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Mechanism of Ionic Action in the Melanophore System of Fish I. Melanophore-concentrating Action of Potassium and Some Other Ions

With 10 Text-figures

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(*Communicated by* H. KINOSITA)

That potassium and other alkaline ions cause concentration of granules within the fish melanophores was first observed by Spaeth (1913) on the melanophores of the scale isolated from the killifish, *Fundulus heteroclitus*. In his subsequent papers (Spaeth 1916, 1917) he reported that alkaline earths were also found to have an analogous effect on the same kind of melanophores.

Since then, several investigations have been made on the action of ions on the chromatophores of various species of fishes (Lowe 1917, Wyman 1924a, b, Meyer 1931, Matthews 1931, Smith and Smith 1935, Kamada and Kinosita 1944, Miyoshi 1952). Moreover, making use of these responses, attempts have been made to study the mechanism of pigment migration within the pigment cells (Spaeth 1916, Spaeth and Barbour 1917, Matthews *op. cit.*, Marsland 1944, Kinosita 1953).

However, considerably little information is available regarding the mode of actions of the stimulating ions on the motor mechanism of the melanophore system. The first interpretation on this mechanism was given also by Spaeth (1913). He concluded from the observations on the melanophores of isolated scale, that they respond to ions directly without any possible intervention of the nervous action. Ever since, none of the alternative theories has been postulated against his view (Spaeth and Barbour *op. cit.*, Wyman 1924a, Marsland *op. cit.*, Nagahama 1953, Ueda 1955).

In the present investigation, an attempt was made to elucidate the effect of ionic stimulation by observing the responses of both innervated and denervated melanophores within the caudal fin of a fish.

MATERIAL AND METHODS

Material used was goby, *Chasmichthys gulosus*, collected from the tide pools near the Misaki Marine Biological Station. Experiments were carried out exclusively on the melanophores in the skin of the caudal fin, and not on those in the isolated scale.

The physiological solution, in which the melanophores keep their dispersed state, was a mixture of 0.25 M NaCl, 0.25 M KCl, 0.17 M $CaCl_2$, and 0.17 M $MgCl_2$ in a volume ratio of 100 : 3.5 : 1.5 : 2.4, pH being adjusted to 7.2 by $NaHCO_3$ (Yamamoto 1949). The chemical stimulation to induce the concentration of melanin granules was given by applying externally the salt solutions prepared to be isotonic with the physiological saline (0.25 M NaCl eq.). In some experiments 10^{-5}—10^{-6} M solution of adrenaline (epinephrine) made up with the physiological solution was used to induce maximal response. pH of these stimulant solutions were adjusted to be 7.2 mainly by $NaHCO_3$, although $KHCO_3$ was used in the case of KCl solution.

So long as the solutions are applied from the surface of the skin, the presence of the epidermal layer through which the agents must migrate before acting upon the melanophore largely affects the melanophore response (Nagahama *op. cit.*). Therefore, it is more desirable to make them act more directly without intervention of the epidermal layer. For this purpose a special preparation which may be called "isolated split preparation" for convenience was made, and was used in some of the experiments (sections B, C and D in Experimental). Under the binocular dissecting microscope a long strip of fin including 2—4 rays was isolated from the caudal fin, and by means of fine needle and forceps, this piece of fin was carefully split into two symmetrical halves on either side of the median plane. This can be more or less easily performed, since each fin-ray consists of two lateral halves, and the connective tissue between both surfaces of the fin is sufficiently loose and soft to be torn off without any perceptible injuries on the derma in which melanophores lie. As there is no necessity for stimulant ions penetrating through the epidermal layer, the reaction time for the initiation of melanophore response is remarkably short* in contrast with the case of intact fin or even of the simply isolated piece of fin (Fujii 1958).

Attempting to record the response quantitatively, the photoelectric method (Hill, Parkinson and Solandt 1935, Smith 1936) was adopted in the experiments described in sections B and C. Light, transmitted through the circular area** on the interradial membrane of the isolated split preparation, was thrown on the photocell attached to the microscope. The photoelectric current was magnified by means of one step amplifier, and was led to a short period D'Arsonval galvanometer (period : 0.25 sec. ; sensitivity : 5.3×10^{-8} A). An ex-

* In the case of 10^{-5} M adrenaline stimulation, the mean reaction time (± standard deviation) for the concentration response was (14.8±3.7) seconds. (number of tests : 40, room temperature : 25—29°C)

** Usually 70—300 melanophores were found within the area, having a diameter of about 900μ.

amination of the relationship between luminosity and galvanometer deflection proved it to be almost linear throughout the range of the present determinations.

Experimental

A. *Preliminary experiments for obtaining the denervated melanophores.*

Using the caudal fin of goby, it was rather easy to obtain an area in which the melanophores were free from the chromatic nerve innervation (Wyman 1924a, Parker and Porter 1933, Abramowitz 1935). At first a transverse incision was made with a piece of safety-razor blade across two fin-rays together with their interradial membrane near the base of the fin. As is well known, the operation was followed by the appearance of a dark band extending from the cut to the posterior end of the fin. In the material used in the present study, the discernible duration of the band reached a week or more, which is considerably longer in contrast with that of *Fundulus* (Parker 1935). Disconnected from cell bodies, the chromatic nerves in this area must inevitably lose their function in due course of time. Time course of functional degeneration as well as that of regenerative growth could be examined by testing the responses of melanophores to electrical stimulation as is shown in the following lines.

After being kept in sea water for a given period of time, the fish was decapitated and was mounted in a vessel for microscopic observation of the caudal fin. Perfusion with the physiological solution brought about a well-marked dispersion of all the melanophores of the fin. If A.C. stimulation was applied through a pair of fine Ag–AgCl electrodes placed on the cut end of the spinal cord, innervated melanophores soon responded by concentration. Melanophores on the caudal band, on the other hand, did not show the concentration response so long as the process of nerve regeneration had not commenced. With the progress of nerve regeneration the band melanophores within the corresponding region recovered the ability to respond by concentration. Usually, the regeneration began about 2 weeks after the administration of the cut (20°C), although the time of onset was not so definite among individuals and was according to the temperature.

Functional degeneration of the concentrating nerve can also be submitted to the test easily by A.C. stimulation through the electrodes placed on the band. With stimulation strong enough to cause a rapid concentration of the melanin granules within the innervated melanophores, the nerve free cells did not show any signs of concentration response. If the response was also observed among the band melanophores, it was concluded that the nerves peripheral to the incision were still functional. The total disappearance of the nervous function usually took place 6—9 hours after the operation (26°C). Sustained stimulation on the cut end of the spinal cord or on the fin-rays in the neighbourhood of the denervated band brought about the gradual fading of the dark band from the margin. This may be attributable to the lateral invasion through the tissue of the concentrating neurohumor secreted from the nerve endings within the innervated area. On removal of the stimulation the pigments in

innervated cells promptly began to disperse, and soon regained the initial dispersed state. Meanwhile, the band melanophores pursued a process in reverse of that in the case of electrical stimulation; namely, the dispersion response could be seen at first on the melanophores along the border, and then, this darkened area spread towards the axis of the band. When the rapid dispersion response comparable with that of innervated cells was regained among the band melanophores, it was concluded that the regeneration of the dispersing nerves must have been completed. It was found that both kinds of chromatic nerves, concentrating and dispersing, began to regenerate approximately at the same time; the result is in agreement with that described by Abramowitz (*op. cit.*) on *Fundulus* and *Ameiurus*.

Before the time when the concentrating nerves lost their function (6—9 hours) and after 3—5 hours after the operation, the decapitation of fish was usually followed by the appearance of an enduringly blanched band showing a sharp contrast with the surrounding darkened area. The fact that the infliction of a second incision a little posterior to the previous one does not cause the dispersion response in the peripheral part reveals that the functional degeneration of dispersing nerves had already been completed. Thus, it can be concluded that the dispersing nerves lose their activity about 3—5 hours after the operation (26°).

From these preliminary experiments, and under apprehension of the possible effect of the chemical degradation accompanying the process of degeneration, the experiments on denervated melanophores have been carried out mostly on those fishes submitted to the operation 3—10 days previously.

B. *Responses of innervated melanophores.*

By making use of the isolated split preparation, it was possible to found out that, save the well-known K ions, Li^+, Rb^+, Cs^+, Sr^{++}, and Ba^{++}, among the alkaline and alkaline-earth ions tested, induced the concentration response of the melanophores of *Chasmichthys*. On the other hand, melanophores kept in the isotonic solution of NaCl, $CaCl_2$, or $MgCl_2$ maintained their dispersed state.

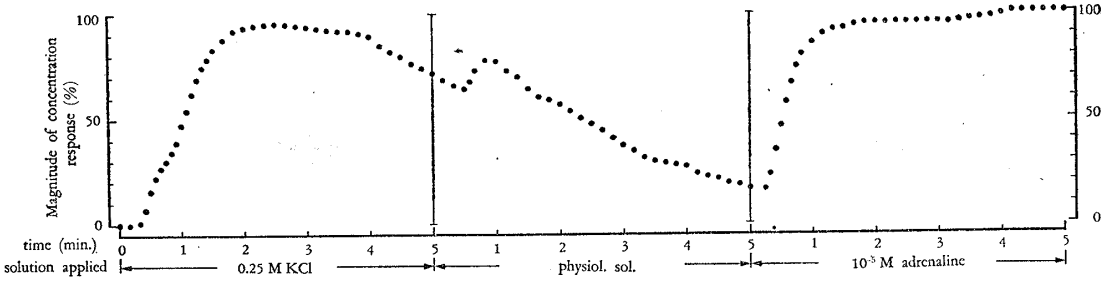


Fig. 1. Photoelectric record of the typical response of the melanophores of goby to K^+. Body length: 57 mm; room temp.: 28°C. Magnitude of concentration response is represented in percentage of the difference between galvanometer deflection corresponding to maximal dispersion and that in the case of maximal concentration. The same is also available for Figs. 2—4.

Figure 1 exhibits a typical example of the response to K^+ of the melanophores

within a split preparation. Reaction time, or the time required from the beginning of the application of the test solution to the initiation of the concentration response, was remarkably short, e.g., 20 sec. in the case shown in Figure 1. The figure also illustrates that the attainment of the maximal concentration was followed by the gradual dispersion of the melanophore pigments. Analogous phenomena of secondary dispersion were observable in the case of other stimulant ions. These facts, together with the temporary concentration observable at the beginning of dispersion as an effect of physiological solution, will be dealt with later.

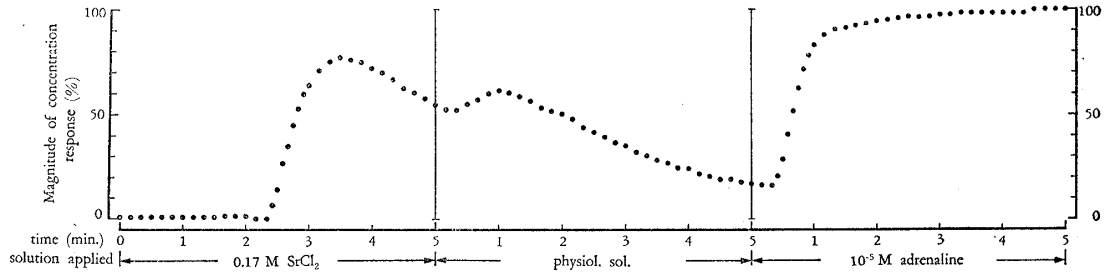


Fig. 2. Photoelectric record of the typical response of the melanophores of goby to Sr^{++}. Body length: 59 mm; room temp.: 28°C.

As a typical example of the responses of innervated melanophores to divalent cations the record of the concentration response induced by Sr^{++} is shown in Figure 2. Except for the more or less longer reaction time, the response was qualitatively analogous with that in the case of K ions.

The effect of other concentration-inducing ions was also similar to that of K^+ or Sr^{++} though the response to Li^+ accompanied a remarkably long reaction time and relatively low rate of concentration (Fig. 3).

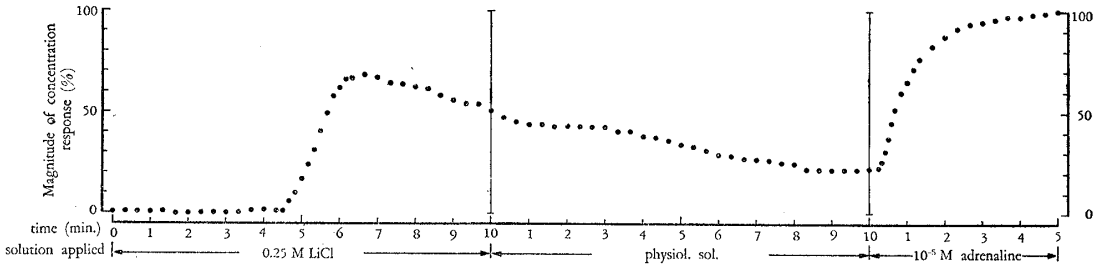


Fig. 3. Photoelectric record of the typical response of the melanophores of goby to Li^+. Body length: 55 mm; room temp.: 28°C.

The principal characteristics of the responses of innervated melanophores to the concentration-inducing ions tested are summarized in Table 1. The extent of response is represented by the maximal deflection of the galvanometer during the test in percentage of the galvanometer deflection attained after 5 minutes' standing in 10^{-5} M adrenaline solution.

If the reaction time is conveniently taken to represent the effectiveness of the stimulant ions to cause the melanophore response, it is possible, from the

data given in Table 1, to arrange these ions according to their effectiveness in the following order; K^+, Rb^+, Ba^{++}, Cs^+, Sr^{++}, Li^+. The reliable positions in the series of Ba^{++}, Cs^+ and Sr^{++} could not be determined because of the insignificance of difference.

Table 1

Numerical representation of the concentration response of the innervated melanophores of *Chasmichthys gulosus* induced by chloride salts of some alkalis and alkaline-earths

Fishes of 50—66 mm long were used. For details see text.

Solution applied	Reaction time (in sec.)	Extent of response (in percentage)	Number of determinations	Temperature (in °C)
0.25 M LiCl	268 ±56	80.3±15.5	10	26 —29
0.25 M KCl	22.5± 4.0	79.1±14.9	10	25 —28.5
0.25 M RbCl	29.8± 3.2	32.6±13.1	5	27 —28.5
0.25 M CsCl	94 ±27	63.2±19.6	10	26 —28.5
0.17 M $SrCl_2$	106 ±20	77.8±10.3	10	25 —28.5
0.17 M $BaCl_2$	90 ±19	89.6±10.0	10	26.5—29

C. *Responses of denervated melanophores.*

So as to avoid the possible action of the diffusible neurohumor, a special care has been taken, in the preparation of the denervated split piece, not to leave any innervated area connected with it. The responses of these denervated melanophores were recorded photoelectrically as was already mentioned.

In spite of the fact that the denervated melanophores show hypersensitivity to adrenaline (Smith 1941, Parker 1942, Fujii *op. cit.*), none of the alkaline and alkaline-earth chlorides examined could induce the concentration response (e.g. Fig. 4).

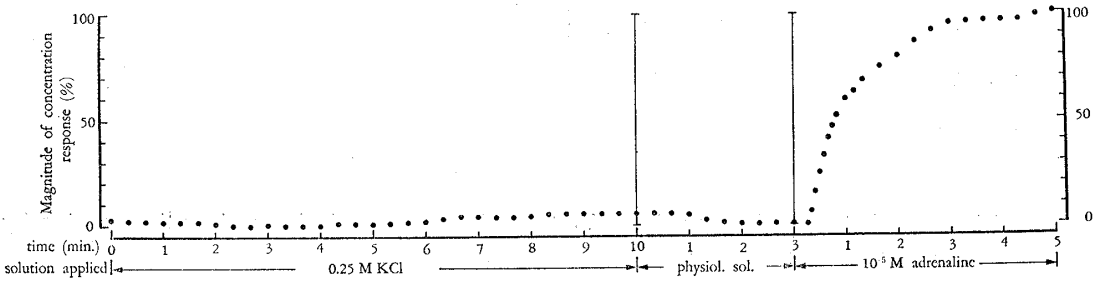


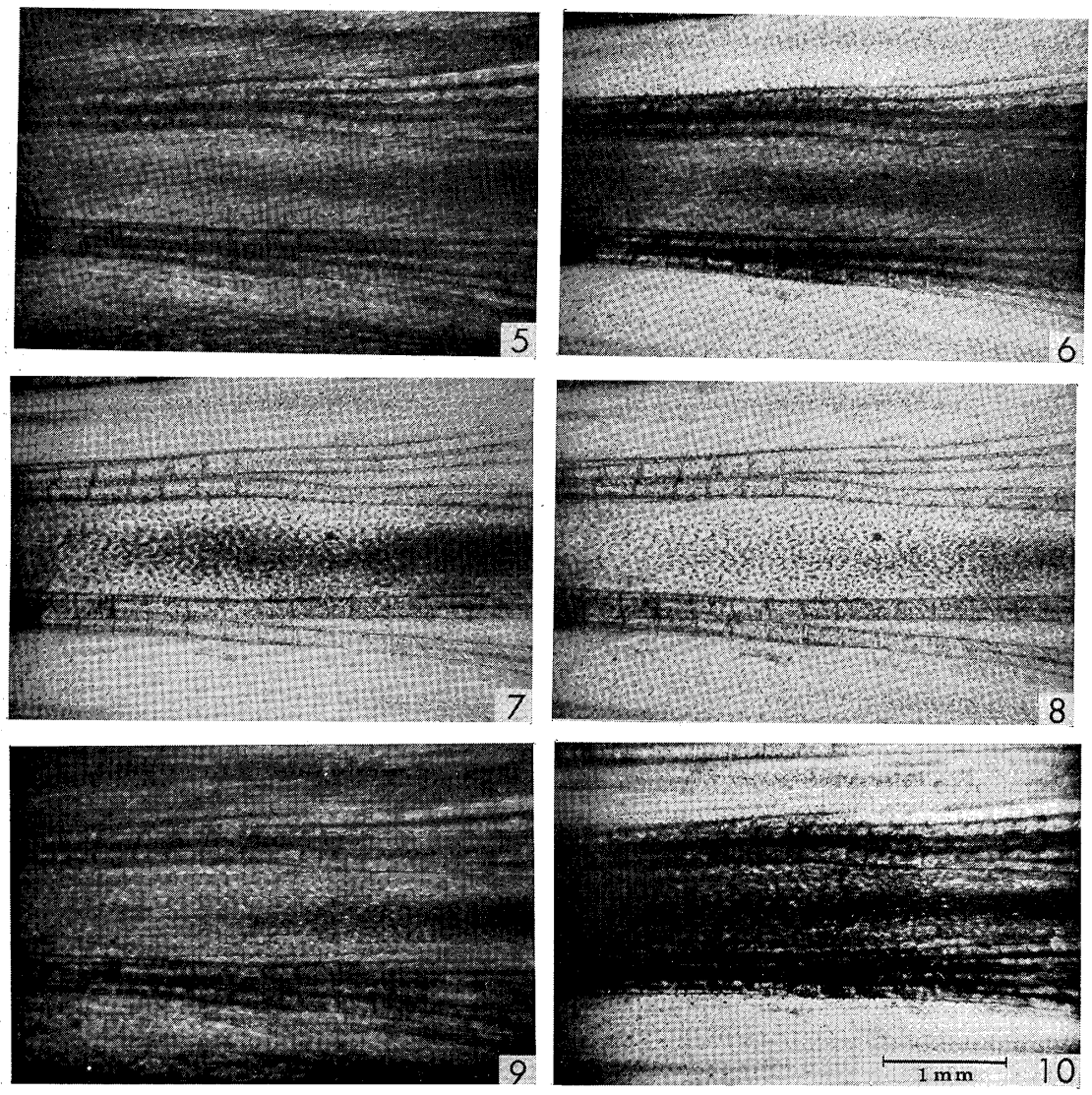
Fig. 4. Photoelectric record of the typical response of the denervated melanophores of goby to K^+. Body length: 57 mm; room temp.: 27°C.

The fact that these melanophores which were sensitive to adrenaline failed to respond to those ions seems to suggest that the seat of action of these two kinds of stimulants may be quite different, and that there might be some intervention of physiological mechanism in the process of the ionic stimulation of the melanophores. This concept is confirmed in the next section.

D. *Mode of action of concentration-inducing ions.*

Isolated pieces of caudal fin containing 4 fin-rays, having a denervated band on the middle of it, were mainly used. In some cases, split preparations from the denervated band and the adjacent innervated area were also employed. Such a preparation was mounted on a perfusable vessel constructed on microscope stage. After equilibration with physiological saline, the perfusate solution was changed to one of the stimulant solutions.

Responses to K^+ of the melanophores within a piece of caudal fin simply



Figs. 5—10. Responses to K^+ of the innervated and denervated melanophores in an isolated piece of caudal fin of goby. Body length: 52 mm; room temp.: 27.5°C. Fig. 5: Initial dispersed state in physiological solution. Figs. 6, 7 and 8: 7, 15 and 27 minutes after the application of isotonic KCl solution respectively. Fig. 9: Full recovery after 90 minutes' application of physiological solution. Fig. 10: Test by A.C. stimulation on the proximal part of the preparation ascertaining that the regeneration of the concentrating nerves had not yet commenced.

isolated from the tail are shown in Figures 5—10. The innervated melanophores on the interradial membrane shown on both upper and lower sides of these figures showed concentration response sooner or later. By this time, those within the denervated area failed to do so (Fig. 6), but in the course of time those on the margin of the band began to show concentration response. Thus, the blanched area gradually spread towards the axis of the band (Figs. 7 and 8). After sufficiently long equilibration with the physiological solution (Fig. 9), A.C. stimulation was given on the proximal part of the preparation through a pair of Ag-AgCl electrodes, in order to confirm that within the band, the regeneration of the concentrating nerves had not yet commenced. Figure 10 shows the resulting concentration of innervated cells together with the irresponsiveness of the denervated ones showing the latter cells to be free from nerve supply.

Similar results were obtained in the action of the other ions. In the case of the merely isolated pieces, the reaction of the innervated cells was remarkably retarded. In split pieces, however, it occurred much more rapidly. Although, due to the secondary dispersion observed after continued application of concentration-inducing ions (*vid.* section B), spreading of the blanched area into the denervated band was discernible only with considerable difficulty, high power observation on the border of the band revealed that the sequences are quite the same in the cases of these ions.

When the scale isolated from *Oryzias latipes* or *Gambusia* sp. was partially treated with KCl solution, concentration response of the melanophores was seen only in the treated area, and never propagated into the rest of it. From this fact, Nagahama (*op. cit.*) concluded that the conduction system such as the melanophore-concentrating nerve does not play any part in the mechanism of K^+ stimulation. Ueda (*op. cit.*) reaffirmed his view on the scale melanophores of *Oryzias*, and further, on the melanophores within an isolated piece of caudal fin of *Gambusia*.

The analogous tests on *Chasmichthys* were carried out on the isolated fin-ray or on the split piece from a caudal fin, applying the isotonic KCl or other stimulant solutions locally by means of a micropipette. Here again, it was found that only the melanophores in the treated area respond, suggesting that these agents may cause concentration not through the conduction system, but through some structure situated close to the melanophore.

DISCUSSION

Spaeth (1913) was the first to explain that when an isolated scale of *Fundulus* was immersed in solutions of various potassium salts, concentration response of melanophores took place first at the margin of the scale where epidermis and derma had been torn off, and gradually proceeded inwards until all the melanophores within the scale had shown concentration response. He concluded that this might be due to the gradual centripetal diffusion of ions beneath the epidermis. Wyman (1924a) stated that epidermis is impermeable to ions from the fact that when the salt solutions were applied directly on the tail or on the body surface of *Fundulus*, no effects could be seen at all. This may also

be in agreement with Spaeth's view that ions penetrate most readily from the lesion. Nagahama (*op. cit.*), on the other hand, claimed from the observation on the scale melanophores of *Oryzias*, *Gambusia* and of *Cyprinus* that K ions may travel through the epidermis and reach the tissue space surrounding the melanophores.

It is true that there is a very slow penetration through the epidermal layer. However, the following observation by the author on *Chasmichthys* and on *Oryzias* may support Spaeth's interpretation: first, when dipped in the stimulant solutions, melanophores around the newly inflicted incisions showed most rapid and remarkable response in the cases both of the fin and of the body surface; secondly, on the isolated piece from the fin as well as on the isolated scale, the ionic action appeared at first on the margin, and gradually spread inwards. These facts may be explained rather easily, if it is assumed that ions may readily invade from the lesion where the tissue are supposed to be more easily permeable.

The isolated split piece from the caudal fin, conveniently used in the present study, was designed to abolish this troublesome participation of epidermis in the study of the ionic action, and has proved itself to be useful as expected. In addition to the rapid commencement of the response, all the melanophores within the preparation responded almost synchronously. These advantageous features made it possible to measure the response quantitatively and to compare the effectiveness of various ions.

The photoelectric records of the responses of innervated melanophores have shown the importance of two distinct phenomena, i.e., the secondary dispersion observable after the prolonged application of the stimulant solutions, and the temporary concentration observable at the beginning of the dispersion response caused by the effect of physiological solution. The former may be attributable to the adaptation of the melanophore system to the stimulant ions. The fact that adrenaline which is regarded to act directly on melanophore (Lieben 1906, Wyman 1924a, Parker 1934, Fujii *op. cit.*) concentrated the pigments without accompaniment of this phenomenon suggests that the seat of the adaptation development is not the melanophore itself, but may be a mechanism closely related to the secretion of concentrating neurohumor. Furthermore, from the fact that stimulant ions induce concentration response without the intervention of the conduction system it may be concluded that the adaptation develops at the endings of the concentrating nerves. The phenomenon of secondary concentration mentioned above also seems to be related to the adaptation at the concentrating nerve endings, although the minute exposition of the sequence cannot be given here.

Spaeth's first interpretation on the mode of action of the ionic stimulation (Spaeth 1913) was based on the above-mentioned observation on the centripetal spreading of the blanched area within an isolated scale. Since the isolation of the scale from the body surface would accompany a severance of the chromatic nerve-fibres, their torn end must inevitably be exposed to the surrounding solutions. If the ionic stimulation had been established there, a synchronous reaction should have followed among the melanophores. He concluded, there-

fore, that ions act directly without any interecalation of the nervous mechanism.

Lowe (*op. cit.*) immersed the embryos of brook trout in solution of inorganic salts. From the fact that melanophores respond both to Na^+ and to K^+ he concluded that the ions might travel through the epidermis and act directly on the cells.

Wyman (1924a) could not find any effect on melanophores, when the solution of KCl or NaCl was applied directly to the body surface or to the caudal fin of *Fundulus*, and concluded that this might be entirely due to the impermeability of the epidermis to the ions. Actually, he confirmed Spaeth's results concerning the action of K and Na ions on the isolated scale to which the ions could readily invade owing to the lesion given to the margin.

A pharmacological study on the nature of chemical stimulant by Spaeth and Barbour (*op. cit.*) also came to the same conclusion. They found that following the treatment with ergotoxin, adrenaline which otherwise cause vigorous concentration response induced the dispersion response of melanophores, while the application of KCl gave rise to the usual concentration response. They concluded, therefore, that the seat of concentration-inducing action is different for these two kinds of drugs, and that K ions may act directly upon the melanophores, independent of the nervous mechanism.

In his paper on the pigment displacement in the melanophore of *Fundulus*, Marsland (*op. cit.*) insisted on the direct action of K ions from the observation that K ions induced the concentration response of the denervated melanophores as well as of the innervated ones.

As is mentioned in the previous section, Nagahama (*op. cit.*) proved from the observation on the scale melanophores of *Oryzias*, *Gambusia*, and of *Cyprinus* that the melanophore-concentrating nerve does not play any role in the action of K ions. He observed that the treated area where the melanophore pigments were made to concentrate by the action of K ions never spread over the rest of the scale. From this result he concluded that the melanophore-concentrating neurohumor also does not participate in the ionic action. Ueda (*op. cit.*) came exactly to the same conclusion as Nagahama's, using the melanophores of the fin of *Gambusia* and of the isolated scale of *Oryzias*.

Contrary to their observations, however, local application of K and other concentration-inducing ions on the isolated fin of *Chasmichthys* brought about a blanched area having rather indistinct boundaries which during the prolonged treatment gradually spread into the rest of the area. This may be attributable to the invasion of the melanophore-concentrating neurohumor secreted within the treated region, though there might be a little possibility of being due to the turbulent flow of stimulant solutions, or to the diffusion of ions through the tissue space.

Similar phenomenon of the spreading of the blanched area was by far clearly observable within the denervated band, when a preparation having the band through the middle of it was immersed in one of the stimulant solutions (Figs. 6—8). Since the sequence is quite similar to that observed in electrical stimulation (*vid.* section A in Experimental), it can safely be said that also in the case of ionic stimulation, the concentrating neurohumor secreted within the

innervated region may invade into the band bringing about the pigment concentration within denervated cells. Together with the fact that denervated melanophores are non-sensitive to concentration-inducing ions, it necessarily follows that ionic action can be manifested at least through the secretion of the neurohumor. From these observations, and recollecting that the chromatic nerves take no part in the ionic action, it may be concluded that K and other concentration-inducing alkaline and alkaline-earth ions act on the endings of the concentrating nerves to induce the secretion of neurohumor, which, in turn, causes the concentration response of melanophores.

The discrepancy seen between the conclusion reached by the former investigators and that of the present study seems largely to be due to their failure to regard the possibility that ions might act not directly but on the neighboring structure. Consequently, the greater part of their observations can be reasonably explained by the interpretation presented here. Marsland's observation that the denervated melanophores as well respond to K ions, however, is quite inconsistent with that in the persent investigation. Since his observation was performed on a small piece from the tail fin, so selected that about half the area was derived from the denervated band, neurohumoral invasion into the denervated region might have induced the concentration response of the melanophores there. It might also be possible that the regeneration of the chromatic nerves had already been completed within the denervated area.

Summary

The mode of action of the ions which induce the concentration of melanin granules within melanophores of the tail of goby, *Chasmichthys gulosus*, was studied.

1. A photoelectric method for the quantitative recording of the melanophore response to chemicals was described.

2. Among the alkaline and alkaline-earth ions tested, Li^+, K^+, Rb^+, Cs^+, Sr^{++} and Ba^{++} were found to be effective in inducing the concentration response of the melanophores.

3. According to effectiveness these ions could be arranged in the order of K^+, Rb^+, Ba^{++}, Cs^+, Sr^{++}, Li^+, though the difference among Ba^{++}, Cs^+ and Sr^{++} was insignificant.

4. Denervated melanophores failed to respond to these ions.

5. The caudal band became narrower after prolonged application of these ions on the tail. This may be attributable to the invasion of the melanophore-concentrating neurohumor secreted within the innervated area.

6. Local application of these stimulant solutions on the isolated fin-ray caused, in the long run, connentration response of melanophores over the treated area. This may exclude the possibility of these ions acting through the nerve.

7. It was concluded that the concentration-inducing ions act not directly upon the melanophores, but on the concentrating nerve endings to induce the secretion of the neurohumor which, in turn, brings about the concentration of

the melanophore pigments.

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