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PHYSIOLOGICAL RESPONSES OF FROG MELANOPHORES IN VITRO[1]

PAUL A. WRIGHT

Department of Zoölogy, University of Michigan, Ann Arbor, and Marine Biological Laboratory, Woods Hole, Massachusetts

THE ability of many cold-blooded animals to mimic the shade of their environment by shifting the position of pigment in dermal chromatophores is well known to almost every student of general zoölogy. In view of the important role which chromatophores, but particularly melanophores, play in contributing to the survival of many forms of life, it is surprising that these melanin-bearing cells have not received more attention as physiological units. In surveying the literature on animal chromatics, one is impressed by the emphasis which has been placed upon such aspects as the nature of control of melanophores, responses of animals to varieties of shades and colors of background, the number of chromatophores involved in a color response, etc., and how little notice has been paid to the cellular reactions of the chromatophores or melanophores themselves. We have been given much information, comparatively, about the nervous versus the humoral control of pigment cells, the number and kinds of nerve-fiber connections to melanophores,

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the number and origin of neurohumors contributing to an animal's color reaction, while we lack an understanding of the cellular processes initiated by the humoral or nervous activity which bring about color change.

The older view of chromatophoral or melanophoral activity pictured pigment cells like amoebae or phagocytes, putting out dendritic processes filled with pigment (expanded state) and drawing them in again to become a rounded cell during the opposite phase (contracted or punctate state). It now appears (Matthews, 1931) that dendritic processes are permanent possessions of the cell and that pigment granules merely move out into preformed cytoplasmic branches (now called "dispersed" instead of "expanded" state) and back again to a centrally located clump ("concentrated" instead of "contracted" state). The idea that change in position of melanophoral pigment is effected by sol-gel changes within the cell was proposed by Redfield (1918) and has been substantiated by the pressure and centrifugation studies of Marsland (1944). Using isolated scales of *Fundulus*, which show pulsating activity upon treatment with NaCl and $BaCl_2$, Marsland found that increased atmospheric pressure caused melanophores to cease pulsations, remaining in the dispersed condition. Upon release of pressure, the cells reinstituted regular rhythmic cycles. Centrifugation (70,000 g) of melanophores in the dispersed state caused melanin granules to be distributed toward the direction of gravity, but pigment in punctate melanophores could not be moved, even with forces up to 125,000 g. Drawing analogies from similar work on other effectors, Marsland concluded that cytoplasm in melanophores with dispersed melanin is in the form of plasma sol, and in punctate melanophores it is plasma gel.

It is the purpose of this communication to present the results of several kinds of experiments designed to characterize further the type of cellular response involved in melanophoral activity and to determine something of the sources of energy for pigment migration in frog skin, with the hope that some insight might be gained into the real mechanism of hormonal action on melanophores. Although a small part of the data presented here has appeared previously in abstract form (Wright, 1946, 1947, 1951, 1952*a*, *b*, 1953; Wright and Sabal, 1952), a full account augmented by recent information is now in order, to clarify some of the basic concepts of melanophoral physiology.

MATERIAL AND METHODS

Skins of small frogs (40–60 gm.) were found in preliminary experiments to be the most satisfactory for the in vitro method used in all this work. For the most part, *Rana pipiens*, obtained from both Wisconsin and Vermont, were the donors, but *R. clamitans* from Vermont were substituted on two occasions when *pipiens* were not available. There seemed to be no detectable variation in the responsiveness of melanophores of the two species. Excised skin of *R. catesbiana* quite unexpectedly proved to be unsuitable for these tests because their melanophores were refractory to any kind of hormonal stimulation under the conditions of these experiments. Presumably, the thicker skin of *catesbiana* made it essentially impermeable by passive diffusion to intermedin and other hormones.

Although frog skin contains several types of chromatophores in addition to the abundant supply of melanophores, most, if not all, of the color change comes about from the movement of melanin (Parker and Scatterty, 1937). If light is passed through excised skin, then, the

percentage of transmission will furnish a direct indication of the state of melanin dispersion or concentration, thereby allowing an accurate graphic depiction of melanophoral activity. The photoelectric technique used throughout these studies to estimate dispersion of melanophoral pigment has been described in detail previously (Wright, 1948). Briefly, the method consists of placing patches of femoral skin between Bakelite frames especially designed to fit into the rectangular absorption cells of the industrial model Klett-Summerson photoelectric colorimeter and following the degree of pigment dispersion or concentration as indicated by colorimeter readings. Melanin dispersion reduces light transmission through the skin, and colorimeter values consequently rise, while concentration of melanin allows greater light transmission, and colorimeter readings accordingly drop. Because of variability in thickness of the skin, density of melanophores, and integumentary pattern, each skin will give its own characteristic range of colorimeter values. A thin skin obtained from a smaller frog in which melanophores are somewhat widely separated will show very low readings when all melanophores are punctate. Similarly, a thicker skin from a larger frog in which melanophores are more closely packed will give a much higher reading, even though all melanin is concentrated. The reading obtained from a fully blanched thick skin may be (although it usually is not) higher than that read from a thoroughly darkened thin skin. Since actual colorimeter values have no meaning in themselves, only changes in colorimeter readings are reported in this communication.

Instead of using Petri dishes with 50 ml. of fluid for storing skins between readings, as originally described, it was found convenient to keep the frames containing skins in extra absorption cells of the type supplied with the colorimeter. Use of absorption cells for this purpose has the advantage of allowing free circulation of fluid on both sides of the skin, but it was necessary to reduce the volume of solution to 40 ml. Readings were taken simply by placing the entire assembly in the colorimeter. Pigment moves sufficiently slowly in frog melanophores that readings taken at 15-minute intervals give a clear representation of melanophoral behavior.

Pituitary glands from adult frogs were used as the source of intermedin (the vertebrate melanophore-darkening hormone). No attempt was made to separate the intermediate lobe from the anterior and posterior portions of the gland. To avoid individual variation as far as possible, fifteen to twenty glands were collected at a time, and from this pool a concentrated extract in Ringer's solution was made and kept frozen between experiments. The various units of intermedin (Wright, 1954) were secured from the concentrated stock by dilution.

EXPERIMENTAL

I. RESPONSE TO INTERMEDIN

Frog skin is sensitive at room temperature to as little as the equivalent of 0.001 of a pituitary gland from one frog (one-fifth of a frog skin unit) dissolved in 40 ml. of Ringer's solution, in that a measurable, though somewhat unpredictable, amount of darkening is produced during a 90-minute exposure to solutions of this strength. Increasing the potency of hormone to the equivalent of 0.1 of a pituitary gland (20 units, Fig. 1) in the same volume of Ringer's fluid produces a maximal darkening response, while dosages intermediate between the two figures effect correspondingly submaximal responses. Submaximal darken-

ing during exposure to hormone is obtained also when amounts of intermedin in excess of 20 units are used (Fig. 2), and this observation is interpreted as being a reversal or inhibitory effect of hormone overdosage.

Skins darkened by intermedin blanch when fresh Ringer's fluid is substituted for intermedin-Ringer's solution, and the rate of paling bears a direct relation-

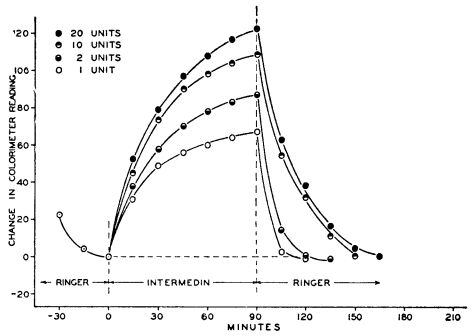

FIG. 1.—Responses of melanophores in excised frog skin during and following exposure to small and intermediate dosages of intermedin. Each curve represents an average of the behavior of eight different tests.

ship, within limits, to the concentration of the intermedin solution to which the skin had been exposed. After contact with dilute solutions of intermedin, paling in Ringer's fluid begins immediately; but after exposure to 100 units of intermedin or more, blanching is preceded by further darkening. In seeking an explanation for this seemingly unorthodox behavior, it should be remembered that in vitro technique involves passive diffusion of hormone molecules into and out of the skin and that inhibitory concentrations of intermedin will be diluted to more effective levels while the hormone is in the process of being removed by Ringer's medium.

The intermedin molecule is thought to be a polypeptide (Abramowitz *et al.*, 1943), and for that reason it is interesting that this compound with such a relatively large molecular size diffuses so readily into and out of the skin. When using concentrations of hormone ranging up to 40 units in 40 ml. of Ringer's solution, there appears to be little evidence that the melanophores or other cells of the skin in any way "capture" intermedin molecules. Much less is there any indication that intermedin enters into permanent chemical combination with cellular constituents, for the hormone leaves the skin as readily as it entered. If skins are exposed to 100 or 200 units of intermedin (dosages which may well be considered within the unphysiological range), however, the blanching process is erratic and

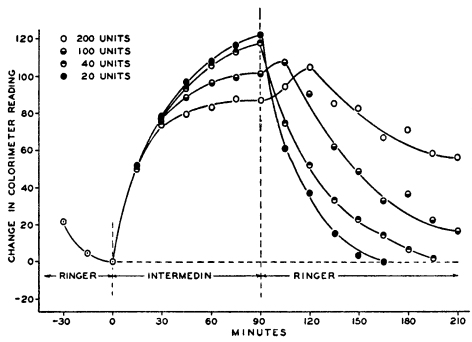

FIG. 2.—Responses of melanophores during and following exposure to intermediate and large dosages of intermedin. Each curve is an average of eight different tests.

may even fail to go to completion. Just what interpretation should be placed on this behavior is uncertain. Possibilities are that some irreversible reaction does occur in the presence of a great excess of hormone or that polarity or permeability of cell membranes has been upset by hormone overdosage. The suggestion has been made that large amounts of hormone may overstimulate cellular activity so that exhaustion ensues, leaving the melanophore with insufficient energy to complete the paling process. This explanation seems unlikely in view

of the fact that epinephrine, as will be seen more fully later, forces paling of a skin which is slow to occur in Ringer's solution alone after exposure to 100 or 200 units of intermedin. Epinephrine, of itself, is not known to introduce new energy into a system, and so it appears that the necessary energy for full blanching has not been depleted by the action of excessive intermedin but rather that excessive hormone has somehow inhibited or slowed a normal cellular process leading to paling.

The fact that a small amount of intermedin effects minimal darkening and that a larger dosage induces a full response raises the question of whether melanophoral activity exemplifies the "all-or-none" law. Is partial darkening the result of submaximal response of all melanophores, or have a few reacted fully and others not at all? A photoelectric colorimeter cannot distinguish between these two alternatives, and direct microscopic observation was therefore required to show that the former theory is correct. In a skin partially darkened with a nearly minimal dosage of intermedin (0.4 unit) examined microscopically, all melanophores were seen to have assumed about the same degree of melanin dispersion, none were found with fully dispersed pigment, and only rarely was a completely punctate melanophore discovered. The nonapplicability of the "all-or-none" law is seen also in the reversibility of melanophoral activity at any point in the darkening or paling curves. During 15 minutes' exposure to an optimal dosage of intermedin (20 units) the skin darkens the equivalent of perhaps 50 or 60 colorimeter units, but returns to the pale condition within the next 15 minutes if placed in Ringer's fluid. Darkening begun under the influence of intermedin, then, is stopped at once as soon as the hormone is removed by Ringer's medium. Melanophores appear to be unlike muscle cells, therefore, in that they need not complete a physiological cycle, once they are stimulated to activity.

Another aspect of the effect of intermedin concentration concerns the unitage of hormone required to raise a skin to a maximal level of darkening as opposed to that amount necessary to maintain the maximally darkened state. The question has often been asked whether a smaller dosage of hormone would keep a skin in the totally darkened state, once it had been brought into this condition by an optimal intermedin titer. To test this possibility, a skin was darkened with 20 units of intermedin until a constant colorimeter value was obtained (105 minutes) and was then immersed in a solution containing but 1 unit of intermedin. The skin promptly assumed within 15 minutes the degree of darkening characteristically produced by 1 unit (about 50 per cent of the original darkening), showing that a higher hormone level is necessary to maintain complete pigment dispersion. It cannot be, then, that intermedin triggers a cellular mechanism which can proceed without hormonal influence from point of initiation. Instead, continuous action of intermedin is required not only to disperse melanin but also to keep it totally dispersed.

Interesting variations in the pattern of melanophoral responses may be obtained by subjecting intermedin solutions to boiling prior to immersing the skin preparations. In the experiments described here, concentrated pituitary solutions were boiled for 3 minutes, cooled, and diluted to the desired dosage. It will be noted (Fig. 3) that darkening curves are not significantly different from those obtained with unboiled hormone, but that blanching time after exposure to intermediate dosages of boiled

intermedin is considerably lengthened. Indeed, paling never occurs following darkening with 40 units or more of heat-treated hormone. It appears that the intermedin molecule has been altered by high temperature, not in its ability to darken melanophores, but in properties which permit it to leave the cell, once it has exerted its effect. If intermedin is potentiated by heat (Langdrebe and Waring, 1941), one would expect to be able to detect smaller quantities of

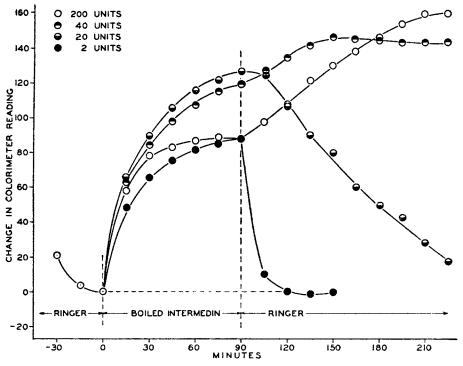

FIG. 3.—Melanophoral responses during and following contact with pituitary extracts which had been boiled. Compare curves with those obtained with natural hormone (Figs. 1 and 2).

boiled hormone than of natural hormone. Isolated skin is insensitive to the difference, however, for minimal dosages (one-fifth unit) of both boiled and natural hormone give indistinguishable darkening responses, and one-tenth unit of intermedin of either kind does not elicit a response.

II. RESPONSE TO EPINEPHRINE

A striking, and possibly the most significant, effect of epinephine on frog melanophores is observed when the hormone is applied to skins already in the blanched or "resting" condition, after intermedin has been allowed to wash out of the excised skin. If epinephrine's effect were the same as that produced by lack of intermedin, it would be expected that epinephrine added at this time would exert no noticeable paling influence. Skins free of intermedin, placed in epinephrine (1–100 parts in 10,000,000 Ringer's fluid, Fig. 4) do show a further blanching of as much as 35 colorimeter units, however. It is as though epinephrine had brought paling to completion, a feat which the simple lack of intermedin falls short of doing. When the skins were removed to Ringer's solution, colorimeter readings tended to return to the "base-line" or "resting" condition.

These data raise the question immediately of what constitutes the blanched state of frog skin. If melanophores assume a punctate condition after intermedin has been removed from the skin, how can they be rendered any more

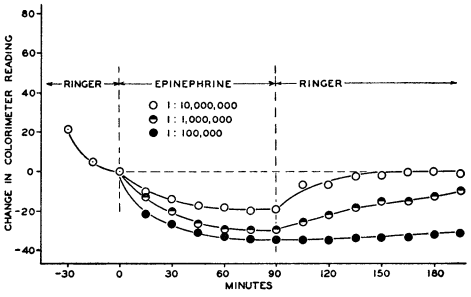

FIG. 4.—Melanophoral responses during and following exposure to solutions of epinephrine. Each curve is an average of four tests.

punctate by epinephrine? The answer is found in microscopic examination of skins under the two conditions, where it is seen that pigment clusters in melanophores responding to epinephrine are almost perfectly rounded, while pigment in melanophores exposed only to Ringer's fluid would still be considered punctate, but pigment is gathered in somewhat unevenly contoured aggregations, and occasionally a melanophore with dispersed pigment is found. Epinephrine is known to induce vasoconstriction, of course,

but it is difficult to see how a small change in the size of dermal blood vessels could account for so great a change in colorimeter value. Pigment in other chromatophores is equally dispersed under the two experimental conditions, and no nonmelanophoral changes, therefore, could be responsible for the difference in colorimeter readings. Apparently, then, a further paling in response to epinephrine does take place, or, as Marsland (1944) pictures melanophoral activity, there is a greater contraction of plasma gel under the influence of epinephrine than that which follows the withdrawal of intermedin alone.

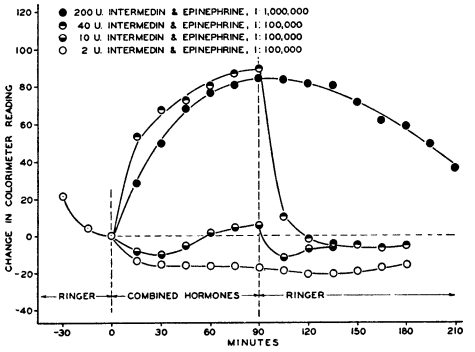

FIG. 5.—Curves obtained from melanophoral activity upon exposure to combinations of epinephrine and intermedin. Compare with appropriate curves in Figs. 1, 2, and 4.

III. RESPONSE TO COMBINATIONS OF INTERMEDIN AND EPINEPHRINE

Results of representative tests using intermedin and epinephrine in combination (Fig. 5) show that melanophores apparently respond to both hormones, the reaction curve depending upon the relative concentration of the two hormones. If a large dosage of intermedin (e.g., 200 units) and a small titer of epinephrine (1:1,000,000) are combined, the skin darkens nearly as much as it would have with intermedin alone. Note, however, that paling following immersion in the combination of hormones is not preceded by further darkening, as it is after exposure to intermedin alone. With the situation reversed (i.e., a larger dosage of epinephrine, 1:100,000, and 2 units intermedin), the blanching properties of epinephrine are hardly affected by the presence of intermedin. Except when very large amounts of intermedin and dilute solutions of epinephrine are used, skins darkened with combinations of intermedin and epinephrine blanch in Ringer's fluid in a fraction of the time required when intermedin alone is used. Epinephrine, in this case, seemingly accelerates a process which would ordinarily occur of its own accord in time.

Epinephrine (1:100,000) added to a skin darkened with 40 units boiled intermedin brings about a rapid and complete melanin concentration, the reaction being quite indistinguishable from that observed when natural intermedin is used. It appears, then, that boiled pituitary extract has not irreversibly inhibited energy processes which lead to blanching. These processes do not occur in Ringer's solution alone following exposure to heated intermedin but may proceed normally with the aid of some extra "push" which epinephrine provides.

IV. EFFECT OF TEMPERATURE ON MELANOPHORAL RESPONSE

For these experiments, absorption cells were immersed in water baths designed to operate at a fixed temperature $\pm 1°$ C. Intermedin solutions and Ringer's fluid were brought to the desired temperature before being used in these tests. Readings were taken as usual by removing the absorption cells from the water baths and placing them in the colorimeter.

A look first at the effect of temperature

variation upon the darkening curves of frog skin (Fig. 6, *A*) shows at once that both the rate of darkening and the degree of melanin dispersion in response to the same dosage of intermedin may vary greatly with temperature. Both maximal rate of response and maximal darkening are obtained at 37° C. (an experiment conducted at 44° C. gave results identical with those obtained at 37° C.), and successively diminishing rate and total response are observed as the temperature is reduced to 25°, 14°, and, finally, to

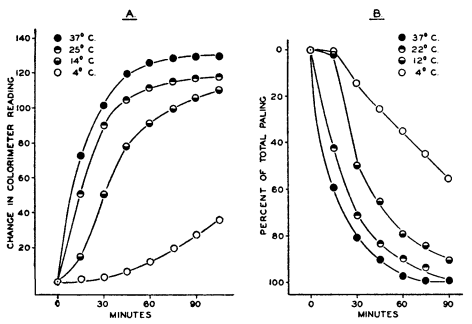

FIG. 6.—*A*, darkening curves obtained with 20 units intermedin at various temperatures. *B*, blanching curves obtained at various temperatures after maximal darkening with 20 units intermedin at 22° C.

4° C. It is evident that high temperature has no inhibitory effect, at least, on melanophoral pigment dispersion, despite the statement found rather generally in the literature that warmth favors the blanched state of intact frogs. If frogs have a tendency to be pale regardless of background at high temperatures, it would seem that the explanation must lie in some failure of intermedin secretion rather than in lack of response in the melanophores themselves. Conversely, very low temperature (4° C.) is conducive to a slow rate of darkening, and, during the time of these experiments, total darkening was not even approached. Again, these results are at variance with the classical view that cold favors the darkened state.

To test the effect of temperature on blanching following exposure to intermedin, the limits of responsiveness of each skin were determined first by allowing complete blanching in Ringer's solution and then by maximally darkening the skin with 20 units of intermedin at 23° C. Blanching curves at various temperatures (Fig. 6, *B*) in Ringer's solution appear very much as expected. The Ringer's fluid was changed every 30 minutes to offset any possibility of intermedin accumulating in the medium as the hormone washed out of the skin. At 4° C. melanin concentration occurred slowly and, by the end of 90 minutes, had progressed only 55 per cent of the way to complete blanching on the basis of the previous determination. The delay in onset of blanching at 4° and 12° is unexplainable but may be due, at least partially, to a change in skin permeability rather than entirely to altered melanophore responsiveness. Blanching at 37° was significantly more rapid than at 22°, but it is surprising that a greater difference did not appear in the curves for the two temperatures. Room temperature (22° C.), then, comes reasonably close to providing optimal conditions for both blanching and darkening, in rate as well as in totality of response.

V. EFFECT OF CHANGE IN HYDROGEN-ION CONCENTRATION

Two sets of experiments have been performed in which the pH of the Ringer medium was altered. Although these tests are insufficient to allow any definite conclusion as to the effect of pH on melanophoral activity, the results do give an indication of the importance of controlling the hydrogen-ion concentration. The phosphate-bicarbonate buffering of the Ringer's formula used throughout

this work was sufficient to keep the pH variability between 6.9 and 7.4 in all the experiments reported thus far. Nevertheless, it seemed important to know the effect of mildly acidic and basic solutions not only on unstimulated melanophores but also on their responses to intermedin and epinephrine.

By adding appropriate amounts of NaH_2PO_4 and Na_2HPO_4 (quantities insufficient to alter the osmotic properties of Ringer's fluid), a range of pH values between 6.2 and 8.2 was obtained. When blanched frog skin was placed in these solutions, the melanophoral state remained unchanged, an essentially constant value being maintained, as would have been the case in unaltered Ringer's fluid. Darkening upon addition of intermedin appeared identical to that occurring when ordinary Ringer's solution was used, as was also paling when skins were placed in fresh Ringer's of the same pH as that used for darkening. Blanching as induced by epinephrine was also typical.

Skins with punctate melanophores placed in a solution of succinic acid (0.001 M, pH 5.4) in Ringer's fluid, however, showed darkening to a degree comparable with that effected by about 1 unit of intermedin. Blanching upon removal to Ringer's at pH 7.2 was incomplete, indicating a partially irreversible effect of low pH. When the pH of succinic acid solution was raised to 6.4 and 7.4 by the addition of NaOH, no melanin dispersion occurred. Thus it appears that the darkening influence of succinic acid is an effect of hydrogen-ion concentration and is not due to a peculiarity of the succinic acid molecule itself.

VI. METABOLIC STUDIES ON MELANOPHORES

Although the use of general and specific inhibitors falls short of providing a complete picture of metabolic events, they are handy tools in the initial stages of investigation. Since there has been no previous inquiry into the sources of energy for, or the type of metabolic reaction accompanying, melanophoral activity, it seemed advisable to assay a few of the metabolic poisons in common usage, in the hope of revealing some part of the general nature of melanophoral metabolism.

One of the principal points to be tested concerns the necessity for oxygen, transferred by the cytochrome oxidase system, for either melanin dispersion or concentration. In this connection it is interesting that Lerner (1952) found that excised frog skin responded to intermedin in a vacuum, indicating that, for darkening at least, oxygen was not required. It has also been reported (Wright, 1952*b*) that frog skin consumed oxygen at the same rate when immersed in Ringer's fluid alone as it did in intermedin or epinephine solution. In the present work cyanide and azide were used to poison the cytochrome oxidase system, thereby largely preventing the use of oxygen. Isolated skin immersed in 0.001 M KCN or 0.001 M Na_3N in Ringer's responded maximally to 10 units of intermedin during a 90-minute exposure, and it blanched in 45 minutes when transferred to a fresh solution of Ringer's containing the same concentration of cyanide or azide. It appeared, then, that the energy for melanophoral activity came from some anaerobic system or, at least, that oxygen was not immediately involved in the single darkening and paling processes.

But what of the requirement for oxygen in repeated darkening and blanching? Might it not be that melanophores could complete one cycle of activity by making use of anaerobic processes but that continued activity in the presence of cyanide or azide might soon result in

exhaustion and the necessity for replenishment by aerobic oxidative reactions? To test this possibility, skin was immersed in 0.001 M KCN or 0.001 M Na_3N in Ringer's, to which 10 units of intermedin were added, and darkening was allowed to proceed for 30 minutes. Substitution of fresh cyanide- or azide-Ringer's effected complete blanching in another 30 minutes, whereupon intermedin was added again, and the cycle continued in this manner. Thus the skin was exposed continuously to cyanide or azide and alternately to intermedin and the lack of it. After 4 hours of such treatment the melanophores were still as competent as they had been at the start of the experiment, and their behavior was essentially the same as that of control skin treated in the same way but not exposed to cyanide or azide. It seems unlikely, therefore, that aerobic reactions contribute directly to energy for pigment migration.

The use of phosphate-bond energy in muscular contraction has now become well known, as has also the part which glycolysis plays in providing energy for replenishment of high-energy phosphate. If melanophores use phosphate energy as muscle cells do, then its seems likely that poisoning with glycolysis-inhibiting compounds would arrest color change at some point in the darkening or paling cycle. As with muscle tissue, it would be expected that melanophores poisoned with iodoacetate or fluoride might well show some activity while the reserve of phosphate energy was being expended but that pigment migration would soon cease after depletion of phosphate energy. Since it was not known whether blanching or darkening might be affected by glycolytic poisons, a schedule of continuous exposure to poison but alternate contact with intermedin (30 minutes) and the lack of it (30 minutes) was used, as with the cyanide and azide experiments. Upon exposure to 0.001 M sodium iodoacetate and 10 units of intermedin in 40 ml. of Ringer's fluid, normal darkening occurred during the half-hour interval (Fig. 7), but blanching during the next half-hour after removal of intermedin was only about 60 per cent of that achieved by the control skin. Darkening occurred again after another addition of intermedin, but blanching following this second darkening was negligible. Alternate treatment with intermedin and the lack of it after this point in the test produced no further change, and the skin was rendered permanently dark. As had been postulated, melanophores poisoned with iodoacetate had been able to show limited activity, and it seemed reasonable to suspect that inhibition of glycolysis had been responsible for the cessation of pigment movement.

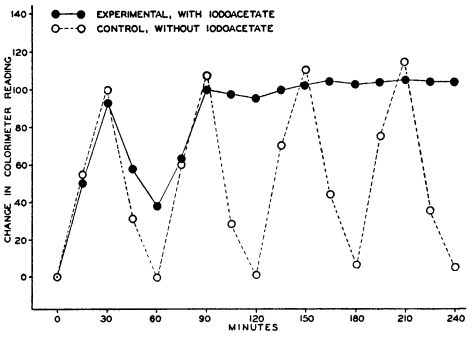

FIG. 7.—Behavior of melanophores in presence of iodoacetate to alternate exposure to 10 units of intermedin (30 minutes) and to the lack of intermedin (30 minutes). Note that a partial paling follows the first immersion in intermedin but that subsequent blanchings are inhibited.

But iodoacetate is also known to interfere with the reactivity of sulfhydryl groups of some enzymes, and the objection might well be raised that the inhibiting effect of iodoacetate on color change was not necessarily due to its action in halting glycolysis. Cysteine in

appropriate concentration has the property of restoring the SH activity of enzymes in the presence of iodoacetate, however, and so the experiment just described was repeated with the addition of cysteine (0.001 M). The results were unchanged from those obtained with iodoacetate alone, strengthening the view that the effect of iodoacetate in these experiments was one of glycolysis inhibition and that the ability to perform glycolysis is a requirement for complete blanching of frog skin.

Subsequent to the foregoing, it was discovered that skins exposed for the standard 90 minutes to 10 or 20 units of intermedin plus 0.001 M iodoacetate do not blanch in Ringer-iodoacetate medium but, instead, remain unexplainably dark for some 30–45 minutes, and then very slowly pale, but only to about the 60 per cent level observed above. If a darkened skin is removed from the Ringer-iodoacetate-intermedin mixture to Ringer-iodoacetate-epinephine (1: 100,000) solution, the skin is "forced" to pale very rapidly, but still only to about the 60 per cent level characteristic of iodoacetate poisoning. If the same skin is darkened again for a 60- to 90-minute period by intermedin, it does not respond a second time to epinephrine or to the lack of intermedin but remains immovably dark.

Addition of sodium fluoride (0.001 M) to the alternate darkening and paling system described caused the skin to pale a little less during each successive blanching period. After four color cycles in fluoride solution, however, the melanophores were still capable of about 40 per cent of total blanching. The same results were obtained with 0.001 M malonate, but, interestingly, sodium fluoracetate (0.001 M) had no inhibitory effect on either darkening or blanching. It is difficult to find an explanation for the difference between the partial inhibition imposed by fluoride and malonate and the complete inhibition of iodoacetate. Presumably, none of the poisons allows glycolysis to go to completion, but it may well be that melanophores find a way around this inhibition in the case of fluoride and malonate but not in the presence of iodoacetate.

Isolated skin immersed in a 0.1 per cent solution (0.003 M, pH 8.0) of triphenyltetrazolium chloride (TTC) in Ringer's fluid did not darken in response

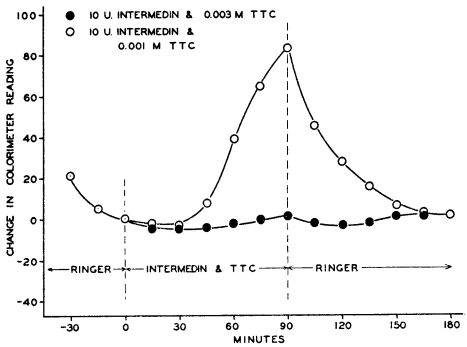

FIG. 8.—Responses of melanophores in the presence of triphenyltetrazolium chloride (TTC). Note total inhibition of darkening by 0.003 M TTC, but partial response in the presence of 0.001 M TTC.

to 10 units of intermedin; in fact, a slight paling, suggestive of the action of weak epinephrine, occurred (Fig. 8). The same concentration of TTC had no effect on the blanching of skin maximally darkened with intermedin. Unlike the activity of epinephrine, 0.003 M TTC was just as capable of inhibiting 20 or 200 units of intermedin as it was 10 units, indicating that TTC affects the responsiveness of the melanophore and does not enter into chemical relationship with intermedin. A concentration of 0.001 M TTC only partially prevented the darkening influence of 10 units of intermedin, and 0.0001 M TTC was ineffective. The darkening-inhibitory capacity of 0.003 M TTC was abolished by addition of 0.001 M suc-

cinic acid, which reduced the pH to 5.6, but was reinstituted when this mixture was brought to a pH of 7.5 by NaOH. Presumably, there is a change in the reactivity of the TTC molecule at low pH. There was indication that TTC did not exert an effect through its characteristic reaction of forming red (reduced) formazan, for there was no change in color of the solution during these experiments. Furthermore, photoreduced and freshly prepared solutions of TTC were equally effective in inhibiting the response to intermedin.

Because of the action of colchicine in preventing the formation of the spindle (a gelation process) during mitosis, the alkaloid was considered to hold some promise as a melanophore poison. Skins immersed in 0.1 per cent colchicine in Ringer's solution darkened normally in response to 10 units of intermedin, but remained dark when restored to Ringer's fluid. These results seemed similar on the surface to those obtained with moderate or large dosages of boiled pituitary extracts, or iodoacetate, but were distinguished by the response to epinephrine. Treatment of a darkened, colchicine-poisoned skin with epinephrine (1:100,000) in Ringer's fluid resulted in no blanching whatsoever. It appears, then, that colchicine poisoning effects an irreversible change, inhibiting whatever blanching reactions may ordinarily be set in motion either by the withdrawal of intermedin or by epinephrine.

In carrying the investigation of colchicine action further, it was shown readily that the alkaloid of itself had no melanophore-darkening properties. Blanched skins immersed in 0.1 per cent colchicine in Ringer's fluid remained at their resting level for periods of at least 90 minutes. If the skins were then rinsed or soaked for 1–3 hours in Ringer's fluid (with several changes) and then placed in intermedin solution (10 units in 40 ml.), they darkened as usual during the next 90 minutes, but did not blanch in Ringer's fluid. Their behavior was exactly as though they had been exposed to a combination of intermedin and colchicine, even though their only contact with the alkaloid preceded the 90-minute exposure to intermedin. Colchicine, by some unknown reaction, obviously can affect a subsequent paling even 3 hours before darkening has been instituted.

It is interesting that colchicine applied after a skin has been darkened by intermedin alone does not affect blanching, and the paling curve is indistinguishable from that obtained in Ringer's solution alone. The explanation for this surprising observation is not apparent, but it is tempting to speculate that colchicine is barred from the melanophore while the cell is decreasing its intermedin titer.

DISCUSSION

One of the oldest controversies in the field of animal chromatics concerns the active and resting states of melanophores, dating from the time of Brücke (1852) and continuing up to the report of Parker (1940). In discussing this point, most workers find it convenient to draw analogies between the behavior of melanophores and that of other effectors, particularly muscle fibers (e.g., Spaeth, 1916). In the days of these discussions, muscular physiology was much less understood than it is today, and it was customary to speak of a contracting or contracted muscle as "active" and a relaxed or relaxing muscle as "resting." Today we are aware that equally important chemical processes go on during both contraction and relaxation, and we cannot speak of one process as being active and the other passive. No consensus has ever been reached as to

whether the darkened or pale phase of melanophores constituted the active condition, but Wyman (1924) could not be convinced that either view was correct. He was led to propose that melanophores were active when pigment was moving in either direction and were resting when pigment was motionless.

Although the studies of melanophores and metabolic inhibitors are in their infancy, they seem to show that Wyman was near the truth. The iodoacetate tests indicate strongly that melanophores require energy—linked in some manner to glycolysis—for purposes of concentrating pigment. The partial inhibition imposed by fluoride and malonate also serves to strengthen this view. On the other hand, the results of experimentation with tetrazolium salt suggest that energy exchange or enzyme transfer has been blocked by TTC, thereby inhibiting melanin dispersion. Because of the comparative instability of both iodoacetate and TTC, it would be impossible to use them in any long-term experiment for determining energy requirements necessary to hold melanophoral pigment in either the dispersed or the concentrated state. Some other means will need to be devised to determine just how much "resting" a melanophore does while not engaged in moving pigment, but it seems more than probable that the "expensive" energy (Parker, 1940) is required for the changing of color.

From the inhibition studies again, the role of intermedin in melanophoral activity emerges in a different light, i.e., not only that of activator but also, and perhaps primarily, that of inhibitor. Intermedin may be pictured as the activator while frog melanophores progress from the pale to the darkened state. Such activation is prevented by TTC, although the mechanism for this inhibition is not known. But if the inference from the use of iodoacetate is correct, intermedin in fostering the darkened state is actually an inhibitor of glycolysis, the process whose energy seems to be required for complete paling. It may well be that the different degrees of darkening produced by varying dosages of intermedin are traceable to different degrees of glycolytic inhibition. Continuing this same line of reasoning, large dosages of hormone may turn out to be somewhat less effective in darkening because of being less effective in inhibiting glycolysis. It is not uncommon for large dosages of hormones (in general) to do the reverse of what smaller amounts bring about.

A consideration of the behavior of melanophores in response to epinephrine serves to uphold this scheme of possible intermedin action, for the adrenal hormone has long been associated with facilitating glycolysis. The rapid blanching of darkened skins, as well as the further paling beyond the "resting" state effected by epinephrine, may well be thought of as due to accelerated glycolysis. Similarly, the curves obtained from the use of epinephrine and intermedin in combination are explainable in terms of the glycolysis-accelerating action of epinephrine opposing the glycolysis-inhibiting capacity of intermedin. It goes almost without saying that the temperature and pH studies at least do not provide contrary evidence. A variation in pH is known to affect the reactivity of many enzyme systems, and it is very possible that low pH may not be conducive to glycolysis.

Until more is known of the biochemical reactivity of colchicine, no statement can be made about the measure of support which use of this alkaloid may lend to the glycolysis theory of blanching. At present, data from colchicine experiments seem most useful in supporting

the idea (Redfield, 1918; Marsland, 1944) that melanin concentration involves a contraction of plasma gel. This statement is largely deductive from the action of colchicine in preventing the formation of the spindle (gelation) in mitosis. For the time being, the only plausible explanation for the action of colchicine on melanophores is that in some way it prevents cytoplasmic gelation and that this process is essential for, or indeed is, melanin concentration.

SUMMARY

Studies have been made of the effects of hormone concentration, of variation in factors of the physical environment, and of metabolic inhibition on melanophoral activity of excised frog skin in Ringer's medium. A photoelectric method was used to estimate the degree and rate of melanin dispersion or concentration in color cells.

Increasing titer of intermedin produces a progressively greater response up to 20 units. Larger dosages of hormone are partially inhibitory. Blanching is very markedly slowed after skins are exposed to moderate dosages of boiled pituitary extract and is inhibited after exposure to large amounts of heated intermedin.

Epinephrine opposes the action of intermedin, producing a very rapid concentration of melanin and paling of the skin. Experiments are described in which combinations of epinephrine and intermedin were used.

Both blanching and darkening are very much slowed at 4° C., occur more rapidly at 14° and 22° C., and most rapidly at 37° C.

Variation of the pH of Ringer's fluid between 6.5 and 8.5 did not affect the responsiveness of frog skin to intermedin or epinephrine. There was some indication that a pH of 5.4 may induce some darkening in the absence of intermedin.

Sodium iodoacetate partially inhibited the first paling of skin darkened with intermedin, and after a second darkening in the presence of iodoacetate, blanching was totally inhibited. Malonate and fluoride produced only 60 per cent inhibition of blanching after four successive darkenings.

Triphenyltetrazolium chloride was found to inhibit the responsiveness of melanophores to intermedin.

Exposure of skins to a mixture of intermedin and colchicine prevents paling, which would normally follow intermedin-induced darkening.

A brief discussion is presented of the implications of these results on an up-to-date concept of melanophoral physiology.

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EFFECTS OF GONADECTOMY AND HYPOPHYSECTOMY ON PRE-SPAWNING BEHAVIOR IN MALES OF THE GOBIID FISH, BATHYGOBIUS SOPORATOR[1]

WILLIAM N. TAVOLGA[2]

Department of Animal Behavior, American Museum of Natural History, New York

As in other vertebrates, the endocrine secretions of the gonads of teleosts are known to affect morphological characteristics. "Nuptial" or breeding colors, gonopodia of viviparous poeciliids, the ovipositor of the bitterling, and many other structures associated with reproduction have been described as being under the direct control of gonadal hormones. In some cases the gonadotrophic function of the pituitary has been included as a direct or indirect factor in the development of these secondary and accessory sexual characteristics. Most of the reports on the teleost fishes have dealt with the effects of hormone administration, and a few have utilized the techniques of gonadectomy and hypophysectomy.

Despite the fact that the spawning behavior of a large variety of teleostean species has been described, the data available on the relationships of the endocrine glands with reproductive activities are sparse, inadequate, and contradictory. The present report is a corollary to a descriptive study of the spawning behavior in the gobiid fish, *Bathygobius soporator* (Cuvier and Valenciennes) (Tavolga, 1954). These data represent an approach to the investigation of the internal mechanisms involved in sex discrimination and courtship behavior in this species.

SOURCE AND MAINTENANCE OF MATERIAL

The specimens used in this work were collected from tide pools and shallows along the Intracoastal Waterway in the vicinity of Marineland, Florida. The ani-

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[2] Public Health Service Research Fellow of the National Institute of Mental Health.