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# NERVOUS CONTROL OF CHROMATOPHORES IN TELEOST FISHES

## II. THE INFLUENCE OF CERTAIN DRUGS IN THE MINNOW (*PHOXINUS PHOXINUS* (L.))

BY J. D. PYE*

*Department of Zoology, Bedford College, University of London*

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### INTRODUCTION

Electrical stimulation of the central nervous system of teleosts gives paling of the skin by aggregation of pigment within the chromatophores. There is now considerable evidence (reviewed by Parker, 1948) that the fibres which mediate this response are adrenergic, or at least sympathomimetic in type.

Administration of ergotamine to the minnow reverses the chromatic response, so that electrical stimulation causes pigment dispersal, thus darkening the skin (Giersberg, 1930; von Gelei, 1942; Pye, 1964*a*). Giersberg argued that this phenomenon represents a suppression of the melanophore-aggregating system (the paling or *W* fibres) and so demonstrates the action of an opposed cholinergic melanophore-dispersing system (darkening or *B* fibres). The evidence of von Gelei for the pathways of these fibres has been invalidated by further experiments (Pye, 1964*a*), which have also eliminated the possibility of humoral influences, for instance by the stimulation of adrenal tissue. But it is still not certain whether ergot really does demonstrate the existence of a double opposed innervation; Gray (1955) has suggested that ergot may in fact reverse the influence of a single adrenergic motor system.

There is some precedent for this suggestion. Goodman & Gilman (1956) have reviewed the action of ergotamine and concluded that in mammals sympathomimetic responses may not only be suppressed but are often reversed by this drug, although the effect is generally greater for blood-borne adrenalin than for the noradrenalin released as a transmitter at adrenergic nerve endings. Barbour & Spaeth (1917), Spaeth & Barbour (1917) reported that the melanophores of scales removed from the killifish (*Fundulus heteroclitus* (L.)) showed distinct pigment aggregation on immersion in concentrations of adrenalin as low as $2 \times 10^{-8}$. Following exposure to ergotamine, the response to adrenalin was reversed to a pigment dispersion. This has been confirmed for several other genera by further workers (Gray, 1955). However, Wyman (1924) was unable to support reversal by the injection of drugs into intact *Fundulus*, and Giersberg (1930) remarked that injection of adrenalin into ergot-treated minnows resulted in normal aggregation.

In view of this confusing situation, the problem has been re-examined in *Phoxinus*. Investigations were made of the effects of injecting adrenalin or noradrenalin, and of

* Present address: Department of Zoology, King's College, Strand, London, W.C. 2.

the immersion of isolated skin fragments in solutions of these drugs, all with and without prior treatment with ergot. Some simple experiments on another adrenergic depressant (Rogetine) and a cholinergic depressant (atropine) are also reported. No individual fish was subjected to more than one experiment. Freshwater teleost Ringer was made according to the formula given by Young (1933).

*Injection experiments*

Minnows weighing about 3·5 g. and swimming on either black or white backgrounds when injected intraperitoneally with 0·1 ml. of Sandoz Femergin (containing 0·05 mg. of ergotamine tartrate in placebo—a dose of 14 mg./kg.) assumed within $\frac{1}{4}$ hr. a pale colour, slightly darker than that of normal fish on a white background. Normal colouring was not regained for 24–48 hr. but, except for a tendency to be hyperexcitable, the fish otherwise behaved in an apparently normal fashion. This dose was the same as that used in obtaining reversal of electrical responses (Pye, 1964*a*) and was made the standard treatment in the present experiments.

Twelve fish were placed in dishes on a black background. Eight were given a dose of ergot and after an hour were the expected pale colour. Then four of these and the four untreated fish were each injected with 0·1 ml. of a $2 \times 10^{-4}$ solution of Adrenalina B.P. in Ringer (6 mg./kg.). The four fish which received only adrenalin paled completely for an hour and recovered completely during a further hour. No difference could be detected at any time between those which received ergot only and those with both ergot and adrenalin; the latter group showed no further paling and no darkening until all eight recovered from the ergot after 24–48 hr.

The same experiment was repeated using 0·1 ml. of a saturated solution of adrenalin in Ringer. Adrenalin-only fish became fully pale in 15 min. but recovered within $2\frac{1}{2}$ hr. The ergotized fish also became fully pale when given this large dose of adrenalin, but after $2\frac{1}{2}$ hr. had recovered the normal colour of ergot-treated fish. The response in these cases was slight since the fish were already paled by ergot, but no sign of any dispersive response was detected at any time.

The experiment was repeated again on twelve further fish, using doses of 0·1 ml. of a solution containing $2 \times 10^{-4}$ parts of L-noradrenalin in Ringer. This alone produced complete paling with recovery in 3 hr.; noradrenalin following ergot produced no further response and the fish were indistinguishable from those which had received ergot only.

Thus the standard ergot treatment, as used for nerve stimulation experiments, appeared to suppress the action of both adrenalin and L-noradrenalin injected in mild doses, although a strong dose of adrenalin still induced melanophore aggregation. No reversal of this aggregation response, i.e. no melanophore dispersion, was evoked at any concentration.

*Experiments on isolated skin*

Barbour & Spaeth immersed melanophore-bearing scales of *Fundulus* in solutions of Ringer containing $3 \times 10^{-4}$ parts of ergotamine tartrate or $10^{-4}$ parts of adrenalin. These concentrations were both much higher than those experienced by the melanophores of intact fish during the foregoing injection experiments.

When scales of *Phoxinus* are removed with forceps they seldom carry any melanophores with them, so that it was not possible to reproduce precisely the technique of

Barbour & Spaeth. However, it was found that if strips of skin were carefully cut from the flanks of anaesthetized minnows the melanophores remained active for some hours in dishes of Ringer solution, and their responses to various stimuli could be observed with a low-power microscope. It was necessary to include a certain amount of subcutaneous tissue with the skin if the melanophores were not to be damaged by the dissection. Allowance must therefore be made for the slower rate of diffusion of drugs from the solution to the effector cells than in the isolated scale preparations. Also, presumably because leaching was ineffective in Ringer, the responses to drugs were usually irreversible and each experiment, unlike those of Barbour & Spaeth, could be performed only once on each specimen.

Several pieces of skin were each divided by sharp scissors into four pieces. Two were transferred to a $10^{-4}$ solution of Adrenalina B.P. in Ringer and all the melanophores showed complete aggregation in 7–10 min. The other two were left in Ringer as controls and showed no aggregation for several hours. In other experiments half the pieces were placed in Femergin ($5 \times 10^{-4}$ ergotamine tartrate in placebo); all melanophores aggregated completely in 30 min., while the controls in Ringer showed no change. Then all were quickly washed in clean Ringer and transferred to $10^{-4}$ adrenalin in Ringer. The ergot-treated pieces showed no further responses while the controls aggregated completely in 5 min.

Finally as a control on the possible effects of the ergot placebo (sodium chloride and tartaric acid), a solution of $5 \times 10^{-4}$ crystalline ergotamine tartrate in Ringer was prepared. This produced a light flocculent precipitate which dissolved on the addition of a little tartaric acid. A similar amount of tartaric acid was added to the control Ringer solution. In ergot, all the melanophores aggregated as before, while in Ringer + tartaric acid, a wide range of melanophore conditions was produced. In $10^{-4}$ adrenalin, the ergot-treated specimens showed no further response but the controls aggregated rapidly and completely.

All these experiments were repeated using L-noradrenalin instead of adrenalin, but the results were identical. Again there was no indication that ergot caused a reversal of the normal pigment-aggregating response.

*Injection followed by isolation of skin*

It is possible that the immersion of skin fragments in ergot solution was not equivalent in its influence to the injection of this solution into intact fish in the nerve-stimulation experiments. Combined experiments were therefore performed. The normal dose of ergotamine (0·05 mg.) was injected intraperitoneally. After 1 hr., when the fish were characteristically pale on a black background, they were anaesthetized and pieces of skin were removed. Control fish were injected with a similar volume of Ringer 1 hr. before being anaesthetized. When skin fragments from each were immersed in $10^{-4}$ adrenalin in Ringer complete and rapid aggregation occurred in both cases. Similar results were obtained when L-noradrenalin was used instead of adrenalin.

These fish had been treated initially in exactly the same way as those which showed reversal of response to electrical stimulation of the nervous system, but in every case the melanophores removed to adrenalin or noradrenalin solutions showed slight further (maximum possible) pigment aggregation.

*Some experiments with Rogetine*

Rogetine (C-7337 marketed by CIBA Ltd) was selected as an alternative adrenergic depressing agent. Trapold, Warren & Woodbury (1950) stated that this drug has a highly specific sympatholytic action (in mammals), but in certain cases can reverse responses to sympathomimetic drugs. In the absence of previous records of the effects of Rogetine on colour change in teleosts a fairly extensive series of tests was performed (Pye, 1961). The dose finally adopted as standard consisted of 0·125 mg. in 0·1 ml. of Ringer (36 mg./kg.). This induced maximal darkening within 15–30 min., regardless of background colour, with recovery over 12 hr. Pale Rogetine-treated fish were obtained by dissolving the drug in a crude extract of one minnow pituitary gland, containing melanophore-aggregating hormone, or by leaving the fish on a white background for some hours to allow partial recovery. No responses could be evoked by electrical stimulation of the superficial ophthalmic nerve in either of these conditions although 24 hr. after injection such responses were again normal. Thus Rogetine appears to suppress all activity in chromatic nerve tracts while leaving the melanophores free to respond to the humoral influence of large doses of pituitary extracts.

The influence of Rogetine on responses to injections of adrenalin was tested as follows. Fish were injected with Rogetine on a white background and when fully darkened were transferred to a black background. Control fish received injections of the same volume of Ringer. Thirty minutes after the first injection all fish were given 0·1 ml. of $2 \times 10^{-4}$ adrenalin in Ringer; all paled quickly and completely and, even during recovery, both groups of fish were indistinguishable in colour. There is no evidence from these tests that Rogetine influences in any way the normal action of adrenalin on melanophores.

*Some experiments with atropine*

This drug, stated by Goodman & Gilman (1956) to be a specific blocking agent for post-ganglionic cholinergic fibres (in mammals), has been employed fairly extensively in investigations of colour change in fishes. Barbour & Spaeth (1917) found that immersion in solutions containing $10^{-3}$ to $10^{-5}$ parts of atropine sulphate produced pigment dispersion in the melanophores of isolated scales from *Fundulus*. Wyman (1924) obtained dispersion by injecting intact *Fundulus* with 2·5 mg. of atropine sulphate. Smith (1931*a*) injected *Fundulus* with 0·039–0·55 mg. and reported no change on a black background but an intermediate colour for all doses on a white background. This was the exact opposite to the responses found by the same author to injections of ergot. Larger doses induced complete darkening regardless of background but were lethal. Smith (1931*b*) reported similar results for *Phoxinus*.

In the present experiments 0·5 mg. of atropine sulphate (143 mg./kg.) was found to be lethal, but 0·1 mg. (28 mg./kg.) produced intense dispersion in 15 min., fading over about 7 hr. No obvious ill-effects were noted at this dosage. As with Rogetine, pale fish could be obtained by the addition of a crude extract of one minnow pituitary gland to the injection. These results do not confirm those of Smith for this drug.

Electrical stimulation of the superficial opthalmic nerve in atrophine-treated fish produced normal melanophore aggregation on the head and snout. Responses were a little slower than in untreated fiish, but nerve threshold voltages were unchanged

On cessation of stimulation, the melanophores rapidly recovered to the fully dispersed state. Slowness of response did not therefore seem to be caused by decreased excitability of the nerve fibres but by some antagonism to their peripheral influence.

### *Responses to local temperatuse changes*

Smith (1931*a*) reported that the administration of ergotamine reversed the local temperature responses of otherwise intact *Fundulus*, whereas atropine in sublethal doses did not have this effect. He argued that the temperature-sensitive chromatic reflex, whose presence he proposed, must therefore be mediated through adrenergic fibres. These would be suppressed by ergot, leaving the melanophores free to act as independent effectors. The properties and significance of normal temperature responses will be discussed in more detail in a later paper (Pye, 1964*b*).

Minnows injected intraperitoneally with 0·1 ml. of Sandoz Femergin were tested under urethane anaesthesia. The application of fragments of melting ice to the skin produced considerable, but not quite complete, local melanophore dispersion. When regions of the skin were warmed to 35° C., two fish out of four showed distinct further paling, while a third was already so pale that further aggregation might not have been seen. The fourth showed no detectable response. All responses were reversible and repeatable. These effects are the opposite of those shown by normal fish and therefore support the observations of Smith.

The effect of Rogetine was also investigated at the standard dose of 0·125 mg. Under anaesthetic no responses could be elicited either by high or by low temperatures. Further fish were then injected with a dose of Rogetine together with the extract of one minnow pituitary gland. Four such pale fish were anaesthetized. Marked but incomplete melanophore dispersion was then produced both by warming and by cooling regions of the body surface. In one fish respiratory movements ceased during exposure to 35° C.; the temperature was then lowered to 20° C. but no recovery of melanophore condition occurred until breathing was restarted. The significance of this will be made clear in a later paper (Pye, 1964*b*).

Finally a dose of 0·1 mg. of atropine sulphate was tested but no thermal responses could be evoked in this state. The melanophores were again biased by the addition of pituitary extract to produce a pale atropinized fish. Now both warming and cooling produced complete and reversible melanophore dispersion. In one case cessation of respiratory movements suspended the responses for a time but resuscitation restored them. These results do not support Smith's findings in atropinized *Fundulus*. The close similarity to the effect of Rogetine is remarkable since these two drugs are supposed to produce highly specific but directly opposed effects in mammals.

## DISCUSSION

Administration of ergotamine to minnows reverses the chromatic response to electrical stimulation of peripheral motor nerves; the present experiments give no indication that ergotamine also reverses the chromatic responses to adrenalin or noradrenalin. Although responses to these agents may be suppressed, sufficiently large doses still produce an aggregation of melanophore pigment. This might be taken as support for the presence of a double innervation, involving melanophore-dispersing

fibres (presumably cholinergic) as proposed by Giersberg. However, the results obtained with Rogetine are not consistent with this argument.

Rogetine is stated to be a more specific sympatholytic agent than ergot (in mammals) yet it abolishes all chromatic responses to electrical stimulation of motor nerves, even in the artificially paled fish. This suggests that there is but a single, adrenergic innervation. Similarity of the responses to injections of Rogetine and atropine, and differences from the responses to ergot, may possibly be explained by assuming that both are direct responses of the melanophores acting as independent effectors. But this argument cannot be extended to the thermal responses.

It is also possible that the chromatic motor fibres of fishes, although 'adrenergic' in type, produce at the effector ending neither adrenalin nor noradrenalin, but some other sympathomimetic transmitter substance. This might have chemical affinities to the more common agents but be more susceptible to reversal of its effect by ergot.

It may also be mentioned here that ergotamine treatment suppresses the melanophore dispersion normally produced by section of the peripheral chromatic nerves, at least for the superficial ophthalmic nerve and for tail-band sections. This cannot be reconciled simultaneously with the ideas of Parker (that cutting stimulates *B* fibres) and of Giersberg (that ergot only eliminates the *W* fibres).

The critical experiment would be to perform nerve-section in an ergotized fish which had been darkened by some other agency, to see if the effect of nerve-section is actually reversed by ergot, as is that of nerve stimulation. If this were so, both Parker and Giersberg would have to be refuted. Unfortunately no suitable 'sympathoneutral' darkening agent was available at the time of the above experiments and possible further aggregation of small areas in the already pale ergotized fish could not be gauged with absolute certainty.

The only possible conclusion from these experiments supports that of Nicol (1952) who stated that the use of stimulating and depressing drugs on the autonomic nervous system of teleost fishes produces largely inconsistent results. It seems that deductions from the use of such drugs by analogy with their known effects in mammals are 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.

## SUMMARY

1. Responses of melanophores to injections of adrenalin or L-noradrenalin have been examined with and without prior injection of ergotamine.

2. Responses of the melanophores of isolated skin preparations in solutions containing adrenalin or L-noradrenalin have been examined with and without prior injections of, or direct exposure to, solutions of ergotamine.

3. No reversal of response could be found in any of the above cases.

4. Responses of melanophores following injections of Rogetine (an alternative adrenergic blocking agent) are quite unlike those after ergot but closely resemble those after atropine.

5. It is concluded that it is unjustified to assume that drugs produce similar effects in fishes and in mammals. The case for double innervation of melanophores is therefore still unproven.

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