STUDIES IN THE PIGMENTARY SYSTEM OF CRUSTACEA. IV. THE UNITARY VERSUS THE MULTIPLE HORMONE HYPOTHESIS OF CONTROL

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Since the first demonstration by Perkins (1928) that the physiological activity of crustacean chromatophores is regulated by a hormone originating from the eye-stalks, numerous studies have confirmed and extended his results (for a review of the literature see Hanström, 1937). It was later shown (Kleinholz, 1934, 1936) that the retinal pigments of crustaceans were also under endocrine control, being affected by the same eye-stalk extracts which contracted [2] the chromatophores of the integument.

On the basis of independent chromatophoral response to various colored backgrounds, Brown (1935*a*) concluded that a number of different hormones were concerned in the regulation of color change. Abramowitz (1937*b*), on the other hand, has questioned the necessity of more than one hormone in explaining the pigmentary activity of crustaceans. When the evidence available from studies of the retinal and integumentary pigments is considered, it appears that at least two different hormones might be involved. For example, while the chromatophores of *Palaemonetes vulgaris* are maximally concentrated [2] and the distal retinal pigment is in the position of complete light-adaptation in animals kept on an illuminated white background, on an illuminated black background the red chromatophores are maximally dispersed but the distal retinal pigment is still in the light-adapted position. If both types of pigment cells were controlled by the same hormone, then absence of the chromatophorotropic hormone from the circulation (evidenced by the dispersed integumentary chromatophores in animals maintained on an illuminated black background) should also result in a position of the distal retinal pigment characteristic for complete dark-adaptation.[3] It is obvious, on the other hand, that the different be-

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[2] The terms "contracted" and "expanded" in reference to the condition of the pigment within chromatophores are used synonymously with the concentrated and dispersed state.

[3] Kleinholz and Knowles (1938) have shown that the position of the distal retinal pigment is directly proportional to the ratio, $\frac{\text{intensity of incident light}}{\text{intensity of reflected light}}$.

havior of these two components of the pigmentary system could be explained on the basis of differential threshold sensitivities to the same hormone.

Diurnal rhythms in the pigmentary system of crustaceans, persisting under constant external conditions, afford an opportunity to test the question of the unitary or multiple hormone hypothesis. Three crustaceans are known in which the integumentary chromatophores behave in such rhythmic fashion: a macruran, *Hippolyte varians* (Gamble and Keeble, 1900); an isopod, *Ligia baudiniana* (Kleinholz, 1937*a*); and a brachyuran, *Uca pugilator,* (Abramowitz, 1937*a*). When individuals of these three species are kept in constant darkness the animals become pale at night and darken again during the day. Welsh (1930*a*, 1935) and Kleinholz (1937*b*) have shown that similarly persistent cyclic activity of the retinal pigments occurs in macrurans and brachyurans when maintained under constant conditions of illumination. On the basis of the unitary hormone hypothesis the threshold of the retinal pigment must be lower than that of the integumentary pigments; persistent rhythm in the integumentary chromatophores of a crustacean should therefore also be accompanied by a persistent cyclical behavior in the activity of the retinal pigments. This paper reports the results from experiments designed to determine whether pigmentary activity in crustaceans is to be explained on the basis of one or of more than one hormone.

These studies were conducted in part at the Marine Biological Laboratory of the United Kingdom, Plymouth, England, and in part at the Stazione Zoologica, Naples, Italy. To the Directors of these laboratories, Dr. Stanley Kemp and Professor R. Dohrn, and to the staffs of these institutions I am greatly indebted for generous facilities and kind assistance. I am also obliged to the committee for permission to occupy the Jacques Loeb Memorial Table at Naples.

The crustaceans used in these studies were *Crangon vulgaris, Hippolyte varians,* and *Leander adspersus.*

Materials and Methods

Black- and white-painted dishes were used as backgrounds to obtain individuals with expanded and contracted melanophores (*Crangon*); these vessels were illuminated from above by a 60-watt electric lamp at a distance of 30 inches.

It was desirable in some experiments to observe the color reactions of blinded individuals.[4] Two methods were used to incapacitate

[4] Blinding may be of two kinds (Kleinholz, 1937*a*); incapacitating the retina, whereby only the sensory elements are affected, and total ablation of the eye-stalks, resulting in removal of the chief functional source of eye-stalk hormones.

the retinas of *Crangon:* in one, an opaque mixture of plaster of Paris was moistened into a paste and spread over the entire eye-stalk, while in the second the retinal portion of the eye-stalk was destroyed by cautery. In removing the eye-stalks of *Crangon* it was found advisable to proceed in two stages, allowing a day or two to elapse before ablation of the second eye-stalk.

Blood "transfusions" from black-background-adapted [5] individuals to white-background-adapted [5] shrimps and *vice-versa* were effected by withdrawing blood from the pericardial sinus of donors (approximately 0.1 cc. could thus be obtained from a single individual) by means of a hypodermic syringe, and injecting 0.05–0.07 cc. into the abdominal musculature of the recipients.

Extracts of the rostral region of *Crangon* were prepared in an attempt to duplicate the results of Koller (1928). Both eye-stalks of the donor were first ablated at the base to avoid the possibility of including fragments of Hanström's sinus gland; the rostral region was then excised with a second pair of scissors as indicated in Fig. 1 of Koller's (1928) paper. The excised portions from 10 black-adapted *Crangon* were triturated for 3 minutes in 1.0 cc. of filtered sea water or crustacean perfusion fluid (Pantin, 1934). One-tenth cc. of the clear supernatant extract was injected into the abdominal musculature of the white-adapted recipients which were then returned to their white backgrounds for observation. Precautions were taken to prevent contamination of the fluids used for injection; separate syringes and needles were used in taking blood from white- and from black-adapted individuals; a third hypodermic syringe was used exclusively for injecting extracts prepared from the rostral regions. Before use the syringes were washed in tap-water, immersed in bichromate-sulfuric acid solution for 12 hours, flushed in running tap-water for 12 hours, rinsed several times in distilled water, 95 per cent alcohol, ether, and dried in air.

Destruction of the rostral region, claimed by Koller to be the source of a melanophore-dispersing hormone, was performed by cautery in the manner prescribed by him (1928). The exoskeleton in the region indicated on his Fig. 1 was excised and a hot needle used for surface and deep cautery; the replaced square of excised exoskeleton was usually kept in position by the blood clot which formed around the edges; in some cases coagulation of the blood exuding around the wound was

[5] The terms "black-background-adapted," "black-adapted" and "black" are used to describe individuals in which the melanophore pigment has been dispersed as a result of the animal's having been kept on an illuminated black background; "white-background-adapted," "white-adapted," and "white" are similarly used to describe the concentrated condition of the melanophores in animals kept on an illuminated white background.

hastened by drawing the cautery needle along the margins of the incision.

Hippolyte varians which had been kept in darkness and in light were killed in hot water (80° C.) during the day ("day light" and "day dark" eyes) and at night ("night light" and "night dark") to determine whether a persistent diurnal rhythm existed in the movements of the retinal pigments. Subsequent histological treatment of the eyes followed the method described in an earlier paper (Kleinholz, 1937*b*). Blinding operations, similar to those performed on *Crangon,* were made on *Hippolyte;* retinas were destroyed by cautery or were ablated with iridectomy scissors; entire eye-stalks were removed after first crushing the base to prevent excessive hemorrhage.

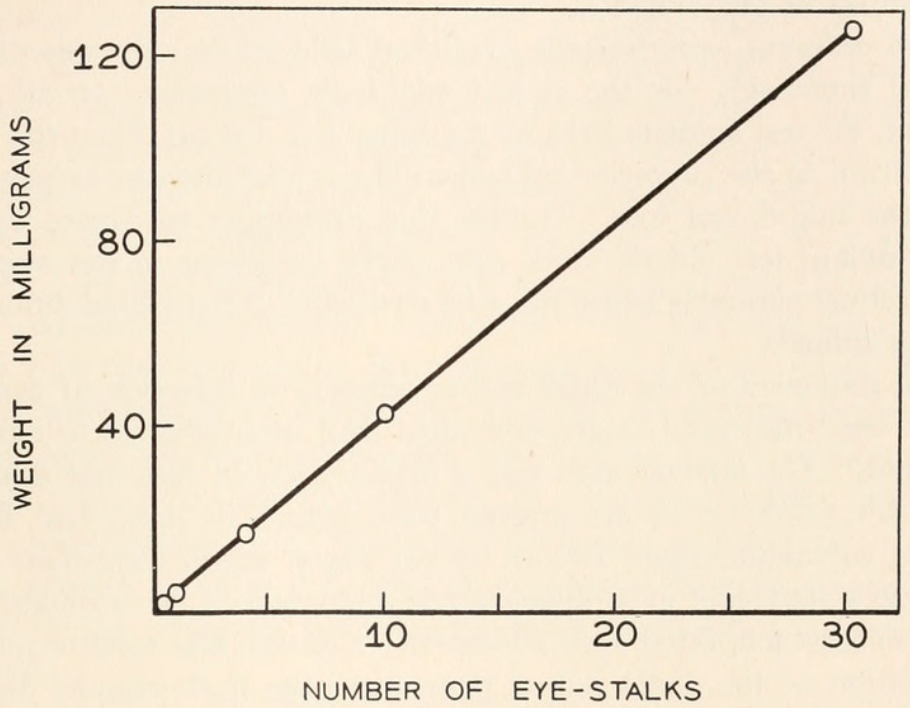


FIG. 1. The relation between the number of eye-stalks and their "wet weight" in milligrams.

Leander adspersus was used in the quantitative studies for the determination of threshold concentrations of hormones for the retinal and integumentary pigments. The procedure was standardized; stalk extracts were prepared from animals which measured exactly 1.9 cm. in the midline from the tip of the rostrum to the posterior margin of the cephalothorax. This proved the most accurate method of assuring the use of eye-stalks of the same size. Earlier attempts to measure the eye-stalk itself were unsatisfactory. That the method used was justified is shown by the accompanying curve (Fig. 1) in which the relation between the number of eye-stalks and their "wet" weight (the stalks were rolled over filter paper to remove excess moisture) is plotted. It

is reasonable to expect that Hanström's sinus gland or whatever glands are responsible for the source of the pigment-activating hormones would show a similar relation to the size and number of eye-stalks used in preparing the extract.

The stalks were excised from animals which had been kept on an illuminated white background, thereby insuring maximum hormone content (Kleinholz, 1936; Abramowitz, 1937*a*), and were triturated in a small mortar with 0.5 cc. of cold-blooded Ringer's solution. The resulting tissue suspension was brought to a boil and Ringer's solution added to make 1.0 cc. of clear extract which could be diluted to desired amounts. Fresh preparations were made for each daily series of experiments. Exactly 0.04 cc. of extract were injected into the abdominal musculature of test animals.

Two different experimental conditions held in the determination of minimal thresholds for the retinal and body pigments. In all cases, however, the test animals were of the same size, 1.6 cm. from the tip of the rostrum to the posterior margin of the cephalothorax, as measured along the mid-dorsal line. Despite this precaution to assure the use of a standard test object, there were slight variations in the responses of the retinal pigments which must be attributed to individual differences between animals.

The responses of the distal retinal pigment to injection of eye-stalk extract were measured in animals which had been adapted to darkness overnight. The method used was a modification of that first described by Welsh (1930*b*). Four prawns were placed in individual 500-cc. beakers, containing about 200 cc. of sea water, which were then transferred to a dark-box in a photographic dark-room. An ordinary compound microscope, fitted with a micrometer ocular, was used to measure the position of the distal retinal pigment in the dark-adapted animals. The source of illumination was a sub-stage microscope lamp over the face of which had been fitted a light-mask with a circular opening 1 cm. in diameter. The intensity of the light was further reduced by using a blue glass filter. Preliminary trials showed that no proximal migration of the distal pigment occurred in the eyes of dark-adapted test animals when they were kept for as long as 20 minutes in the rays of this lamp.

The cell was made of colorless celluloid, 4 inches square, with two openings in the walls to allow circulation of sea water. The bottom of the cell was covered to a depth of 1 cm. with a black paraffin mass, except for a free circular area for light to pass into the microscope and a shallow trough to receive the body of the experimental animal. A prawn could be kept in position, with its head projecting over the opening, long enough for a reading to be made by placing a small glass

slide on the telson and another on the rostrum. After each reading the animal was returned to its beaker.

In determining the minimal threshold of the body pigment to eye-stalk extracts, standard animals from which both stalks had been previously removed were used. Although *Leander* possesses red, yellow and white chromatophores, only the red ones were watched closely in their responses to stalk extract. Observations of the chromatophores were made by means of a binocular dissecting microscope.

Background Responses of Crangon

Koller's studies of chromatophoral activity in *Crangon vulgaris* were the first to present evidence in favor of the multiple hormone hypothesis in the pigmentary behavior of crustaceans. He reported (1927) that blood from dark individuals, when injected into white-adapted *Crangon,* resulted in darkening of the recipients. After cauterizing the rostral region and injecting extracts prepared from tissue of this region (1928) he concluded that an organ in this vicinity was the source of the melanophore-dispersing hormone which he called "expantin." He also confirmed Perkins' results and named the chromatophore-concentrating principle "contractin." Various American investigators (Perkins and Snook, 1931; Kropp and Perkins, 1933; Brown, 1935*a*) were unable to confirm the presence of a chromatophore-dispersing hormone in the crustaceans they studied. Beauvallet and Veil (1934) reported a slight darkening in some cases when *Palaemon squilla* were injected with large doses of rostral extract. This section of the present study is a description of results obtained in an investigation of the reported melanophore-dispersing hormone in *Crangon vulgaris.*

The accompanying Table I is a summary of the responses obtained in these experiments. It is evident that, in normal animals, the melanophore pigment is concentrated on an illuminated white background and maximally dispersed on a black one and in darkness. In individuals whose retinas had been opaqued or destroyed by cautery, the melanophores were always maximally dispersed, regardless of the background on which the animals were kept, resembling in this respect those of shrimps maintained in total darkness. These results eliminate the possibility of a direct response of the melanophores to light and to darkness. The behavior of the melanophores in *Crangon* from which the eye-stalks had been removed is slightly different; instead of the maximum dispersion, such as found in normal shrimps kept in darkness and in individuals with incapacitated retinas, the melanophores of stalk-less animals

were in a condition intermediate between concentration and dispersion. Such individuals, although darker than *Crangon* adapted to white backgrounds, were readily distinguishable from those with incapacitated retinas (Fig. 5).

The white chromatophores behaved quite differently. The guanophores were dispersed in all *Crangon* (normals, individuals with both eye-stalks ablated, and those with incapacitated retinas) which were maintained in an illuminated environment, regardless of the color of the background, and were concentrated in those shrimps kept in total dark-

TABLE I

The responses of the chromatophores of *Crangon vulgaris* to various conditions of illumination. *E.S.*, both eye-stalks ablated; *R.C.*, both retinas cauterized; *R.O.*, both retinas opaqued; *C*, pigment concentrated; *D*, pigment dispersed; *I*, pigment intermediate between concentration and dispersion; *Irreg.*, pigment irregular; (*K*), results obtained by Koller.

Illumination	Condition of animals	Chromatophore pigment	
		Black	White
White background	Normal	*C*	*D*
White background (*K*)	Normal	*C*	*D*
White background	*E.S.*	*I*	*D*
White background	*R.C.*	*D*	*I–D*
White background	*R.O.*	*D*	*D*
Black background	Normal	*D*	Irreg.
Black background (*K*)	Normal	*D*	*C*
Black background	*E.S.*	*I*	*D*
Black background	*R.C.*	*D*	*D*
Darkness	Normal	*D*	*C*
Darkness	*E.S.*	*I*	Irreg.
Darkness	*R.C.*	*I*	*C*
Darkness	*R.O.*	*D*	*C*

ness. Similar results were reported by Perkins (1928) and by Hanström (1937) for *Palaemonetes vulgaris* and by Stephenson (1934) for *Leander serratus*. Stephenson and Hanström explained this as a direct response of the guanophores to light and to darkness, although the latter investigator admitted the possibility of a reflex connection between additional photoreceptors (a persistent nauplius eye, Hanström, 1937) and secondary sources of a guanophore-contracting hormone (Brown, 1935*a*). Experiments designed to test these possibilities have been performed by Mr. F. G. W. Knowles of Oxford University and will be published elsewhere.

The Rôle of the So-called "Rostral Organ" in the Chromatic Physiology of the Melanophores in Crangon

Koller (1927) reported that blood of a black *Crangon,* when "transfused" into one in which the melanophores were contracted, induced dispersion of the melanophore pigment within 30 minutes. This led him to postulate the presence of a melanophore-dispersing hormone, the source of which he later (1928) believed to be an organ in the rostral region.

The results of my experiments to test for the presence of a melanophore-dispersing principle are summarized in Table II. When 43

Table II

Results from injecting blood and control solutions into white-adapted *Crangon. Bb,* blood from black-adapted individuals; *Bw,* blood from white-adapted individuals; *Rb,* extract of rostral region from black-adapted shrimp; *S,* filtered sea-water; *P,* crustacean perfusion fluid (Pantin, 1934). A.M., experiments run in the morning from 9–12 o'clock; P.M., experiments run from 2–10 o'clock in the afternoon and evening.

Substance injected	A.M.			P.M.			Summary		
	Number injected	Number darkening	Number not changing	Number injected	Number darkening	Number not changing	Total injected	Total darkening	Total not changing
Bb.....	19	1	18	24	11	13	43	12	31
Bw.....	24	2	22	30	11	19	54	13	41
Rb.....	15	4	11	23	18	5	38	22	16
S.....	—	—	—	10	8	2	10	8	2
P.....	10	0	10	10	2	8	20	2	18

white-adapted individuals were injected intramuscularly, each with 0.05 cc. of blood from black *Crangon,* only 27 per cent of the recipients darkened to an appreciable extent. In a control series, blood from white-adapted *Crangon* was injected in similar fashion into 54 white-adapted individuals; a like proportion, 22 per cent, darkened. The results indicate that the darkening induced by blood transfer is a non-specific response. This belief gained further support from the following observations: injection of extracts of the rostral regions, prepared from dark individuals, into 38 white-adapted *Crangon* induced darkening in only slightly over half the number of animals; filtered sea water and crustacean perfusion fluid were able in some cases to effect dispersion of melanophore pigment when injected into white-adapted individuals.

An apparent difference in the facility with which dispersion of the melanophore pigment could be induced was noticed between injections

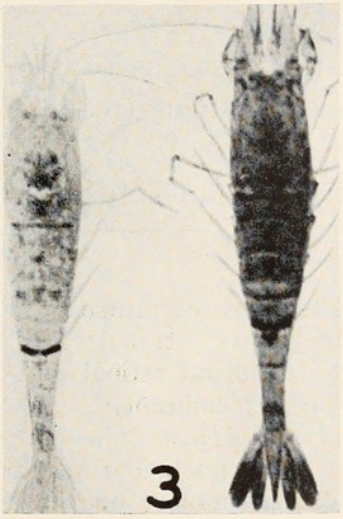
which were made in the morning and those which were performed in the afternoon and evening. No explanation for this situation is offered at present. During the period these experiments were performed (July to September), the pigmentary behavior of animals on the black and white backgrounds was perfectly normal all through the day; if the results from morning and evening injections are due to a cyclical release of some metabolite (or hormone) into the circulation as evening approaches, the normal chromatic behavior of individuals on white backgrounds gave no external indication of such internal changes.

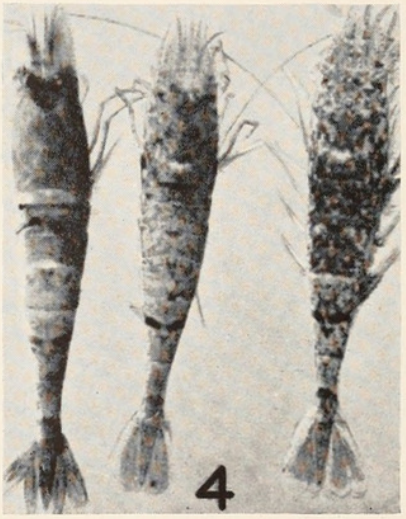
The results of blood transfer in these experiments are thus seen to differ greatly from those reported by Koller. The latter author found that blood from dark individuals when injected into 46 white-adapted *Crangon* induced melanophore dispersion in 95 per cent of the cases, while blood from white-adapted individuals, used as a control, had almost no effect (1927, pp. 237–239).

To locate the origin of the melanophore-dispersing principle, Koller (1928) attempted local destruction of the regions suspected of this endocrine function. He reported that superficial cautery of a delimited area in the rostral region resulted in a permanent loss of the animal's ability to adapt to black backgrounds; *Crangon* operated in this manner remained light in appearance because of a complete concentration of the melanophore pigment.

My attempts to duplicate Koller's experiments were unconvincing in their results. In none of the 16 *Crangon* in which cautery of the rostral region was superficial did the melanophores become permanently and maximally concentrated; within a few minutes after the operation the dispersed melanophore pigment of black-adapted individuals became temporarily concentrated to an intermediate condition, but the pigment granules soon became dispersed again. Different results were obtained by deep cautery of the same region: of 69 shrimps thus treated, 9 became permanently pale, the melanophores being maximally contracted regardless of the fact that the animals were maintained on an illuminated black background (Fig. 4). *In each of these 9 individuals, however, equilibratory and swimming movements were grossly abnormal, the animals performing circus movements when swimming, and resting ventral side uppermost on the bottom of the container.*

The results from these experiments, while partly confirming those of Koller, do not lend ready support to his belief in the presence of two hormones. In the tests described above, the results from blood "transfusions" are seen to be too irregular to accept as evidence in favor of a melanophore-dispersing hormone in the circulation. The total ineffectiveness of superficial cautery of the rostral region, and the





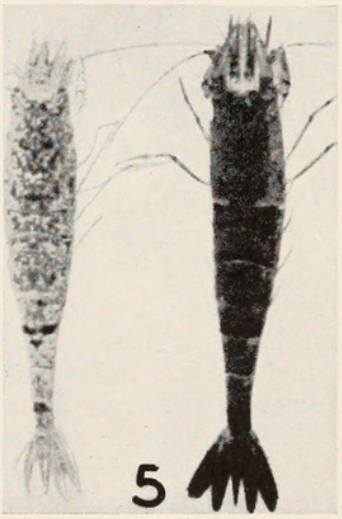


PLATE I

The color reactions of *Crangon vulgaris*.

FIG. 2. Extract was prepared by triturating the rostral regions from 10 black *Crangon* in 1.0 cc. of filtered sea water; 0.05–0.07 cc. were injected into each of 10 white-adapted shrimps at 9:00 P.M. One hour later the maximum effect was recorded by this photograph.

FIG. 3. The normal color responses to white (on the left) and black (on the right) backgrounds.

FIG. 4. The two animals on the left were individuals in which the rostral region had been deeply cauterized, resulting in permanent pallor; both eye-stalks had been removed from the shrimp on the right, resulting in an intermediate condition of the melanophores.

FIG. 5. Both eye-stalks were removed from the animal on the left. Both retinas were destroyed by cautery in the specimen on the right; as a consequence the melanophores were completely dispersed.

fact that deep cautery in the same region resulted in only 13 per cent positive results must also be regarded with caution. The few cases of "successfully" cauterized animals were abnormal in their equilibrium and swimming movements, indicating injury of the central nervous system possibly accompanied by interference in the regulation of Hanström's sinus gland.[6] More critical evidence will be needed before the existence of a melanophore-dispersing hormone, originating in the rostral region, can be unqualifiedly accepted.

Persistent Cyclic Activity in the Pigmentary System of Hippolyte [7]

As mentioned briefly in the introductory section, the existence of persistent cyclic activity in the pigmentary system of various crustaceans when the individuals are maintained under constant environmental conditions, presents an opportunity to subject to critical test whether one hormone or more than one hormone is involved in the regulation of these effectors. Gamble and Keeble (1900) first reported a periodic contraction of the chromatophores in *Hippolyte varians* when these animals were maintained under constant conditions of illumination. It was decided to test the validity of the unitary hormone hypothesis, as presented by Abramowitz (1937*b*), by examining the positions of the retinal pigments in this animal under the various experimental conditions previously used in this procedure (Welsh, 1935; Kleinholz, 1937*b*). According to the unitary hormone hypothesis, the threshold for the

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PLATE II

Ommatidia from the eyes of *Hippolyte* to show the positions of the retinal pigments. *DL*, "day light" eye; *DD*, ommatidium from "day dark" retina; *NL*, "night light" retina; *ND*, unit from a "night dark" eye; *D*, distal retinal pigment; *R*, reflecting pigment; *P*, proximal pigment; *B*, basement membrane.

Figs. 6 to 9. Ommatidia from the retinas of *Hippolyte varians*. The pigments are in the positions characteristic for adaptation to light and to darkness; there is no persistent diurnal rhythm. Note that the reflecting pigment migrates in two directions in the light-adapted eye; in one, it forms a small cap above the distal retinal cells and in the other it moves below the basement membrane.

Fig. 10. "Day light" eye of *Hippolyte pleuracantha*. The three retinal pigments are in the typical light-adapted positions. The reflecting pigment is different from that in most prawns hitherto studied in that it has migrated almost wholly above the distal retinal cells instead of below the basement membrane.

Fig. 11. "Day dark" eye showing the persistence of diurnal rhythm. The reflecting and proximal pigments are in the dark-adapted position but the distal retinal cells remain in the position normal for a light-adapted retina.

Figs. 12 and 13. "Night light" and "night dark" ommatidia. These show the ordinary responses to light and to darkness.

[6] Bethe (1898) and Roeder (1937) have shown that the supraesophageal ganglion in arthropods exercises an inhibitory influence on many reflex activities.

[7] A preliminary report describing the results of part of the material included under this section has been published in collaboration with Dr. J. H. Welsh (1937).

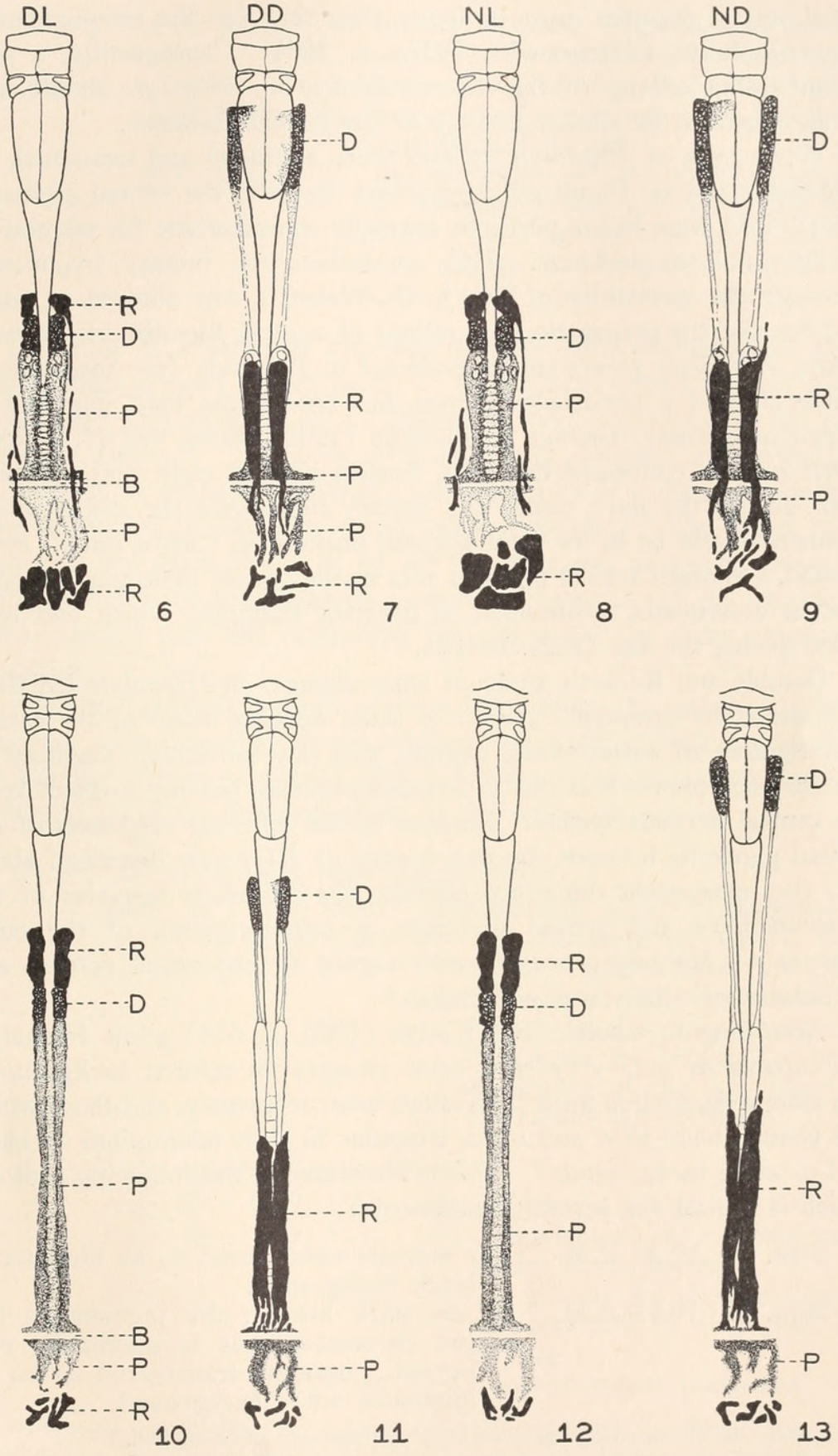
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PLATE II

distal retinal pigment must be lower than that for the integumentary chromatophores (Abramowitz, 1937*a*, p. 356). Consequently, a persistent cyclic activity of the chromatophores in *Hippolyte* should also be accompanied by similar activity of the retinal pigments.

When eyes of *Hippolyte varians* were sectioned and examined, no evidence could be found of a persistent rhythm; the retinal pigments (Figs. 6–9) were in the positions normally characteristic for adaptation to light and to darkness. This contradicts the unitary hypothesis. Through the generosity of Dr. J. H. Welsh, I was allowed to study and describe his preparations of retinas of another member of the same genus, *Hippolyte pleurocantha,* collected in Bermuda (on *Sargassum*). These showed a persistent rhythm, but not of the kind expected in *Hippolyte varians;* Gamble and Keeble (1900) stated that *H. varians,* when kept in continued darkness, became pale at night and remained dark during the day; under the unitary hypothesis the distal retinal pigment should be in the light-adapted position in "night dark" eyes; instead, the distal retinal pigment was in the typical light-adapted position in individuals, maintained in constant darkness, which had been killed during the day (Figs. 10–13).

Gamble and Keeble's study of color changes in *Hippolyte* led them to conclusions essentially similar to those held by many of the earlier investigators of metachrosis, namely, that any particular condition of the chromatophores was due to nervous impulses passing to them from the central nervous system. Because of the differing responses of the retinal pigments between the two species of *Hippolyte* described above and the unexpected difference between the periodical behavior of the integumentary and retinal pigments, a reinvestigation of the color changes of *Hippolyte varians* with regard to endocrinal control and persistent periodicity was undertaken.[8]

According to Gamble and Keeble (1900, p. 614) adult *Hippolyte* are capable of only very slow color changes on colored backgrounds. On close examination most individuals were refractory, and those which did change were slow and often irregular in their adaptations to black and to white backgrounds.[9] This is illustrated in the following protocol which is typical for several experiments:

Sept. 1.	10.30 P.M.	Four animals were placed on an illuminated black background.
Sept. 2.	10.45 A.M.	All are dark brown; the pigment in the red chromatophores is maximally dispersed. Animals transferred to an illuminated white background.

[8] Only the chromatophores containing red pigment were studied.

[9] Minkiewicz (1908), however, reported that *Hippolyte varians* underwent adaptive color changes in response to changes in background.

12.10 P.M. One animal is green (chromatophores are punctate); the remaining three are light brown (chromatophores are dispersed).
4.00 P.M. All four prawns are brown with the chromatophores dispersed.
10.15 P.M. All four are yellowish-olive in color but the red chromatophores are maximally dispersed. The lighter tint must be due to a change in the yellow chromatophores which can't be seen clearly.

Injection of crustacean eye-stalk extracts into normal dark *Hippolyte* effects a rapid concentration of the pigment within the red chromatophores, accompanied by a diffusion of blue pigment into the surrounding tissues.[10] The same chromatophoral response can be elicited by injection of extracts prepared from the stalks of a different species; doses of 0.01–0.03 cc. of a preparation containing 5 eye-stalks from *Hippolyte* triturated in 0.2 cc. of sea water as well as similar doses of a preparation of 2 eye-stalks of *Leander serratus* in 0.5 cc. sea water, when injected into test *Hippolyte,* brought about the same chromatic condition.

Normal *Hippolyte,* as well as those from which the retinas had been removed, were examined to see whether the cyclic activity reported by the English authors persisted. The following summaries show that this nocturnal condition is independent of the retinal receptors:

A. Normal individuals with intact retinas

Sept. 9. 5.30 P.M. Nine animals placed in darkness.
10.45 P.M. All are complete or partial nocturnes. In the latter the chromatophores are punctate or slightly stellate in the anterior half up to the "hump" or point of flexure of the tail; those of the telson are dispersed.

B. Animals with both retinas removed

Aug. 27. 11.30 P.M. Seven prawns placed in darkness.
Aug. 30. 5.00 P.M. All are nocturnes, the red chromatophores being punctate.
Sept. 1. 12.30 A.M. In five prawns the chromatophores are punctate, in two they are stellate.
10.00 P.M. All 6 survivors are nocturnes.

[10] Gamble and Keeble (1900) have called this chromatic condition "nocturne" because they found it appearing in normal animals each evening as darkness fell.

In all reports dealing with metachrosis in the macruran crustaceans, removal of both eye-stalks has resulted in a permanent darkening of the animals due to the dispersion of the chromatophoral pigments; this results because of loss of the sinus glands, the source of the chromatophore-concentrating principle. It therefore follows that, if the nocturnal color state is effected by release of the eye-stalk hormone, removal of both eye-stalks should prevent the appearance of this chromatic condition in animals thus operated. The following summary shows such an assumption to be incorrect:

C. Animals with both eye-stalks removed

Aug. 30.	10.00 A.M.	One eye-stalk removed from each of 10 *Hippolyte;* animals placed in darkness.
	11.15 P.M.	All 10 are nocturnes; the red chromatophores are punctate.
Aug. 31.	11.00 A.M.	The second eye-stalk removed from each; 7 survivors were returned to the dark box.
Sept. 1.	12.15 A.M.	3 are nocturnes; chromatophores are punctate.
		2 are light brown; chromatophores are intermediate.
		2 are brown; chromatophores dispersed.
	10.00 P.M.	In 3 the chromatophores are punctate; in 3 they are intermediate; and in 1 they are dispersed.
Sept. 2.	10.25 P.M.	4 are partial nocturnes as in "B" above; in 1 the chromatophores are intermediate; and in 2 they are irregular between the intermediate and dispersed state.

Brown (1935*a*) found that injection of extracts of the central nervous system (exclusive of the eye-stalks) concentrated the chromatophoral pigments and has cited the persistence of the chromatic rhythm in *Hippolyte varians* from which both eye-stalks have been removed as additional proof of the existence elsewhere than in the eye-stalks of glandular tissue capable of secreting the chromatophore-concentrating principle. At present this view is not conclusively supported by experimental evidence. For, in spite of Brown's results and the report by Hanström (1937) that a persistent nauplius eye is present in *Palaemonetes* (thus presenting the possibility of a functional photoreceptor with reflex connections to a source of the chromatophore-concentrating principle), there is no positive physiological proof that such a system

is able to function. In fact, the available evidence contradicts this possibility; *Palaemonetes* from which both eye-stalks have been removed lose their ability to adapt to changes from black to white backgrounds and remain permanently dark because of the dispersion of their chromatophoral pigments.

It is not necessary to consider the results presented in the above summaries on *Hippolyte* as due to a functional reflex between photoreceptors and glands; the nocturnal coloration of *Hippolyte* can be more simply explained as due to a direct effect of darkness on the pigment cells. Thus, these prawns would be dark in color when illuminated and would be nocturnes when placed in darkness. Were it not for the persistent cyclic activity of the chromatophores described by Gamble and Keeble, the accounts of chromatophoral behavior presented above would confirm this point. Normal *Hyppolyte* and individuals whose retinas and eye-stalks had been removed were therefore placed in darkness and examined several times during the day to eliminate the effects due to the reported periodic phenomenon. The chromatic condition of the operated and normal animals was always the same in individuals which had been kept in darkness; the prawns were either total or partial nocturnes with the chromatophoral pigments concentrated. When such pale animals were removed from the dark-box and placed in diffuse sunlight or under the rays of a 60-watt electric lamp they became completely dark within 10 minutes; microscopic examination revealed maximum dispersion of the chromatophores. These results indicate that the integumentary chromatophores of *Hippolyte* can respond directly to changes in light-intensity. Gamble and Keeble's observations of persistent cyclic activity of these effectors are not confirmed. Their report that these prawns remained dark during the day when kept in darkness and paled only as night fell seems probably due to the fact that light conditions in their experiments were not constant.[11]

Threshold Concentrations of Eye-Stalk Hormone for Retinal and Integumentary Pigments in Leander adspersus

The method of analysis used with *Hippolyte* having proved inconclusive, it was decided to measure actual threshold concentrations of the eye-stalk extract for the retinal and integumentary pigments. The standardization of the procedure has been described under "Methods." The data for the responses of the distal retinal pigment to various con-

[11] To obtain the condition of constant darkness they covered the vessels in which their animals were confined with a double thickness of black cloth; these containers may not have been completely light-proof.

centrations of eye-stalk extracts are arranged in Table III and are plotted in the curves shown in Fig. 14. It can be seen that injection of 0.04 cc. of an extract containing 0.1 eye-stalk in 1.0 cc. of Ringer's solution is one which gives a slightly perceptible response of the distal retinal pigment. More dilute extracts and control injections of Ringer's solution effected no discernible migration of this pigment over a period

TABLE III

The responses of the distal retinal pigment in animals injected with various concentrations of eye-stalk extract. The concentration is expressed in the fraction or number of eye-stalks in 1.0 cc. of cold-blooded Ringer's solution; the distance migrated in micra is the average for the total number of eyes studied.

Conc. of extract	0.1	0.2	0.5	1.0	4.0	10.0	15.0
No. measured	16	12	18	12	6	6	6
Minutes after inj.							
0	0 μ	0 μ	0 μ	0 μ	0 μ	0 μ	0 μ
15	19	15	40	79	87	73	100
30	22	46	73	104	150	137	—
35	—	—	—	—	—	—	156
45	17	41	73	100	159	—	171
50	—	—	—	—	—	171	—
60	14	36	49	96	139	—	—
65	—	—	—	—	—	—	170
75	14	22	28	90	132	—	—
80	—	—	—	—	—	174	—
90	—	21	28	61	105	—	—
95	—	—	—	—	—	—	152
105	10	18	27	36	66	—	—
120	—	—	24	24	57	—	—
125	—	—	—	—	—	109	—
135	—	—	14	—	—	—	112
140	—	—	—	—	13	—	—
155	—	—	—	—	—	61	—
165	—	—	5	—	—	—	73
195	—	—	—	—	—	—	59

of 30 minutes. This concentration of stalk extract can therefore be labelled the minimal effective dosage for the distal retinal pigment. This is equivalent to 0.016 mg. (wet weight) of eye-stalk.[12]

[12] Abramowitz and Abramowitz (1938, p. 281), after making several assumptions, find the effective dosage for the melanophores of *Uca* to be 0.000016 gamma of hormone. By making the same assumptions the minimal threshold for the distal retinal pigment of *Leander* is 0.0008 gamma and that for the red chromatophores is 0.00004 gamma. It is probably preferable, however, in describing effective dosages, to express these in terms of dry weight of eye-stalk.

The curves show that with increasing concentration of stalk extract the amount of migration of the distal retinal pigment increases until a maximum is reached (between 4 and 10 eye-stalks per 1.0 cc.). This is the maximum threshold concentration; although more concentrated extracts do not affect the amount of migration of the distal retinal pigment, there is some indication that "recovery" or return of distal pigment cells to the position characteristic for the dark-adapted eye is

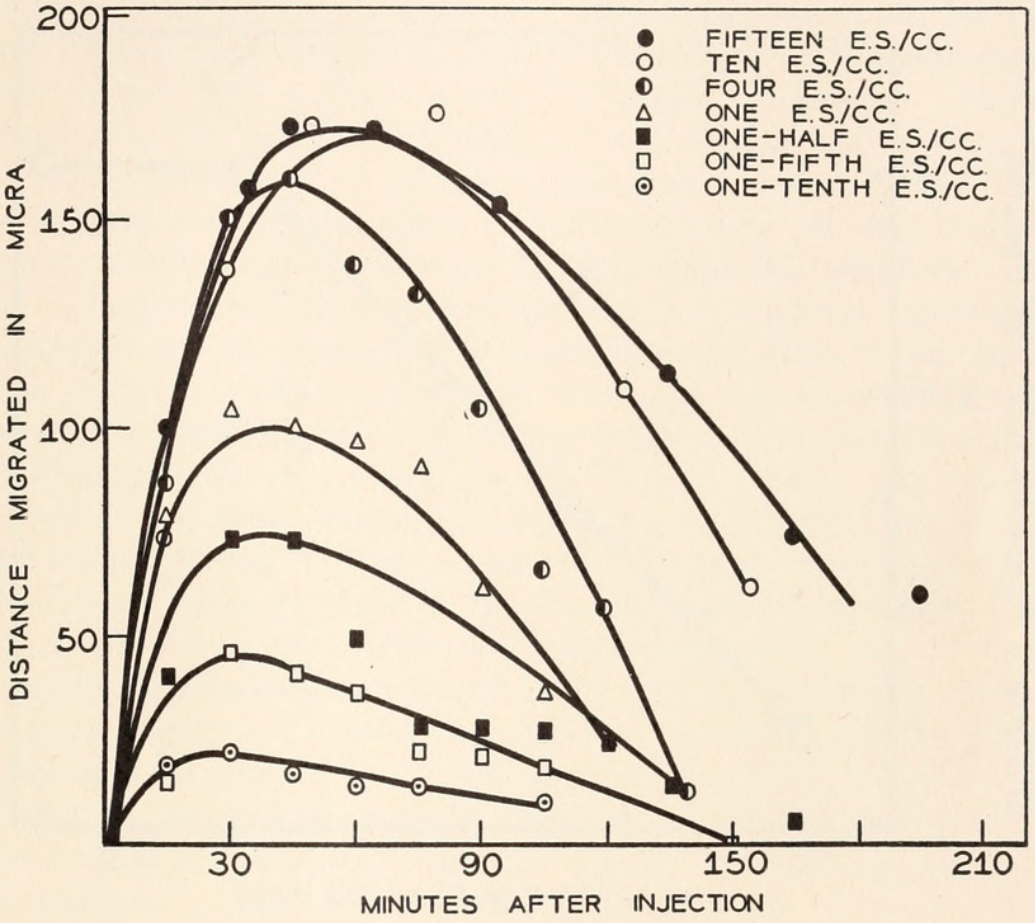

FIG. 14. The rate of migration of the distal retinal pigment cells in dark-adapted *Leander adspersus* in which have been injected various concentrations of eye-stalk extract. The distance migrated was measured from the cornea to the distal margin of the pigment cells.

further delayed; this situation is probably dependent on the rate of elimination or destruction of the injected hormone.

The relation between amount of migration of the distal retinal pigment and the concentration of the injected eye-stalk extract is shown better in Fig. 15, where the distance migrated in micra (obtained from the curves in Fig. 14) is plotted as a function of the logarithm of the concentration, 30 minutes after injection. At this (and at a 45-minute)

interval the relation is a direct proportion until the maximum migration is attained at the maximum threshold concentration, the effect of supra-maximal concentrations being reflected in the levelling-off of the curves. This is in agreement with the known observations (Welsh, 1930*b*; Kleinholz, 1936) that migration of the distal retinal pigment to the light-adapted position requires between 30 and 45 minutes. A direct proportion is also shown when similar curves are drawn for intervals 15 and 75 minutes after injection.

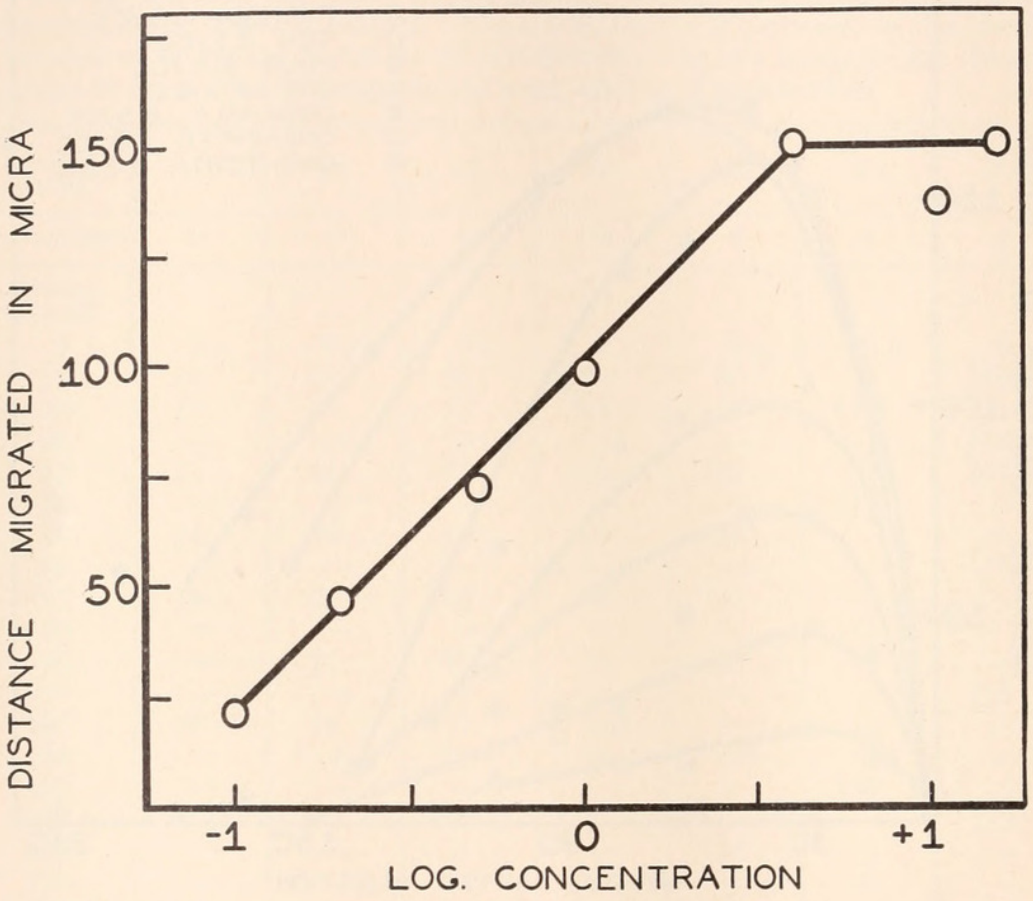

FIG. 15. The relation between migration of the retinal pigment and the concentration of the injected stalk-extract. The values for the position of the pigment were calculated from Fig. 14.

In determining the minimal threshold of the integumentary chromatophores, groups of 5 standard prawns which had been blinded a week previously by amputation of both eye-stalks were each injected with 0.04 cc. of the prepared stalk-extracts. With concentrations of 1.0, 0.5, and 0.2 eye-stalks per 1.0 cc. there was maximum concentration of the chromatophoral pigment within 5 minutes after injection, accompanied by the appearance of the characteristic diffuse blue coloration; the erythrophores of animals treated with these extracts remained

punctate for 1 hour or more before the stellation indicative of approaching pigment dispersion was discernible. When concentrations of 0.05, 0.02, and 0.01 eye-stalks per 1.0 cc. were used, the chromatophores became punctate within 5 minutes but darkening began between 30 and 40 minutes after injection in each of the 5 test prawns in a series. In the final series 5 *Leander* (both eye-stalks amputated) were injected standard doses of extract containing 0.005 stalks per 1.0 cc. of Ringer's solution; ten minutes after injection the chromatophores in 2 of the prawns were completely punctate, in 1 animal they were variable between punctate and stellate, and in the remaining two the chromatophores ranged from partial stellation to complete dispersion. Thirty minutes after the injection darkening of the animals was evident; at the end of 1 hour four of the five animals were dark while the fifth was still intermediate in color. Control injections of cold-blooded Ringer's solution had no effect on the chromatophoral pigments. Injection of extracts of this concentration into normal prawns maintained in darkness had no discernible influence on the position of the distal retinal pigments. This concentration (0.005 eye-stalks per 1.0 cc.) is equivalent to 0.0008 mg. wet weight of eye-stalk and represents the minimal threshold for the integumentary chromatophores. The lower threshold of the chromatophores is thus about one-twentieth that of the distal retinal pigment.[12] These values do not support the unitary hormone hypothesis which requires that the threshold for the retinal pigment be lower than that for the body chromatophores.

Additional physiological evidence shows that the retinal and integumentary effectors react differently to darkness. In most macruran crustaceans thus far studied, the retinal pigments of animals maintained in darkness are found in the position characteristic for that state (except in those cases where a persistent cyclic activity affects one or more of these pigments); this is also true of *Leander adspersus*. In total darkness, however, the red chromatophores of this prawn are completely punctate and the animal is pale in appearance; similar chromatophoral behavior was noticed with *L. serratus*. This response is not a direct effect resulting from the exclusion of light, for individuals from which both eye-stalks had been removed and which had darkened in consequence remained unchanged in color when kept in total darkness. The concentrated state of the red chromatophoral pigments in darkness is therefore dependent upon release of the chromatophore-contracting principle from the eye-stalks. The importance of this situation is fully recognized by Abramowitz (1937*a*, p. 363): "The key to the whole situation lies in the behavior of the red and yellow chromatophores in animals maintained in darkness. If Brown is correct in noting that

the more usual condition of the red and yellow pigments of animals maintained in darkness was slight dispersion, and that a long sojourn in darkness resulted in the same condition of these pigments as that occurring when animals were adapted to a red background, there is little reason for postulating separate autocoids for the body and retinal pigments. . . . If the earlier observations of Perkins that the red and yellow pigments are contracted in darkness is correct, the existence of separate hormones would be clearly indicated.[13]

When the responses of the retinal pigments during their persistent cyclic activity are analyzed (Kleinholz, 1937*b*, Table I), no conclusions on the number of hormones concerned in regulating the activity of these effectors can be made. The responses are such that either more than one hormone is involved, or that there is one hormone to which the retinal pigments react independently because of variations in threshold levels. If the latter case is true, then there is no constancy in the relative reactivity of these pigments in different species.

From similar evidence, Brown (1935*a*, 1935*b*) has presented the argument in favor of the multiple hormone hypothesis. The same criticism, namely that the independent responses of the various integumentary chromatophores may be due to different threshold levels to one or two hormones, can be applied to Brown's observations. Brown's explanation has been criticized by Abramowitz (1937*b*), who was led by the results from a study of the comparative physiology of crustacean metachrosis to advocate the unitary hormone hypothesis.

The data presented in the above sections conclusively show the inadequacy of the unitary hormone hypothesis in explaining the behavior of the retinal and integumentary pigmentary effectors. It is clear from the experimental observations that at least two hormones are necessary to reconcile the known facts. The problem as to the actual number of hormones involved in regulating the crustacean pigmentary effectors is far from settled. It can be solved only when these substances have been prepared in pure form and their action on the various components of the pigmentary system tested.

Summary

1. Experiments were performed to determine whether one or more than one hormone is involved in the control of the crustacean pigmentary system.

[13] Brown (1935*a*) and Perkins (1928) studied *Palaemonetes vulgaris*. Hanström (1937), using the same species, confirmed Perkins' observations of the conditions of the chromatophores in animals kept in darkness.

2. Transfusions of blood from black- and from white-adapted individuals into white-adapted *Crangon* result in the darkening of an equally low percentage of the test animals. Injection of rostral region extract produced darkening in slightly over 50 per cent of the injected animals.

3. Superficial cautery of the rostral region has no permanent effect on color mutability. Deep cautery in 69 animals resulted in 9 individuals which became permanently pale. In each of these 9 *Crangon,* however, swimming and equilibratory movements were abnormal. It is suggested that the deep cautery injured the supraesophageal ganglion, resulting in interference with the regulation of Hanström's sinus gland.

4. The validity of the unitary hormone hypothesis was subjected to a biological test by a study of the retinal and integumentary pigments in *Hippolyte* which has been reported to undergo a diurnal rhythm in the activity of the integumentary effectors.

5. No evidence of persistent activity could be found in the retinal pigments of *H. varians;* the eyes of *H. pleuracantha* showed a persistent cyclic rhythm of the distal retinal pigment, but this was not in the phase expected in *H. varians.*

6. Further study showed that the reported periodicity of color change in *H. varians* was due to a direct effect of darkness on the body chromatophores.

7. The threshold limits of the retinal and integumentary pigment cells to eye-stalk hormones were determined in *Leander adspersus.* The lower threshold limit for the distal retinal pigment was found to be equivalent to 0.016 mg. (wet weight) of eye-stalk; that for the integumentary pigment was 0.0008 mg. These values do not support the unitary hormone hypothesis which requires that the minimal threshold for the retinal pigments be lower than that for the body chromatophores.

8. The responses of the distal retinal pigment to various concentrations of stalk extract are plotted in curves which show a direct relation between the response and the injected dosage.

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