

STUDIES ON PIGMENT-ACTIVATING SUBSTANCES IN ANIMALS

I. THE SEPARATION BY PAPER ELECTROPHORESIS OF CHROMACTIVATING SUBSTANCES IN ARTHROPODS

By Sir Francis G. W. Knowles, Bart.,

Marlborough College, Wiltshire

David B. Carlisle

The Plymouth Laboratory

and Marie Dupont-Raabe

Laboratoire de Zoologie de la Sorbonne, Paris

(Plates I and II in colour and Text-figs. 1-6)

INTRODUCTION

It has been established that many substances can bring about a change in position of pigments in crustacean chromatophores (Florey, 1951). The extracts of certain animal tissues (pituitary, crustacean sinus-gland and post-commis-sure organs, insect brain and corpora cardiaca) are especially active (Brown, 1940; Knowles, 1953; Dupont-Raabe, 1952; Thomsen, 1943); there is evidence that these tissues intervene in the normal colour change of the animals which possess them and that their products may properly be considered as hormones. On the other hand, many species which do not themselves possess chromatophores (oligochaetes, molluscs, and many insects) have nevertheless been shown to contain substances in their tissues which will, after injection into crustaceans, initiate pigment movements (Scharrer, 1954). It has not yet been ascertained whether these pigment-activating substances chemically resemble normal colour-change hormones or whether the pigment movements they produce are pharmacodynamic effects irrelevant in the study of colour physiology.

Many workers have made reciprocal-injection experiments in their studies of colour-change, and have found that for the most part the pigment-activating hormones are not species-specific, a fact which might seem to indicate some degree of chemical resemblance between chromactivating substances, though a number of observations have indicated that a chemical identity is unlikely (Brown, 1944). The interpretation, however, of the early reciprocal-injection experiments is complicated by the fact that many workers have referred to pigment changes after the injection of extracts without specifying with sufficient precision which pigments responded. The colour patterns of crustaceans

are very complex, and it is now recognized that chromatophores with apparently identical pigments may differ physiologically in relation to their position on the body (Brown & Wulff, 1941; Knowles, 1955) and that, for instance, an injection may be followed by the concentration of some red pigments but by the dispersal of others. Injection and extirpation experiments have given rise to the suspicion that in a single crustacean species there may be a number of distinct chromactivating substances, each playing a specific role in the changing colour pattern of the individual. The probability of many colour-change hormones in a single individual makes comparison by reciprocal injection experiments difficult to assess, and it is clear that the ultimate resolution of the problem of the number and the identity of the various pigment-activating substances which have been extracted from animal tissues must await chemical separation and purification.

Chemical studies on the pigment-activating substances of crustaceans have hitherto been restrained by the difficulty of obtaining adequate material for analysis. For this reason the attempts by Abramowitz (1940) to purify the crustacean eye-stalk extract did not yield conclusive results (Brown, 1944). The techniques of paper chromatography and of paper electrophoresis overcome to some extent the problem of the separation of minute quantities of substances for study, and we have therefore used these methods in the present studies on pigment-activating substances. In this preliminary paper we give an account of experiments designed to provide at least a partial solution to two fundamental problems in the endocrinology of colour-change in arthropods, namely (1) whether there are chemical similarities between the pigment-activating substances which have been extracted from the sinus-glands and the post-commissure organs of crustaceans and from the brain and the corpora cardiaca of insects, and (2) how many pigment-activating substances are present in a single crustacean or insect species.

MATERIALS AND METHODS

Animals

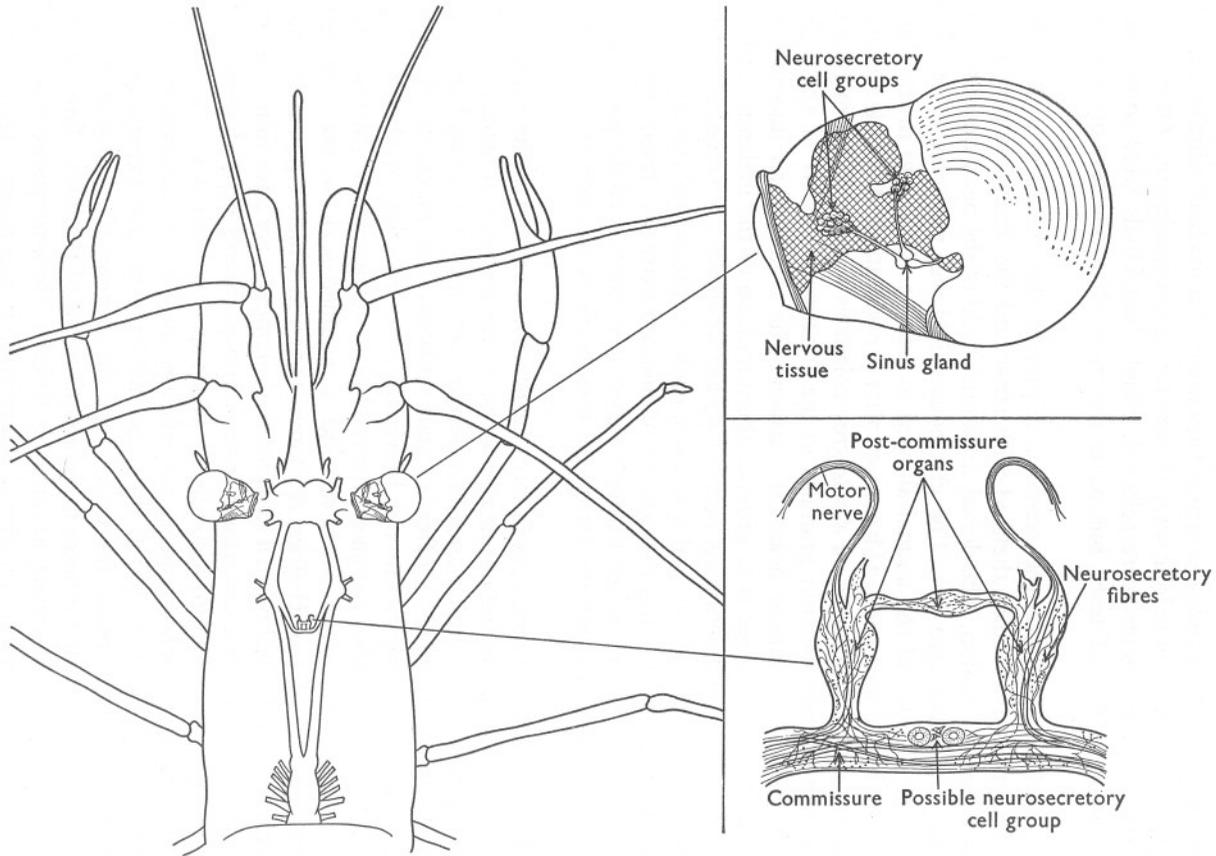
Three species of animals were used for testing the chromactivating potency of extracts, the crustaceans *Leander serratus* and *Crangon vulgaris* and the stick insect *Carausius morosus*, although others were used in some of the experiments as sources of extracts. *Leander* and *Crangon* were taken from various habitats near Plymouth and Roscoff, and stored until required for use in the aquarium tanks in running sea-water. *Carausius* were taken from the colony maintained by one of us (M.D.-R.) at the Sorbonne, and from that in the insect house of the Zoological Society of London.

Leander and *Crangon* were prepared as test subjects for this work by eye-stalk ablation. One eye of a male or non-ovigerous female was removed at the base by cutting through the arthrodial membrane with sharp-pointed scissors;

no precautions of sterility or of cauterization were taken. Twenty-four hours later the other eyestalk was removed in the same way. Animals were used after a further 24 h had elapsed or at any time later. Individuals which matched each other perfectly in colour were chosen for each experiment. Only those with dispersed pigments were selected. Single individuals were sometimes used for a number of tests, but never for more than one test on any one day.

The brain of *Carausius* was removed to prepare this animal as a test subject. The animal was anaesthetized with ether and the operation performed under Ringer's solution. The dorsal chitinous shield of the head was removed and the brain dissected out. The chitinous shield was replaced, to be held by clotting blood, and the animal allowed to recover. *Carausius* in the sixth or seventh instar were used, 24 h or more after the operation. After the removal of the brain *Carausius* takes on a pale grey colour as a result of the migration of the hypodermal melanin granules to the innermost depths of the cells, thus leaving less pigment near the surface. The result of an injection of chromactivating material is to provoke the migration of the melanin towards the surface so that the animals become darker in colour. A subjective scale of degree of darkening assessed macroscopically on the animal has been used throughout in determining the activity of injected extracts. Injections were made of about 0.05 ml. saline through a glass hypodermic needle passed in a forward direction through the arthrodial membrane at the base of one of the legs.

Colour change in *Leander*, as in most Crustacea, is a result of the migration of pigment in hypodermal chromatophores. There are several main types of these, and apparently similar ones on different parts of the body may behave in quite dissimilar manners. After eyestalk ablation the pigments of most chromatophores disperse, but not maximally; the injection of chromactive extracts produced either a greater dispersal or a concentration. In assessing colour change in *Leander* we have used a modified scale of the accepted chromatophore index of Hogben & Slome (1931), in which 1 represents full concentration and 5 full normal expansion of pigments; we have found, however, that under certain conditions some chromatophores expand some way beyond the normal state to a condition which we have called 5+, in which adjacent chromatophores appear, superficially at least, to anastomose (see Knowles, 1955, fig. 12). Using this scale we have in each experiment endeavoured to assess under the microscope the degree of expansion of the various types of chromatophores in different regions of the body. Our observations were made on as many as nineteen different chromatophore types, but only a few of these will be considered in any detail in this paper. Assessments were made 15, 30, 60 and sometimes 120 min or more after injection. Injections were made through a no. 28 steel hypodermic needle passed forward through the arthrodial membrane between the terga of the third and



Text-fig. 1. Diagram illustrating the approximate positions of the sinus glands and post-commissure organs of *Leander serratus*.

fourth abdominal segments, slightly to one side of the mid-line; the needle thus entered through muscle and was passed forward, directed towards the mid-line and slightly dorsally, till the point came to lie in the pericardium. 0.05 ml., or a little more, was injected each time into the main blood stream in this way, and the lateral muscle blocks served, by their contraction, to seal the hole, and so to reduce bleeding.

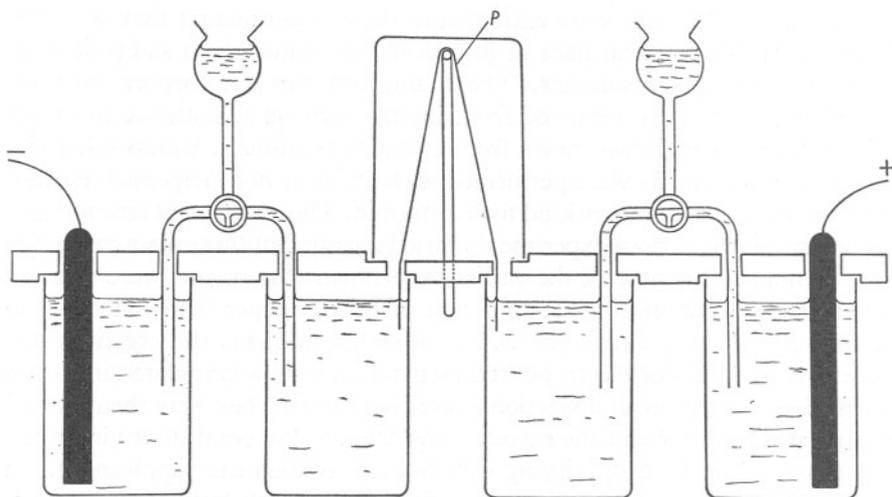
Preparation of Extracts

Four different organs were extracted in these experiments; they were the brain and the corpora cardiaca of insects and the sinus gland and post-commissure organs of crustaceans. The brain, and also the corpora cardiaca, which lie near it were removed from insects without anaesthesia and from animals which were intact, never from eyestalkless animals. Unless otherwise stated, all experiments were performed with males or non-ovigerous females. Each dissection of whatever kind took 4-10 min. The subsequent treatment of the organs for the various experiments varied greatly, but three main categories may be noted. In some of the earlier experiments the organs were crushed with forceps direct on to the central spot of the dry paper strip which was to be used for electrophoresis (*v. i.*). In other experiments the organs which were to be used in one electrophoretic separation were accumulated in a drop of distilled water until all dissections were completed. They were then ground up in a small mortar and the extract transferred to the point of origin on the paper strip, drop by drop, drying with hot air between each application. In all the later experiments the strip was first wetted with buffer solution, extractions were made in the same solution and applied to the wet paper immediately before electrophoresis; the extracts were not allowed to become dry at any time. All three methods were used in experiments on each of the four organs.

Electrophoresis

Electrophoresis was carried out upon filter-paper in two kinds of apparatus. In the earlier experiments at Plymouth the paper strip, 3 cm wide, was draped over a horizontal glass support and hung down on either side into the buffer vessels (Text-fig. 2). In the apparatus used at Roscoff the paper strips, several of which could be used simultaneously, were stretched horizontally. A more fundamental difference was that in the power pack used at Plymouth the current was stabilized, but the voltage allowed to fluctuate somewhat, while in that at Roscoff the voltage was stabilized but the current rose sometimes as much as 100% during the course of the experiment. Since it is not possible to stabilize both at the same time, it appears of more theoretical importance to stabilize the voltage, but in practice stabilization of either leads to adequate uniformity of results. In all runs phosphate buffer was used,

N/15 or N/30. Unless otherwise stated, the pH was 7.5, each run at Plymouth lasted 16 h (less at Roscoff, but variable) and the length of paper was 28 cm. The extract which was to be separated by electrophoresis was applied to the central point of the paper, midway between the electrodes. In some of the later experiments a spot of LD-leucine was placed alongside to act as a marker; this was stained with ninhydrin at the end of the run and gave some measure of the constancy of conditions from one run to the next. Whatman's paper no. 1 was used.



Text-fig. 2. A diagrammatic section through the electrophoresis apparatus used at Plymouth; *p*, the filter-paper strip.

After electrophoresis the paper was cut into sections, usually after preliminary drying with hot air. In the earlier experiments the band extending 0.5 cm on either side of the origin was cut out and numbered 0. The remaining portions of the paper were cut transversely into five equal parts, 2.7 cm wide, on each side of the origin, numbered -1 to -5 at the cathode and +1 to +5 at the anode. The arrangement of the pieces was thus:

anode +5 +4 +3 +2 +1 0 -1 -2 -3 -4 -5 cathode.

In some experiments the region numbered -1, as it proved of exceptional interest, was further subdivided into three pieces, each 0.9 cm wide, numbered $-\frac{1}{3}$, $-\frac{2}{3}$, and -1. In the experiments performed at Roscoff, bands 1 cm wide on each side of the origin were cut out separately and numbered +0 and -0; the other bands, each 2 cm wide, started correspondingly farther out from the centre than in the Plymouth experiments. These various bands were eluted with 0.2 ml. of an appropriate saline or sometimes distilled water

for a period of 15–30 min. The eluates were then injected, as described above, and the results recorded.

Further details of special methods used are mentioned in the experimental sections.

THE POST-COMMISSURE ORGANS

The effect of an injection of a post-commissure extract into an eyeless prawn differs significantly from the effects produced by the injection of a sinus-gland extract. A sinus-gland extract concentrates the small red and the large red chromatophores of the body and of the tail and is generally without effect on the white pigments (sometimes a slight concentration is observed after injection). During the first half hour after injection the effects on the large red chromatophores and on the small red chromatophores are indistinguishable, maximum contraction being produced in both type of chromatophore, but later the large red chromatophores begin to re-expand before any change in the small red chromatophores can be observed. On the other hand, injection of a post-commissure extract is followed by a contraction of all chromatophore pigments (including the white pigments), which lasts for 5–10 min., but thereafter the small red chromatophores of the body and certain red chromatophores of the tail begin to expand and in due course become more expanded than they had been before the injection. The large red and the white chromatophores, however, remain contracted for an hour or longer. Brown & Klotz (1947) reported that they could extract two substances from the commissure of *Crago* (= *Crangon*) by making use of differential solubility in alcohol. One of these (which they called CBLH) concentrated dark pigments in the body and tail of *Crago*, and the other (CDH) in the absence of the first, darkened the animal by promoting expansion of the dark chromatophores of the body and of the tail. We have by electrophoresis separated these two substances and have shown that there are also other chromatophorotropically active substances in the post-commissure organs.

The electrophoresis of post-commissure organ extracts was carried out at Plymouth and at Roscoff. In the Plymouth experiments care was taken to ensure that the current did not fluctuate during an experiment, but stabilization of the voltage was not complete. A relatively low current, ranging from 0.2 mA to 1 mA/cm width of paper was applied in this first series of experiments. In a later series of experiments, carried out at Roscoff, higher voltages, higher current strengths and shorter times were employed than in the first series and the apparatus used delivered a stabilized voltage, but the current increased during the course of an experiment. Each extract of the post-commissure organs was generally prepared from that portion of the post-commissure complex posterior to the commissure, namely the two post-commissure nerves and their lamellae (Knowles, 1953); in none of the later experiments

did the extract contain any commissure material. The post-commissure organs used were dissected from large male individuals or non-ovigerous females, measuring 50–55 mm in length.

Plymouth Experiments

A series of experiments was carried out at Plymouth during January and July–August, 1954. In the preliminary experiments some of the commissure material was included with the post-commissure organs when the extracts were made. Each extract contained ten post-commissure organs and the extract was allowed to stand at room temperature while the organs were being dissected, an operation which generally took up to an hour to complete. The extract was then applied to the centre of a strip of Whatman no. 1 paper and dried under an electric hair-drier; after this operation the paper strip was subjected to electrophoresis, sometimes immediately, but usually a period of about $\frac{1}{2}$ h was allowed for equilibration before the current was switched on. In later experiments at Plymouth the organs from five individuals were used each time and the extracts were applied immediately to the paper: they were not allowed to stand at room temperature.

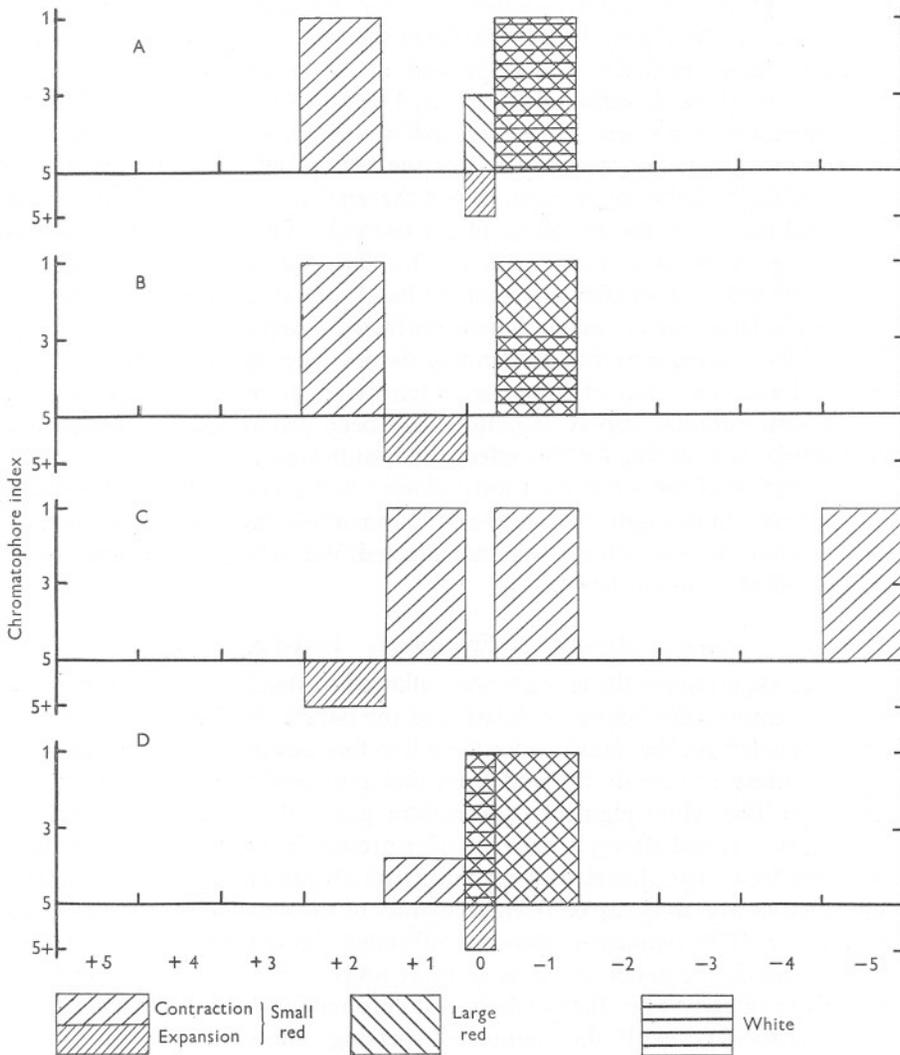
After electrophoresis the strip was dried once more under an electric hair-drier and then cut into portions each 2.7 cm long, with the exception of the central portion, containing the point of application of the extract, which was only 1 cm long. Each portion was then eluted separately in distilled water and the resultant solutions were injected into prawns from which the eyes and their stalks had been removed a week previously. The resultant effects are shown graphically at Text-fig. 3, in which the generally accepted chromatophore index has been modified slightly to give a means of expressing an expansion of pigments greater than that normally found in eyeless individuals. This extraordinary expansion is referred to as 5+.

Immediate Application (Text-fig. 3A)

In three experiments the organs were macerated first in water and then in methanol. Aqueous and alcoholic portions were applied separately to point of origin immediately after removal from the body and the resultant extract was applied within a few minutes to the paper which was then dried. It was hoped in this way to reduce to a minimum the possibility of chemical change of the extract before electrophoresis began. Electrophoresis was carried out at pH 7.5, 320–350 V, current 0.3 mA/cm, applied for 16 h.

In each of these experiments three portions of the paper yielded active extracts after electrophoresis (Text-fig. 3A), namely +2, zero and –1, and the effects of these extracts were different.

An injection of the –1 extract was followed by a contraction of all the chromatophores of the body and of the tail, namely the large red, the small red and the white chromatophores. The effects of this extract resembled those



Text-fig. 3. Diagrams of the results of typical experiments with injection of post-commissure extract into *Leander*, after electrophoresis. In each graph the horizontal axis represents the length of the paper strip, cut into numbered portions (see section on methods), and the vertical axis the chromatophore index after 60 min, in the prawns which were injected with the eluates of the corresponding fragment of paper. Only striking departures from the initial chromatophore index at the beginning of the experiment are recorded; minor variations are omitted. A, after immediate extraction, pH 7.5, 18° C, 16 h, 320-350 V, 0.3 mA/cm; B, organs left 1 h in water at room temperature before extracting, pH 7.5, 18° C, 16 h, 230-250 V, 0.2 mA/cm; C, organs left 4 h in water at room temperature before extraction, pH 7.5, 18° C, 16 h, 430-460 V, 0.5 mA/cm; D, extract boiled, pH 7.5, 18° C, 12 h, 230-250 V, 0.2 mA/cm.

produced by the -1 extract yielded by sinus gland and corpus cardiacum extracts but differed from them by reason of the presence of the white-pigment activator. The substance at -1, present in the three organs enumerated, we have called the A-substance (Carlisle, Dupont-Raabe & Knowles, 1955).

An injection of the zero extract was followed by a contraction of the large red chromatophores of the body and of the tail and of the white chromatophores of the body but by an expansion of the small red chromatophores of the body and the tail, especially those of the uropods. The general effect of this injection was to produce an individual with a dark body and with a scarlet tail; during the first 10 min after injection the bands of colour on the body turned blue as the large red chromatophores contracted sharply and a blue pigment diffused from them into the surrounding tissues; later this blue pigment disappeared and the bands of colour were hardly apparent. A maximally dark prawn with minimal display of pattern had been produced. We have called the material responsible for this effect the B-substance.

An injection of the +2 extract was followed by the contraction of the small red chromatophores only; the large red chromatophores remained dispersed and the white chromatophores were not affected. We call such a substance with a single effect an α -substance.

Delayed Application Experiments (Text-fig. 3B, C)

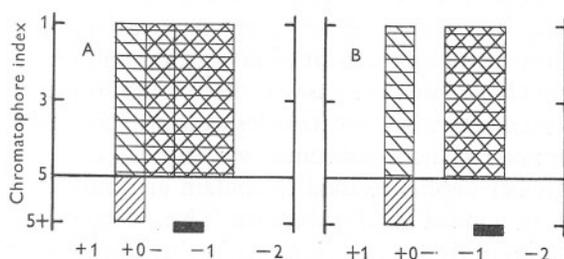
In four experiments the extracts were allowed to stand for an hour or more at room temperature before application to the paper. In one experiment the extract was left on the damp paper for 6 h before electrophoresis began.

The pattern of activity differed from that previously described in several ways. (1) The white pigment concentrator gradually disappeared from its position at -1, and after 3 h was no longer present in a detectable concentration. Sandeen (1950) has already reported that a white pigment-concentrating substance slowly disappeared from an extract of the tritocerebral connectives of *Uca*. (2) The pigment-dispersing substance becomes more mobile and moves towards the anode as far as 4 cm or more from the point of application of the extract. The effect of large red chromatophore concentration previously associated with the pigment-dispersing substance was no longer present, and one may speculate whether the large red chromatophore concentrating substance was originally linked to the pigment dispersing substance, and that the rupture of this link rendered the dispersing substance more mobile. If, this is so, the B-substance contains two components active on red pigments; one ('B_x') disperses red pigments; the other ('B_C') concentrates the red pigments of the large red chromatophores. (3) The -5 portion of the paper yielded an extract which concentrated the small red chromatophores. This additional α -substance had not been detected in the immediate extraction experiments and it seems likely to be a disintegration product of some larger molecule.

Roscoff Experiments

The first series of experiments having shown that when fresh extracts were used the greater part of the substances with effect on the chromatophores were relatively immobile under the conditions used in the experiments, a further series of experiments were undertaken with the express intention of separating those relatively immobile substances. A higher voltage and current were used but for a shorter time; and the zero portion of the paper was cut into two portions, one on each side of the centre, in order that any slight migration might be detected.

In the second series of experiments the extracts, which were made in buffer solution, were not allowed to become dry at any time; they were applied to *damp* paper, which was then immediately subjected to electrophoresis. A spot of DL-leucine was added at the edge of the paper in these experiments to serve as a marker.



Text-fig. 4. As Text-fig. 3, but showing two experiments performed at Roscoff. The black rectangle represents the position taken up by a spot of DL-leucine. pH 7.8, 18.5° C, 5 h, 320 V, 0.8 mA/cm.

After electrophoresis the still damp paper was cut into portions and extracted at once with sea water, while a thin strip along the edge of the paper was dried and stained with ninhydrin to detect the leucine. The immediate application method was used in each experiment.

The most significant feature of these later experiments was the complete separation of the A- and the B-substances (Text-fig. 4), and the demonstration that the B substance bears a slight negative charge, even when both B_X and B_C effects are produced. Another interesting feature of these later experiments was the distribution of the white-pigment-concentrating substance. At 320 V and 0.8 mA/cm it did not apparently move far from the point of application and sometimes not so far as did the A-substance; its distribution in this case strengthens the suspicion that it is not normally bound to the A-substance. If it is bound to any other substance, it would seem more probable that it may be bound to the B-substance, for in a boiled extract it is found associated with the B- rather than with the A-substance (Text-fig. 3D). This suggestion would also help to explain the otherwise somewhat baffling

distribution of the white-pigment-concentrating substances in the experiment illustrated in Text-fig. 4A. The distribution becomes more reasonable if we suppose that some of the white-pigment-concentrating substance has become detached from the B molecule but that some still remains, but we have not yet sufficient evidence to establish that the white-pigment-concentrating substance forms part of a larger molecule.

Environmental and Sex Differences

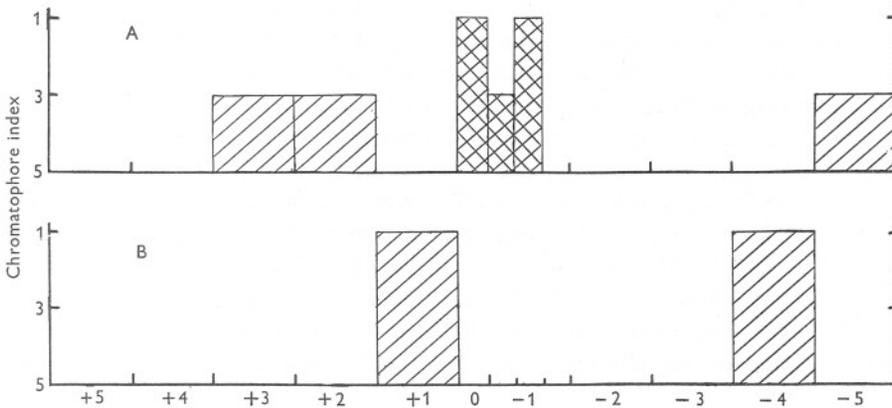
Most of the experiments have been carried out using extracts prepared from male individuals or from a mixture of males and non-ovigerous females, but we have sometimes perforce used females only. The prawns have generally been gathered close inshore in water 1-6 m in depth, but once prawns were used which had come from greater depths—40 or more metres. We have not yet sufficient data to dogmatize, and it is hoped that an investigation of environmental and sex differences, if indeed they do exist, may form the subject of a later publication, but it is worth putting on record here that the electrophoretic patterns which we have so far described may only be typical of shallow-water, male animals. As we have shown, these seem to contain a mixture of A- and B-substances, but when only females were used in the preparation of the extracts little or none of the B-substance was found. On the other hand, male prawns from greater depths seemed to contain an abundance of the B-substance, but little or none of the A-substance. These deeper-water prawns were noticed to be different from those gathered in shallower waters, being almost scarlet in colour and with maximal expansion of the small red chromatophores.

THE SINUS GLAND

The general pattern of these experiments resembled that of the experiments already described. Fresh and stored extracts were used from prawns of both sexes gathered from various localities in the Plymouth area. Normally ten glands were used for each extraction, but in later extractions the number was sometimes smaller.

Immediate Application Experiments (Text-fig. 5A)

In these experiments the glands were dissected out under isotonic saline, immediately crushed on to the spot of origin on the filter-paper strip and dried with a blast of hot air. Each dissection took 4-7 min. As soon as all the glands



Text-fig. 5. As Text-fig. 3, but extracts of sinus glands. Band -1 was subdivided into three. A, after immediate extraction; extract derived from large, blue, non-ovigerous females, pH 7.5, 18° C, 16 h, 320-350 V, 0.3 mA/cm; B, organs left for 12 h in water before extraction, pH 7.5, 18° C, 12 h, 320-350 V, 0.3 mA/cm.

had been applied in this manner the paper strip was placed in the electrophoresis apparatus and, after equilibration, the current was switched on. In such a run the distribution of chromactive substances was similar to that found with extracts of the post-commissure organs, except that no consistent effect was observed on the white chromatophores, nor was any sign apparent of any substance tending to provoke expansion of the small red chromatophores (Text-fig. 5A). About the middle of the -1 band (which was frequently subdivided for elution into smaller sections) was a substance provoking strong concentration of all red pigments, but with a rather stronger action on the small red than on the large red chromatophores. At zero a substance effective in concentrating red pigments was sometimes found, but we have not sufficient evidence that this is a distinct substance. At about +2 and -5 were found small quantities of more labile substances—the α -substances—acting only on

the small red chromatophores. These α -substances appeared similar in all respects to those of the post-commissure organ extracts.

In a small number of experiments a slight concentration of the pigment of the white chromatophores was found after injection of material from about -3. This may be an artifact, for the white chromatophores are directly light-sensitive and may be affected by slight alterations in the intensity of illumination (Knowles, 1940). We have, however, sometimes observed a similar effect after injection of whole fresh extracts of sinus gland (see also Knowles, 1939).

Delayed Application Experiments (Text-fig. 5B)

For these experiments the first gland dissected out was placed in 0.1 ml. distilled water, and each succeeding gland added to this. When all the dissections were completed the material was ground in a small mortar, with addition of a further 0.1 ml. water, and the extract applied, after a variable interval of time, to the dry paper by successive applications with a capillary pipette. Each successive application was dried with a blast of hot air. The residue, after the aqueous extract had been transferred to the paper, was extracted further with methanol, which was then applied to the same spot on the paper.

When an extract was kept for some hours at room temperature before application to the paper the electrophoretic pattern obtained was quite different from that found with fresh extracts (Text-fig. 5B). Little or no chromactivating substance was found at zero or -1 under these conditions, but an abundance of the α -substances was found at -4 or -5 and at +1 or +2, with a strong concentrating effect upon the small red chromatophores.

CORPORA CARDIACA AND BRAIN OF INSECTS

For the comparative study of the chromactivating substances of the corpora cardiaca and brain of insects we have chosen a species, *Carausius morosus*, which itself shows colour changes. The active substances in the extracts of these organs, after separation by electrophoresis, were tested on the living insect and on the crustaceans *Leander serratus* and *Crangon vulgaris*.

Whole extracts of the brain and of the corpora cardiaca of insects are each active upon the pigments but their effects differ, both upon insects and upon Crustacea. Whole extracts of corpora cardiaca provoke a strong concentration of the red chromatophores of *Leander* (Thomsen, 1943; Brown & Meglitsch, 1940) and the black of *Crangon* and *Ligia* (Dupont-Raabe, 1952), but their activity is only moderate upon the pigments of *Carausius*: after injection of a total extract of corpora cardiaca the animals do not take on the black colour characteristic of complete darkening, but pass only from light grey to dark grey. Total extracts of the brain, on the other hand, are completely inactive on the pigments of *Leander*, but act very strongly on the pigments of insects and

possess some degree of activity on the black pigments of crustaceans such as *Crangon* and *Ligia* (Dupont-Raabe, 1949, 1951, 1952).

The conditions of electrophoresis were as described above. Ten organs were usually used for each separation. Dissections were made rapidly under Ringer's solution ('Rabbit Ringer'); the organs immediately after injection were placed in a small quantity of distilled water, ground up and transferred directly, or sometimes after boiling, to the paper strip.

Corpora cardiaca

Injection of eluates, of different portions of the paper strip after electrophoresis, into *Leander* revealed the existence of a band at -1 which was very strongly active upon the small and large red chromatophores. It provoked a strong contraction of all the red chromatophores of the body and tail, but had no effect on the white pigments. In its effects and in its electrophoresis migration it seems comparable to substance A present in extracts of the post-commissure organs and sinus glands of *Leander*. At pH 7.5 eluates of the part of the paper near the origin and 1 cm towards the cathode (0 and $-\frac{1}{3}$) were often rather strongly active, especially upon the small red chromatophores. At pH 5.0 the migration of this substance towards the cathode was much stronger: it was not present in portions 0 and $-\frac{1}{3}$, but revealed its presence at -1 and -2 .

Besides substance A, another substance is evidently present in extracts of corpora cardiaca—a more mobile substance which provokes concentration of only the small red chromatophores. After electrophoresis at pH 7.5 for 16 h at 0.3 mA/cm, 320–350 V the band corresponding to this substance was to be found at $+2$ and $+3$. With a higher current and voltage (1 mA/cm, 540–580 V) it migrated to $+5$. This substance may correspond to the α -substance found at $+2$ in sinus gland and post-commissure organ extracts, representing a disintegration product of the A-substance. Such an hypothesis is supported by the fact that this α -substance cannot be detected if electrophoresis is performed with a boiled extract (in which presumably the enzymes are destroyed), which, however, retains the A-substance, at -1 in full strength.

Substance A is responsible for the action of corpora cardiaca extracts upon the melanophores of *Crangon*. In a series of experiments in which the eluates of the central portions of the paper strip were injected simultaneously into *Leander* and *Crangon* the responses obtained, though different in kind—concentration of the red pigments in *Leander* and of the black in *Crangon*—were always coincident.

The identification of that substance of the corpora cardiaca which is active upon the pigments of insects offers some difficulties, and the results are not yet clear. It will be recalled that total extracts of corpora cardiaca provoke only a moderate darkening of insects, and after electrophoresis, with its attendant losses of activity, the concentration in the eluates is lower and the

reactions obtained still weaker, so that the delimitation of the zones of activity is difficult. The positions of the active zones, so far as they have been determined, sometimes coincide with substance A and substance α , but sometimes we have obtained no response at -1 , while positive responses have been observed with the eluates of portions of the paper inactive upon crustacean pigments, notably $+1$ and -4 . Thus it seems improbable that it is the A- and α -substances in total extracts of corpora cardiaca which influence the insect pigments, the more so as in the only two exploratory experiments we have performed with sinus gland extracts we have not found an exact correspondence in position of substances active upon insects and Crustacea. In later experiments, performed at Roscoff, with a higher voltage and an apparatus permitting several simultaneous runs, a detailed comparison was made, in the central region of the paper from $+2$ to -2 , between the positions of substances active upon insects from extracts of the corpora cardiaca and brain. These experiments showed clearly that the tritocerebral chromactivating substance of the brain (substance C) which was so strongly active upon the pigments of the insect, was not present in extracts of corpora cardiaca.

The Brain

Electrophoresis of extracts of the brain at pH 7.5 revealed the existence of a substance of very low mobility, which was to be found on both sides of the origin ($+0$ and -0). This substance brought about a maximal darkening of the insect, analogous to that obtained with total extracts, and also a concentration of the melanophores of *Crangon*, an effect which was, however, not like that of substance A of the corpora cardiaca. It is totally inactive on pigments of *Leander*. The position of this substance on the paper did not coincide with either substance A or substance B and we have called it the C-substance.

It appears probable that, as with substance A, the C-substance may decompose into more mobile substances of lower molecular weight; parts of the paper situated rather far from the point of application of the extract often showed quite strong activity upon *Carausius*, particularly $+3$, $+4$ and $+5$. We may call this tentatively the γ -substance(s). It will be interesting in the future to compare in detail these substances with the mobile α -substances of the corpora cardiaca which are active upon insects and to establish if they are similar or perhaps identical.

No portion of the paper provoked concentration of the red pigments of *Leander*: substance A does not exist in the brain. Injection of total extracts had revealed no activity, but this might have been due to the existence of masking by an antagonistic substance; this possibility appears to be eliminated by the results of the electrophoresis experiments. Nor has any substance been found in the brain of insects which provokes expansion of any pigments of *Leander* or *Crangon*.

The insects thus possess at least two different chromactivating substances: substance A in the corpora cardiaca and substance C in the brain, localized in the tritocerebral region (Dupont-Raabe, 1954). It is difficult to pronounce any opinion on the origin of the A-substance, present in the corpora cardiaca, and upon its significance in the physiology of the insect, since its presence does not seem to be necessary for the normal colour changes. It does not seem to have its origin in the pars intercerebralis of the brain, where are situated the cells from which the internal cardiac nerves run; nor in the lateral protocerebral neurosecretory cells. Extracts of these parts dissected out from the brain, and of the internal cardiac nerves, are quite inactive, even after boiling, upon the pigments of *Leander* and those of the insect. Perhaps substance A is a transformation product of substance C, arriving at the corpora cardiaca by migration along a third pair of tritocerebral-cardiac nerves whose existence has only just been discovered (Dupont-Raabe, 1955). Yet a single experiment performed with a *Carausius* deprived of its brain 5 days previously has shown no diminution in the content of A-substance in the corpora cardiaca; this would suggest (though this is against the current of present day speculation) that the cells of the corpora cardiaca themselves may elaborate substance A.

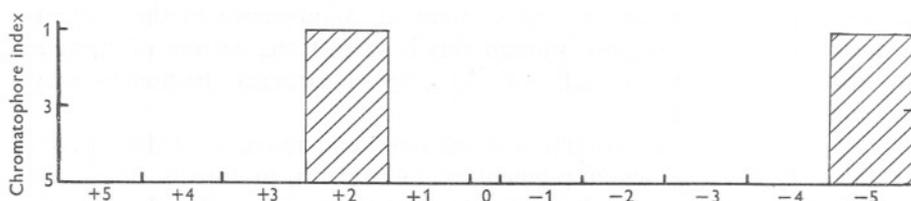
It is possible that the substances of the brain and above all of the corpora cardiaca which bring about pigmentary movements in insects, intervene equally in the regulation of other physiological phenomena, such as heart-beat, water metabolism or the genesis of pigments—all phenomena whose regulation certainly involves the brain and corpora cardiaca (Wigglesworth, 1954; Cameron, 1953; Stutinsky, 1953; L'Hélias, 1955)—and that a chromactivating potentiality is possessed in various degrees at times (perhaps incidentally) by a precursor and by different products of disintegration.

DIALYSIS EXPERIMENTS

In these experiments extracts were dialysed against distilled water or saline through a variety of semipermeable membranes. No significant variations were observed when the following membranes were used: British Cellophane Company's cellophanes PT 600, PT 400, PT 300; British Cellophane Company's wet cellulose film ref. no. 2/468/EHD/WR; two different samples of synthetic sausage skin. All these membranes allowed the passage of eosin, methylene blue (or at least some component of it), and a pink constituent of trypan blue, but failed to allow the passage of congo red. For experiments the extract selected was placed in a cavity slide and covered with a membrane; 0.25 ml. of distilled water or of saline was then placed on top of the membrane and the whole covered with an inverted Petri dish. Under these conditions an hour's dialysis sufficed for the passage of detectable quantities of chromactivating materials from the fresh whole extracts of sinus gland of *Leander*

serratus, *Homarus vulgaris*, *Carcinus maenas*, *Maia squinado* and *Cancer pagurus* from fresh whole extracts of post-commissure organs of *Leander serratus* and of corpora cardiaca of *Carausius morosus*. When, however, extracts of the brain of *Carausius* were dialysed the dialysate was much less active than the residue; it produced its action more quickly after injection, but the action was ephemeral, disappearing after 2-3 h, while that of the residue persisted for at least 4 h.

It was evident in these preliminary experiments that not all the material in the extracts was able to pass through the membranes which we used, for the activity of the dialysate was frequently different in kind from that of the residue. This was especially noticeable with extracts of the post-commissure organs, where apparently the B-substance did not pass, for the dialysate had a concentrating effect on the red pigments while the residue had a strong dispersing effect (Pl. II, fig. 10).



Text-fig. 6. As Text-fig. 3, but extract derived by leaving intact post-commissure organs in saline for $\frac{1}{2}$ h and then removing them from the saline without maceration; saline used without further treatment. Note absence of the A-substance. pH 7.5, 18° C, 16 h, 380-430 V, 0.4 mA/cm.

In order to determine which fractions were and were not able to pass the membranes dialysis was performed on the eluates of various portions of filter-paper strips after electrophoresis of the extracts. It was found that the A- and B-substances and the C-substance were incapable of passing the membranes (Pl. II, fig. 12). The more transient α -substances which are to be found at about +2 and -5 on the papers and the γ -substances readily passed the membranes. These results hold for extracts of sinus gland, post-commissure organ and corpora cardiaca and insect brain.

With the intention of using the cell membranes as the semi-permeable membrane for dialysis experiments, post-commissure organs were dissected out carefully with a portion of the commissures and connectives attached. These were allowed to stand in isotonic saline for half an hour and then the saline was subjected to electrophoresis. The resulting pattern (Text-fig. 6) lacked substances A and B, possessing only the more labile α -substances. In further experiments the dissection was performed as before and the preparation left to stand for five minutes in saline. When this saline was injected

it was found to contain no detectable chromactivating substances. Fresh saline was then added to the preparation and the connectives stimulated with a square-wave stimulator (100 c.p.s., 1.5–2 V) for 2 min only. Some of the saline was then subjected to electrophoresis and some of it injected direct into *Leander*. This showed a strong effect in concentrating the red pigments. The electrophoretic pattern once more lacked the A- and B-substances; the α -substances, however, were present in abundance. It appears likely, therefore, that the chromactivity of the dialysates we have tested is due to the α -substances (and γ -substances) and that the A-, B-, and C-substances, by reason of the size of their molecules or the charges they bear, do not readily pass the membranes.

DISCUSSION

It is evident that paper electrophoresis provides a precise means of dealing with the very small amounts of pigment-activating substances that are present in insect and crustacean organs. Our preliminary studies have shown that the method provides a simpler and cleaner separation than the method of differential solubility in alcohol adopted by Brown and his collaborators, while at the same time supporting their contention that a number of different chromactivating substances are present in crustaceans. Brown & Klotz (1947) showed that the alcohol-soluble fraction of the commissure, which they called CBLH, concentrated the body chromatophores of *Crago* but was without effect on the tail chromatophores; it seems probable that this may correspond to the 'A-substance' which we have found in the extracts of the post-commissure organs as well as in the sinus-gland and the corpora cardiaca. The alcohol-insoluble fraction of the commissure (CDH) darkened both the body and the tail of *Crago*; it seems likely that this is the B-substance that we have isolated from commissure organ extracts. Clearly therefore our paper-electrophoresis studies thus far corroborate the work of Brown & Klotz, but in addition they provide evidence that other pigment-activating substances besides the A- and the B-substance may be present in extracts of the sinus-gland and the post-commissure organs.

The early work on the 'eyestalk hormone' suggested that it was chemically a very stable substance. Abramowitz (1940) found that boiling, even in HCl, appeared to increase the activity of the extracts used. Perkins & Snook (1931) reported that desiccated eyestalks would retain their activity for long periods of time. Our electrophoresis studies suggest that this appearance of stability can be to some extent illusory. While it is true that sinus-gland and post-commissure organ extracts of *Leander* still retain pigment activating potency after some hours in solution at room temperature it is clear that the active substances present after some hours are not those which are present in the neurohaemal organs immediately after they are removed from the body. It has already been reported that the white-pigment activator disappears fairly

rapidly from *Uca* commissure extracts which are allowed to stand at room temperature and that the black-dispersing factor disappears, though more slowly (Sandeen, 1950). Our experiments suggest, moreover, that a red-pigment concentrator (the A-substance) becomes chemically transformed if the extracts are allowed to stand at room temperature, although what appear to be the transformation products (the α -substances) still retain some red-pigment-activating potency. We did not find α -substances after electrophoresis of boiled extracts; this agrees with Sandeen's observation that brief boiling retarded the disappearance of activity from *Uca* extracts.

We have not yet determined whether the α -substances are normally produced and released into the blood of *Leander*; if in fact they are the true hormones and the A-substance is a precursor. The greater mobility of the α -substances and the fact that they pass more readily through a cellophane membrane than do the A- and B-substances supports the idea that they would, if present in the animal, pass freely into the blood-stream, but we cannot yet with certainty state that they are normal blood-borne substances; the evidence suggests that they are fragmentation products of the A- or B-substances, but it is possible that they may be artificially produced during the preparation of the extracts. The results of the experiments in which the connectives were stimulated electrically support the idea that these α -substances are indeed among the actual blood-borne hormones, while the A and B substances are not, but they offer no proof of this. It is certain, however, that immediate extraction of a fresh organ gives different results from a slightly more delayed extraction, and any consideration of earlier work on the chemistry of the eye-stalk extracts and deductions from injection experiments should be reviewed in the light of this knowledge. It is clear that any future studies on the chemical analysis of chromactivators must distinguish between true hormones and precursor substances. The mode of preparation of extracts would seem to be all-important in this respect.

It is interesting to find that the sinus-gland and the post-commissure organs of crustaceans possess a chromactivating substance that resembles physico-chemically a substance present in the corpora cardiaca of insects. Hanström (1940), Brown & Meglitsch (1940) and Thomsen (1943) showed that extracts of the corpora cardiaca concentrated the red pigments in crustacean chromatophores but they did not bring forward any chemical evidence to suggest that the corpora cardiaca substance resembled the sinus-gland substance. Indeed, Brown (1944) has suggested that the chromactivating content of the sinus-gland and the corpora cardiaca may differ. In his experiments in collaboration with C. M. Stuter (unpublished) he claimed that the results indicated that the sinus-glands contained a precursor of the pigment-activating hormone but that the corpora cardiaca did not. The position of the pigment-activating substances after electrophoresis of sinus-gland and corpora cardiaca extracts indicates that the A-substance in the insects and the crus-

taceans may be chemically similar though as yet we cannot prove identity. The significance of the A-substance in insects is not yet clear, unless, as our electrophoresis studies have suggested, it may itself be a precursor of other substances.

Our studies have shown that the behaviour of the brain pigment-activating substance of *Carausius* after electrophoresis differs from any pigment-activating substance that we have separated from the neurohaemal organs of crustaceans. It is also clear that this substance, which we have termed the C-substance to distinguish it from the crustacean chromactivating substances, is ineffective when injected into *Leander*. It does, however, bring about pigment-concentration in the chromatophores of *Ligia* and *Crangon* (Dupont-Raabe, 1952). We may therefore speculate whether the C-substance is absent from crustaceans, or whether it is a physiologically active colour-change hormone of some species. It is perhaps significant in this respect that *Crangon* and *Ligia* differ from *Leander* but resemble *Carausius* in the presence of melanin in their pigmentary effector systems.

It is interesting to find that the method of paper electrophoresis separates from crustacean organs more than one substance which concentrates red pigments in the chromatophores. This finding suggests that both precursor and active substances may be present in extracts of crustacean neurohaemal organs, and that the precursor substances too have some pigment-activating potency. Such a concept would provide a possible explanation for the presence of pigment-activating substances in organs of species which do not themselves possess chromatophores.

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SUMMARY

Pigment activating substances in the prawn *Leander serratus* and the stick insect *Carausius morosus* have been compared. Paper electrophoresis has been used successfully to separate substances in extracts. A substance which we have called substance A appears to be present in extracts of sinus glands and post-commissure organs of *Leander* and in extracts of corpora cardiaca of *Carausius*: it seems very similar or possibly even identical in all three types of extracts. It stimulates contraction of all the red pigments of the body of *Leander*. It is incapable of passing the semipermeable membranes which we

have used, and possesses a low mobility at pH 7.5. There is evidence suggesting that it is not the definitive hormone which is released into the blood, but a precursor which is split before release. What appear to be disintegration products of substance A are present in extracts and increase in amount at its expense if an extract is left standing. These substances—the α -substances—pass freely through a dialysis membrane and have a high mobility at pH 7.5. Only the α -substances are released under the effect of electrical stimulus of the commissure when the post-commissure organs are lying in a saline bath. The α -substances affect only the small red chromatophores of *Leander*.

Another substance of low mobility was found only in extracts of post-commissure organs. This substance B concentrated the pigment of the large red chromatophores only and expanded the pigment of the small red chromatophores of the body and tail. It is antagonistic to substance A. It is incapable of passing a dialysis membrane.

Substance C is present only in extracts of the brain of *Carausius*. This substance provoked a darkening of *Carausius* and concentration of the black pigments of *Crangon*, but was without effect on *Leander*. Substance C has low mobility and cannot pass a dialysis membrane.

At least two chromactivating substances are present in *Leander*, and two in *Carausius*. The mechanism of colour change appears to be totally different in the two species, but *Carausius* possesses one of the substances which is concerned in the colour change of *Leander*, and another of its chromactivating substances is active upon *Crangon*, which, unlike *Leander*, possesses melanophores. It may be that melanin is under the same control in both insects and crustaceans.

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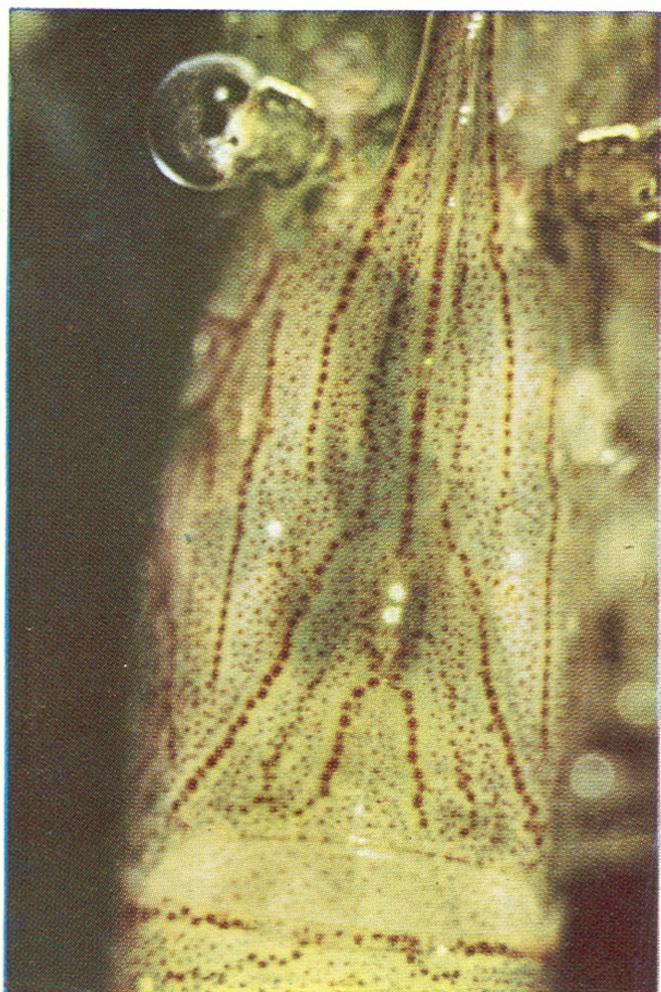
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EXPLANATION OF PLATES

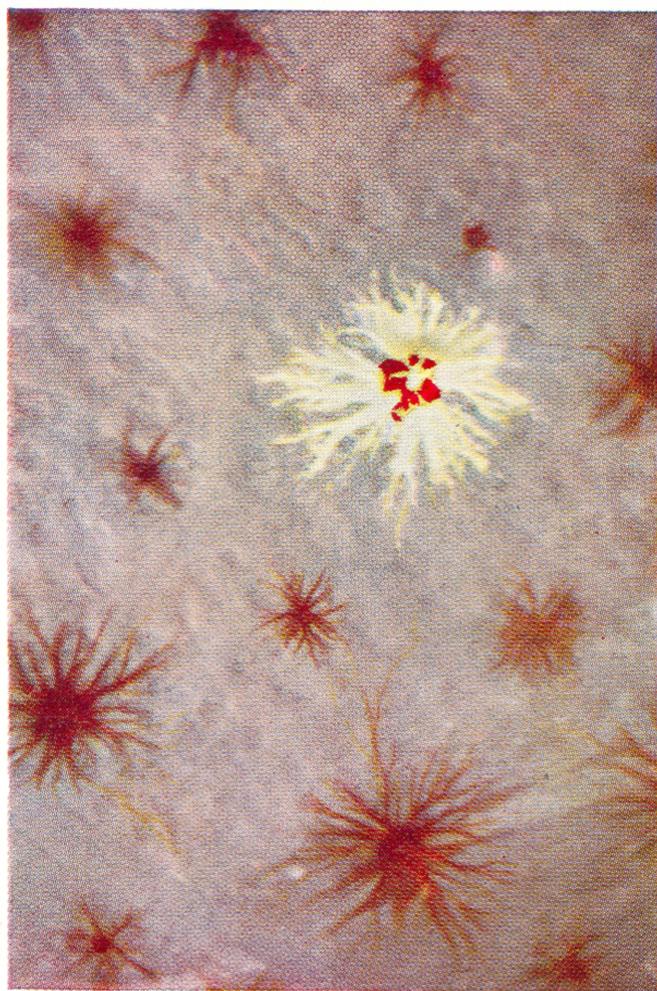
Colour photographs of *Leander serratus*, taken on Kodachrome film (Figs. 1 and 2) or on Ferraniacolor film (Figs. 3-12) with electronic flash.

PLATE I

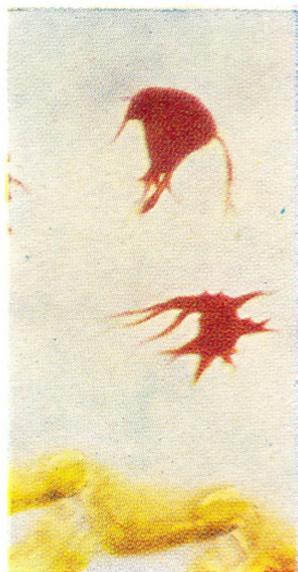
- Fig. 1. A dorsal view of the cephalothorax of *Leander* illustrating some of the chromatophore types—the white reflecting chromatophores, the large red-yellow chromatophores forming the bands, and the small red-yellow chromatophores between.
- Fig. 2. A group of expanded chromatophores (chromatophore index 5) from the cephalothorax; white, large and small red, and faintly under each red chromatophore its yellow component; note the red component of the white chromatophore.
- Fig. 3. The edge of one of the uropods, showing all pigments moderately dispersed (chromatophore index 4-5).
- Fig. 4. Two large red chromatophores of one of the uropods, moderately contracted (chromatophore index 2).
- Fig. 5. The same two chromatophores moderately expanded (chromatophore index 4-5).
- Fig. 6. An eyestalkless *Leander serratus* which had been injected $\frac{1}{2}$ h previously with 0.05 ml. sea water (control).
- Fig. 7. A similar animal which had been injected with an extract of one sinus gland in 0.05 ml. sea water—concentration especially of small red chromatophores, thus enhancing the pattern.
- Fig. 8. A similar animal which had been injected with an extract of one post-commissure organ in 0.05 ml. sea water—concentration especially of the large red chromatophores of the bands, thus obscuring the pattern.



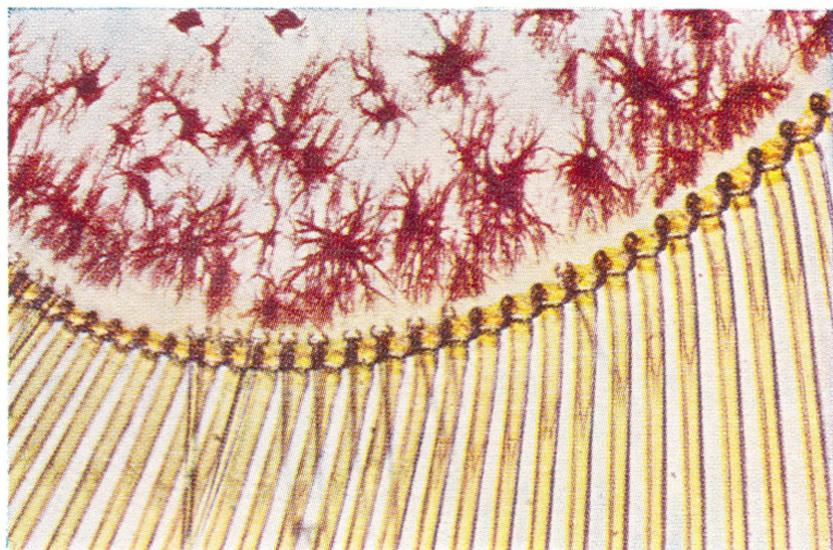
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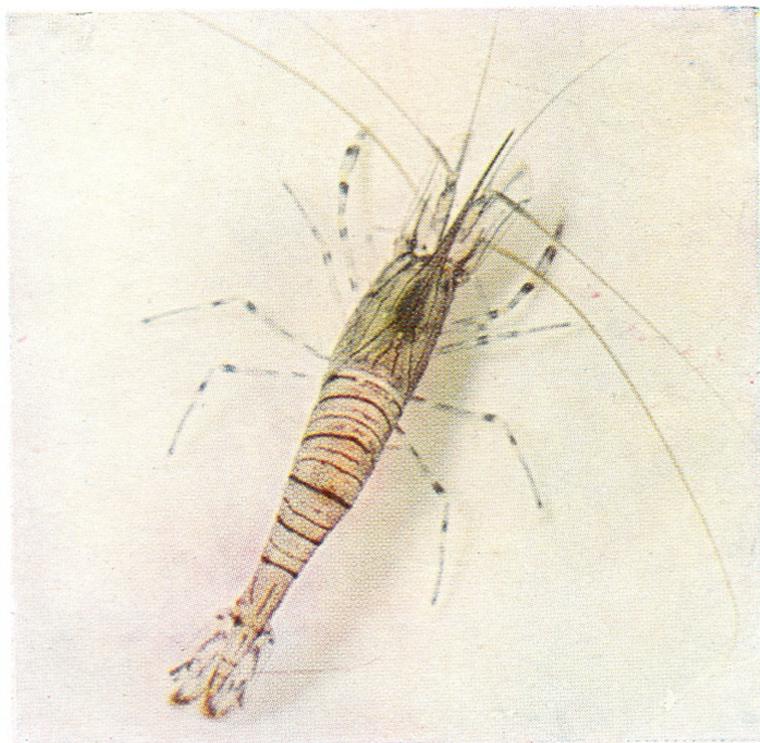
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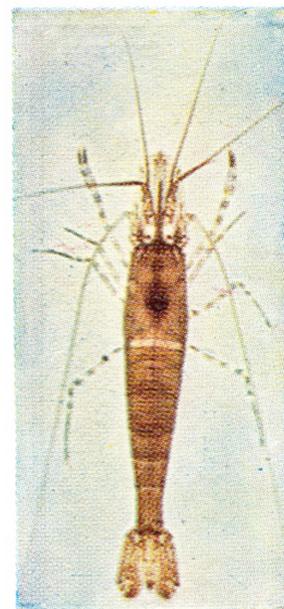
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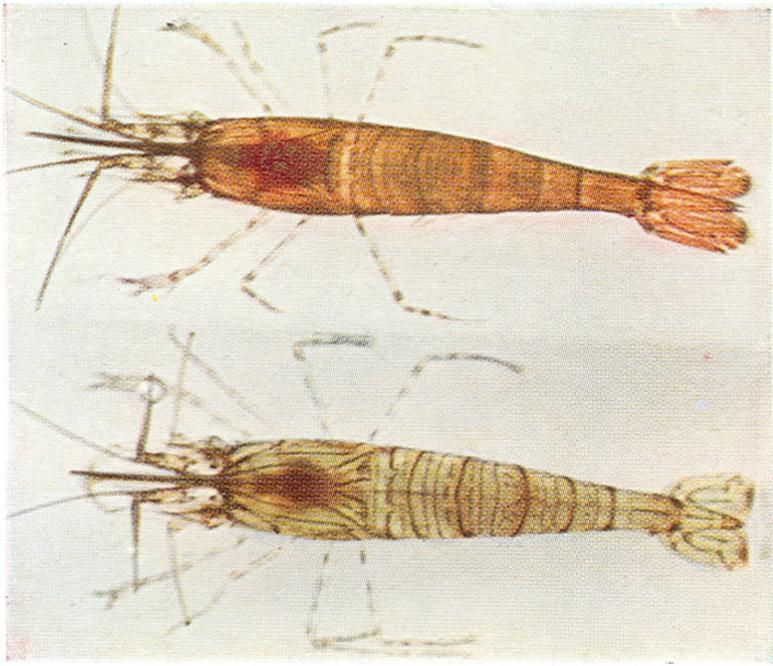
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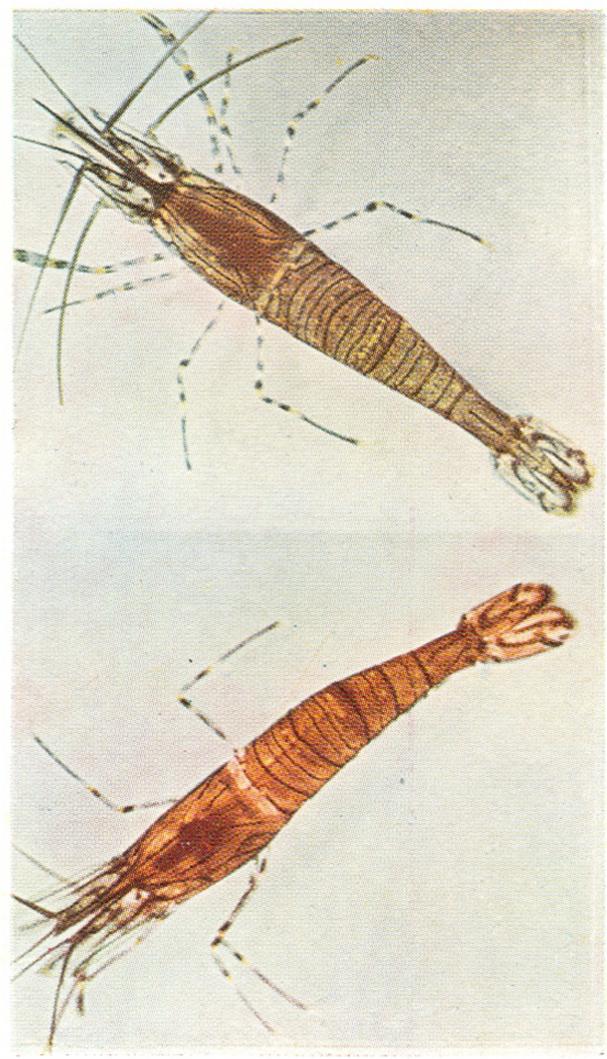
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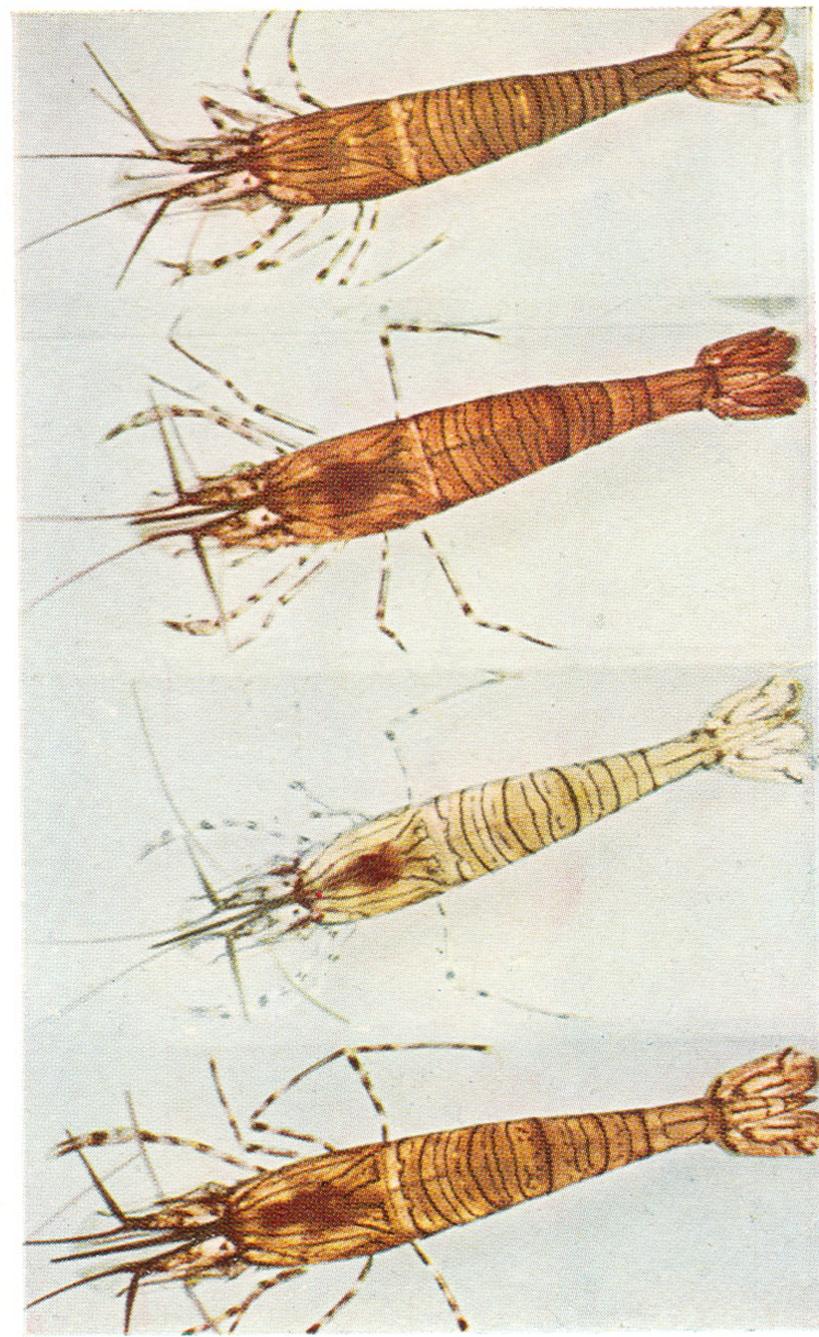
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10



12



+2

0

9

-1

-2



11

PLATE II

- Fig. 9. Electrophoretic separation of chromactivating substances from an extract of post-commissure organs. Under the conditions of this experiment substance B remains at 0, producing a hyperexpansion of the small red chromatophores to 5+ but a concentration of the large red chromatophores; substance A migrates to -1, concentrating all the red chromatophores. Photograph taken 30 min after injection.
- Fig. 10. Separation of chromactivating substance by dialysis. Half an hour before this photograph was taken the left animal had received the fraction of a post-commissure organ extract that had passed through a cellophane membrane: this fraction contained *a*-substances; the animal on the right received the fraction that did not pass through the membrane: the effect is predominantly that of substance C.
- Fig. 11. Separation of chromactivating substances by dialysis. The animal on the left received the fraction of a sinus gland extract which had passed through a cellophane membrane; the right-hand animal that which had not passed through.
- Fig. 12. As Fig. 11, but using the eluate of the -1 fraction of the paper strip after electrophoresis of the sinus gland extract: the A-substance is incapable of passing the cellophane membrane.