THE PIGMENTS OF THE DECAPOD CRUSTACEA.

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PRELIMINARY.

THERE are several investigations on the pigments of different Crustacea; thus Maly [12] described a red and a yellow pigment in the eggs of *Maia squinado*, Halliburton [5] in the course of his paper on the Blood of the Crustacea described a red pigment in *Astacus*, *Nephrops*, etc., Krukenberg [7] discussed some of the characters of the coloration,

while Moseley [13] and MacMunn [11] have studied the spectroscopic characters of certain of the pigments; notwithstanding this there is no general investigation on the pigments of the shell. The matter is of considerable interest because, as is well known, while red pigments are very common in the group, they tend to predominate in the deep-sea forms, those inhabiting moderate depths being very frequently blue or green. Again, as is most familiar in the common lobster, the blue or green colours turn red on the application of heat, acids, alcohol or of many other reagents; but the exact nature of the change is unknown. Krukenberg [7] called the blue pigments lipochromogens, and believed that they readily underwent change, then giving rise to the red (lipochrome) pigments. Pouchet [15] called them the soluble blues or cyanic series, and apparently considered that their destruction allowed the previously invisible red pigments—the xanthic series—to become apparent. The object of the present investigation was to find if possible the relation between the two series, and to study the characters of the pigments. The work was carried on first in the Physiological Laboratory, Surgeons' Hall, and later in the Laboratory of the Royal College of Physicians, Edinburgh. I have to express my obligations to the Council for permitting me to occupy a place in the Laboratory, and especially to the Superintendent, Dr D. Noël Paton, for much kind advice and assistance throughout.

Crustacea investigated.

The animals chosen were *Homarus vulgaris*, the common lobster, *Nephrops norwegicus*, the Norway lobster, and *Astacus nobilis*, the red-clawed variety of the fresh-water crayfish. The colours of the three are very different. In the lobster the shell is a deep blue-black colour, the underlying hypodermis being bright red. In *Astacus* the shell is of a greyish-brown colour, tending to become red in places and especially on the lower surface of the penultimate segment of the chelæ. The hypodermis is bright blue, or violet or reddish, and in many places is clearly visible through the almost transparent shell. In *Nephrops* the shell is orange and the hypodermis red.

It has long been known that the red or orange pigments colouring these structures are lipochrome pigments, although there are no complete chemical investigations on the subject.

Characters of the Lipochrome Pigments.

1. *Definition of Lipochrome Pigments.*

The term lipochrome was introduced in 1882 by Krukenberg [7] to denote a series of pigments exhibiting the following characters:— They are colouring matters which vary in tint from red to yellow or greenish yellow, are readily taken up by fats, and in their natural condition usually occur dissolved in fats, become bluish-green to deep indigo when treated with concentrated sulphuric or nitric acid, give a spectrum with one, two, or perhaps even three bands in the blue or violet, and which are further characterised by the fact that they are not destroyed by the process of saponification, and are soluble in chloroform, ether, alcohol, carbon disulphide, benzol, and in fatty and ethereal oils. The term has been widely accepted in place of the older lutein which was employed in a less extended sense.

2. *Methods and Results of previous Investigators.*

Maly [12] worked at the pigments of the eggs of *Maia*. He found that the eggs contained two pigments, a red and a yellow, and he describes three methods of separating them. He found that the pigments could be obtained by extracting the ova with a cold aqueous solution of albumin. The watery solution was then coagulated by boiling after the addition of a drop of acetic acid, and the coagulum was extracted first with petroleum ether and then with carbon disulphide. The first solvent became yellow and the second deep red. Maly however admits that this is not conclusive, as the effect of carbon disulphide is to heighten the colour of these pigments, but states that the yellow solution left on evaporation a yellow pigment and the red one a red pigment.

As a modification of this method he directly extracted the eggs with petroleum ether, and then, after pouring off the ether, extracted the eggs with water. The red watery solution gave no bands, the petroleum ether showed a feeble band in the neighbourhood of the *F* line.

His second method was to treat an alcoholic extract of the eggs with pure animal charcoal, and allow it to stand for three or four hours and then filter. The solution filters yellow, the red pigment being, he says, detained on the filter.

His third method was to add warm saturated baryta water to the alcoholic extract, when the red pigment is completely precipitated as a baryta compound while the yellow is not. The compound is washed with alcohol in which it is insoluble, and then with alcohol containing dilute acid, by means of which the pigment is obtained pure. Similar compounds are formed by the addition of caustic soda or potash, lime or magnesia. The yellow pigment does not form such compounds. Of the yellow pigment Maly has little to say: he admits the extreme difficulty in obtaining it pure, says that it gives the blue colour with concentrated nitric and sulphuric acids with more difficulty than the red, but gives the spectra of the two pigments as the chief means of distinguishing them. The red pigment he says gives a broad band round *F*, the yellow two bands, one at *F* and one between *F* and *G* —a statement which is obviously inconsistent with that made as to the spectroscopic characters of the yellow petroleum ether solution obtained in his first method.

Investigators since Maly have either confined themselves wholly to spectroscopic work or have employed a modification of his third method as a means of separation of the red and yellow pigments. Thus Krukenberg, who repeated Maly's observations on *Maia*, extracted the eggs with alcohol, and saponified by Kühne's method. This consisted in adding a 20 % solution of caustic soda in the proportion of 1 to 50, driving off the alcohol by heat, adding water, and, after boiling for some time, a strong solution of common salt. He then extracted the soap with petroleum ether repeatedly, and afterwards with ether. The first solution according to him contained Maly's vitellolutein and gave a two-banded spectrum, the second—Maly's vitellorubin—gave a single band. Krukenberg speaks, however, in an exceedingly doubtful way of these two pigments, and considers that there is probably in the eggs a third pigment—zoonerythrin, which gives no bands in its spectrum. In another investigation on the pigments of the Crustacean *Virbius* he got a *one*-banded spectrum with an alcoholic extract, but the residue after evaporation of the alcohol on being dissolved in ether gave a *two*-banded spectrum.

The only other important chemical investigations appear to be the recent ones by Zopf (1892). Zopf studied the pigments of the little fresh-water Crustacean *Diaptomus bacillifer*. His method was similar to that of Krukenberg. He extracted in a mixture of hot alcohol and ether, evaporated off the ether, added caustic soda, boiled the solution to remove the alcohol, and then added excess of

common salt. The soap was digested with petroleum ether which became yellow. This yellow solution gave a two-banded spectrum, and left on evaporation a yellow pigment which gave a blue colour with concentrated nitric or sulphuric acid.

The soap was washed with ether, and then with dilute sulphuric acid, and then again with ether which became reddish-yellow, gave a single broad absorption band, and left on evaporation a red pigment which gave the usual blue colour with strong acid. He gives some other characteristics of the red pigment; among them that the sodium compound is soluble in ether, petroleum ether, benzol and carbon disulphide, and is readily precipitated by adding caustic soda to an alcoholic solution of the pure pigment, the pigment being reformed on the addition of acid. This obviously introduces a difficulty into his method, for if the sodium compound of the red pigment is soluble in petroleum ether and is present in his soap, how can he prove that the yellow petroleum ether extract contains nothing but the yellow pigment? My own observations make me believe that this is an insuperable difficulty, but further criticism may be left until these observations have been detailed.

3. *Observations on the Lipochrome Pigments.*

In the three Crustaceans investigated it was found that lipochrome pigments occur in each case in three situations, (*a*) in the shell, (*b*) in the hypodermis, and (*c*) in the ova, and can in every case be extracted by means of alcohol. It may be convenient to remark at once that in the three animals, *Homarus*, *Astacus* and *Nephrops*, the pigments seem to be identical, whatever the apparent difference of tint.

(*a*) *The shell.*—The shell of *Nephrops* is naturally orange-red and the shells of the other two become orange-red when boiled with water. In all cases on decalcification with dilute acid the orange tint is lost and the colour becomes pure red. The undecalcified shell, especially in *Nephrops*, yields little pigment to cold alcohol, and even on boiling gives only a yellowish solution. After decalcification, the shell yields some pigment to cold alcohol, and on boiling a bright orange-red solution is formed. Unless therefore the removal of the lime salt produces merely a physical change, it would seem that the action of the acid has a direct effect upon the pigment.

The orange-red extract of the decalcified shell was in the early experiments saponified by Kühne's method, but this was later found unnecessary and the following simple method was adopted. A few

drops of caustic soda were added to the alcoholic extract, and the whole heated on the water-bath, water being added if necessary. Usually in the course of a few minutes there was a copious precipitate of pigment of orange-red colour, the solution being left a clear yellow colour. The precipitate was collected on a filter, washed with cold alcohol in which it is insoluble, and then, with dilute acetic acid, which completely changes the colour from orange to dull red. The pigment is now readily soluble in cold alcohol, forming a pink or red solution, and is probably identical with Maly's vitellorubin and Moseley's[13] crustaceorubin. As already described by the former it forms compounds with the alkalis and alkaline earths, all of which compounds are insoluble in cold alcohol and are of an orange-red colour. The fact that the lime compound is orange in colour and insoluble in cold alcohol suggests that the colour of the shell in *Nephrops* is not due to the pure pigment but to the lime compound. This would at least explain the colour-change during decalcification, and the fact that the undecalcified shell yields so little pigment to alcohol.

When caustic soda is added to the alcoholic solution of the shell, and the orange-red precipitate separates out, the solution is left a clear yellow colour. This solution does not yield any precipitate even when the alcohol is completely removed by boiling, and the solution acidified. It is not precipitated on the addition of salt, and is not removed from the solution by shaking in a separation funnel with petroleum ether. From the descriptions of Maly, Krukenberg and Zopf it was at first concluded that this yellow pigment was a yellow lipochrome. All these investigators state that the yellow pigment does not form compounds with alkalis or alkaline earths, but there is some difficulty in making out from their descriptions what becomes of the pigment in alkaline solutions. The yellow pigment obtained above is certainly soluble in caustic soda, and cannot be precipitated from the solution by any means known to me. Not infrequently on shaking the yellow solution with ether, the ether extracts some pigment, and on evaporation leaves an orange mass which may give the blue colour with nitric and sulphuric acids. The whole of the colour can never be extracted in this way, and I am of opinion that in the cases where the blue colour was obtained from the residue, it was due to a trace of crustaceorubin not completely precipitated by the caustic soda, and not to the yellow pigment. Later in connection with the ova more detailed reasons will be given for the view that this yellow pigment is not truly a lipochrome.

The yellow pigment can also be obtained by boiling the shell with caustic soda for a short time, when it passes into the solution.

(*b*) *The hypodermis.*—From the hypodermis of the three forms alcohol or ether similarly extracts the pigment, forming with the first reagent an orange-red solution, with the latter a yellow or orange one. The largest amount of pigment is contained in the hypodermis of the lobster, which is a very brilliant red; that of *Astacus* in the natural condition is blue, but it turns red on the addition of either alcohol or ether.

The hypodermis in each case exhibits some very curious colour-changes with the two reagents mentioned. When the skin, *e.g.* of the lobster, is placed in ether, the ether becomes first yellow, then orange, or if much pigment be used orange-red, the tissue itself also turning orange. If the ether be poured off and the skin allowed to dry, it recovers its red colour. When the skin is boiled with methylated spirit or absolute alcohol, the alcohol becomes orange-red and the skin a dull red; prolonged boiling completely removes the pigment, forming a dark orange-red to cherry-red solution. These colour-changes were at first ascribed to differential extraction, but of this no evidence could be obtained. The alcoholic solution was treated with caustic soda as before, and as before an orange-red precipitate fell, leaving a yellow solution; the two pigments appeared to be identical with those of the shell.

The explanation of the changes observed when the hypodermis is treated with different solvents is found in the characters of the red pigment. This pigment is red in the dry state when pure, but turns orange on being treated with ether and yields an orange solution. If the yellow or orange solution be allowed to evaporate, the red colour reappears as the last drops of ether pass off. The same phenomenon is noticeable in the case of petroleum ether which gives a pure yellow solution. The observation is of importance because as we have already seen Maly and Zopf regarded a pure yellow solution in petroleum ether as evidence of the existence of a yellow pigment; my observations go to prove that the conclusion is absolutely untrustworthy. There seems to me to be little doubt that Zopf's petroleum ether contained traces of the sodium compound of the red pigment, which in small amount, or after fading, may appear yellow.

Another important point about the red pigment, and one which, although known to Maly, has been neglected by all subsequent writers, is its ready solubility in solutions containing proteid. A direct ex-

traction of the hypodermis of the lobster with water gives a pure red solution, which is perceptibly deeper in tint if a trace of a neutral salt be added in order to dissolve globulins. Further, the red pigment is not only soluble in solutions containing native proteid but also in those containing albuminates. Thus a direct extraction of the hypodermis in water is coagulated on boiling, the pigment being precipitated with the albumin, but if a few drops of caustic soda are added, then alkali-albumin is formed and the pigment remains in solution, even on boiling, with the proteid. From such solutions the pigment is not precipitated by any reagent which does not also precipitate the proteid.

(*c*) *The ova.*—The ova of the lobster were the only ones investigated. In the natural condition they are of a green colour, but are turned red by a great number of reagents. The methods of obtaining the pigments were the same as before and showed that here again two pigments existed, a red and a yellow; the yellow was however present in much larger amount than in the shell or hypodermis.

4. *Characters of the Red Pigment.*

The red pigment, which may be called by Moseley's name of crustaceorubin, is bright red in mass in the dry condition. It dissolves in ether and petroleum ether to form in each case when dilute a pure yellow solution, in alcohol to form a pink to red solution, in benzol or chloroform to form a bright pinkish-red solution. In the pure state it is exceedingly unstable, whether in the dry state or in solution, fading even in darkness within a day or two at most. The solutions in benzol seem to be the most persistent, but this is probably due to their brighter colour, which renders the fading less obvious than in the case of alcoholic solutions. Pigment dissolved in benzol loses with great rapidity the power of giving a blue colour with strong acid. This instability is characteristic only of the pigment when obtained pure by the decomposition of the sodium compound, solutions containing the red pigment mixed with the yellow being relatively stable.

The dry pigment gives with concentrated sulphuric or nitric acid a brilliant blue colour, which especially in the case of nitric acid is extremely evanescent.

Further, the pigment forms compounds with caustic soda or potash, and with lime, baryta and magnesia. The compounds are orange-red in colour, but the pure red pigment is instantly regenerated on treating them with dilute acid. The compounds are insoluble in cold alcohol, but are soluble in ether, petroleum ether (slightly) and in benzol; they

are quite insoluble in alkaline solutions either hot or cold, but are readily soluble in solutions of the albuminates. From these they are precipitated by any agent which precipitates the albumin.

The red pigment is soluble both in fats and in albuminous solutions; in the Crustacea mentioned it does not seem to occur in connection with fat.

As to the spectroscopic characters, in a strong solution the pigment absorbs all the spectrum except the red and a little of the green, but in a dilute solution there is a very ill-defined band in the neighbourhood of *F*, as already described by Halliburton and Krukenberg. With a spectroscope of wide dispersion the band is very faint and cannot be accurately measured. The centre appears to be between 495 and 500.

Besides occurring in the situations mentioned the pigment is found, as described by Halliburton, in the blood; here it is unmixed with the yellow pigment.

5. *Characters of the Yellow Pigment.*

About the yellow pigment it is not possible to say very much as it is exceedingly difficult to obtain pure. It occurs in the shell, hypoderm and ova, but in largest amount in the ova. It is readily soluble in ether but little soluble in cold alcohol or petroleum ether, so that a cold alcoholic extract of the shell or hypoderm contains only a trace of it. On saponification of an alcoholic extract obtained by boiling, the yellow pigment remains in the caustic solution, and is not precipitated by the addition of salt or on acidification. The addition of acid causes a change in the colour of the solution from pure yellow to a brownish colour, but there is no precipitation. The pigment is apparently to a slight extent removed by ether from the caustic solution, but it is difficult to prove that it is not merely traces of unprecipitated red pigment which are taken up by the ether. If the yellow caustic solution be evaporated by means of heat, the pigment chars, but if it be allowed to evaporate spontaneously yellow crusts of caustic soda are left coloured with the pigment. The pigment may be then dissolved by hot alcohol or ether, in both of which it forms a yellow solution. The dry pigment is yellow in colour, and gives no blue colour with concentrated sulphuric or nitric acid. With nitric acid it gives a peculiar and characteristic reaction, which is recognisable even when it is mixed with the red pigment. An extraction of the ova with ether, for example, gives on evaporation red pigment more or less intermixed with oily yellowish drops; the addition

of nitric acid gives a brilliant blue colour, which fades in a few moments, then the oily drops float to the surface, the acid becomes bright yellow, and there is a sudden, often violent effervescence with evolution of nitrous fumes; as these clear away the oily drops are seen to be a brilliant green colour. If the amount of yellow pigment be small, the acid becomes orange without the development of the green colour. With the ordinary "pure" nitric acid of the laboratory, the reaction is slow—two to three minutes may elapse before the green colour developes, but nitrous acid produces it instantly. The change is evidently one of oxidation.

On account of the above characters, I do not regard this yellow pigment as a lipochrome. The confusion seems to have arisen from the extreme difficulty of separating it from the red lipochrome and from the fact that the red lipochrome forms pure yellow solutions in ether and petroleum ether.

The statement made above as to the insolubility of the yellow pigment in petroleum ether may seem inconsistent with the statements made by Maly and Krukenberg as to the difference between the spectra of the petroleum ether extract and the ether extract in the case of *Maia*, but the accounts of the spectra given by these two investigators are so difficult, and in some respects so contradictory, that it seems not improbable that some of the results were due to changes in the red lipochrome under the influence of light.

The Pigments of the Digestive Gland.

The pigments of the so-called liver or digestive gland were investigated only in *Homarus* and *Nephrops*.

(*a*) *Nephrops*. The digestive gland in *Nephrops* is of a yellow colour. To cold methylated spirit it yields little pigment, but when boiled with it the spirit becomes a clear yellow colour, turning turbid on cooling. As cold methylated spirit extracts a pigment of apparently identical characters from the liver of the salmon, I believe that the solubility depends upon the nature of the fat with which the pigment is associated rather than upon the pigment itself. The salmon liver contains abundant oleïn, which is readily dissolved by cold alcohol and carries the pigment with it; the Crustacean digestive gland apparently contains little oleïn, the fats present do not dissolve in cold alcohol, and the pigment comes down with the fats on cooling a boiling solution.

The pigment is very readily soluble in ether, in which it forms a

clear yellow solution. If the hot alcoholic solution be saponified by caustic soda, or the ethereal solution by metallic sodium, there is obtained in each case a perfectly colourless soap, showing that the red lipochrome is absent from the liver. In the first case the pigment remains in the caustic solution and in the second in the ether. By the evaporation of the latter solution the pigment is obtained pure and is of a yellow colour. It gives no trace of the lipochrome reaction, but strong nitric acid gives the green reaction already described. The same reaction is given by the yellow oily drops obtained by the evaporation of the ether extract before saponification.

(*b*) *Homarus.* In the lobster the digestive gland is a greenish-brown colour; if placed in methylated spirit it becomes yellow at the surface, and greenish in the deep parts. In ether it becomes green at the surface and brownish yellow deeper in. These appearances are due to the fact that alcohol dissolves out a green pigment which is insoluble in ether, and ether a yellow pigment similarly insoluble in alcohol. The yellow pigment is identical with that of *Nephrops* or with the yellow pigment of the shell, hypodermis and ova. The green pigment is perhaps merely an oxidised form of the yellow one. The evaporation of the green alcoholic solution yields merely a brownish residue, which gives no colour reaction with nitric acid. On the saponification of the green alcoholic solution the pigment remains in the caustic solution, which is however only very slightly coloured.

Both the yellow and green pigment seem to be included under Krukenberg's term "hepatochrome" and MacMunn's[10] term "enterochlorophyll," but their chemical characters do not appear to have been studied before. MacMunn describes the "bile" of the lobster as giving a spectrum with a band in the red, and he states that he found a similar band in the hypoderm extract, which he regards as a proof that the liver pigment is converted into the red lipochrome. The liver extracts examined by me showed no bands but merely a diffuse absorption of the violet end.

Relations of the Pigments.

The above observations seem to prove that while the red pigment found in the shell, hypoderm, and ova of the Decapod Crustacea has been correctly described as a lipochrome, the yellow pigment which can be obtained from the same situations, does not give the lipochrome

reaction, but gives a peculiar and characteristic coloration with nitric acid. It is further identical with the yellow pigment of the liver. This is a point of considerable interest, for according to Cuénot[3] the pigment of the liver in these Decapods is comparable to a true bile pigment, inasmuch as it is habitually eliminated with the fæces to which it imparts their normal colour. A portion of this pigment must therefore be eliminated with the cuticle at the moult instead of by means of the gut.

As to the relation between the red and yellow pigments it is not as yet possible to speak with much certainty. Although the yellow pigment can be obtained from the shell and hypoderm by extraction with ether, caustic soda or boiling methylated spirit, there is little evidence that the yellow pigment as such plays much or any part in the coloration of these tissues in the living condition. The hypodermis of the lobster contains only beautifully branched *red* chromatophores, so that if the yellow pigment exists as such it must be mixed with the red. Further, this hypodermis forms with water or dilute saline solutions a pure red solution with no trace of yellow colour, which filters without leaving any yellow pigment behind. If this albuminous solution be precipitated by the addition of ammonium sulphate, and the precipitate extracted with ether, the ether becomes pure yellow. The yellow extract contains the red pigment intermixed with the yellow. This is shown by the addition of nitric acid, which gives first the blue lipochrome colour, and later the green colour due to the yellow pigment.

Thus, although the hypodermis in the fresh condition gives no evidence of the existence of a yellow pigment, yet this can be extracted from it by means of cold ether. Of this there are three possible explanations, (1) the yellow may be present in too small amount to affect the colour of the living tissues, (2) it may be the result of the modification of a very sensitive chromogen, (3) it may be produced by the modification of part of the red pigment. On the whole the third hypothesis seems the most probable. It is confirmed by the fact that the blood contains the red pigment unmixed with the yellow, and also by the tendency to oscillate between red and yellow which is exhibited by many Crustacea. Many of the Crustacea, especially the smaller and more delicate forms, vary in colour according to the intensity of the illumination, tending to be red in darkness and yellow in light. The change is associated with changes in the shape and colour of the contractile chromatophores. The colour-change is

exceedingly difficult to understand unless there is an intimate relation between the red and yellow pigments.

If the conclusion that there is such an intimate relation be correct then we have the interesting result that apparently a red lipochrome may be built up from an effete "bile" pigment. It would be premature to inquire to what extent this is a common origin for these puzzling lipochrome pigments.

As to the names of the red and yellow pigments, it seems on the whole desirable to retain Moseley's term crustaceorubin for the red one, as the terms tetronerythrin (Wurm[18]) and zoonerythrin (Bogandow[2] also employed for it have been used by different authors in very different senses. For the yellow pigment, until further investigation has cast more light upon its affinities, Krukenberg's term hepatochrome may be profitably retained, in allusion to its occurrence in the liver or digestive gland.

Characters of the Blue and Green Pigments.

Next as to the blue and green pigments of the Crustacea. As already mentioned Krukenberg has stated his belief that these pigments are merely compounds of lipochromes, lipochromogens he calls them, but he was unable to obtain them in solution. In the literature of the Crustacea generally, however, these pigments are usually sharply contrasted with the lipochromes as pigments of the cyanic series, or the soluble pigments, as contrasted with the "fixed" lipochrome pigments which occur in branched contractile pigment cells. The present investigation entirely supports Krukenberg's view.

1. *Pigments of the shell.*

The shell of the lobster as is well known is usually a deep blue-black colour, the soft and imperfectly calcified regions, like the antennæ and swimmerets, tending to become orange-red. A curious exception to this rule is found in the fact that the soft cuticle forming the joints of the great claws is often a pure blue colour. The blue-black shell further turns orange-red when boiled, or exposed for a long time to the action of cold methylated spirit, turns pinkish-red when exposed to the action of acid, and fades to a reddish colour if exposed to the action of light, as in dry museum specimens.

(*a*) *Method of obtaining a solution.* Cold water extracts no pig-

ment from the calcified shell. If, however, the fresh shell be scraped so as to remove the red hypoderm and placed in dilute hydrochloric acid (about 0·1%) and allowed to stand, a blue solution can be obtained. The first addition of the acid is followed of course by an energetic evolution of carbonic acid, while the solution becomes pale pink. The pink colour is due to the presence of small amounts of lipochrome pigment, which dissolves in the acid solution on account of the presence of traces of albumin dissolved from the shell. After standing for some time, however, the hydrochloric acid becomes completely converted into calcium chloride, and the solution becomes neutral or alkaline, and acquires a blue colour. If the operation be performed in a beaker, there is frequently a separation into two layers, a superficial pink acid one, and a basal alkaline blue one. As the blue colouring matter dissolves out, the shell becomes greenish or brownish-orange, but still acquires a reddish colour on heating.

A similar blue solution is obtained by treating the shell with a dilute solution of ammonium chloride, or a very dilute solution of ammonia, but in both these cases the results are somewhat uncertain, and the solution is exceedingly liable to undergo decomposition.

(*b*) *Properties of the blue solution.* The blue solution thus obtained is exceedingly unstable, and is turned pink by a large number of reagents. It always contains proteid, as shown by the xanthoproteic reaction, but often only a trace.

1. *Heat.* When heated on the water-bath, the colour turns violet and then pink at a temperature of from 45°—50° C., and the colour does not return on cooling. On further heating there is sometimes a precipitation of proteid coloured pink by the lipochrome pigment, but in other cases alkali-albumin is formed and the lipochrome remains in solution with it even on boiling.

2. *Acids and alkalis.* The blue solution is exceedingly sensitive to the action of acids, turning pink on the addition of a few drops of dilute hydrochloric acid. Not only the mineral acids but the weaker organic ones like citric and acetic effect the same change. Carbolic acid and crystals of thymol also destroy the blue colour, which renders it exceedingly difficult to preserve the solutions. The addition of alkalis to solutions reddened by acid does not restore the blue colour.

The direct addition of alkalis to the blue solution produces different results according to the alkali employed, and the nature of the solution.

Ammonia added in small quantities to a solution containing calcium chloride, gives a blue precipitate which tends to turn pink on exposure

to the air; added to a solution containing ammonium chloride it produces no effect.

Caustic soda gives a copious precipitate of lime mixed with pink lipochrome, the blue colour being entirely lost.

3. *Salts.* The addition of excess of ammonium sulphate to a solution obtained by means of ammonium chloride completely precipitates the pigment as a bright blue precipitate, but the solution obtained by means of dilute hydrochloric acid is exceedingly difficult to precipitate with ammonium sulphate. No precipitate was obtained with excess of sodium chloride or magnesium sulphate.

4. *Reducing agents.* The action of a stream of sulphuretted hydrogen or of coal gas on the blue solution was usually to discharge the blue colour, but the action was slow and doubtful. A stream of carbonic acid had no effect.

5. *Oxidising agents.* A solution of hydrogen peroxide had no effect on the blue solution.

6. *Absence of copper.* The blue precipitate obtained from the solution gave no reaction for copper when heated on platinum wire in the bunsen flame, nor did the blue solution give any trace of a black precipitate with sulphuretted hydrogen. The same tests were tried with the shell after ignition with a negative result.

7. *Action of alcohol and ether.* The blue precipitate when treated with cold alcohol or ether turns pink instantly, the pink dissolving in the alcohol or ether. When alcohol is added to the blue solution the latter turns pink, the pink pigment being partially precipitated with the proteid.

The pink pigment obtained in all these ways from the blue solution is readily soluble in alcohol or ether, and gives all the characters of the red lipochrome—crustaceorubin—discussed in the first part of this paper.

2. *Pigments of the hypodermis.*

As already mentioned the hypodermis of *Astacus* usually contains a considerable amount of blue pigment; this is readily dissolved by water or better by dilute saline solutions, and gives the same reactions as those given by the blue solution obtained from the shell of the lobster. There are marked individual variations in the amount of blue pigment contained in the skin of *Astacus*, some specimens containing little or none. The shell of *Astacus* is so thin that the colour of the hypodermis has a considerable effect on the coloration.

3. *Pigments of the eggs.*

The eggs of the lobster when contained in the ovary are bright green, and when found attached to the swimmerets are very dark green. In both cases they yield if treated with water a bright green turbid solution, which clears on the addition of ammonium chloride. The eggs themselves are turned orange-red with great rapidity if treated with alcohol, ether, dilute acid, hot water, etc. If preserved in a dilute solution of formalin they turn red also but much more slowly; thymol or carbolic acid added to a watery extract of the ova also turns it red. The impossibility of finding a preservative agent which does not effect this change is a great difficulty in the way of a study of the colouring matter, as except in very cold weather the ova decompose very rapidly. The green solution is more unstable than the blue one obtained from the shell, but resembles it in all its reactions. Caustic soda or potash turns it red at once but ammonia does not, and gives no precipitate. A solution containing ammonia usually however turns red on standing. The clear solution obtained by crushing the ova with a dilute solution of ammonium chloride sometimes deposits yellow-orange oily drops and then turns pale blue. The blue solution gives all the characters of that obtained from the shell. The oily drops seem to consist of fat mixed with both red and yellow pigments.

The above observations seem to show that the green colour of the ova is due to a mixture of the blue pigment of the shell with the yellow hepatochrome and apparently traces of crustaceorubin.

Nature of the Blue Pigment.

As to the nature of the blue pigment, the observations detailed above seem definitely to exclude the hypotheses that the change from blue to red is due to oxidation or reduction, and that the blue pigment is a copper-containing compound. The constant association of proteid with the blue pigment, might suggest that this is an albuminous pigment derived from the hæmocyanin of the blood. This is however negatived by the fact that the amount of proteid is exceedingly variable and often very small. The presence of proteid in the solutions is, I think, explained as follows. The blue pigment readily undergoes changes which convert it into a red lipochrome, and we have already seen that this red lipochrome is remarkable for the

readiness with which it forms compounds with alkalis and alkaline earths, these compounds being insoluble in pure water but readily soluble in solutions containing proteid. I am of opinion that the blue pigment is a compound of the red lipochrome, which is insoluble in pure water but is removed from the coloured tissues by any reagent which also removes traces of proteid.

As to the nature of the substance with which the red lipochrome is united it must obviously be exceedingly unstable. If a piece of blue shell is boiled with water, the water becomes strongly alkaline, as does also water in which the green eggs are boiled. If the filtered alkaline solution be warmed with a few drops of caustic soda, a volatile alkali is given off, which gives Nessler's test for ammonia; the alkali is not given off on boiling without caustic soda. As in the case of the shell or the blue solution, the solution becomes alkaline as the blue colour disappears, there is a strong presumption that the alkaline substance is produced by the decomposition of the blue pigment. The strongly alkaline water in which portions of the shell have been boiled contains a large amount of proteid. If the solution be exactly neutralized a considerable amount of proteid is precipitated, and the addition of alcohol produces a further precipitate. If this precipitate be filtered off, and the solution evaporated, a crystalline residue is left behind, but this is always mingled with a brownish substance of unknown composition, which, like the crystals, is soluble both in alcohol and water.

Now ammonia itself, like other alkalis, unites with crustaceorubin to form a compound which is soluble in a solution containing proteid, but this compound is not blue but somewhat orange-coloured, so that the blue pigment cannot be due merely to a compound of this kind. From the somewhat "fishy" smell given off during the boiling of the shell in caustic soda, it seems not improbable that the volatile alkali is not ammonia, but a substituted ammonia, *e.g.* trimethyamine. It was therefore thought that the blue pigment might be a compound of a lipochrome with trimethylamine. If this were so, however, one would expect that the introduction of the alkali, obtained by warming the shell with caustic soda, into a solution of the red lipochrome would give the blue pigment. This was tried with the beautiful red solution obtained by treating the hypodermis of the lobster with water, but gave a negative result. This fact may merely prove that all the conditions necessary for the synthesis were not fulfilled, but the other fact that the alkaline solution does not give off a volatile

alkali except after the addition of caustic soda, leads me rather to believe that the blue pigment is the result of a combination between a complex organic base and a lipochrome, the base being decomposed or modified by boiling, and giving off a volatile alkali on heating with caustic soda. Now the interest of this result lies first in the fact that there is in the muscle of the lobster a complex unstable substance, which is according to Krukenberg of the nature of a lecithin, and which readily decomposes on boiling; and secondly that according to Krawkow[6] chitin itself is formed from the union of a carbohydrate with a nitrogenous substance, perhaps a member of the ammonia group. Krawkow indeed describes an evolution of ammonia from the tissues of the crab after the moult. If it be correct that the blue pigment is formed from the union of an organic base and a lipochrome, this would seem to suggest that there is some connection between the little known substance in the muscle and the formation alike of the blue pigment and of chitin. I hope to make some further observations on this subject, but the above at least shows that Krukenberg was correct in regarding the "cyanic" pigments of Crustacea as compounds of a lipochrome, and it seems also to prove that the compound is of the nature of a union between an extractive of the muscle and the pink lipochrome.

These observations do not shed very much light on the reason why the blue pigment should be almost invariably absent from deep-sea forms, but it should be remembered that the chitinous coat in these cases is exceedingly delicate and slightly developed. If it be true that the base in the blue pigment has some connection with the formation of chitin, then the absence of the pigment and the diminished power of forming chitin may be associated. Further, although I am not at present able to make any statement as to the nature of the readily decomposable substance in the muscle of the lobster, yet if Krukenberg be correct in regarding it as of the nature of lecithin or protagon, then the continued presence of green and blue pigments in the eggs of the deep-sea Crustacea is not very remarkable. There can be little doubt that this substance, whatever its nature, is associated with the formation of the blue pigment, and bodies like lecithin tend to occur in connection with yolk.

Relations of Crustacean Pigments.

In Faxon's account of the Crustacea collected by the U.S. Steamer "Albatross" there is a very interesting description of a deep-sea prawn (*Benthesicymus tanneri*). This prawn was usually found to be of a blood-red colour with blue spots on the anterior abdominal segments, but one specimen was taken in which these segments were unsymmetrically marked in blue and yellow in an extraordinary fashion. Faxon recalls the old (1872) observations by Pouchet on *Leander serrator*, a prawn in which the colour changes from red through blue to yellow according to the intensity of the illumination, and suggests that in *Benthesicymus* the apparent abnormality may be due to change of colour produced during the passage from the darkness of the ocean depths to the light of the surface. Faxon considers that this case shows that the "cyanic" pigments persist even in the deep-sea forms, and appears to lay especial stress on the expansion of the chromatophores in the absence of light as the important factor in the production of the deep-red of the abysmal Crustacea. So far as I understand him, he considers that the blue pigments still exist in these forms, but are concealed by the expanded chromatophores. Now in forms with a transparent cuticle, the chromatophores doubtless play an important part in varying coloration, but it is difficult to believe that their movements alone account for the changes from red to blue so obvious in many Crustacea. Bateson [1] speaks of finding Copepoda living under similar conditions of which some had red egg-sacs and some blue, and the fresh-water cray-fish sometimes appears in a full blue variety (9 and 17). Even in the hypodermis of an individual cray-fish there are always areas which are pure blue, areas which are purplish, and others which are pure red, the colour seeming to depend on the amount of blue pigment present rather than upon the condition of the chromatophores. These facts seem to point to the conclusion that the blue pigment is formed or destroyed under certain conditions of the tissues from the pink lipochrome of the chromatophores; what these conditions are however is still uncertain. The observations by Pouchet and Faxon as to the relations between blue and yellow colours, taken in conjunction with those already described in this paper as to the relations of the red and yellow pigments seem to me to prove that that conversion of the red into a yellow pigment which seems to take place so readily after death, also tends to occur in the living tissues, especially under the influence of light.

Summary.

The above observations show that the Crustacea investigated contain in their shells, hypodermis and ova, a red lipochrome pigment. In the case of the two former structures this is either accompanied by a small amount of a yellow pigment, or more probably the red is an exceedingly unstable pigment, and tends under the influence of certain reagents, and especially in the presence of heat, to become converted into the yellow. The yellow pigment appears to be identical with one which normally occurs in the digestive gland, and is in part eliminated with the fæces.

Further, the red lipochrome very readily forms combinations with alkalis and alkaline earths, the compounds being orange in colour and almost insoluble in cold alcohol. As the undecalcified shell of *Nephrops* is orange and yields little pigment to cold alcohol, while the decalcified shell is pink and yields its pigment very readily to cold alcohol, it seems not improbable that some such lime combination of the pigment exists in it. If this be so, it probably explains in part the fact that the deep-sea Crustacea tend to be blood-red in colour, as do also some of the small and more delicate surface forms. In both these cases the cuticle contains exceedingly little lime, and the pigment apparently exists in the uncombined form when it is bright red. As is well known both these kinds of Crustacea become decolorised in spirit very rapidly, even when not exposed to light.

Finally, this red lipochrome unites also with an organic base apparently derived from the muscle, and thus gives rise to the blue pigment of *Astacus* and *Homarus*.

I conclude therefore that the colour variations of Crustacea can all be referred to chemical variations in the yellow hepatochrome of the liver which forms the central pigment of the group. This may become modified into a red lipochrome, which may directly colour the shell, or may in association with lime give rise to an orange colour. The red lipochrome may further unite with an organic base to form a blue pigment, while a mixture of this blue pigment with unaltered yellow gives rise to a green colour.

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