ECHINOCHROME, A RED SUBSTANCE IN SEA URCHINS.

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INTRODUCTION.

My interest in echinochrome arose from studies in permeability. In the same way that haemolytic agents cause haemoglobin to leave the red blood corpuscles, so do cytolytic agents cause echinochrome to leave the cells containing it. R. Lillie is of the opinion that this is due to the action of the cytolytic agent in increasing the permeability of the cell surface.

In the elaeocytes, wandering cells of the body fluid of Arbacia punctulata, the cytoplasm is crowded with spherical chromatophores. Some of these may be colorless, but usually they are colored bright red with echinochrome. Similar chromatophores, though not so close together, occur in the eggs. In the unfertilized egg they are evenly distributed throughout the cytoplasm. But after fertilization, the chromatophores all migrate to the surface within half an hour. During cleavage of the egg, they are massed in the cleavage furrows. The pigment occurs also in the test of this sea urchin, and gives the animal the characteristic color, which varies from a bright red (especially in young individuals) to a dark red, and may be almost black in old specimens.

In reference to the fact that the pigment may be caused to leave the chromatophores and pass into the cytoplasm and thence into the medium, the following questions may be asked: (1) How is the pigment held in the chromatophores? (2) What is its function? (3) What is its chemical nature? The present paper is concerned with these questions.
Echinochrome

HISTORICAL.

Echinochrome was studied spectroscopically by McMunn, who found it in the elaeocytes of the sea urchins, *Strongylocentrotus lividus, Amphidolus cordatus, Echinus esculentus*, and *E. sphaera*. The spectrum showed faint absorption bands, which varied with different solvents and different reactions of the same solvent. McMunn thought that he noticed changes in the spectrum on the addition of powerful reducing agents, such as stannous chloride, and concluded that echinochrome functioned as an oxygen carrier. However, the absorption bands in its spectrum are difficult to make out except in absolute alcohol (or glycerine) and in this solvent I observed that stannous chloride caused a precipitation of the pigment, which interfered with the examination.

A. B. Griffith attempted an elementary analysis of the substance. He dried the elaeocytes and extracted them with chloroform, benzol or carbon bisulphide. On evaporation of the solvent he analyzed the substance without further purification, although evidently it contained many impurities. From four analyses, he deduced the formula $C_{102}H_{99}N_{12}FeS_{12}O_{12}$, which would make $C = 67.8$ per cent, $H = 5.5$ per cent, and $N = 9.3$ per cent. He states that on boiling with mineral acids it is transformed into haemato-porphyrin, haemochromogen and sulphuric acid ($E + \text{acid} = 2CmH_nN_4O_4 + CmH_nNF_3O_3 + H_2SO_4$). Griffiths agrees with McMunn that echinochrome is an oxygen carrier, and states that the oxygen is held rather firmly, and in nature is removed only by the reducing action of the cell containing the pigment.

EXPERIMENTAL.

The pigment in the elaeocytes, eggs and tests of Arbacia, shows no absorption bands, but after extraction it shows very similar bands in its spectrum to those described for echinochrome by McMunn. He published drawings of the spectra and measured the wave lengths corresponding to the edges of the bands. It is well known that bands become broader as the solution is more concentrated, and for that reason I measured the wave length of a line of the spectrum corresponding as nearly as could be determined to the center of each band. By taking the mean between the wave lengths of the edges of the band in McMunn’s data I have compared his with mine. The discrepancies may be accounted for,

first by the fact that the mean is not the exact center of the band in a prism spectrum, and secondly there is a personal equation in observation. I found the pigment extracted from elaeocytes, eggs or tests to give about the same spectra, though a few isolated observations seemed to vary. These might have been due to decomposition products with different spectra.

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<tr>
<th></th>
<th>Ether</th>
<th>Absolute Alcohol</th>
<th>H₂O</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Neutral</td>
<td>+HCl</td>
</tr>
<tr>
<td>My data...</td>
<td>5296</td>
<td>4844</td>
<td>5304</td>
</tr>
<tr>
<td>McMunn...</td>
<td>5312</td>
<td>4848</td>
<td>5370</td>
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Neither McMunn nor Griffiths succeeded in crystallizing echinochrome. Dr. A. P. Mathews had observed that on the addition of iodine in potassium iodide (KI₃) crystals form easily. In 1910 I obtained quantities of these crystals, but did not succeed in recrystallizing them without great loss by the formation of amorphous masses. The iodine compound in absolute alcohol showed an additional, but very dim band in the spectrum (wave length 5628 or 5696). It crystallized in red or orange needle-like crystals, triangular in cross section, sometimes rhombic in side view and often forming rosettes. They were but slightly soluble in water unless hot or containing acid, soluble in absolute alcohol (the rhombic crystals seeming more soluble than the needles) and slightly soluble in ether. If a solution in water is shaken with ether the latter is not colored. If an alkali is added to the KI₃ solution no crystals are formed (due to combination of the base with the echinochrome) but HCl does not prevent their formation.

Some of this iodine compound which was kept for several months in a dry state became more soluble in ether and crystallized in flat thin, red or orange rhombic plates. Perhaps the substance had decomposed with the liberation of iodine, for I succeeded in crystallizing the mother substance and obtained the same plates, in addition to red or orange needles, never triangular in cross section, but sometimes forming rosettes.

I extracted echinochrome from the tests with strong, slightly acidulated alcohol and purified it by repeated precipitation with
alkali and solution in acid alcohol, and filtration. Finally I dissolved the precipitate in water plus HCl, filtered and shook the solution with ether. The ether did not remove all of the echinochrome and the formation of haptogen membranes caused much loss of material. The ether was evaporated at room temperature, as heat seemed to decompose the substance. Occasionally a few crystals formed at the edges of the solution but the main mass of the residue was amorphous.

The next season (1911) I tried to purify echinochrome without the use of acids or alkalies. The body fluid of the sea urchins was allowed to clot and the elaeocytes thus obtained were placed directly into acetone, which extracted the pigment. The extract was filtered and evaporated at room temperature. The residue was washed with carbon tetrachloride (which does not easily dissolve echinochrome) to remove fats, and again dissolved in the smallest quantity of acetone and filtered to free it from traces of lecithin. This solution was evaporated, dissolved in absolute ether and filtered to remove salts, evaporated to constant weight and analyzed by Dennstedt’s method. A mean of two analyses gave: C = 51 per cent, H = 7.7 per cent. The echinochrome purified by precipitating with alkali gave C = 53.3 per cent, H = 4.4 per cent, N = 1.5 per cent. The nitrogen was determined by Kjeldahl’s method and therefore may not be reliable, since the constitution of the molecule is unknown. Traces of sulphur and phosphorus, possibly due to impurities were found, but no iron. The ether-soluble crystals from spontaneous decomposition of the iodine compound gave C = 57.9 per cent, H = 6.5 per cent.

It was stated above that echinochrome is precipitated by alkalies in alcohol. I precipitated echinochrome with NaOH in 95 per cent alcohol and washed in the same alcohol to remove the excess of NaOH. From the amount of NaOH that was neutralized by the pigment I concluded that it combined with from 18 to 25 per cent of Na. Analysis gave C = 31.5 per cent, H = 6 per cent, Na = 19.5 per cent. Therefore we may say that the echinochrome behaves as an acid, or else is amphoteric. The former view is
supported by the fact that on passing an electric current through the aqueous (colloidal) solution, the echinochrome shows a negative charge (is anodic) and again, if histological sections are placed in such a solution the acidophile portions are stained more strongly than the remainder. In fact its behavior is very similar to that of a weak solution of eosin, except that it is very easily washed out by alcohol.

However the substance is probably amphoteric (the acid character being stronger than the basic) since its aqueous solution is precipitated by phosphomolybdic and phosphotungstic acids but not by tannin.

From the analyses given above it would seem that no one has succeeded in obtaining echinochrome in a reasonably pure state. It is very unstable and probably breaks up into a host of decomposition products all having practically the same spectrum. If it is kept in the dry state for a great length of time, or is evaporated on a bath not over 50° for a shorter time, part of it becomes insoluble in ether but not in alcohol.

When heated in the combustion tube it first melts, then boils and sublimes as crystals on the top of the tube, then very soon turns brown and chars. After being crystallized from a solution in ether the crystals often become smaller and irregular in outline. Perhaps the crystals evaporate or lose water of crystallization, but I think that both these possibilities are improbable. The crystals may decompose into an amorphous substance.

On first obtaining crystals, I feared that they were crystals of some other substance merely colored by echinochrome, but this seems impossible from later observations.

In extractions made for the purpose of studying the lipoids in Arbacia eggs, red or brown substances (echinochrome or its decomposition products) appear in every fraction, rendering analysis difficult and indicating the instability and wide solubility of the substance.

In order to test the statement that it is an oxygen carrier I separated the cells from 50 cc. of body fluid by the centrifuge, and mixed them with sea water to make 50 cc. This suspension, and 50 cc. of sea water as a control, were placed in two similar graduated tubes. The air was pumped out for six hours (until the water boiled), air was then admitted and the tubes sealed. They
were shaken one-half hour and the volume of air measured at atmospheric pressure. The suspension had lost 1.25 cc., the control only 0.8 cc. In another experiment the suspension lost 0.95 cc. and the sea water 0.8 cc. It was thought that in the absence of oxygen the cells would take the oxygen from the echinochrome. However no color change could be observed with the naked eye or the spectroscope, and the greater absorption of air by the suspension may have been entirely due to oxidation in the cells. In similar experiments, with an aqueous solution of the pigment, and distilled water for a control, and using pure oxygen, the two tubes gave the same absorption, as shown by two examples:

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<th>Oxygen absorbed by H₂O</th>
<th>Oxygen absorbed by echinochrome</th>
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<tr>
<td></td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.15</td>
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The question, how echinochrome is held in the chromatophores, cannot be fully answered. The chromatophores when free from pigment are highly refractive and stain strongly with the intravitam stain, neutral red, and when fixed they stain strongly with Delafield's haematoxylin, indicating a lipoid nature. The pigment may be in solution in the lipoid.

The fact that the spectrum is different (shows no bands) in life from the spectrum of the extract may indicate chemical combination of the pigment with the chromatophores. The fact that echinochrome stains acidophile tissue may show a possible mode of such combination, if it be found that the chromatophores contain bases. However I do not think we can rely on the spectroscopic evidence, for the absorption bands are very faint in aqueous solution unless it be alkaline, and the cells containing the pigment interfere with the passage of light and make the observation difficult. I have never seen absorption bands in echinochrome extracted from the fresh cells with distilled water. The same statement is made by McMunn. If the substance is held by chemical combination why does it come out so easily?

The same argument may be made against the possibility that the echinochrome is held in the chromatophores because it is more soluble in them than in water. When the cell is stimulated mechanically or chemically the pigment comes out of the chromato-
phores with explosive rapidity. The cell need not be killed to accomplish this. The mere act of normal fertilization causes some of the chromatophores in the egg to lose their pigment.

The only alternative hypothesis I know of is, that the pigment is manufactured in the chromatophore, and cannot normally get out because the surface of this body is impermeable to it. An increase in permeability of the chromatophore allows the pigment to escape. Such an increase in permeability might be due to an aggregation change in the colloids of the limiting membrane or surface film.

Echinochrome is held in the chromatophores of the sea urchin's cells probably in the same way that chlorophyll is held in the chromatophores of the green plant cell.
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