THE CHROMATOPHOROTROPIC HORMONE OF THE CRUSTACEA: STANDARDIZATION, PROPERTIES AND PHYSIOLOGY OF THE EYE-STALK GLANDS [1]

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STANDARDIZATION

The need for a method of determining quantitatively the amount of hormone in a given extract of a gland is obvious in chemical or quantitative physiological work dealing with endocrine systems. Although the existence of such a system for the pigmentary effectors of crustaceans has been known for almost ten years, no reliable method for the standardization of the eye-stalk hormone has been devised. Navez and Kropp (1934), and Kropp and Crozier (1934) suggested that the eye-stalk hormone may be standardized by the *Avena* coleoptile method since they found that the eye-stalk extract accelerates the growth rate of *Avena* coleoptiles. These authors employed water extracts of the eye-stalks of *Palæmonetes*. Since many other substances in addition to the chromatophore hormone are present in a water extract of the eye-stalks, it is not certain that the growth effects on plants are due actually to the chromatophore hormone. On the basis of biochemical evidence, Carlson (1936) suggested that the growth reactions of plants to the eye-stalk extract was due to the presence of some substance other than the chromatophore hormone. If this is true, it would then appear that Kropp and Crozier have not been measuring the chromatophore hormone but possibly some other substance present in a water extract of the eye-stalks.

Consequently, it was felt that the most reliable way of assaying the chromatophore hormone would be to measure the response of the tissue (the chromatophores) which this hormone normally affects.[2] A convenient laboratory animal on which a method of assay may be devised is the fiddler crab, *Uca*. In the vicinity of Woods Hole, two species, *U. pugilator* and *U. pugnax*, are abundantly present. Both these spe-

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[2] Cf. Burn, J. H., 1930. *Phys. Rev.*, **10**: 146.

cies contain black, red, white and yellow integumentary chromatophores. Only occasionally, however, are red chromatophores observed in *U. pugnax*. The extreme color change of *U. pugnax* ranges from jet black to pale yellow (Figs. 3, 4) and of *U. pugilator* from dark brown to cream white (Figs. 5, 6). The melanophores are the chief agents of this change although the movements of the other pigments are instrumental in producing the end result. Megašur (1912) demonstrated that extirpation of the eye-stalks of *Uca* resulted in the production of the pale phase of its color change due to melanophore contraction. Carlson (1935, 1936) confirmed this observation and interpreted it as an effect specifically due to the loss of the endocrine glands contained in the eye-stalks. An extract of the extirpated eye-stalks when injected into a pale (blinded) animal produced the dark phase resulting from melanophore expansion. Carlson showed further that if 0.1 cc. of a solution containing the extract of 1 eye-stalk of *Uca* in 1 cc. of water was injected into a blinded [3] crab, the melanophores became expanded within 2 hours, remained expanded for about 4 hours, and finally contracted so that the animal resumed the pallor previous to injection. This is specifically a hormonal reaction. There appears to be no other way whereby the melanophores of a blinded *Uca* may be induced to expand.

Preliminary Experiments

The statements of Megašur and Carlson concerning the effects of blinding and of injection of eye-stalk extracts were first confirmed (Figs. 3, 4, 5, 6). The typical response of a blinded animal following the injection of an extract of its eye-stalks in a dose equivalent to 1/20 of a single stalk may be conveniently divided into four phases:

(*a*) The first perceptible response.

This response is evidenced by the beginning of melanophore expansion and occurs from 15–20 minutes after the injection has been made.

(*b*) The attainment of the maximal effect.

After the first perceptible response has occurred, the melanophores become maximally expanded within one hour.

(*c*) Duration of melanophore expansion.

Following the attainment of the maximal effect, the melanophores remain fully expanded for about 3¼ hours.

(*d*) The period during which the melanophores contract again.

Four and one-half hours following the time of injection, the

[3] The term *blinded* will be used throughout this paper to designate the condition of specimens whose eye-stalks have been completely removed.

melanophores begin to contract, and within the following half-hour, the animal becomes again pale, so that the melanophores are in the same condition as that previous to the injection.

Preliminary experiments were therefore designed to determine the relation between each of the four phases of the response and concentration of hormone. A sea water extract was prepared from 280 eye-stalks of *Uca pugnax* and a series of the ten following dilutions was made: 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.015 E.S.[4] per cc. of solution. Several hundred specimens of *Uca pugnax,* blinded two days previously, were employed as test animals. All animals were of equal size and weight (3.5 grams). The injection volume in all experiments was 0.1 cc. Fifty animals in groups of 5 constituted the experimental material for each test. The tests were carried out by injecting each group of 5 animals with but one of the 10 dilutions so that each animal in the first group received a dose of 0.8 E.S. in the second group 0.4 E.S., in the third 0.2 E.S., etc. It was found that the time at which the first perceptible response occurred was independent of the concentration of hormone. In all ten groups the first signs of melanophore expansion occurred 15–20 minutes following the time of injection, although at the two lowest concentrations (0.0015 E.S. and 0.003 E.S.) this time was slightly increased. The attainment of the maximal effect was likewise found to be independent of concentration when concentrations higher than 0.003 E.S. were used. Concentrations below this value did not produce the maximal response. The duration of melanophore expansion, however, was found to be proportional exponentially to the concentration of hormone employed (doses above 0.003 E.S.). The time during which the melanophores became contracted again was, like the first two phases of the response, independent of the concentration.

The significant point of these experiments is the demonstration that the duration of melanophore expansion varies as a function of concentration. The times for the completion of the other three phases of the response are for all practical purposes constants, independent of concentration provided the doses employed are greater than 0.003 E.S. In order to measure the response of the animals to a given concentration, it is therefore necessary to determine only the time of injection and the time at which the animals become again pale. The difference in these two readings is obviously the duration of the response plus 1.75 hours,

[4] The letters E.S. will be used throughout this paper as an abbreviation for the extract equivalent to a given number of eye-stalks. For example, 1 E.S. indicates that the extract is equivalent to that obtainable from one eye-stalk; 0.5 E.S. denotes an extract equivalent to half that obtained from one eye-stalk, etc.

which may be used as a constant equal to the sum of the times of the other three phases of the response.

Variables in the Method of Assay

There are several important variables which must be controlled completely if such a method of assay is to be used.

(1) *Physiological Uniformity of the Responsive Tissue.*—The black chromatophores were used as the sole criterion in obtaining readings since they are the chief instruments in determining the appearance of the animal with respect to paleness or darkness. In blinded specimens of *Uca* the pigment in all melanophores of the appendages and body is uniformly and extremely concentrated, and remains so indefinitely regardless of environmental conditions which would provoke expansion in a normal animal. Thus it appears certain that the responsive tissue in all test animals is in a physiologically uniform state.

TABLE I

Size of eye-stalk in relation to hormone concentration

Size of Eye-stalk	Injected Dose	No. Tested	Average Weight of Test Animals	Average Response
			grams	*hours*
Small (animal weight, 1.7 grams)	0.025 E.S./cc.	8	3.5	3.75
Medium (animal weight, 2.8 grams)	0.025 E.S./cc.	8	3.6	4.09
Large (animal weight, 5.1 grams)	0.025 E.S./cc.	8	3.5	4.88

(2) *Concentration of the Injected Hormone.*—This variable is easily controlled since the method of preparing the extract unless otherwise stated was maintained constant. The eye-stalks were cut off and ground in a small mortar in the amount of sea water necessary for any particular concentration. This extract was brought to a boil so that a coagulum (presumably the tissue proteins) formed. The solution was filtered, and the filtrate made up by the addition of unboiled sea water to the desired concentration. About 0.6 cc. of the sea water was usually lost during the process of boiling and filtration. This resulted in a slightly hypertonic extract, but this factor is immaterial for the purpose of the experiment (cf. Carlson, 1936). The solution was allowed to cool to room temperature, usually about one-half hour elapsing, and the injection made. While the concentration of the hormone in terms of numbers of eye-stalks can be controlled quite accurately, the size of the eye-stalks used in making an extract of a certain concentration could not be controlled so easily. Larger eye-stalks may contain large glands and hence more hormone, as shown by Table I. To control this factor,

the extracts were prepared from eye-stalks of animals of a definite and uniform size.

(3) *Size of the Test Animal.*—This variable proved to be a very disconcerting factor at the beginning of the work. It was reasonable to suppose that large animals would show a smaller response to a given dosage than small animals because they would contain a greater amount of the responsive tissue, and because the hormone would be subjected to greater dilution. That this is plausible is shown by Table II.

Consequently, in order to avoid the use of a curve for the calibration of response as a function of the size of the test animals, assay experiments were always carried out on animals of uniform weight.

(4) *Individual Variation.*—This variable is probably the most significant of those already mentioned because it is not easily controlled. Since it is almost impossible to carry out all experiments on the same animal, the best method would be to use sufficient numbers of animals

TABLE II

Size of test animal in relation to response

Hormone Concentration	No. of Animals Tested	Average Weight	Average Response
		grams	*hours*
1.0 E.S./cc.	10	4.36	4.13
1.0 E.S./cc.	6	3.86	5.56
1.0 E.S./cc.	10	1.55	7.83

and to treat the data statistically. In performing an assay, from 15 to 20 animals of the same size and weight were injected with the same volume and concentration of hormone and the average response taken. The standard deviation of the arithmetic mean was calculated and determinations made to see if the differences in the response at different concentrations were really significant.

(5) *Volume of Injected Dosage: Place of Injection.*—Several experiments made to determine the most suitable volume of hormone to be injected led me to adopt finally 0.05 cc. as the standard injection volume. Carlson used 0.1 cc. but while this is satisfactory for *U. pugnax* and very large specimens of *U. pugilator,* it seemed to be too great a dose for the size of crabs employed in most of my experiments. The most convenient region for the injection of the hormone into the body spaces was found after many trials on different regions of the body to be through the soft tissue forming the joint between the coxipodite and the protopidite of the walking legs. This method allows for speed and

accuracy of injection for, using a 27-gauge hypodermic needle, one can inject 20 crabs within 2 minutes.

Method Finally Adopted

Upon the basis of these preliminary experiments, the standardization of the eye-stalk hormone was completed using blinded *Uca pugilator* [5] as a test animal. One hundred and fifty such specimens, whose eye-stalks were extirpated two days previously, were arranged in ten groups of 15 animals each. All animals were of uniform size and weight ($2.46 \pm .01$ gram). A stock solution of hormone was made by extracting the extirpated eye-stalks as previously described. A series of 10 concentrations ranging from 5 E.S./cc. to 0.03 E.S./cc. was prepared. Five-hundredths of a cc. of these concentrations was used as the injection volume in all cases. Each of the 15 animals in a particular group received 0.05 cc. of a particular concentration, so that each of the ten groups of animals was injected with but one of the ten various known concentrations. The difference between the time of injection and time at which the animals became pale again was noted. The results of this experiment are shown by Fig. 1. The experiment was repeated five times, and although the points obtained did not fit the curve as well as those of Fig. 1 do, it was generally true that the magnitude of the response was exponentially proportional to concentration.

It can be seen from Fig. 1 that the relation between the complete response of the melanophores and the concentration of hormone is least sensitive over the range of concentrations above 1.0 E.S./cc. and most sensitive over the range from 0.06 E.S./cc. to 1.0 E.S./cc. This sensitive range is re-plotted in Fig. 2, which shows that there is a linear relationship between the duration of melanophore expansion and concentration of hormone.

Figure 2 was therefore used to establish the *Uca* unit. Two possibilities are open. One can choose an arbitrary response near the midpoint of this curve and designate this value as one *Uca* unit. Assays would therefore be made by repeated dilutions of an unknown until the resulting response equals that chosen to represent one *Uca* unit. A second possible method would be to inject 0.05 cc. of an unknown concentration, obtain the average response and read off from Fig. 1 the concentration in terms of *Uca* E.S./cc. which gives the same response. The latter procedure was adopted because both amount to the same thing provided the unknown concentration falls within the sensitive

[5] *Uca pugilator* was substituted for *U. pugnax* as the standard test animal because it is much more uniform in weight and size and because the rate of mortality and the frequency of autotomy are much lower than in *U. pugnax*.

range.[6] The response (4.9 hours) produced by 0.05 cc. of an extract of 1 E.S. of *U. pugilator* (2.46 grams) per cc. of solution was designated as one *Uca* unit.

Hormone Content in the Eye-Stalks of Various Crustaceans

In estimating the hormone content in the eye-stalks of various crustaceans, the following method was used: a number of eye-stalks

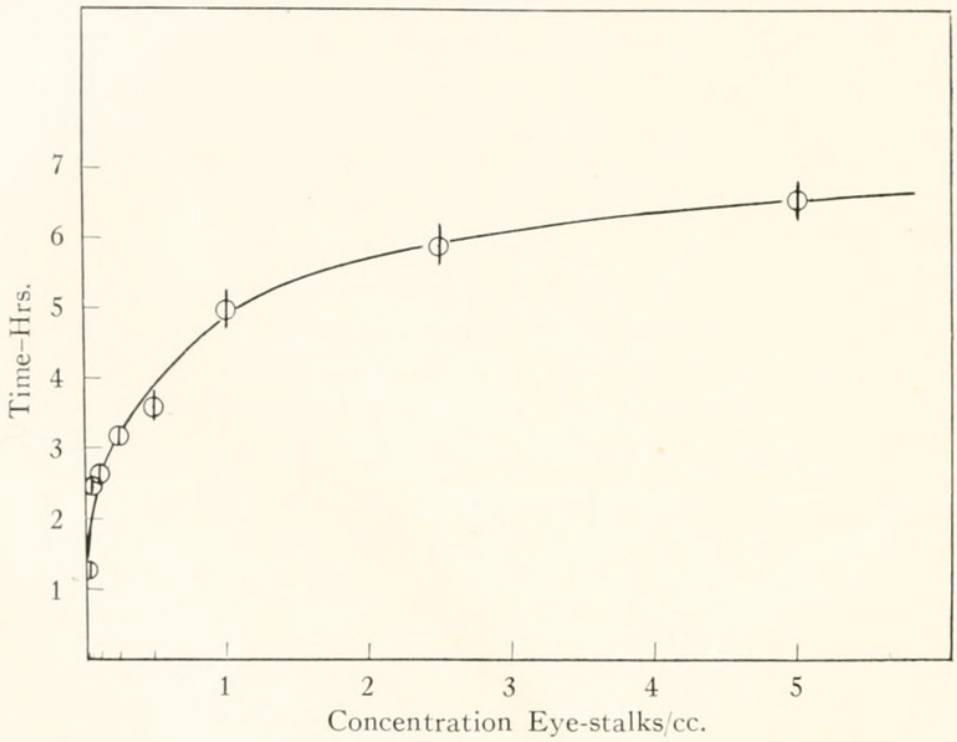


Fig. 1. Curve showing the relationship between the concentration of the hormone and the complete response of *Uca pugilator*. The points are averages of 15–20 animals, over 150 animals being used in this particular experiment. The solid bars represent values equal to twice the standard deviation of the arithmetic mean.

were cut off, extracted with sea-water, boiled, filtered, and in all cases the solution was made up so that 1 cc. contained the extract equivalent

[6] This range is sensitive enough to detect differences in response produced by a concentration of X, and one of 2X. The significant differences between some of the points along this range are much greater than that required by the standard equation $\frac{\text{S.E.}_1 - \text{S.E.}_2}{\sqrt{\sigma^2_1 + \sigma^2_2}} = \text{or} > 3$. The sensitivity of the curve would be increased by obtaining more points along this range, and by using extremely large numbers of animals to cut down the size of the standard error.

to 1 eye-stalk.[7] Five-hundredths of a cc. of the solution was injected into each of 15 animals and the average of the responses taken. The concentration in terms of *U. pugilator* E.S./cc. which produces the same average response was read off from Fig. 1. The hormone content of one eye-stalk of various crustaceans as compared with one eye-stalk of *U. pugilator* (1 unit) is given in Table III. The extracts were always made during the day and from animals which had been maintained under illumination (daylight).

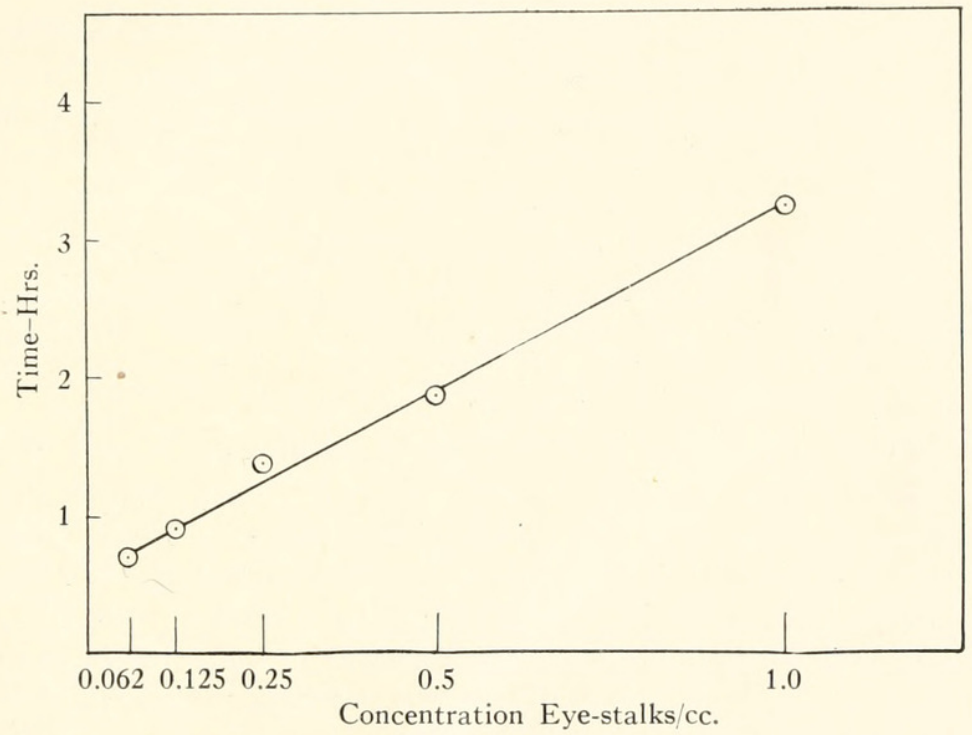


FIG. 2. Curve showing relationship between the duration of melanophore expansion and concentration of hormone (values replotted from Fig. 1). Theoretically, the curve should go through the 0 point.

EXTRACTION AND PURIFICATION

Although ten years have elapsed since the eye-stalk hormone was discovered, very few chemical properties of the hormone are known. Perkins showed that the hormone was soluble in water, and that it was resistant to boiling. Carlson (1936) and Abramowitz (1936*a*, *c*) demonstrated that it was soluble in alcohol but insoluble in ether. Carlson also added that the hormone was not destroyed when treated for short periods of time with acid or alkali.

[7] Unfortunately this proved to be a mistake because a few readings obtained in assaying the potency of the eye-stalk extracts of certain crustaceans were not within the sensitive range, and therefore cannot be considered as accurate readings.

Since a method for standardizing the hormone has been devised, an attempt was made to purify the substance. One thousand eye-stalks of *Uca pugilator* were dried and pulverized. The total activity of the dry powder was 1000 *Uca* units, or 0.6 *Uca* units per mg. of dry powder. This material was extracted several times with small volumes of light petroleum ether in order to remove a red carotenoid pigment which was found to contaminate the subsequent fractions. The ether solution was washed with small amounts of distilled water. The water layer was then added to the residue insoluble in ether and the ether layer being only slightly active was discarded. The residue which was insoluble in ether was then extracted three times with distilled water. The water solution was boiled and filtered, and the filtrate dried in a current of

TABLE III

Hormone content of the eye-stalks of various crustaceans

Average Weight of Animals	Species	*Uca pugilator* Units
grams		
2.46	1 E.S. *Uca pugilator*	1.0
3.3	1 E.S. *Uca pugnax*	1.0
11.0	1 E.S. *Pagurus pollicaris*	1.25
309.0	1 E.S. *Homarus americanus*	1.20
2.0	1 Head *Hippa talpoida*	0.25
0.38	1 E.S. *Crago borealis*	0.25
219.0	1 E.S. *Carcinus mœnas*	1.25
80.0	1 E.S. *Cancer irroratus*	5.0
0.4	1 E.S. *Palœmonetes vulgaris*	0.36
15.0	1 E.S. *Uca minax*	1.5
127.0	1 E.S. *Libinia dubia*	4.0

warm air. The dried material was washed with small amounts of chloroform to remove traces of the red pigment, and the chloroform washings being only slightly active were discarded. The dried filtrate was then extracted with 95 per cent ethanol, and the alcohol solution was centrifuged. The alcohol-soluble fraction was decanted and dried. Both the alcohol-soluble and alcohol-insoluble fractions were active, the latter being much less so than the former, and hence was discarded. The material soluble in 95 per cent ethanol was then dissolved in hot absolute alcohol and precipitated by the addition of ether. The activity of the material soluble in absolute alcohol was approximately two *Uca* units per mg. Further attempts to precipitate the material proved unsatisfactory because of the exceedingly small yield. The loss in total

activity was about 60 per cent. The material which is soluble in absolute alcohol is devoid of pigment and is apparently protein-free.[8]

Properties of the Hormone

The eye-stalk hormone is readily soluble in water, but not completely soluble in ethanol or methanol. It is only slightly soluble in acetone, and insoluble in organic solvents such as benzin, chloroform, or ether. The hormone is thermostable, but is destroyed by oxidation. It does not decompose when boiled with HCl or NaOH in a 1 per cent solution for short periods of time. If the hormone is boiled for 2 hours with NaOH, the activity is completely destroyed. The hormone adsorbs easily to various substances present in crude extracts of the eye-stalks. If the eye-stalks are extracted with benzin, and the benzin-soluble material (chiefly pigment) and the benzin-insoluble material tested in equivalent doses, only the benzin-insoluble is found to be active. However, if the benzin-soluble material is concentrated and then tested, some activity will be found. The same phenomenon was observed when working up sea-water extracts of the eye-stalks of various crustaceans. These extracts were dried, and the dried material extracted with 95 per cent ethanol. Two volumes of acetone were then added to the alcohol solution to precipitate the salts, which settle out in crystalline form. The salt crystals were also found to be slightly active. The observations indicate that the hormone adsorbs very readily. The hormone may be kept in a water solution in the refrigerator for some time without appreciable loss of activity. However, it is destroyed slowly when kept in a water solution at room temperature.

Physiology of the Eye-Stalk Glands

One of the basic problems in endocrine research is to determine the factors which affect the production of a hormone and the mechanism which regulates its release into the circulation. The standardization of the eye-stalk hormone has made it possible to investigate the physiology of the eye-stalk glands, for the amount of hormone in the stalks of animals maintained under special conditions can now be determined. The presence or absence of the hormone in the circulation can be determined quite easily by observing the states of the chief chromatophores with the aid of a microscope. This can be illustrated by the pigmentary reactions of *Uca* and *Palæmonetes,* the two animals chosen for this investigation. Perkins (1928) and Brown (1933) have shown that the contraction of

[8] The alcohol-soluble material gave negative results when tested with Millon's reagent and with the Xanthaproteic test.

the red and yellow chromatophores of *Palæmonetes* is due to the presence of the hormone in the blood stream, and that the expansion of these chromatophores results from the absence of the hormone. The situation is just reversed in *Uca* for in the brachyurans the eye-stalk hormone expands the melanophores and the erythrophores when it is circulating through the animal, while its absence results in the contraction of these chromatophores.

Palæmonetes

Forty specimens of *Palæmonetes vulgaris* of uniform size and weight were separated into four equal groups. One group was placed in a black vessel, another in a white, a third group in a yellow and a fourth in a blue. The vessels were then placed under the illumination of two 75-watt electric bulbs. The animals were supplied with a continuous current of fresh sea water, and left undisturbed for 8 hours. A fifth group of 8 animals were placed in total darkness for a day. At the end of 8 hours, the eye-stalks of each group under illumination were extracted with sea water and each of the 4 extracts assayed to determine the amount of hormone present. The eye-stalks of the animals placed in total darkness were extracted in darkness on the following day. The results of this investigation are listed in Table IV.

TABLE IV

Hormone content in the eye-stalks of Palæmonetes under various environmental conditions

Number of Eye-stalks Tested	Condition	Average Weight of Animals	*Uca* Units/E.S. of Palæmonetes
		grams	
20	Black-adapted	0.25	0.46
20	White-adapted	0.29	0.50
20	Yellow-adapted	0.30	0.49
20	Blue-adapted	0.25	0.47
16	Darkness (day)	0.28	0.25

This experiment was repeated three times and the same result was obtained in each case. The amount of hormone present in the eye-stalks of animals kept under illumination was twice that obtained from animals maintained in darkness. This is confirmatory of Kropp and Crozier's finding that stalk extracts of animals kept in darkness did not depress the growth rate of *Lupinus* as much as extracts made from animals exposed to light. It is also in agreement with the results of Klein-

holz who found that extracts from animals kept in the darkroom produced weaker responses in the retinal pigments than extracts of animals under illumination. Equally significant is the finding that the amount of hormone in the eye-stalks of animals showing a continuous release of the hormone (white-adapted group) was the same as that of animals showing no, or a subminimal release of the hormone into the circulation (black-adapted group).

This situation becomes understandable when the functional cycle of an endocrine gland is considered. The normal physiology of an endocrine gland consists chiefly in synthesizing and storing a hormone, and finally releasing it into the circulation. These three processes may conceivably be controlled separately, or may be controlled uniformly or in part by the same mechanism. In *Palæmonetes,* as in some other crustaceans, the endocrine glands (Blutdrüse of Hanstrom, 1934) of the eye-stalk are innervated from the cerebral ganglia. The functional innervation of a gland would therefore afford a simple mechanism for the release of the hormone into the circulation.

The results listed in Table IV can be readily explained upon the basis of relative rates of hormone synthesis and hormone release. In darkness, there is no release of the hormone into the circulation as indicated by the inactive position of the distal pigment cells of the retina, and by the expansion of the red integumentary chromatophores (Brown, 1935*b*).[9] The rate of synthesis of the hormone must also be decidedly reduced as evidenced by the assays of the eye-stalks. In the presence of light, however, hormone synthesis is greatly increased, and this effect is produced regardless of the background over which the animals are kept. Hormone synthesis, therefore, is due to incident light and is not primarily dependent on reflected light. This accelerating effect of incident light may therefore be termed the primary effect.

The release of the hormone, however, is definitely brought about by light reflected from backgrounds such as yellow or white. This is shown by the contraction of the red and yellow chromatophores of the integument and by the inward migration of the distal cells of the retina under such conditions. The release of the hormone into the circulation is maximal. However, in the black-adapted animals there is no release or only a sub-minimal release of the hormone into the circulation as

[9] The condition of the red pigment in *Palæmonetes* maintained in darkness does not seem to be definitely settled. Perkins (1928) stated that the red pigment was concentrated in complete darkness. Brown repeats this statement (1933) but later (1935*b*) is of the opinion that the red pigment is expanded in shrimps kept in darkness.

indicated by the expanded state of the red chromatophores.[10] Yet under these two conditions, the amount of hormone in the eye-stalks is the same.

This situation is understandable if we consider that the white background reflex produces not only a maximal release of the hormone (and acceleration of synthesis due to the primary effect) but also a concomitant increase in the rate of synthesis so that there is a balance between a high rate of synthesis and a high rate of release. This balance must be attained since *Palæmonetes* retains a transparent hue for months

TABLE V

Hormone content during diurnal rhythm

Condition	*Uca pugilator*			*Uca pugnax*		
	Phase	Number of Eye-stalks Extracted	*Uca* Units/E.S.	Phase	Number of Eye-stalks Extracted	*Uca* Units/E.S.
Retina extirpated 1 week previously						
Day, light	Black	10	0.98	Black	10	0.90
Day, dark	Black	8	0.94	Black	10	1.61
Night, light	Pale	26	1.30	Inter.	18	1.00
Night, dark	Inter.*	10	0.96	Pale	8	0.91
Normal animals						
Day, light	Black	30	1.0	Black	10	0.95
Day, dark	Black	10	2.0	Black	10	0.97
Night, light	Pale	10	1.0	Inter.	18	0.93
Night, dark	Inter.	10	0.90	Pale	10	0.99
1 eye-stalk removed 3 days previously						
Day, light	Black	16	0.95			
Night, light	Pale	16	0.95			

* The abbreviation "inter." is used to designate the intermediate condition in coloration between black and pale.

if kept continually over an illuminated white background. If the white background reflex effected only a maximal release, one would reasonably expect that over a long period of white background-adaptation, the level

[10] The hormone is probably released in amounts and at a rate which constitutes a sub-minimal stimulus for the integumentary chromatophores. Some release must occur because Kleinholz (1935) writes that the retinal pigments of *Palæmonetes* are under control of the eye-stalk hormone, and in animals kept on a black background the retinal pigments are in their active state. Hence, if the same hormone affects both the retinal cells and the integumentary chromatophores, it must follow that the amount of hormone released in illuminated black-adapted animals is minimal for the eye-pigments but sub-minimal for the body pigments.

of synthesis would be outstripped by the rate of release. The glands would therefore become exhausted and the animals would become dark due to chromatophore expansion. This apparently never occurs. In the black-adapted specimens, the release of the hormone is proceeding slowly and consequently, a balance is also obtained between a lower rate of synthesis and this lower rate of release. The fact that the amount of hormone in the eye-stalks is the same in both white-adapted and black-adapted specimens is due simply to the primary effect of incident light.

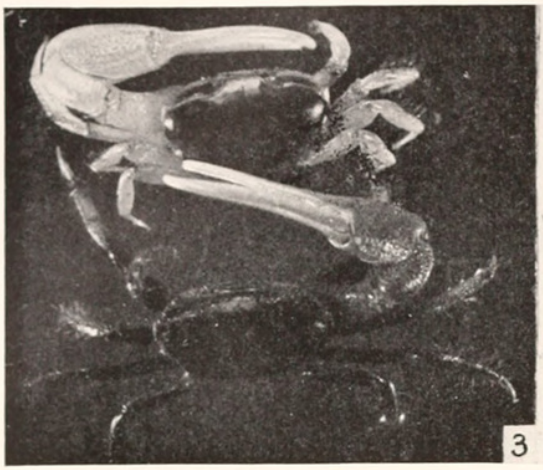
This interpretation of the physiology of the eye-stalk glands may be summarized as follows: Incident light (as opposed to light reflected from backgrounds) induces an acceleration of hormone synthesis but exerts only a sub-minimal release of the hormone into the circulation. This effect of light may be called the primary effect, and occurs in specimens placed on any background provided overhead illumination is present. The white background response, however, is due to the combination of the primary effect of incident light and of the effect of reflected light, which is to produce a maximal release of the hormone and a concomitant increase in the rate of synthesis.

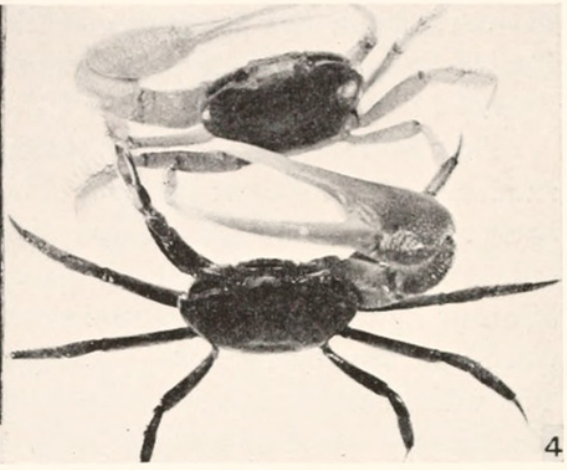
Uca

Uca differs from *Palæmonetes* in that it undergoes a periodic change in color and that background adaptation is lacking. Both *Uca pugilator* and *pugnax* show a periodicity of color change. The diurnal rhythm of *Uca pugnax* was described by Megašur (1912). The animals are black by day, pale by night, and this daily cycle repeats itself regardless of background or of light intensity. I have confirmed these statements and have extended them to *Uca pugilator*. When the eye-stalks are removed the rhythm is permanently abolished (at least until regeneration of the stalks takes place) and the animals remain pale regardless of background or light intensity. Periodicity is therefore controlled by a rhythmical release of the hormone into the circulation, the release occurring every 12 hours.

Several animals (*Uca pugnax*) were maintained upon an illuminated white background from Oct. 20–Nov. 14, 1935. During the day they were jet black in color, but at 5:30 P.M. they began to pale so that at 7:00 P.M. all the animals were pale (lemon yellow in color). At about 8 A.M. on the following morning the animals turned black again. The same was true of specimens maintained from Oct. 20–Nov. 14, 1935, in total darkness. Background adaptation in *Uca* seems therefore to be lacking. Periodicity is the chief factor in its color change.

Complete extirpation of both stalks leads to permanent destruction of the rhythm. Extirpation of but one eye-stalk does not impair the periodicity. One eye-stalk is therefore sufficient for the continuance of normal chromatic activity. If the retinal portion of both eye-stalks is cut off cleanly by a sharp scalpel, the rhythm is likewise interrupted.





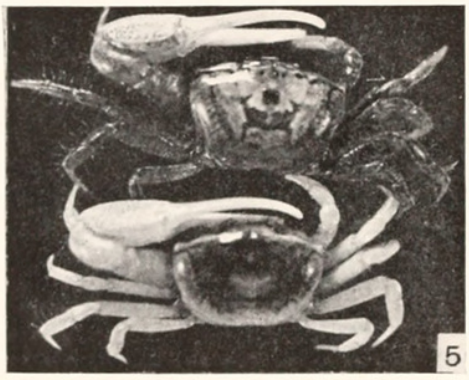


PLATE I

All photographs are from life and one-half life size.

FIG. 3. Two specimens of *Uca pugnax* photographed on a black background. Above, a specimen whose eye-stalks had been extirpated a day previously; and below, a normal animal in daylight.

FIG. 4. Same as Fig. 4, but photographed upon a white background.

FIG. 5. Two specimens of *Uca pugilator*. Above, a blinded animal 2 hours after injection of an extract of its extirpated eye-stalks. Below, an uninjected blinded specimen.

FIG. 6. Same as Fig. 6, but photographed upon a white background.

Such animals when maintained in the light on a white background remain black during both day and night, for 3 or 4 days. Similarly prepared animals maintained in total darkness remained black during both day and night for 3 days. After this period of time the rhythm appeared again but was quite erratic for a day or two, after which it appeared to

be normal again. During the past summer, I repeated some of these experiments with large numbers of animals (*Uca pugilator*). In constant light the periodic change in color occurred at 10–11 P.M. and at 9 A.M. Moreover, only about 70 per cent of the animals showed periodic changes.

The hormonal content of the stalks of selected animals under various phases of their diurnal rhythm was determined, and the results given in Table V. Animals of equal weights were used. The average weight of *Uca pugilator* was 2.5 grams, of *U. pugnax* 3.5 grams.

The results listed in Table V show that the amount of hormone present in the eye-stalks is the same whether the animals are in the pale phase of their rhythm or whether they are in the dark phase. There are some variations in the quantity of hormone extracted under different states, but these are statistically insignificant. As in *Palæmonetes,* the quantity of hormone extracted from the eye-stalks is the same in conditions during which the hormone is being continually secreted or is absent from the blood stream. This situation may be similarly interpreted. The diurnal rhythm of *Uca* is therefore an external expression of a diurnal release of the hormone into the circulation. During the 12 hours of release, the rate of production of the hormone may be increased to keep pace with its secretion into the blood.

The mechanism which controls this diurnal release differs from the release mechanism of *Palæmonetes.* The latter is definitely brought about by environmental factors (white background reflex), while in *Uca,* the diurnal release proceeds regardless of environmental factors. Furthermore, the theoretical increase in rate of synthesis is dependent on light in *Palæmonetes,* and is independent of light in *Uca,* since the diurnal rhythm proceeds in total darkness. The common feature shared by both these forms is that the release is probably controlled nervously. This is not as certain in *Uca* as it is in *Palæmonetes,* but the fact that the Blutdrüse in *Uca* are innervated and the observation that their normal physiology may be temporarily disturbed by destruction of the retinal portion of the eye-stalks seem to point towards the presence of a functional innervation. At any rate, it is difficult to imagine that the diurnal rhythm is an intrinsic property of the gland cells, for an inherent diurnal synchronism of all the cells in a gland would be somewhat surprising. Furthermore, if this conception is true, one would reasonably expect to find variations in the content of hormone during various phases of the rhythm since the glands would secrete during the day and synthesize during the night. The data in Table V show that this does not occur. It would be more satisfying to regard the blood glands as being regulated by a diurnal flow of nerve impulses. In either case, the ori-

gin of the diurnal rhythm is unknown, for one is now left without an explanation of the cause of a diurnal flow of nerve impulses. The intermediary step in the cycle is clear, however, for this is merely a diurnal release of the hormone into the circulation. The change in body coloration is but the external expression of this diurnal release.

A final experiment was performed to determine if a compensation of either hormone synthesis or release is made by the intact eye-stalk when its fellow is removed. One eye-stalk was removed from each of 32 specimens of *Uca pugilator* and 3 days later, the remaining eye-stalks were extracted and assayed. During this 3-day interval, the diurnal rhythm proceeded quite normally, the animals being maintained under illumination. The eye-stalks of 16 of these crabs were assayed during the day, the remaining 16 during the night. The results, tabulated in Table V, show that the amount of hormone in the remaining eye-stalk of such "one-eyed" crabs is the same during their dark phase as during their pale phase, and also the same, per eye-stalk, of normal crabs. Compensation, therefore, does not take place. One eye-stalk is quite sufficient to regulate chromatic activity when its fellow is removed.

Discussion

The interpretation just advanced should be considered only as a suggestion for it represents the first attempt to understand the normal physiology of the eye-stalk glands. It should also be stressed that the method of assay is sensitive enough to detect the difference between a concentration of X and one of 2X. Consequently, if slight variations do occur in the hormone content of the eye-stalks, these will not have been detected. Furthermore, inhibiting and activating systems may be present and play important parts in the biochemistry of the hormone.

It should also be emphasized that the theoretical considerations and the interpretations advanced are dependent upon the assumption that the glands in the eye-stalk are the chief if not the sole source of the manufacture of the hormone. This assumption is generally believed to be true, but certain recent observations are opposed to this notion. Hosoi (1934) found that extracts of the ventral nerve cord and male genitalia of *Penæus* produced chromatophore contraction when injected into blinded specimens of *Paratya,* showing that these extracts contained the chromatophorotropic hormone. Extracts of the stomach and muscles were also slightly active, but extracts of heart tissue were decidedly inactive. By a simple method Hosoi calculated that the eye-stalks were about one hundred times stronger in hormone content than the ventral nerve cord or genitalia. These results were criticized by

Kleinholz (1935), who attributed them to the presence of the hormone in the coagulated blood contained in these organs. Kleinholz's criticism seems to be well taken, but it is rather surprising that heart extracts were negative since these should also contain coagulated blood. At any rate, Hosoi does not describe the type of animal from which the extracts were made. If the extracts were prepared from shrimp showing the phase in which the eye-stalk hormone was being released into the circulation, Kleinholz's criticism would seem very justifiable and the importance of Hosoi's results would be uncertain. If the extracts were made from black-adapted specimens, or better, from animals whose eye-stalks were amputated some time previously, Hosoi's results could then be taken to indicate that organs other than the eye-stalks may produce the hormone. Brown (1933, 1935*a*) likewise states that extracts of the ventral nerve cord of *Palæmonetes* are active when tested on the chromatophores of blinded specimens. However, he gives no data concerning the phase of the shrimps from which his extracts were prepared nor of the potency of such extracts in relation to an extract of the eye-stalks. It would seem that Kleinholz's criticism of Hosoi's work would apply aptly to that of Brown. It has not been shown conclusively, therefore, by this type of experiment that other tissues may produce the hormone found so abundantly in the eye-stalks.

A second line of evidence bearing on this question has been advanced by Brown (1935*a*). Brown applied heat and electricity to the stubs of the eye-stalks in animals whose stalks were previously removed and observed that such stimuli induced rapid chromatophore contraction. This reaction was interpreted by him to be due to the excitation of endocrine glands of some region of the body outside of the eye-stalks by these heterologous stimuli (heat, electricity). However, this interpretation must be supported by the demonstration that the assumed endocrine glands in some region of the body are actually present, and that the contraction of the chromatophores following such stimuli is actually hormonal.

It can be concluded from this discussion that the eye-stalks are the chief source of the production of the chromatophore hormone. It can also be stated that none of the experiments already mentioned shows conclusively that tissues other than the eye-stalks may produce the hormone. The facts that under illumination blinded *Palæmonetes* remain steadily dark, and that blinded *Uca* remain continuously pale indicate that if other tissues, capable of forming the chromatophore hormone, are present they play an insignificant part in the ordinary chromatic physiology of these animals.

The interpretation of the mechanism of diurnal rhythm in *Uca* differs slightly from that advanced by Young (1935) for the cyclostome, *Lampetra*. *Uca* and *Lampetra* show many features in common. In each animal background adaptation is lacking. Each animal is pale during the night, and dark during day. Each responds to the loss of its chromatophore hormone by complete pallor, and each responds to the loss of its retinas by melanophore expansion, which is not as permanent in *Uca* as in adults of *Lampetra*. The diurnal rhythm of either *Uca* or *Lampetra* may proceed in total and enduring darkness. However, under constant illumination, the coloration of *Lampetra* remains steadily dark while the color rhythm of *Uca* may continue. To account for the periodic change in the coloration of the lamprey, Young suggested that the paired eyes in collaboration with the pineal glands affected the melanophores by a nervous inhibition of the secretion of the melanophore-expanding substance by the pituitary so that the animal becomes pale. This associates the active phase of the daily cycle with the appearance of the pale coloration of the lamprey, that is, with its nocturnal hue. The mechanism advanced for *Uca* places the active phase of the cycle with the appearance of its diurnal hue, but there appears to be no reason why Young's interpretation could not also apply to the situation in *Uca*. What actually happens in either *Uca* or *Lampetra* is the secretion of the melanophore hormone (assuming that we are dealing in terms of one melanophore substance in either case) into the circulation during the day, and its absence during the night. This daily cycle may be due to (1) a diurnal nervous stimulation of secretion, (2) a nocturnal nervous inhibition of the glands which, if not inhibited, would continue to secrete, or (3) to a combination of both. At present, it is difficult to prove which of the three possible mechanisms is correct. The interpretation that the active phase of the daily cycle in *Uca* was associated with the dark phase was made in consideration of the results obtained with *Palæmonetes,* in which the release of the hormone into the circulation is evidently due to a nervous stimulation of the glands.

In conclusion, it would be fair to outline briefly other possible explanations of the physiology of the eye-stalk glands in *Palæmonetes*. The explanation already advanced is based partly on the observations of Brown (1935*b*), which may not be entirely correct, and also takes into consideration the behavior of the retinal pigments on the basis that they are affected by the same hormone. Concerning the latter, there are no indications at present for the existence of two hormones, one for the body pigments, and one for the retinal cells. All that is known now is that the same extract which affects the body chromatophores also

affects the retinal cells (Kleinholz, 1935). The key to the whole situation lies in the behavior of the red and yellow chromatophores in animals maintained in darkness. If Brown (1935*b*, p. 320, but see also p. 328) 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 (2–3 weeks) in darkness resulted in the same condition of these pigments as that occurring when animals were adapted to a red background (cf. p. 319, Brown, 1935*b*), there is little reason for postulating separate autocoids for the body and retinal pigments. Threshold differences would account for the various responses. If the earlier observation of Perkins (1928) that the red and yellow pigments are contracted in darkness is correct, the existence of separate hormones would be clearly indicated (because the chief body pigments would react completely independently of the retinal pigments in darkness and on an illuminated black background), and the entire problem would be greatly simplified. Secretion and synthesis of the retinal hormone would take place only under the action of light; in darkness, release would be abolished and synthesis reduced. For the chief body pigments, it would have to be assumed that the gland produces and releases the hormone continuously, that synthesis is accelerated by incident light regardless of background, and that release is inhibited by a black background. The latter process seems theoretically difficult, for a white background reflects while a black background absorbs light. Finally, the stimulus for release or for inhibition of release may depend on the excitation of certain portions of the retina by a particular ratio of incident to reflected light. All these possibilities are well worth investigation.

Summary

A method for the standardization of the crustacean eye-stalk hormone on the blinded fiddler crab, *Uca,* has been described. The *Uca* unit has been defined as the amount of hormone contained in 1 cc. of solution, 0.05 cc. of which when injected into each of 15 specimens of *Uca pugilator* blinded 2 days previously produces a response whose average duration is about 5.0 hours. The response is measured as the amount of time intervening between the injection of the hormone and the time at which the animals again become pale, an interval during which the melanophores expand, remain expanded for some time, and finally contract. The hormone content in the eye-stalks of various crustaceans was determined. A method for the extraction and purification of the hormone has been described, and some chemical and physical properties of the hormone have been listed.

The amount of hormone extracted from the eye-stalks of *Palæmonetes* is the same regardless of whether the hormone is secreted continuously into the circulation, or whether it is continuously absent, conditions which are brought about by illuminated white and black surroundings respectively. In darkness, there is no release of the hormone into the blood, and a very low content of hormone in the eye-stalks, approximately half that obtained from the stalks of illuminated animals. It is postulated that light, regardless of background, causes an acceleration in hormone synthesis, and that light depending on certain backgrounds such as white, causes a maximal release of the hormone into the circulation with a concomitant increase in rate of production of the hormone. The diurnal color rhythm of *Uca* is an external expression of a diurnal release of the hormone into the circulation. Both release and synthesis are independent of environmental conditions, and it is suggested that they are controlled by a diurnal discharge of nerve impulses from the C.N.S. This discharge, during the day, would exert a 12-hour release of the hormone with a concomitant increased rate in production, the absence of the discharge during night would cut off release and slow down rate of synthesis.

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