NEUROSECRETION AND CRUSTACEAN RETINAL PIGMENT HORMONE: ASSAY AND PROPERTIES OF THE LIGHT-ADAPTING HORMONE[1]

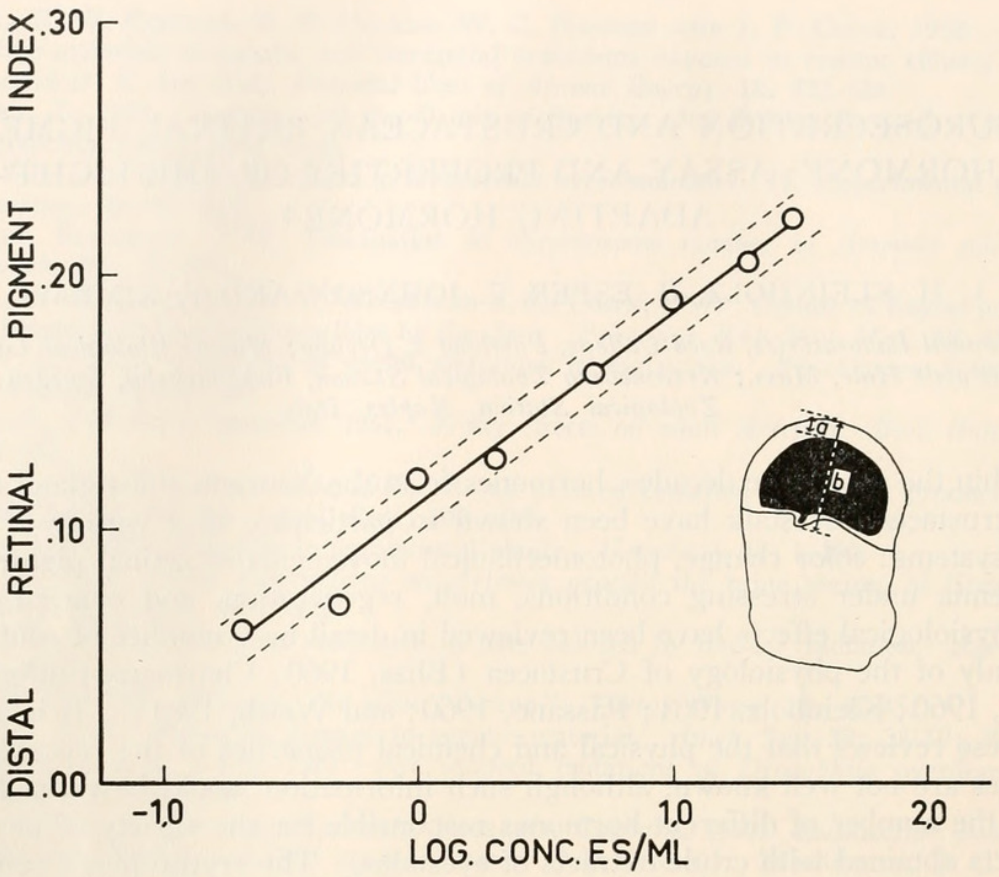
L. H. KLEINHOLZ, H. ESPER, C. JOHNSON AND F. KIMBALL

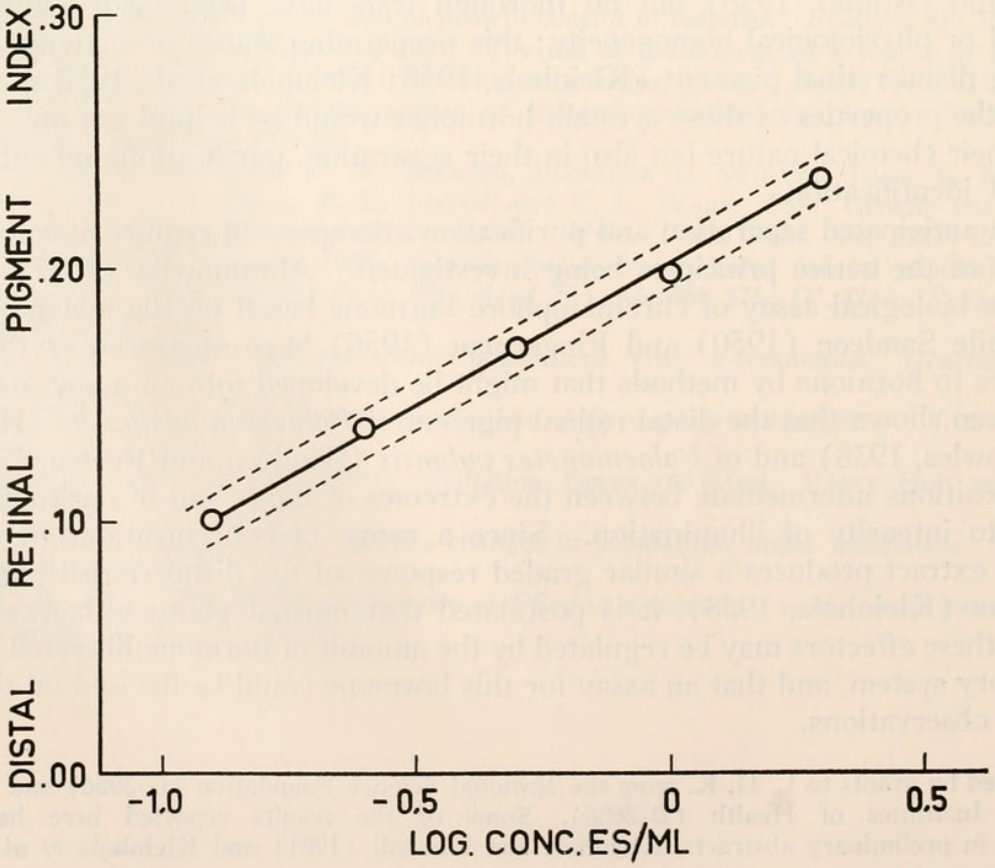
The Biological Laboratories, Reed College, Portland 2, Oregon; Marine Biological Laboratory, Woods Hole, Mass.; Kristineberg Zoological Station, Fiskebäckskil, Sweden; Zoological Station, Naples, Italy

Within the past three decades, hormones from the X-organ-sinus-gland complex of the crustacean eyestalk have been shown to participate in a variety of physiological systems: color change, photomechanical movements of retinal pigments, hyperglycemia under stressing conditions, molt, regeneration, and ovarian growth. Such physiological effects have been reviewed in detail by a number of contributors to a study of the physiology of Crustacea (Bliss, 1960; Charniaux-Cotton, 1960; Florkin, 1960; Kleinholz, 1961; Passano, 1960; and Welsh, 1961). It is apparent from these reviews that the physical and chemical properties of the reported active principles are not well known, although such information would be valuable in resolving the number of different hormones responsible for the variety of physiological effects obtained with crude extracts of eyestalks. The erythrophore-concentrating hormone has been the only one reported as a purified preparation (Edman, Fänge and Östlund, 1958) but no thorough tests have been made either of its chemical or physiological homogeneity; this preparation shows no activity in light-adapting distal retinal pigment (Kleinholz, 1958; Kleinholz *et al.*, 1962). Knowledge of the properties of these eyestalk hormones would be helpful not only in indicating their chemical nature but also in their separation, purification and subsequent chemical identification.

Such anticipated separation and purification attempts will require assay methods for each of the active principles being investigated. Abramowitz (1937) has described a biological assay of chromatophore hormone based on the melanophore of *Uca,* while Sandeen (1950) and Fingerman (1956) have measured erythrophore responses to hormone by methods that might be developed into an assay procedure. It has been shown that the distal retinal pigment of *Palaemon adspersus* (Kleinholz and Knowles, 1938) and of *Palaemonetes vulgaris* (Sandeen and Brown, 1952) assumes positions intermediate between the extremes of light- and of dark-adaptation related to intensity of illumination. Since a range of concentrations of injected eyestalk extract produces a similar graded response of the distal retinal pigment in *Palaemon* (Kleinholz, 1938), it is postulated that normal photomechanical movement of these effectors may be regulated by the amount of hormone liberated into the circulatory system, and that an assay for this hormone could be devised on the basis of these observations.

[1] Aided by grants to L. H. K. from the National Science Foundation (G-3986) and from the National Institutes of Health (B-2606). Some of the results reported here have been described in preliminary abstracts, Kleinholz and Kimball (1961) and Kleinholz *et al.* (1961).


DISTAL RETINAL PIGMENT INDEX
.00
.10
.20
.30
-1,0
0
1,0
2,0
LOG. CONC. ES/ML
a
b


DISTAL RETINAL PIGMENT INDEX
.00
.10
.20
.30
-1.0
-0.5
0
0.5
LOG. CONC. ES/ML

The present report describes dosage-response relations for the light-adapting retinal pigment hormone, and several properties of this principle. Both kinds of examination were undertaken as necessary preliminaries to a systematic attempt at isolating the hormone in pure form.

Materials and Methods

The decapod crustaceans, *Libinia emarginata* Leach (males, weighing 500–550 grams), *Palaemonetes vulgaris* Say (not selected by sex but including a large proportion of ovigerous females, 35–40 mm. rostrum-telson length) and *Carcinus maenas* Linnaeus (males, approximately 5 cm. in maximum carapace width) were donor species whose eyestalks were used to construct dosage-response curves for the distal retinal pigment. *Palaemonetes vulgaris* [2] was the test animal for the first two donor species and *Palaemon adspersus* [2] for the third. Eyestalks from the light-adapted donor species were triturated with a small amount of reagent-grade sand and were extracted with measured amounts of solvent (distilled water for *Palaemonetes* eyestalks, sea water for the others). The tissue suspensions were centrifuged and the supernatants injected into test animals within an hour after the extractions were begun.

Test and control animals, isolated in individual containers, were dark-adapted for 3–10 hours before injection. At timed intervals, 0.05 ml. of the prepared extract was injected into a test animal by the dim light from a red lamp; uninjected control animals were exposed to the same light for comparable periods. Forty-five minutes after injection (Welsh, 1930; Kleinholz, 1936, 1938), response of the distal retinal pigment cells was measured. The slight modification of the Sandeen and Brown (1952) method of recording the response as a "distal retinal pigment index" (DRPI) has been described (Kleinholz *et al.*, 1962). Briefly, the ratio of two measurements (distance from the cornea to the distal margin of the distal retinal pigment, and distance from the cornea to the proximal margin of the dorsal pigment spot shown in Figure 1) furnishes the DRPI. The dosage-response curves are based on a minimum of 10 injected test animals (*i.e.*, 20 retinas) for each concentration of extract.

Stability of distal retinal pigment light-adapting hormone (DRPLH) to drying, heating and freezing was examined. An extract of 10 eyestalks of *Libinia* in 1 ml. distilled water was heated for 2 minutes at 100° C. and centrifuged. Three 100-μl. aliquots of the supernatant were applied to strips of filter paper and dried in a stream of warm air; the paper strips were then stored under vacuum at 20° C. After 1, 6, and 20 days of storage one of the paper strips was eluted for 2 hours with 0.5 ml. distilled water. The eluates, equivalent to concentrations of 2 eyestalks per 1 ml., were tested for activity by injection into dark-adapted *Palaemonetes*.

[2] The systematic nomenclature of these crustaceans has recently undergone revision. The new names were also used in the first report in this series, Kleinholz *et al.* (1962).

Figure 1. Regression of distal retinal pigment index (response) on logarithm of eyestalk concentration of injected extracts. The upper figure is for *Palaemonetes* eyestalk extract, with the standard error of the estimate shown in broken lines. The inset drawing of an eyestalk shows the measurements made for calculating the DRPI. The lower figure is for *Libinia* eyestalk extract. The test species for both figures is *P. vulgaris*.

The effect of heat on activity of retinal pigment hormone was examined by comparing DRPI responses produced by extract of eyestalks dried 2 hours at 110° C. with responses given by extracts prepared from fresh, unheated eyestalks of the same donors. Ablated eyestalks, one from each of 15 light-adapted *Palaemonetes,* were placed in the drying oven. The remaining eyestalks, removed immediately thereafter, were ground and extracted in 1.5 ml. distilled water, centrifuged, and the supernatant injected into dark-adapted *Palaemonetes.* The oven-dried eyestalks were then similarly extracted and tested.

Hormone activity in frozen-dried eyestalks was compared with that in oven-dried eyestalks. One eyestalk from each of 20 *Palaemonetes* was collected and frozen in a small boat of aluminum foil kept on solid CO_2, while the second eyestalks from these donors were dried at 115° C. The frozen eyestalks were lyophilized, after which both sets of eyestalks were stored at room temperature in a vacuum desiccator over anhydrous $CaSO_4$. On the following day extracts were prepared in concentrations of 20 eyestalks per 1 ml. and were tested.

Solubility of retinal pigment hormone in ethanol and in acetone was determined. Sets of eyestalks from light-adapted *Palaemonetes* were dried for 3–7 hours at 115° C. and stored in a vacuum desiccator over anhydrous $CaSO_4$ until used. One set of dried eyestalks was homogenized and extracted with distilled water; the second set of contralateral eyestalks from the same donors was extracted with 100% ethanol that had been dried for the preceding 24 hours over CaO. After centrifugation, each residue was washed with its appropriate solvent. The combined ethanol supernatants were evaporated to dryness at 115° C., and the residue dissolved in distilled water. The homogenized tissue remaining after ethanol extraction was then extracted with distilled water. The final extracts thus represented the original control aqueous extract, the ethanol-soluble extract, and the ethanol-insoluble extract, all now in aqueous solutions whose concentrations were adjusted to 10 eyestalks per 1 ml. Activity of the 100% ethanol fraction was measured on two successive days, the extracts being stored at −20° C. in the interim. The same procedure was used to extract dried eyestalks with 95% ethanol, and with acetone containing 1% glacial acetic acid.

Dialyzability of retinal pigment hormone was tested with Visking cellophane tubing. Distilled water extracts of *Libinia* eyestalks were heated for 2 minutes at 100° C., centrifuged, and 1 ml. of the supernatant, equivalent to 10 eyestalks, was dialyzed at 10° C. against 1 ml. of distilled water. Samples of the dialysate were injected into test *Palaemonetes* after 3 and 24 hours of dialysis; the contents of the cellophane bag were also tested for activity after 24 hours of dialysis.

Gradual inactivation of retinal pigment hormone, found to occur in freshly prepared extracts allowed to remain at room temperature, was suspected of being mediated by tissue enzymes, and the rate of inactivation was examined from this aspect. An extract of 10 eyestalks of *Palaemonetes* in 1 ml. of filtered sea water was prepared; immediately after centrifugation a portion of the supernatant was tested at "zero" hours by injection into 5 dark-adapted *Palaemonetes.* The rest of the supernatant solution, kept at about 25° C., was tested for activity at intervals thereafter of 3, 6, 10.5 and 12 hours. Two modifications in procedure were made to minimize the possible role of micro-organisms in the sea water. In one modification an extract of 40 *Palaemonetes* eyestalks in 4 ml. of *distilled* water was divided

into two parts, one of which was heated at 100° C. for 1 minute. The extracts were then centrifuged and both supernatants tested for activity at "zero" hours. The two solutions, kept at 25–27° C., were tested again 12 and 24 hours afterwards. In the second modification, the supernatant of a centrifuged extract containing 40 *Palaemonetes* eyestalks in 2 ml. of distilled water was divided into two equal portions. One sample was diluted with an equal volume of distilled water, while to the second was added an equal volume of antibiotic solution (10 mg. Parke-Davis crystalline Penicillin G-potassium and 10 mg. Squibb Mycostatin in 50 ml. distilled water). The two extracts and a control consisting of the antibiotic solution were injected into groups of 5 test *Palaemonetes* at "zero" hours; a second test of the two eyestalk extracts was made 11 hours later. The amount of inactivation of the DRPLH was calculated from:

$$100\% - \left(\frac{DRPI_t - DRPI_c}{DRPI_o - DRPI_c} \times 100 \right) = \% \text{ inactivation}$$

where $DRPI_t$ = the average DRPI produced by unheated extract at the various intervals after its preparation; $DRPI_o$ = the average DRPI produced either by extract tested at "zero" hours or by heated extract; $DRPI_c$ = the average DRPI, 0.050, found for a large series of dark-adapted, uninjected control *Palaemonetes*.

The optimum pH for this inactivation was determined and distribution of the enzyme in a variety of tissues was examined. A stock enzyme solution was prepared by homogenizing 200 *Palaemonetes* eyestalks in an ice bath and by extracting the homogenate with small amounts of iced 1% NaCl solution. The supernatant, after centrifugation, was dialyzed for 20–24 hours at 3° C. against three changes of 1.5 liters of 1% NaCl, to remove retinal pigment hormone, and was made to a volume of 2 ml. The retinal pigment hormone substrate was a partially-purified preparation containing the equivalent of 200 eyestalks of *Palaemonetes* per 1 ml. Both preparations were stored at −20° C., samples being removed as needed from the thawed solutions. For subsequent tests, 0.3 ml. of distilled water and 0.1 ml. of the enzyme preparation were added to each of two centrifuge tubes, one of the tubes being heated for 2 minutes at 100° C. to denature the enzyme and serve as a control. Appropriate buffer, 1.5 ml., and 0.1 ml. of the retinal pigment hormone preparation were then added to each tube and the mixtures incubated for 6 hours at 38° C. After centrifugation and checking the pH of the supernatants, hormonal activity was tested, the supernatant containing the undenatured enzyme being injected first, generally within 15 minutes after removal from the incubator. The amount of inactivation of the DRPLH was calculated as described above. The buffers used were: 0.1 *M* succinate, 0.2 *M* borate, and 0.2 *M* Tris maleate, to provide a series of pH concentrations ranging from 5.1 to 9.1.

A number of tissues other than *Palaemonetes* eyestalks were examined for the presence of this hormone-inactivating enzyme. Preparation of the enzyme extract from these tissues was made as described above for eyestalks; quantitative details are summarized in Table II. A volume of tissue brei was placed in each of two tubes, one of which was heated to denature the enzyme. To both tubes were added 1.5 ml. of 0.2 *M* Tris maleate buffer at pH 7.4 and 0.1 ml. of retinal pigment hormone solution. After incubation for 6 hours at 38° C. the mixtures were centrifuged, and the supernatant tested for activity.

The effect of proteolytic enzymes on retinal pigment hormone activity was tested with several preparations. Aqueous extracts of eyestalks in known concentration were heated briefly in a boiling water bath to coagulate eyestalk debris. The supernatant, after centrifugation, was divided into two equal portions, enzyme being added to one while the other served as a control. Incubation at 35–38° C. was for varying periods (Table III), after which both mixtures were immersed in a 100° C. water bath for 1–2 minutes, centrifuged, and the activity of the supernatants tested. At Naples, extract was prepared from eyestalks of *Palaemon serratus* and tested on *Palaemon xiphias;* at Woods Hole, donor and test species were *Palaemonetes vulgaris.* Salt-free crystalline trypsin and chymotrypsin (Worthington Biochemical Co.) and a crystalline chymotrypsin (Armour and Co.) containing 50% ammonium sulfate were the enzymes used.

Observations

1. *Dosage-response relations*

Eyestalk extracts of *Palaemonetes* give the following average DRPI values and the calculated standard deviations when injected into dark-adapted *Palaemonetes* in

Table I

Properties of the light-adapting distal retinal pigment hormone. Activity tests were made on dark-adapted Palaemonetes vulgaris, *as described in the text.* DRPI, *average distal retinal pigment index for a test group and the standard deviation;* ES, *eyestalks.*

Eyestalk extract		Results	
Donor species and concentration	Treatment	Experimental DRPI ± S.D.	Control DRPI ± S.D.
P. vulgaris: 10 ES/ml.	Oven-dried ES vs. control fresh ES	0.215 ± 0.01	0.200 ± 0.02
P. vulgaris: 5 ES/ml.	Oven-dried ES vs. control fresh ES	0.143 ± 0.04	0.181 ± 0.03
P. vulgaris: 20 ES/ml.	Lyophil. ES vs. oven-dried control ES	0.222 ± 0.01	0.229 ± 0.01
P. vulgaris: 10 ES/ml.	100%-EtOH-sol. extract vs. H_2O-extract of oven-dried ES	0.090 ± 0.01	0.227 ± 0.01
	100%-EtOH-insol. extract vs. H_2O-extract of oven-dried ES	0.214 ± 0.01	0.227 ± 0.01
P. vulgaris: 10 ES/ml.	95%-EtOH-sol. extract vs. H_2O-extract of oven-dried ES	0.161 ± 0.04	0.182 ± 0.05
P. vulgaris: 10 ES/ml.	Acetone-HAc-sol. extract vs. H_2O-extract of oven-dried ES	0.049 ± 0.01	0.225 ± 0.01
	Acetone-HAc-insol. extract vs. H_2O-extract of oven-dried ES	0.215 ± 0.01	0.225 ± 0.01
L. emarginata: pre-dialysis 10 ES/ml.	Dialysate after 3 hrs.	0.113 ± 0.02	
	Dialysate after 24 hrs.	0.196 ± 0.06	
	Dialysate after 22 hrs.	0.215 ± 0.02	

TABLE I—(*Continued*)

Eyestalk extract		Results	
Donor species and concentration	Treatment	Experimental DRPI ± S.D.	Control DRPI ± S.D.
	Inactivation at 25° C.:		
	0 hrs.		0.189 ± 0.04
P. vulgaris: 10 ES/ml.	3 hrs.	0.144 ± 0.04	
	6 hrs.	0.114 ± 0.03	
	10.5 hrs.	0.084 ± 0.02	
	12 hrs.	0.049 ± 0.01	
	Inactivation of unheated extract vs. control heated extract:		
P. vulgaris: 10 ES/ml.	0 hrs.	0.208 ± 0.02	0.216 ± 0.01
	12 hrs.	0.118 ± 0.04	0.215 ± 0.01
	24 hrs.	0.100 ± 0.02	0.200 ± 0.02
	Inactivation of extract + antibiotic vs. control without antibiotic		
P. vulgaris: 10 ES/ml.	0 hrs.	0.208 ± 0.02	0.199 ± 0.01
	11 hrs.	0.077 ± 0.01	0.062 ± 0.02
	pH optimum of inactivation; unheated extract vs. extract with enzyme denatured:		
	pH 5.1	0.171 ± 0.03	0.178 ± 0.03
P. vulgaris: ca. 20 ES/ml.	pH 6.0	0.179 ± 0.02	0.226 ± 0.01
	pH 6.7	0.133 ± 0.04	0.195 ± 0.02
	pH 7.3	0.104 ± 0.03	0.205 ± 0.02
	pH 8.0	0.131 ± 0.03	0.222 ± 0.02
	pH 9.1	0.155 ± 0.04	0.161 ± 0.04

the concentrations shown: 30 eyestalks per ml. = 0.223 ± 0.018; 20 eyestalks per ml. = 0.205 ± 0.019; 10 eyestalks per ml. = 0.190 ± 0.031; 5 eyestalks per ml. = 0.161 ± 0.029; 2 eyestalks per ml. = 0.127 ± 0.036; 1 eyestalk per ml. = 0.120 ± 0.022; 0.5 eyestalk per ml. = 0.070 ± 0.015; 0.2 eyestalk per ml. = 0.061 ± 0.019. The relation between these data is linear when concentration of injected eyestalk extract is plotted on a logarithmic scale (Fig. 1). The equation for this relation is: $Y = 0.108 + 0.077 \log X$, where Y is the average DRPI for 10 test animals, and X is the concentration of the injected extract, within the limits of the upper and lower thresholds. The standard error of the estimate is ±0.008 DRPI.

Extracts of *Libinia* eyestalks tested on *Palaemonetes* result in the following average DRPI values and their standard deviations: 2 eyestalks per ml. = 0.235 ± 0.015; 1 eyestalk per ml. = 0.197 ± 0.039; 0.5 eyestalk per ml. = 0.168 ± 0.053; 0.25 eyestalk per ml. = 0.137 ± 0.022; 0.125 eyestalk per ml. = 0.101 ± 0.011. The upper threshold concentration is about 2 eyestalks per ml., because the next higher concentration tested, 4 eyestalks per ml., gives a DRPI of 0.237 ± 0.019. The linear relation resulting from a plot of the average response against the logarithm of concentration has for its equation: $Y = 0.200 + 0.107 \log X$, with the standard error of the estimate being ±0.007 DRPI.

TABLE II

Inactivation of retinal pigment hormone by tissue brei. $DRPI_u$, *average distal retinal pigment index of unheated extract and its standard deviation;* $DRPI_h$, *average distal retinal pigment index of heated control extract and the standard deviation.*

Species	Tissue used	Saline vol. used for making tissue brei	Brei vol. in incubated mixtures	$DRPI_u$	$DRPI_h$	Inactivation
Libinia emarginata	Heart; wet wt. = 0.6 gm.	2 ml.	0.2 ml.	0.140 ± 0.04	0.205 ± 0.03	41%
	Vas deferens from 2 males	2 ml.	0.4 ml.	0.050 ± 0.01	0.212 ± 0.03	100%
	Thoracic muscle = ca. 1 gm. wet wt.	4 ml.	0.4 ml.	0.061 ± 0.01	0.195 ± 0.03	92%
	Hypodermis from 2 carapaces	2.5 ml.	0.4 ml.	0.055 ± 0.02	0.232 ± 0.02	97%
	Blood; 3 ml.	0 ml.	0.4 ml.	0.213 ± 0.03	0.215 ± 0.01	1%
Palaemonetes vulgaris	Ventral nerve cord; 35 animals	1 ml.	0.4 ml.	0.093 ± 0.02	0.197 ± 0.03	72%
Pandalus borealis	50 eyestalks, = ca. 500 mg. dry wt.	2.5 ml.	0.2 ml.	0.151 ± 0.03	0.223 ± 0.02	42%
Mercenaria mercenaria	Adductor muscle, wet wt. = 2 gm.	4 ml.	0.4 ml.	0.059 ± 0.01	0.218 ± 0.03	95%

Similar tests with extracts of *Carcinus* eyestalks on *P. adspersus* as test animal yield the following DRPI responses: 15 eyestalks per ml. = 0.188 ± 0.025; 10 eyestalks per ml. = 0.196 ± 0.029; 5 eyestalks per ml. = 0.190 ± 0.031; 2.5 eyestalks per ml. = 0.178 ± 0.027; 1 eyestalk per ml. = 0.134 ± 0.015; 0.5 eyestalk per ml. = 0.129 ± 0.020; 0.2 eyestalk per ml. = 0.048 ± 0.012; 0.1 eyestalk per ml. = 0.035 ± 0.004. The upper threshold concentration seems to be about 5 eyestalks per ml. If these responses are plotted as a function of the logarithm of concentration of the injected extract the equation for the resulting linear relation is: $Y = 0.134 + 0.096 \log X$, with the standard error of the estimate being ±0.015 DRPI.

2. Stability, solubility and dialyzability of the hormone

Samples of aqueous extract of *Libinia* eyestalks, dried on filter paper strips and stored in vacuum, retain most of their activity. This is shown by average responses of 0.210 ± 0.02; 0.150 ± 0.04; and 0.170 ± 0.02 obtained when eluates from such paper strips made 1, 6, and 20 days, respectively, after storage are tested by injection. The concentration of the eluates (on the assumption that complete elution of hormone had occurred) was 2 eyestalks per ml.; the responses can be compared with the dosage-response curve for *Libinia* in Figure 1. A better controlled examination of the effects of drying on stability of retinal pigment hormone is shown in the next group of experiments. Extracts prepared from oven-dried *Palaemonetes* eyestalks give responses only slightly different from those produced by con-

TABLE III

Effect of proteolytic enzymes on activity of retinal pigment hormone. Prepared extracts, after heat treatment, were divided into two portions, enzyme being added to one and the other serving as control. The animals used were: P.s., Palaemon serratus; P.v., Palaemonetes vulgaris; P.x., Palaemon xiphias. *The crystalline enzymes used were:* T, *trypsin, and* C, *chymotrypsin; the designation in parentheses indicates the commercial source given in "Methods." Results are shown as* DRPI, *average distal retinal pigment index with the standard deviation, and the percentage inactivation, calculated as described.*

Eyestalk extract		Enzyme treatment			Results			
Donor	Conc.	Enzyme	Conc.	Incubation	Test species	DRPI ± S.D.		Inactivation
						Enzyme	Control	
P.v.	10 ES/ml.	T (WBC)	10 mg./ml.	12 hrs. at 35° C.	P.v.	0.136 ± 0.03	0.207 ± 0.02	45%
P.v.	10 ES/ml.	T (WBC)	5 mg./ml.	11 hrs. at 35° C.	P.v.	0.122 ± 0.03	0.193 ± 0.01	50%
P.s.	3 ES/ml.	C (ARM)	5 mg./ml.	19 hrs. at 37.5° C.	P.x.	0.097 ± 0.02	0.200 ± 0.01	70%
P.v.	10 ES/ml.	C (WBC)	8 mg./ml.	4 hrs. at 37.5° C.	P.v.	0.098 ± 0.02	0.204 ± 0.03	69%
P.v.	10 ES/ml.	C (WBC)	8 mg./ml.	13 hrs. at 37° C.	P.v.	0.075 ± 0.01	0.208 ± 0.01	84%

trol extracts of fresh eyestalks; similarly, extracts of lyophilized eyestalks result in test indices much like those obtained with oven-dried eyestalks (Table I).

Solubility studies, with precautions taken to avoid moisture in the solvents and in the eyestalk tissue, show little or no activity extracted by 100% ethanol or by acetone containing 1% glacial acetic acid. On the other hand, 95% ethanol does extract active material from oven-dried eyestalks. Some loss in activity occurred when the 100%-ethanol series (the ethanol-soluble, the ethanol-insoluble and the control aqueous extract) was thawed and tested after storage at −20° C.; the respective DRPI were 0.071 ± 0.01, 0.123 ± 0.04, and 0.188 ± 0.02, and an insoluble residue was present in each thawed preparation.

The hormone readily passes through a cellophane membrane, three hours of dialysis being sufficient to indicate the presence of activity in the dialysate (Table I). After 24 hours of dialysis, the tested dialysate produced a maximum response (compare with the dosage-response curve for *Libinia* in Figure 1).

3. Enzymatic inactivation

Spontaneous inactivation of retinal pigment hormone occurs regularly in extracts of fresh eyestalks, although the rate and degree of inactivation may be variable. Inactivation in one such experiment is shown in Table I, where the average DRPI, 0.189, of the freshly prepared eyestalk extract at 0 hours declines progressively until practically no activity remains 12 hours afterward. The rate of such inactivation is shown in Figure 2, with the percentage of inactivation being calculated as described in the section on methods.

A possible enzymatic basis for this inactivation is shown by tests with identical extracts, one of which is heated at 100° C. for 1 minute and serves as control for the unheated extract (Table I). The unheated extract results in an average DRPI of 0.208 at 0 hours and an average DRPI of 0.100 after 24 hours at 25–27° C., while the average activity values obtained with the heated control extract are not ap-

preciably changed under these conditions. Eyestalk extracts of *Palaemon serratus* (5 per ml.) were tested in like manner on dark-adapted *Palaemon xiphias*. The extracts were divided into two portions, one being heated for 2 minutes at 100° C., and were then incubated at 37° C. for 15 hours. The average DRPI obtained with the unheated extract is 0.104 ± 0.03 (21 test animals), while that from heated extract is 0.193 ± 0.02 (17 test animals). The average DRPI for 26 dark-adapted, uninjected control *P. xiphias* is 0.052. The calculated percentages of inactivation are 68% for the *Palaemonetes* test and 63% for the *Palaemon* test, although incubation temperatures and experimental periods were not identical in the two cases.

Addition of antibiotic compounds to eyestalk extracts before incubation does not prevent loss of hormone activity. A curve constructed from the data in Table I over the range pH 5.1–9.1 shows the optimum for this inactivating enzyme to be pH 7.5. A summary of results from examination of a variety of tissues (Table II) shows that this enzyme is present in all tissues tested except blood.

Incubation of eyestalk extracts for different periods and with varying concentrations of trypsin or of chymotrypsin was made in a number of experiments, five of which are summarized in Table III. Nearly 50% of the activity originally present is inactivated by trypsin, while chymotrypsin brings about between 70–85% inactivation.

Discussion

The construction of dosage-response curves for the light-adapting distal retinal pigment hormone makes available a quantitative biological assay method for this hormone. The accuracy with which such assay can be made, however, will probably depend upon standardization of the procedure in the individual laboratory. The average DRPI values we obtain with control extracts of eyestalks from *Palaemonetes* and from *Libinia* show good agreement with those read from the dosage-response curves. Our suggested assay procedure is to obtain by serial dilution of the "unknown" the concentration producing an average DRPI slightly below the upper threshold response of the test animals. The average DRPI for this dilution and those obtained with two additional dilutions below this upper threshold concentration can then be substituted in the equation for the standard dosage-response curve to find their equivalent concentrations. Calculation of the average concentration of eyestalks in the original extract readily follows. It is evident from the examples reported here that the equations for such standard curves may vary with the species of the eyestalk donor and of the test animal, and it will therefore be necessary to construct such a standard curve for the particular species used.

The interconvertibility of such information from one laboratory to that from another would be aided by defining a physiological unit of hormone activity. For the present this can be done with data resulting from tests with *Palaemonetes* reported here. We therefore define the *Palamontes unit* for distal retinal pigment hormone as that concentration of eyestalks which, when injected into a minimum of 10 dark-adapted *Palaemonetes vulgaris*, measuring 35–40 mm. from rostrum to telson, yield an average DRPI of 0.150, this point being selected because it is about mid-way between the upper and lower threshold concentrations on the standard dosage-response curve. By this definition, 1 *Palaemonetes unit* is contained in *Palaemonetes* extracts having a concentration of 3.5 eyestalks per 1.0 ml. or in

Libinia extracts having a concentration of 0.34 eyestalks per 1.0 ml. After retinal pigment hormone has been isolated in pure form, dosage-response relations of whatever species were being used could be compared with the homogeneous preparation as a reference.

The stability and solubility properties described above show that eyestalks retain retinal pigment hormone activity after being oven-dried or lyophilized. This, and Carlson's (1936) report of chromatophorotropic activity in eyestalks dried and stored over a long period, have been useful in collecting and preparing quantities of eyestalk material for purification. We initially observed some loss in activity, accompanied by the formation of a precipitate, in fractionated eyestalk extracts thawed after storage at −20° C., and have therefore avoided repeated freezing and thawing of such preparations.

The *in vitro* inactivation by tissue extracts and by proteolytic enzymes point out additional interesting features of retinal pigment hormone. The variety of tissue extracts which inactivate the hormone, a pH optimum of about 7.5 for such inactivation, and the fact that the ability to destroy hormonal activity is thermolabile indicate a widely-occurring enzyme or group of enzymes. Whether such an enzyme system has an *in vivo* role in degrading hormone in the normal physiology of the retinal effectors is not known. Similar inactivation of chromatophorotropic hormone, first reported by Carstam (1951) for epidermis and later by Pérez-González (1957) and by Stephens and Green (1958) for a number of other

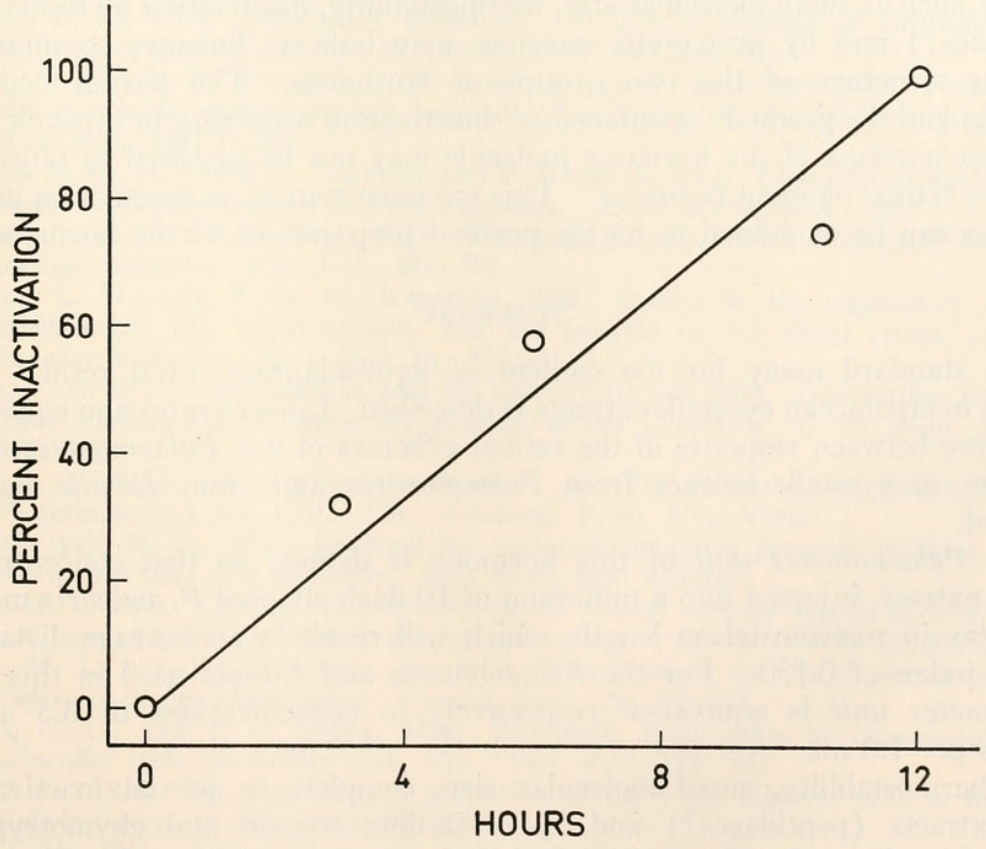


FIGURE 2. Rate of inactivation of distal retinal pigment hormone by enzyme in an eyestalk extract which was allowed to remain at room temperature for 12 hours. Extract was prepared from eyestalks of *Palaemonetes*.

crustacean tissues, may also explain apparent differences in hormone activity reported between boiled and unboiled eyestalk extracts.

The reduction by trypsin and by chymotrypsin of retinal pigment hormone activity described here was also confirmed by Fingerman and Mobberly (1960), after personal communication to them of our results. They too obtain partial loss of hormone activity in their trypsin-treated preparations. We observe a greater amount of inactivation of the retinal pigment hormone by chymotrypsin than by trypsin, but, because we do not yet know with any certainty the chemical nature of retinal pigment hormone, discussion of differences between trypsin and chymotrypsin in their proteolytic action on specific substrate linkages would be little more than speculation at this time. Such differences between trypsin and chymotrypsin may be due to the presence in crude eyestalk extracts of substances differentially inhibiting the two enzymes. Knowles *et al.* (1956) explain the failure of trypsin to inactivate chromatophorotropic hormone in eyestalk extract of *Palaemon* as probably due to inhibitory substances in extract of whole eyestalks, since electrophoretically separated chromatophorotropin is readily inactivated by trypsin. *In vitro* inactivation of chromatophorotropic hormone of *Uca* by trypsin, chymotrypsin, and papain has been reported (Pérez-Gonzáles, 1957; Stephens and Green, 1958).

On the basis of these and other properties, it has been suggested that the activity of chromatophorotropins is dependent on the presence of peptide bonds in the hormone, but the known esterase activity of trypsin and chymotrypsin do not permit this identification with assurance. Similar properties of the retinal pigment hormone, such as small molecular size, thermostability, inactivation by tissue extracts (peptidases?) and by proteolytic enzymes may indicate linkages common to the molecular structure of the two groups of hormones. The partial degradation by trypsin and the gradual "spontaneous" inactivation occurring in eyestalk extracts imply that portions of the hormone molecule may not be essential to physiological activity of retinal pigment hormone. This too must remain as speculation until such properties can be examined in highly purified preparations of the hormone.

Summary

1. A standard assay for the content of light-adapting distal retinal pigment hormone in crustacean eyestalk extracts is described. Linear regression equations for the relation between response of the retinal effectors of test *Palaemonetes* and concentration of eyestalk extract from *Palaemonetes* and from *Libinia* have been calculated.

2. A *Palaemonetes unit* of this hormone is defined as that concentration of eyestalk extract, injected into a minimum of 10 dark-adapted *P. vulgaris* measuring 35–40 mm. in rostrum-telson length, which will result in an average distal retinal pigment index of 0.150. For the *Palaemonetes* and *Libinia* used in this study 1 *Palaemonetes unit* is equivalent respectively to concentrations of 3.5 and 0.34 eyestalks per 1.0 ml.

3. Thermostability, small molecular size, complete or partial inactivation by tissue extracts (peptidases?) and by crystalline trypsin and chymotrypsin are properties of the hormone consistent with a possible peptide structure.

LITERATURE CITED

ABRAMOWITZ, A. A., 1937. The chromatophorotropic hormone of the Crustacea: standardization, properties and physiology of the eye-stalk gland. *Biol. Bull.*, **72**: 344–365.

BLISS, D. E., 1960. Autotomy and Regeneration. *In:* The Physiology of Crustacea (T. H. Waterman, ed.) vol. 1: 561–589. Academic Press, New York.

CARLSON, S. P., 1936. Color changes in brachyuran crustaceans, especially in *Uca pugilator*. *Kgl. Fysiograf. Sällskap. Lund Förh.*, **6**: 1–18.

CARSTAM, S. P., 1951. Enzymatic inactivation of the pigment hormone of the crustacean sinus gland. *Nature*, **167**: 321–322.

CHARNIAUX-COTTON, H., 1960. Sex Determination. *In:* The Physiology of Crustacea (T. H. Waterman, ed.) vol. 1: 411–447. Academic Press, New York.

EDMAN, P., R. FÄNGE AND E. ÖSTLUND, 1958. Isolation of the red pigment concentrating hormone of the crustacean eyestalk. *In:* Zweites Internationales Symposium über Neurosekretion (W. Bargmann, B. Hanström and E. Scharrer, eds.) pp. 119–123. Springer, Berlin.

FINGERMAN, M., 1956. Physiology of the black and red chromatophores of *Callinectes sapidus*. *J. Exp. Zool.*, **133**: 87–105.

FINGERMAN, M., AND W. C. MOBBERLY, JR., 1960. Investigation of the hormones controlling the distal retinal pigment of the prawn *Palaemonetes*. *Biol. Bull.*, **118**: 393–406.

FLORKIN, M., 1960. Blood Chemistry. *In:* The Physiology of Crustacea (T. H. Waterman, ed.) vol. 1: 141–159. Academic Press, New York.

KLEINHOLZ, L. H., 1936. Crustacean eye-stalk hormone and retinal pigment migration. *Biol. Bull.*, **70**: 159–184.

KLEINHOLZ, L. H., 1938. Studies in the pigmentary system of Crustacea. IV. The unitary versus the multiple hormone hypothesis of control. *Biol. Bull.*, **75**: 510–532.

KLEINHOLZ, L. H., 1958. Neurosecretion and retinal pigment movement in crustaceans. *In:* Zweites Internationales Symposium über Neurosekretion (W. Bargmann, B. Hanström and E. Scharrer, eds.) pp. 110–112. Springer, Berlin.

KLEINHOLZ, L. H., 1961. Pigmentary Effectors. *In:* The Physiology of Crustacea (T. H. Waterman, ed.) vol. 2: 133–169. Academic Press, New York.

KLEINHOLZ, L. H., P. R. BURGESS, D. B. CARLISLE AND O. PFLUEGER, 1962. Neurosecretion and crustacean retinal pigment hormone: distribution of the light-adapting hormone. *Biol. Bull.*, **122**: 73–85.

KLEINHOLZ, L. H., H. ESPER, C. JOHNSON AND F. KIMBALL, 1961. Characterization and partial purification of crustacean eyestalk hormones. *Amer. Zoologist*, **1**: 366.

KLEINHOLZ, L. H., AND F. KIMBALL, 1961. Enzymatic inactivation of crustacean distal retinal pigment hormone. *Biol. Bull.*, **121**: 395.

KLEINHOLZ, L. H., AND F. G. W. KNOWLES, 1938. Studies in the pigmentary system of crustaceans. III. Light-intensity and the position of the distal retinal pigment in *Leander adspersus*. *Biol. Bull.*, **75**: 266–273.

KNOWLES, F. G. W., D. B. CARLISLE AND M. DUPONT-RAABE, 1956. Inactivation enzymatique d'une substance chromactive des Insectes et des Crustacés. *C. R. Acad. Sci. Paris*, **242**: 825.

PASSANO, L. M., 1960. Molting and Its Control. *In:* The Physiology of Crustacea (T. H. Waterman, ed.) vol. 1: 473–536. Academic Press, New York.

PÉREZ-GONZÁLEZ, M. D., 1957. Evidence for hormone-containing granules in sinus glands of the fiddler crab *Uca pugilator*. *Biol. Bull.*, **113**: 426–441.

SANDEEN, M. I., 1950. Chromatophorotropins in the central nervous system of *Uca pugilator*, with special reference to their origins and actions. *Physiol. Zoöl.*, **23**: 337–352.

SANDEEN, M. I., AND F. A. BROWN, JR., 1952. Responses of the distal retinal pigment of *Palaemonetes* to illumination. *Physiol. Zoöl.*, **25**: 222–230.

STEPHENS, G. C., AND J. P. GREEN, 1958. Enzymatic inactivation of chromatophorotropic principles from the fiddler crab, *Uca*. *Biol. Bull.*, **115**: 367.

WELSH, J. H., 1930. The mechanics of migration of the distal pigment cells in the eyes of *Palaemonetes*. *J. Exp. Zool.*, **56**: 459–494.

WELSH, J. H., 1961. Neurohumors and Neurosecretion. *In:* The Physiology of Crustacea (T. H. Waterman, ed.) vol. 2: 281–311. Academic Press, New York.