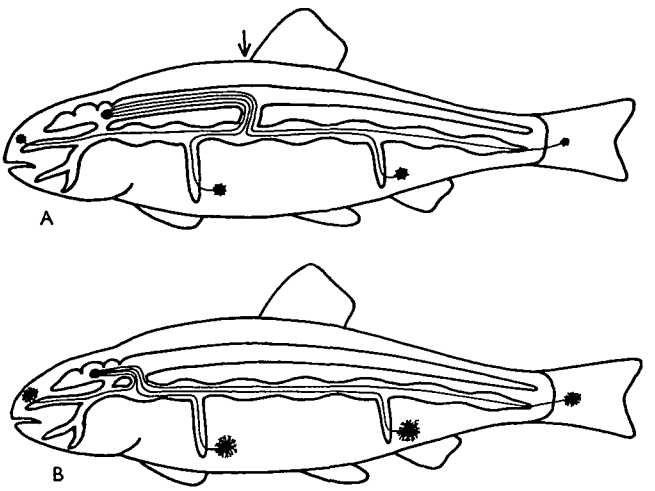

Fig. 1. Simplified diagrams of chromatic-motor pathways within the nervous system of *Phoxinus*. A, Melanophore-aggregating (*W*) fibres, after von Frisch (1911). B, Melanophore-dispersing (*B*) fibres, after von Gelei (1942). The arrow indicates the level of the 15th vertebra.

Giersberg (1930) found that, after administering ergotamine and acetylcholine to minnows, electrical stimulation of the spinal cord produced melanophore dispersion. He argued that ergot suppressed the adrenergic *W* fibres while acetylcholine potentiated a cholinergic *B* fibre system. Von Gelei (1942) combined this technique with crude sections of the sympathetic chain made by introducing a fine knife through the body wall. Melanophore dispersion, produced by a stimulating electrode in the hind-brain or anterior end of the spinal cord, was limited posteriorly by section of the sympathetic chain at any level. This was held to indicate that the *B* fibres emerge from the spinal cord through the first or second rami as shown in Fig. 1B, and run posteriorly through the sympathetic chain alone.

Certain experiments performed by von Frisch (1911), by Healey (1948 *et seq.*) and by Gray (1956) do not support the presence of such tracts. For example, after section of the sympathetic chain just anterior to the 15th vertebra, the anterior part of the body shows no evidence of neural chromatic control whereas von Gelei's *B* fibres should remain intact in this region. Behind such a cut, where von Gelei's fibres should be interrupted, melanophore responses appear to be completely unchanged. Von Gelei omitted to test the effect of stimulation at other sites in the nervous system and to provide controls by repeating his experiments without the influence of ergot. The problems raised by his results are therefore re-examined here.

Further indirect evidence for double innervation of chromatophores has been reviewed by Parker (1948) and Pye (1961). None of it is decisive or relevant to the present investigation, which is concerned solely with the effects of electrical stimulation.

METHODS

Minnows were caught in the London area and showed no signs of the helminth brain parasite *Diplostomum phoxini* (Faust) common in many parts of Great Britain (Erasmus, 1962). Fish weighing about 3·5 g. were selected for experiments and were always tested for normal adaptation to both black and white backgrounds before use. Anaesthesia was induced by immersion in a 1% solution of urethane in tap water and maintained by oral administration of 0·25% solution as described by Healey (1948).

The stimulation apparatus consisted of a square-wave generator followed by a pulse-shaping unit which produced either saw-tooth or rectangular waveforms. The output was fed through a radio-frequency coupling unit and was monitored and measured by a cathode-ray oscilloscope. Stimulating electrodes consisted of 40 S.W.G. silver wire, lightly chlorided, and the 'indifferent electrode' was a heavier silver wire (also chlorided) beneath the body of the fish. The smaller electrode was placed and held in position by a simple ball-and-socket manipulator.

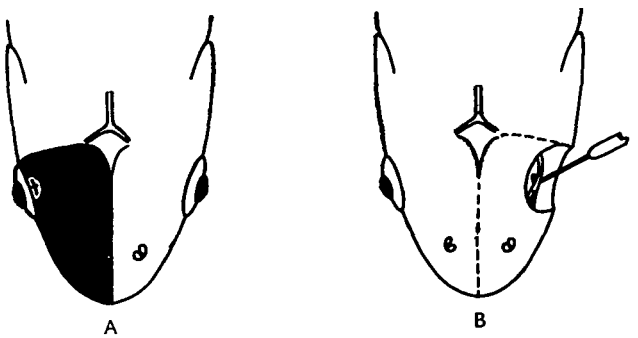

Fig. 2. Diagrams of the local chromatic responses on the head of *Phoxinus*. A, Following section of the right superficial ophthalmic nerve (dispersion). B, Following stimulation of the left superficial ophthalmic nerve (aggregation).

Stimulation of peripheral nerve

In order to assess the properties of peripheral chromatic motor nerves, it was necessary to find a short length of nerve-trunk which could be isolated locally from the body mass. The only nerve which fulfilled this condition and was also sufficiently robust appeared to be the superficial ophthalmic branch of the trigeminal nerve which supplies melanophores on the head and snout. A short incision in the skin of anaesthetized fish, just dorsal to the eye-ball, and slight depression of the eye itself, allowed this nerve to be located and cut; the incision was then sutured by a single stitch of fine nylon and the fish was returned to an aquarium to recover. This procedure was much less severe than that used by von Frisch (1911) and Smith (1931*b*), and produced the same results. Melanophore dispersion occurred in a quadrant of the head bounded by the jaw, the mid-dorsal line and the posterior edge of the orbit (Fig. 2A). The edges of

this patch were always sharply defined at first, but on a white background the darkening faded almost completely in 24 hr.

If the eye were removed, a short loop of the intact superficial ophthalmic nerve could be dissected free of the connective tissues and picked up on a stimulating electrode bent into a hook at the tip. The orbit was then dried with filter-paper and filled with liquid paraffin. Electrical stimulation of this preparation produced complete melanophore aggregation within a few seconds in the same sharply defined area as that darkened by nerve-section (Fig. 2B). Thus stimulation was restricted to the nerve and was not caused by general current spread through the tissues of the head.

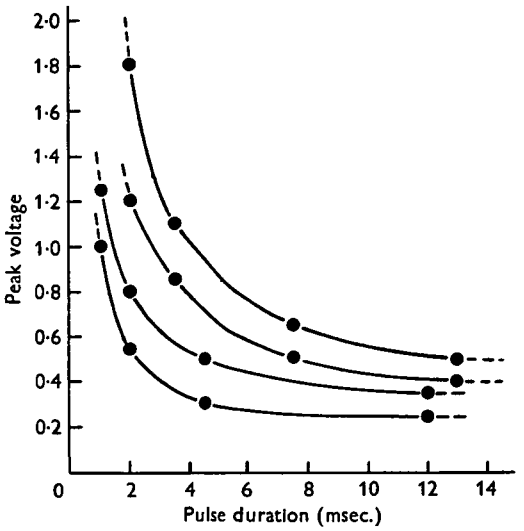

Fig. 3. Threshold curves for electrical stimulation of melanophore-aggregating fibres in the superficial ophthalmic nerve of *Phoxinus*. Uppermost curve, positive (anodal) saw-tooth pulses; second curve, negative (cathodal) saw-tooth pulses; third curve, positive square pulses; and lowermost curve, negative square pulses. Pulse repetition rate 2/sec.

Stimulation curves for a typical preparation are shown in Fig. 3. Anodal stimulation, which in some cases produced slightly lower thresholds than cathodal stimulation, is attributed to the formation of 'virtual cathodes' at the point where the nerve entered the paraffin pool. This is supported by the frequent observation of a tenfold rise of cathodal threshold in ageing preparations (after some hours), probably due to reduced sensitivity in the isolated region of the nerve, while the anodal threshold remained stable. No stimulation could be produced at the indifferent electrode by either polarity of pulse except at very much higher voltages. With saw-tooth pulses (linear rise of voltage followed by an abrupt fall to zero) no rise of threshold with increasing pulse duration could be detected even with pulses very much longer than those shown in Fig. 3. This ruled out the possibility of differential stimulation by selective adaptation, which, it had been hoped, might have demonstrated the action of *B* fibres.

In all cases, changes of pulse repetition rate were found to have no effect on the voltage threshold or on the speed and intensity of melanophore responses, at least between 1 per 2 sec. and 100 per sec. This suggests that the time-constant of temporal

integration of melanophore excitation is extremely long; Gray (1955) has shown that there is little spatial integration within single melanophores. Following section of the nerve on the proximal side of the electrode paling responses were still obtained although the preparation did not remain active for so long. This rules out the possibility of central excitation through the stimulation of sensory fibres in the mixed nerve.

The experiments were repeated on fish which had previously been subjected to section of the sympathetic chain anterior to the level of the 15th vertebra and allowed to recover over a white background (for operative method see Healey, 1948). Section of the superficial ophthalmic nerve now produced only incomplete melanophore dispersion, the edges of the patch were rather irregular and the patch faded in 1–1½ hr. This confirms similar experiments by Gray (1956) on tail-band sections in *Phoxinus* and supports Parker's second-cut results on *Fundulus*. The responses of further minnows with a similar section of the sympathetic chain, and of others which had been previously subjected to anterior spinal section, to electrical stimulation of the superficial ophthalmic nerve were completely normal. The distribution, speed and intensity of the responses in these preparations and the threshold curves obtained were all indistinguishable from those of unoperated fish. That the peripheral motor *W* fibres were still intact at this level, 11–13 days after the initial operation, indicates that there is at least one synaptic discontinuity between the points of section and stimulation. The effector cells were not, therefore, truly denervated but only paralysed.

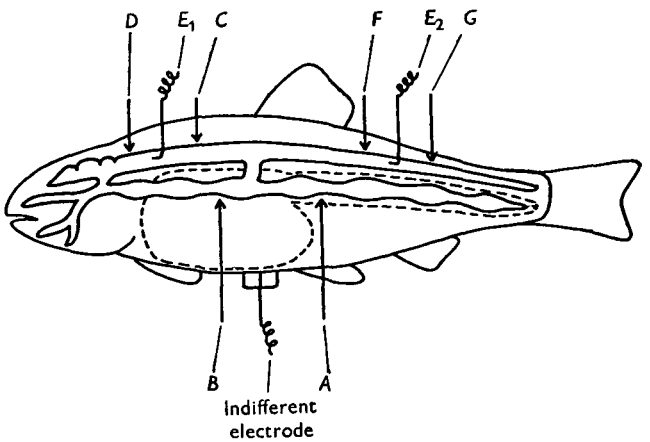

Fig. 4. Diagram of electrical stimulation and nerve-section experiments performed on *Phoxinus*. For explanation of symbols see text.

Central stimulation

Several normal fish were prepared as for spinal section at an anterior level (Healey, 1948). A short length of the spinal cord was exposed and a stimulating electrode was inserted into it as at E_1 in Fig. 4. Stimulation at this site by short, square, negative pulses of 2·0–2·5 V. produced complete melanophore aggregation over the whole body surface. When the active electrode was removed from the spinal cord and placed on muscle tissues at the side of the wound, the melanophores slowly recovered to their resting state, but on replacement of the electrode in nervous tissue the responses

returned. Positive pulses were ineffective at similar voltage levels but bipolar stimulation by two fine electrodes placed close together in the spinal cord was completely effective.

The speed and intensity of melanophore response was markedly dependent on the pulse repetition rate. Above 50 pulses/sec. the response was complete and rapid and showed no variation with pulse frequency, at 10/sec. it was slower but still complete, at 1/sec. the response was sluggish (taking perhaps a minute to completion) and incomplete, while a rate of 1/2 sec. or less seldom produced any visible response. Switching without interruption from 10/sec. to 1/sec. or less was followed by partial or complete recovery to the resting state.

A fine knife was introduced through the body wall to sever the haemal canal (containing the aorta and sympathetic chain) at the level *A* in Fig. 4. No further responses could be evoked posterior to this point and the melanophores dispersed as a result of the nerve-section. Responses anterior to the level of the cut remained normal. A second cut was then made through the sympathetic chain and aorta within the body cavity anterior to the 15th vertebra (*B* in Fig. 4). Again no response was evident posterior to the new cut. Anterior to the second cut all the melanophores dispersed, as is normal after section of von Frisch's tracts, but now *only these* melanophores responded to electrical stimulation of the anterior spinal cord and their aggregation was complete. The melanophores in the middle region of the fish showed no further responses, although their *W* fibre tracts were intact, as indicated by their lack of pronounced dispersion.

Discontinuity of activity at the site of the cuts was always abrupt, producing a well-defined border round the body; aggregation beyond this could only be produced by much higher voltages which gave violent muscle contractions over the whole body. This result suggests that the chromatic fibres of von Frisch's pathways were not being stimulated. In fact the aggregation response showed exactly the same properties as the dispersion obtained by von Gelei. The experiment was performed in every way as von Gelei had described except that no ergot was used. Von Gelei's stimulator was an induction coil with a self-interrupting hammer, suggesting a high stimulus rate, probably 30–50/sec. Such a coil was used for some of the present preparations and at suitable intensity gave results which were indistinguishable from those obtained with a similar repetition rate of square pulses.

In order to locate the pathways involved in these responses, the following experiments were performed, each on three normal fish:

(i) Spinal section was performed at *C* in Fig. 4, followed by 7 days on a white background for recovery and paling of the skin. This directly interrupts von Frisch's pathways. The gap was then carefully dried and filled with paraffin before stimulation at E_1.

(ii) Double spinal sections were made at *C* and *D* to leave a small 'island' of spinal cord about 4–5 vertebrae long into which the electrode was later placed with the same precautions as above (i).

In both these situations the electrical responses were unaffected and were limited by cuts first at *A* and then at *B* as in the intact fish.

(iii) An electrode was placed posterior to vertebra 15 at E_2 (Fig. 4) in a region of the spinal cord generally agreed to have no chromatic function. Paling responses occurred

as before but were limited in their anterior spread by cuts at *A* and *B*; for this reason cut *B* was made first, then *A*. Stimulation was equally effective over a wide range of levels posterior to vertebra 15.

(iv) Spinal section at *F* was followed 7 days later by stimulation at E_2.

(v) Double spinal section at *F* and *G* was followed by stimulation at E_2.

In both these cases the results were exactly the same as in Expt. (iii).

(vi) It is possible that the melanophore-exciting influence was blood-borne since such crude sections of the sympathetic chain always involved section of the aorta. In order to test this, the heart was removed from at least one fish in each of the above experiments. In every case the usual responses were obtained, even after the cessation of respiratory movements.

In all these experiments the response was dependent on the rate of stimulation; it could not be evoked if the electrode were removed to nearby muscle tissue; it was always complete and bore no relation to the responses of the melanophores to section of their normal (von Frisch pathway) motor supply. All fish were carefully examined after death to ensure that spinal sections were complete and to determine their precise positions.

Stimulation after ergotamine treatment

Wyman (1924) and Smith (1931*a*) both reported initial paling followed by an intermediate state in ergotized *Fundulus*. Giersberg and von Gelei stated that intramuscular injection of 0·08–0·15 ml. of Sandoz 'Gynergen' (0·5 mg. of ergotamine tartrate in 1 ml. of placebo) into *Phoxinus* produced an initial darkening for about an hour and then paling regardless of the background colour. Electrical stimulation experiments were performed in the pale phase. For the present experiments Sandoz 'Femergin' was used, but this was stated by the manufacturers to be identical in constitution to Gynergen. However, injection of 0·1 ml. of this product (50 μg. of the salt—a dose of 14 mg./kg.) into London minnows produced a very pale but not completely blanched colour in 5–15 min. regardless of background colour. In a white dish the fish darkened slightly after injection. This was observed in a total of 109 fish, none of which showed an initial darkening phase. The effect of the drug lasted for several hours. No difference could be detected between the effects of intraperitoneal and intramuscular injection; the former was adopted as more convenient and offering less chance of leakage for the relatively large volume of fluid.

One hour after intraperitoneal injection with 0·1 ml. of Femergin, a fish was prepared for stimulation of the superficial ophthalmic nerve. This produced local melanophore dispersion within the area shown in Fig. 2, with no response elsewhere. Responses were reversible, almost complete, independent of pulse repetition rate over a wide range and were evoked at similar voltages to those required to produce aggregation in normal fish which had not been treated with ergot. Threshold was rather variable and the responses slower, taking 1–2 min. to become complete. It was not found necessary to inject acetylcholine in order to evoke this dispersion response.

Stimulation of the anterior end of the spinal cord produced melanophore dispersion over the whole body with the same characteristics as for aggregation in the normal fish; that is the response was dependent on the frequency of stimulation, was restricted by section of the sympathetic chain and aorta, first at *A* and then at *B* (Fig. 4), and was

independent of the blood circulation. No responses were obtained if the electrode was removed from the spinal cord to nearby muscle tissue.

Experiments (i) to (vi) described above were repeated, each on three ergot-treated fish, and in every case the pattern and properties of the melanophore dispersion were the same as those of aggregation in the similarly operated but non-ergotized fish. The same precautions and control procedures were performed as before.

DISCUSSION

The results of von Gelei's experiments are confirmed except that there was no initial darkening on injection of ergot and that acetylcholine was not found to be necessary to invoke the dispersive response. But that author's conclusions regarding the pathways of the dispersing fibres are inconsistent with the finding that a similar response can be evoked from an electrode placed in any level, or even in a small 'isolated' section, of the spinal cord. Moreover, paling may be elicited in exactly the same way if ergot is not present and this is inconsistent with the *W* fibre pathways which von Frisch and others have established by alternative means. New *W* fibre pathways capable of supporting all these results are unlikely to exist and so it must be assumed that the known chromatic motor fibres are being stimulated indirectly in these cases of electrically excited aggregation.

It is suggested that an electrode within the spinal cord might raise the general level of excitation in adjacent sympathetic chain ganglia through any or all of the efferent fibres in the connecting rami. In accord with the generally non-specific nature of sympathetic activity, the excitation might then spread both anteriorly and posteriorly within the sympathetic chain to influence chromatic motor fibres emerging at all levels. This hypothesis is supported by the ill-defined voltage threshold and the strict frequency-dependence of these preparations. These properties are not shared by the peripheral motor fibres in the superficial ophthalmic preparation. Possibly a high rate of stimulation causes a general accumulation of excitatory transmitter substances within the sympathetic ganglia, whereas a low rate results only in more specific synaptic transmission.

According to Nicol (1952) there is some evidence that single chromatic fibres run throughout the length of the sympathetic chain, with collateral branches within each ganglion to the final motor fibres. Such an arrangement could conceivably transmit impulses both orthodromically and antidromically from a site of excitation at any level. If so, one would expect sympathetic effectors other than the melanophores to be affected. No changes could be detected in the heart rate or the iris, but Nicol (1952) states that cardiac accelerator fibres are absent from teleosts, and Walls (1942) that pupillary adaptation does not occur in the Cyprinidae. No other simple observations could be devised.

Whether this hypothesis is correct or not, it does not seem that these experimental results need prejudice von Frisch's conclusions about the paths of *W* fibres. The only possible conclusion is that it is not possible to map the route of chromatic motor fibres in this way and the arguments for von Gelei's *B* fibre pathways must therefore be discarded. The work of Healey and Gray has made it seem likely that, if *B* fibres do exist, they follow the same pathways as the *W* fibres (Fig. 1A).

Whether or not the experiments with ergotamine really do demonstrate the presence of *B* fibres will be examined in a further paper.

SUMMARY

1. Electrical threshold curves have been obtained for the stimulation by square and saw-tooth pulses of peripheral melanophore-aggregating nerve fibres in the minnow, *Phoxinus*. The responses show complete independence of pulse repetition rate over a wide range.

2. The results of von Gelei on the electrical stimulation of the spinal cord in ergotamine-treated minnows have been confirmed but it is found that the melanophore-dispersing activity may pass forwards or backwards from a stimulating electrode at any level of the spinal cord.

3. Identical results have been obtained for melanophore-aggregating activity in non-ergotized fish. All the spinal responses are slow and incomplete at repetition rates below 10/sec.

4. Von Gelei's arguments for mapping the pathways of melanophore-dispersing nerve fibres are shown to be insupportable.

This work formed part of a Ph.D. thesis in the University of London. The author wishes to thank Prof. N. Millott for laboratory facilities, Dr E. G. Healey for helpful supervision and the Medical Research Council for generous financial support during tenure of one of their studentships.

REFERENCES

ERASMUS, D. A. (1962). Distribution of certain strigeid trematodes in Great Britain. *Nature, Lond.*, **195**, 828–9.

FRISCH, K. VON (1911). Beiträge zur Physiologie der Pigmentzellen in der Fischhaut. *Pflüg. Arch. Physiol.* **138**, 319–87.

GELEI, G. VON (1942). Zur Frage der Doppelinnervation der Chromatophoren. *Z. vergl. Physiol.* **29**, 532–40.

GIERSBERG, H. (1930). Der Farbenwechsel der Fische. *Z. vergl. Physiol.* **13**, 258–79.

GRAY, E. G. (1955). An asymmetrical response of teleost melanophores. *Nature, Lond.*, **175**, 642.

GRAY, E. G. (1956). Control of the melanophores of the minnow (*Phoxinus phoxinus* (L.)). *J. Exp. Biol.* **33**, 448–59.

HEALEY, E. G. (1948). The colour change of the minnow (*Phoxinus laevis* Ag.). *Bull. Anim. Behav.* **6**, 5–15.

HEALEY, E. G. (1951). The colour change of the minnow (*Phoxinus laevis* Ag.). I. Effects of spinal section between vertebrae 5 and 12 on the responses of the melanophores. *J. Exp. Biol.* **28**, 297–319.

HEALEY, E. G. (1954). The colour change of the minnow (*Phoxinus laevis* Ag.). II. Effects of spinal section between vertebrae 1 and 15 and of anterior autonomic section on the responses of the melanophores. *J. Exp. Biol.* **31**, 473–90.

NICOL, J. A. C. (1952). Autonomic nervous systems in lower chordates. *Biol. Rev.* **27**, 1–49.

PARKER, G. H. (1934*a*). The prolonged activity of momentarily stimulated nerves. *Proc. Nat. Acad. Sci. Wash.* **20**, 306–10.

PARKER, G. H. (1934*b*). Color changes in the catfish *Ameiurus* in relation to neurohumors. *J. Exp. Zool.* **69**, 199–234.

PARKER, G. H. (1936). Are there antidromic responses in the melanophore system? *Anat. Rec.* **67** (suppl. 1), 37.

PARKER, G. H. (1937). Do melanophores show antidromic responses? *J. gen. Physiol.* **20**, 851–8.

PARKER, G. H. (1948). *Animal Colour Changes and their Neurohumors.* Cambridge University Press.

PARKER, G. H. & ROSENBLUETH, A. (1941). The electrical stimulation of the concentrating (adrenergic) and the dispersing (cholinergic) nerve-fibres of the melanophores in the catfish. *Proc. Nat. Acad. Sci. Wash.* **27**, 198–204.

PYE, J. D. (1961). An investigation of the effects of temperature on the melanophores of some teleost fishes with special reference to chromatic nervous control in *Phoxinus phoxinus* (L.). Ph.D. Thesis, London University.

SMITH, D. C. (1931*a*). The action of certain autonomic drugs upon the pigmentary responses of *Fundulus*. *J. Exp. Zool.* **58**, 423–53.

SMITH, D. C. (1931*b*). The influence of humoral factors upon the melanophores of fishes, especially *Phoxinus*. *Z. vergl. Physiol.* **15**, 613–36.

WALLS, G. L. (1942). *The Vertebrate Eye and its Adaptive Radiation*. Cranbrook Institute of Science, Bull. no. 19.

WYMAN, L. C. (1924). Blood and nerve as controlling agents in the movements of melanophores. *J. Exp. Zool.* **39**, 73–132.