The Colouring Matters in the Compound Ascidian Diazona violacea, *Savigny.*

By Alfred Holt, M.A., D.Sc.

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1. *Experimental Observations.*

The present investigation on the colouring matters of the compound Ascidian *Diazona violacea*, Sav. (= *Syntethys hebridicus*, Forbes and Goodsir), had its origin in an observation of Prof. W. A. Herdman while dredging in the neighbourhood of the Outer Hebrides in 1912.

Some specimens of this rare Ascidian were collected by Prof. Herdman, which showed whilst alive the green tint described by Forbes and Goodsir,* but on placing them in alcohol for purposes of preservation it was found that after a few hours the alcohol had acquired the original green colour of the Ascidian, while the organism itself had changed to violet, a shade nearly complementary to that of the living animal. A description of these specimens has already been published.†

During another expedition to the Hebrides in the summer of 1913, Prof. Herdman obtained so many specimens of this organism that it was possible to use some of the material for an examination into the nature of the green and violet pigments, but a complete study has been impossible owing to the minute quantity of pigment found in any one Ascidian colony, and to the fact that no fresh and living material was at my disposal. The green alcoholic solution obtained from the specimens collected in 1912 had been examined, and a brief account of the results was given in the above-mentioned paper in the Journal of the Linnean Society, but it will be useful to begin by recapitulating them here, and also to add some further information.

The green colour of the solution was not unlike that due to chlorophyll, and it exhibited well marked red dichroism. The absorption spectrum consisted of a broad band in the orange red, which was characterised by a more distinct edge towards the red than towards the yellow, and there was also practically complete absorption at the blue end of the spectrum. The band had the greatest intensity about $\lambda = 620\ \mu\mu$, and the absorption in the blue and violet began at $\lambda = 470\ \mu\mu$, and continued downwards.

Though not identical, this spectrum is not unlike that of true chlorophyll,

* 'Roy. Soc. Edin. Trans.,' vol. 20, p. 307.

† 'Linn. Soc. Zool. Journ.,' vol. 32, May, 1913.

in so far that the general absorption towards the violet begins at almost the same point, and that there is a very definite band in the orange red. The two spectra are shown in the accompanying figure, where their points of agreement and disagreement are more immediately visible.

In 1875, Sorby* obtained a green alcoholic solution from the Gephyrean worm *Bonellia viridis*, which, when examined spectroscopically, gave an absorption spectrum which also resembled chlorophyll in some respects. In neutral solution there was a very pronounced band at $\lambda = 636\,\mu\mu$ and distinct bands at $\lambda = 587$, 520, and $490\,\mu\mu$, but he does not record any general absorption for $\lambda < 470\,\mu\mu$. His spectrum ("bonelleine") is also reproduced in the figure for the sake of comparison.

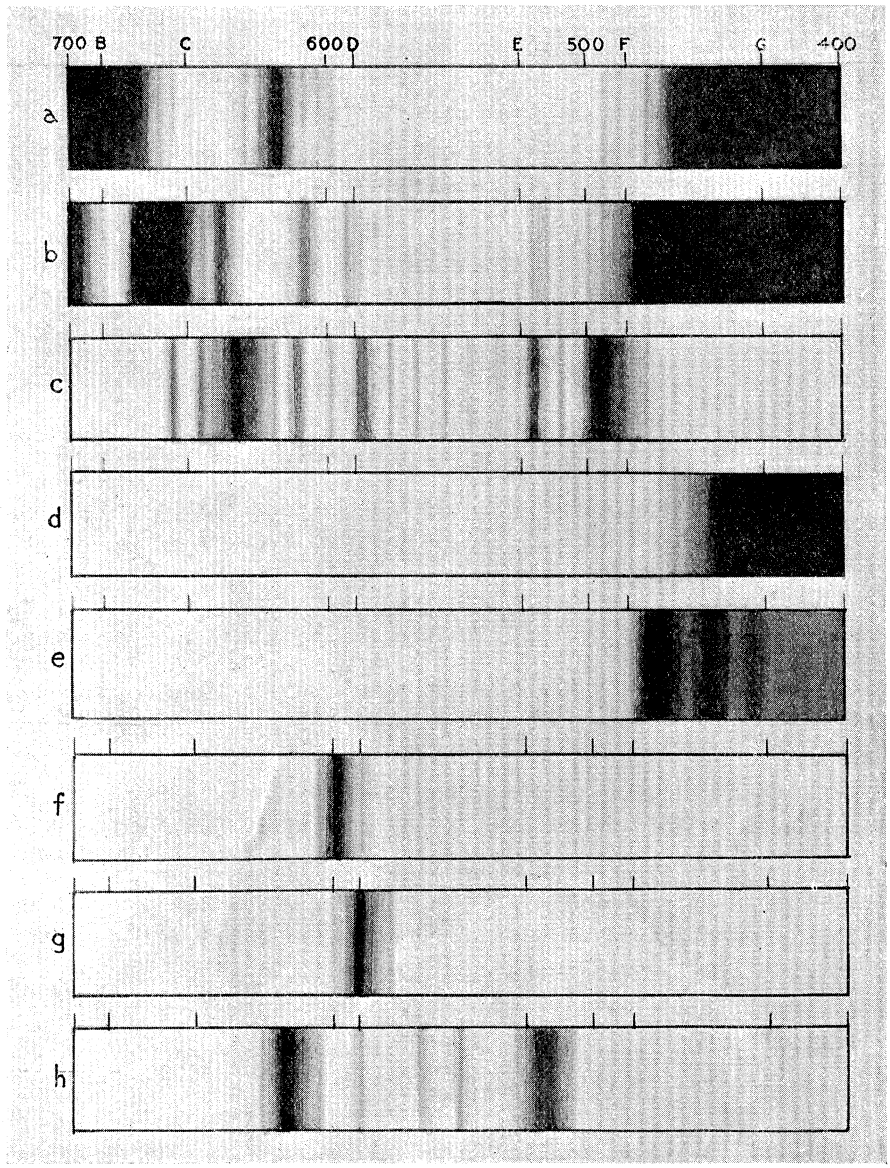
Judging solely from these observations, it appears very probable that the green solutions from *Diazona* and *Bonellia* contain either chlorophyll (for chlorophylls from different sources have not identical spectra), or some closely related chlorophyll body.

On cutting a violet, alcohol-preserved specimen of *Diazona* in two, it was found that the tint only extended a short way (about 1 cm.) beneath the surface, by far the larger mass of the colony (perhaps 20 cm. across) remaining a pale greenish yellow. The coloured outer portion of a specimen was therefore removed, and extracted with absolute alcohol. A blue-green solution was slowly obtained, but only a very minute portion of the violet pigment appeared to pass into solution. On cooling the alcoholic extract the blue-green colour became both paler and more yellow, though the original tint was restored on reheating, while after standing in the cold for some days a very small quantity of a substance having a violet tint identical with that observed in the outer portions of the colony was precipitated, the solution from which it had separated being now yellow-green. Extraction of the inner portions of a colony with absolute alcohol gave a solution of an almost pure yellow tint, scarcely any trace of green being detectable by the eye. No change took place either on cooling or after standing for some time.

The spectrum of the blue-green solution appeared to be similar to that already described, there being absorption in the red, and general absorption in the blue and violet, but the yellow solution from the interior of the colony gave no distinctive band, but only a general absorption of the more refracted rays.

On standing for several days these solutions became somewhat paler in tint, but attempts to concentrate the colour by distilling off the alcohol resulted in such turbidity that spectroscopic observations were impossible. The original green solution obtained from the 1912 specimens has scarcely altered in tint (April, 1914), but on concentration it also becomes turbid.

* 'Quart. Journ. Microsc. Sci.,' vol. 15, p. 167.



a. Diazona (green solution) in alcohol.
b. Chlorophyll in alcohol.
c. Bonelleine, green neutral solution in alcohol. Sorby.
d. Diazona (yellow solution) in alcohol.
e. Chlorophyll (yellow pigment) in alcohol.
f. Diazona (purple pigment) in acetylene tetrachloride.
g. Purpura (purple pigment) in acetylene tetrachloride.
h. Bonelleine, purple acid solution in alcohol. Sorby.

A few of the 1913 specimens had been preserved in formaldehyde solution instead of in alcohol. These had retained their natural green colour, and on treatment with alcohol gave a pale yellow-green solution, the organism itself becoming practically colourless, no trace of purple being observed.

This pale yellow solution showed a faint absorption in the blue and violet, but no band in the orange-red. All the above greenish or yellow solutions were shaken with carbon disulphide to see whether any separation of the colouring matter could be effected. The green 1912 solution after repeated shaking changed to a yellow, or brownish yellow, both the alcohol and carbon disulphide layers being coloured to about the same tint.

The green solution from the outer portion of a colony gave a green carbon disulphide extract, the alcoholic solution becoming yellow. From the strength of the colour it appeared that there was far more green than yellow pigment present. The yellow solution from the inner portion of the colony gave an exactly similar separation, but there appeared to be but little green pigment, as the carbon disulphide became only slightly coloured.

The greenish yellow solution from the formaldehyde-preserved specimens was quite unaltered by shaking with carbon disulphide, this solvent seeming to dissolve no colouring matter.

The green carbon disulphide extracts all showed an absorption band $\lambda = 620\,\mu\mu$ and general absorption for $\lambda < 470\,\mu\mu$, the yellow alcoholic portion exhibiting only a faint general absorption in the blue and violet. No satisfactory chemical observations could be made with any of these solutions. Acids and alkalies gave no very characteristic reactions, as the addition of either merely caused the solutions to become somewhat more yellow. This was particularly the case with alkalies, acids often appearing to have no action. Neutralisation of the alkaline solution did not restore the original colour. Acid or alkaline hydrogen peroxide was also without visible action.

Saturated barium hydroxide solution gave a greenish precipitate with the green solutions, the supernatant liquid being yellow, while with the yellow solution the precipitate had a yellow tint, though the liquid was not completely discolored by the barium salt. There was too little precipitate to try the action of an alcohol-glycerine solution of boric acid.

The unsatisfactory nature of these reactions is mainly due, no doubt, to the great dilution of the solutions employed, but the fact that neither the specimens of *Diazona* nor the alcoholic extracts were fresh may be a contributing factor, for chlorophyll bodies are not very stable.

It may be mentioned that fresh chlorophyll is altered in a non-reversible direction by acids, whereas the pigment from *Bonellia*, as described by

Sorby, changed to purple when strongly acidified, but regained its original shade on neutralisation.

From all the above observations it must be concluded that the green colour of *Diazona* probably results from some chlorophyll-like body. Though the spectra are not exactly those of ordinary plant chlorophyll there is quite a resemblance, and the association of separable green and yellow pigments from the alcoholic solution is also very suggestive.

If it is a chlorophyll body one is driven to the view that the green colour arises from a symbiotic alga, as chlorophyll does not appear to be a likely pigment for a marine animal. In a monograph on the compound Ascidian *Fragaroides aurantiacum*, by Charles Maurice,* the pigmentation of the test is described, and the author concludes that there the yellow pigment cells are in reality algæ (a *Protococcus*), which contain chlorophyll. He comments on the fact that these algæ when free show colours ranging from green to yellow, and that their cells during the period of reproduction resemble most closely the colour and structure of the globules in the test of the Ascidian. There are, however, three possible objections to this view. Firstly, *Diazona* has been collected from a depth of 60 fathoms, and it would appear to be most improbable that sufficient actinic light would penetrate to that depth to cause the formation of chlorophyll. Secondly, there is the evidence of Pizon† that in certain Tunicata which show very similar pigment cells to those of *Diazona* the yellow or yellow-green pigments result from the waste products of the organism, and are gradually excreted from its surface. Chlorophyll would hardly be a waste product. Thirdly, the pigment cells in *Diazona* are far smaller than the algal cells in known cases of symbiosis. The cells appear as minute spheres filled with one or more drops of an oily substance (judging by their high refractive index), and do not appear to show the structure of an algal cell. Until it is possible to work with some fresh, living colonies of *Diazona*, nothing more definite concerning the green pigment can be said than that it resembles chlorophyll in many respects, but is not identical with that ordinarily obtained from plants. It is, however, more like chlorophyll than the green pigment obtained by Sorby from *Bonellia*, and possibly represents algal cells.

Extraction with alcohol having shown that the purple pigment was all but insoluble, some 400 grm. of the organism were worked up on the lines employed by Friedländer in the case of the Mollusc *Murex brandaris*.‡

The material was first ground with sand and then digested for several

* 'Arch. de Biol.,' Liége, 1888.

† 'Compt. Rend.,' 1899, p. 395, and 1901, p. 170.

‡ 'Ber.,' 1909, p. 765.

hours with hot dilute sulphuric acid, fresh quantities of acid being used till the yellow tint at first imparted to it was no longer visible. The mixture of animal matter and sand was then boiled with several portions of water and filtered. It was next extracted with alcohol. Finally, it was treated in a Soxhlet with ethyl benzoate or acetylene tetrachloride. These solvents acquired a fine blue colour, and exhibited a strong purple-red dichroism. On cooling, the solutions gradually deposited a fine purple-black powder, which after recrystallisation and washing with ether did not greatly differ in tint from the violet colour of the Ascidian colony when preserved in alcohol. When quite dry this purple powder had a distinct coppery lustre. It was insoluble in water, alcohol, and ether, but soluble in aniline, pyridine, quinoline, nitro-benzene, ethyl benzoate, and acetylene tetrachloride, though the solubility varied with the liquid. Thus, though easily soluble in hot acetylene tetrachloride, it was almost entirely reprecipitated on allowing the cooled solution to stand for some hours.

In every case the solution was blue with a greenish shade when very dilute, changing to pure blue, and subsequently violet blue, on concentration. When hot both the violet colour and the dichroism were more pronounced.

The absorption spectrum was determined in both hot and cold ethyl benzoate and acetylene tetrachloride.

The ethyl benzoate solution in the cold showed an absorption band with a maximum about $\lambda = 611\ \mu\mu$, though absorption began at $\lambda = 617\ \mu\mu$. When heated, the maximum was shifted to $\lambda = 605\ \mu\mu$, the band being very indefinite towards the green, the total absorption ranging from $\lambda\ 617\ \mu\mu$ to $\lambda\ 598\ \mu\mu$. In acetylene tetrachloride the maximum absorption both when hot and cold was shifted towards the green, the maximum when cold being $\lambda = 606\ \mu\mu$, and when hot $\lambda = 598\ \mu\mu$.

The pigment dissolved in concentrated sulphuric acid to form at first a pinkish solution, which rapidly changed to a dirty purple colour. On standing, or more rapidly on warming, this colour changed to a brown tint with a green shade in it, or if sufficiently strong to a dull green. The pink colour appeared to be of a transient nature, depending for its stability on concentration and low temperature.

Addition of water to the cold acid solution precipitated the pigment, so it must be concluded that it does not form a soluble sulpho-salt, as is the case with indigo, but after heating, the addition of water caused the separation of a dull green flocculent precipitate, not the original pigment.

The colouring matter was insoluble in alkali, but gave a colourless solution with an alkaline reducing agent. Owing to the small quantity available it was impossible to try its action on cotton satisfactorily, but a cotton cloth in

which an Ascidian colony had been wrapped during preservation was found after drying and exposure to air and light to be dyed with a pale pink, not very fast, colour. Qualitative examination showed the presence of a halogen, apparently bromine, for after treating a few milligrammes of the dyestuff by the Carius method a pale yellow silver precipitate was obtained which did not appreciably darken in sunlight and which was slowly soluble in excess of ammonia.

The general behaviour of the colouring matter was thus seen to resemble a dibromindigo, which has been shown by Friedländer to be the dye in the case of the Mediterranean *Murex brandaris.* The chief point of difference appeared to be the bluer shade of tint in all the solvents employed, the greater solubility in ethyl benzoate or acetylene tetrachloride, and except in strong, hot solutions the displacement of the maximum of the absorption band somewhat towards the red.

As living specimens of *Murex brandaris* in quantity were not available, for the sake of comparison, the pigment from the closely related British Mollusc *Purpura lapillus* was therefore examined.

The purple pigment of this mollusc has already been studied by many chemists.*

In the present instance the colouring matter from material collected at Port Erin, Isle of Man, was extracted in exactly the same way as described by Friedländer for *Murex brandaris,* and was obtained in a pure crystalline condition from solution in ethyl benzoate or acetylene tetrachloride.

It will suffice here to say that its appearance and reactions agreed in every particular with the dye from *Murex brandaris*:—66′ dibromindigo. Solutions in various solvents were more red purple than those from *Diazona,* and its absorption band in hot acetylene tetrachloride gave $\lambda = 584\ \mu\mu$. The other three isomeric symmetrical dibromindigos have recently been described by Friedländer,† and their absorption and behaviour in concentrated sulphuric acid are given for reference from the above mentioned paper.

Compound.	λ.	Colour in concentrated sulphuric acid.
	μμ.	
44′ dibromindigo	613	Blue.
55′ ,, ...	621	Blue.
66′ ,, ...	585	Dull violet brown.
77′ ,, ...	606	Greenish blue (peacock blue).

* Bancroft, 'Philosophy of Permanent Colours,' 1803; Negri, 'Gaz. Chem. Ital.,' 1875; Schunk, 'Chem. Soc. Trans.,' 1879 and 1880; Letellier, 'Compt. Rend.,' 1889.

† 'Ann. Chem.,' vol. 388, p. 23 (1912).

It will be observed that while the pigment from *Diazona* in some respects agrees with the 66′ body, in other respects it more resembles the 77′ isomer, which gives blue solutions in solvents, the colour being not unlike that of indigo. Possibly the *Diazona* pigment is some other isomer, or an indigo with a different number of substituted hydrogen atoms, but it is impossible to decide this point without far larger supplies of material.

For the sake of comparison, in the figure (p. 229), the positions of the absorption bands for the violet or blue solutions obtained from *Diazona*, *Bonellia*, and *Purpura* are shown.

2. *Origin and Formation of the Violet Pigment.*

The experimental evidence so far available does not enable one to ascribe any certain origin to the violet pigment nor to account for its development in such different organisms as Mollusca (*Murex* and *Purpura*), Vermes (*Bonellia*), and Tunicata (*Diazona*). Nevertheless it may be useful to collect such evidence as there is at present to hand.

In the case of *Murex brandaris* it seems to be well established that the colour has a photogenetic origin, but in *Murex trunculus* this is not the case, according to Negri (*loc. cit.*). In *Purpura lapillus* the pigment is produced both by the action of sunlight, and by hydrochloric acid in the dark, this latter observation agreeing with the behaviour of *Bonellia viridis* according to Sorby. Further, the pigments produced photogenetically in these organisms are uniformly insoluble in alcohol, while those resulting from the action of acids are soluble. In the case of *Diazona* it is by no means certain that the pigment has a photogenetic origin. Prof. Herdman has recorded the gradual production of violet colour in the living organism under the influence of sunlight, the original yellow-green tint changing first to blue green, then indigo blue, and finally a dull or dirty violet, but he is of opinion that this colour-change attends a moribund condition. There is, however, no evidence that this change would not have taken place in the dark. Natural violet-coloured specimens of the Ascidian have been obtained alive in the Mediterranean, near Naples, and also grey-green specimens which have remained unaltered after preservation in alcohol* but these natural violet specimens do not appear to be healthy, and hence the formation of the dyestuff may accompany or result from a metabolic change. In alcohol the colour is produced in the dark, for the specimens were placed in a closed tank immediately after they were collected. Microscopic examination of an alcohol-preserved specimen shows the purple colouring matter apparently precipitated in the spherical pigment cells of the test, these cells in the inner

* See Herdman, 'Linn. Soc. Journ.,' 1913.

portion of the animal being filled, as already mentioned, with a bright yellow-green oily-looking substance.

It is possible that the action of the alcohol may be merely that of precipitant, for if the dyestuff (which gives a blue solution) were dissolved in this oil the resultant colour would be the green of the living organism.

The chloroplasts, as mentioned above, appear to contain a substance the solution of which in alcohol has an absorption spectrum resembling a yellow chlorophyll body, but it does not follow that they only contain this compound. Some solvent for the indigo derivative may quite possibly be present in them as well.

Now if this solvent is miscible with alcohol its removal would precipitate the colour body in its solid, violet-tinted form. The alcoholic solution, however, would still have a greenish tint, since some of the pigment would dissolve in the alcohol-solvent solution, exactly as in the case of other three-component systems, and so add its blue colour to the yellow of the chlorophyll-like substance.

Hence the presence of pigment in the alcoholic solution need not necessarily imply the existence of a second colour body soluble in alcohol, and this indeed is believed to be the origin of the traces found in some of the alcoholic extracts examined during this investigation. It was remarked that the traces of colouring matter thus obtained were insoluble in absolute alcohol, a fact quite in accordance with the above view, since none of the natural animal solvent would then be present.

It must, however, be pointed out that if the green colour of these extracts was due to the presence of a minute quantity of the violet pigment one would expect the spectrum to exhibit an absorption band about $\lambda = 606$ and not at $\lambda = 620\ \mu\mu$. It is of course a possibility that the animal solvent may shift the absorption band this amount towards the red, though this seems somewhat improbable in dilute solution in alcohol. It is far more likely that the traces of violet pigment found in the alcoholic extract had their origin in a disintegration of parts of the organism during extraction from purely mechanical causes. Though the production of the violet colour could thus be explained when specimens are preserved in alcohol, this precipitation theory seems scarcely sufficient to explain all the observed facts. According to this view the gradual production of the colour as observed by Prof. Herdman in living specimens must arise from its production in such quantity that it can no longer be kept in solution by the solvent in the pigment cells, yet there is no evidence that there is more colouring matter present under these circumstances than when an ordinary green healthy colony is placed directly in alcohol. Further, it affords no explanation why the pigment is produced only

on the exterior, and not throughout the mass of the colony. It may also be remarked that a minute quantity of the pigment causes an intense coloration of its solvents, so much so, that if all the violet colouring matter in a colony was in solution during life the colour of the organism would almost certainly be a bright blue, not yellow green, as it would entirely mask the yellow of the chlorophyll body.

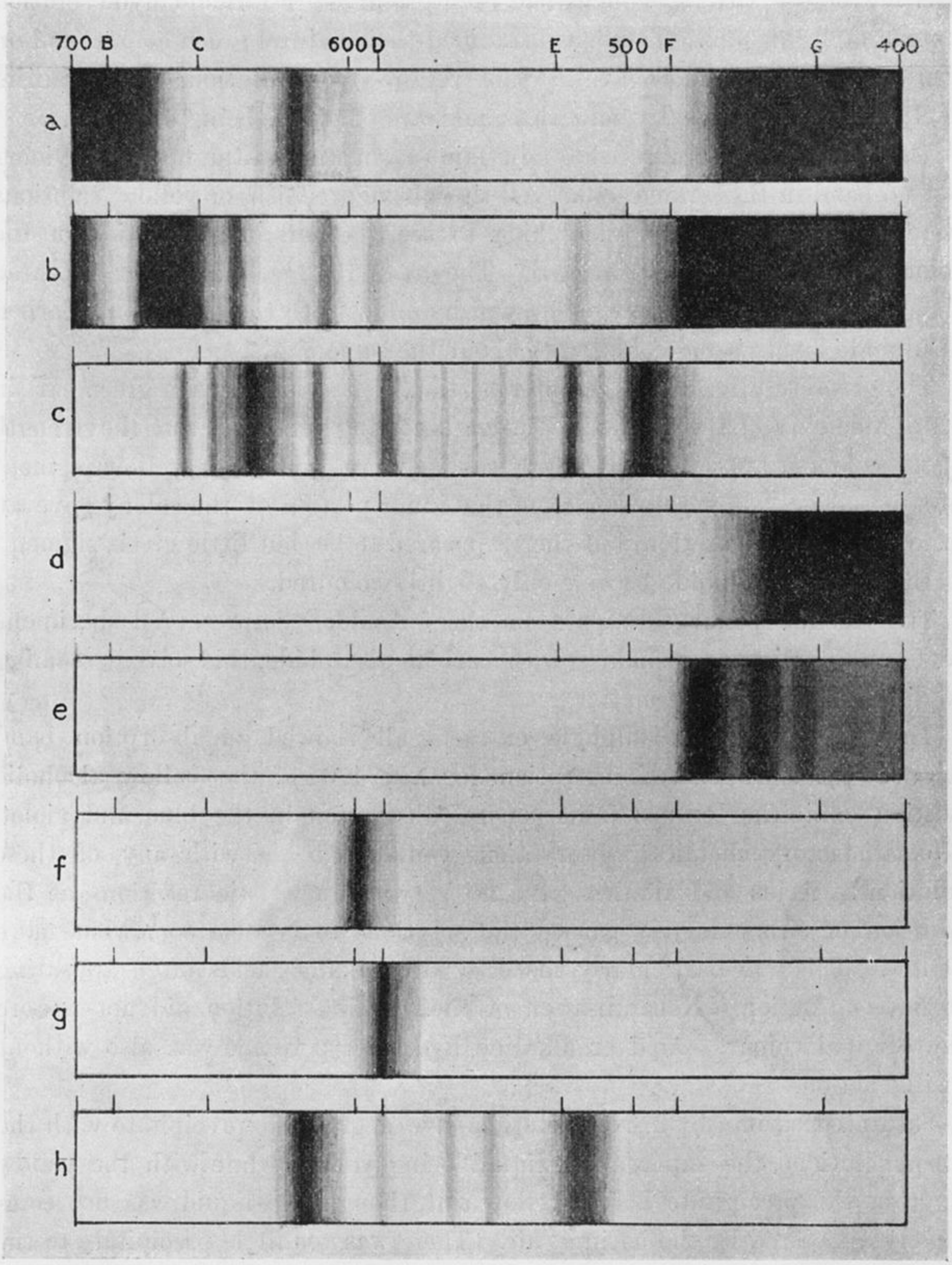
The non-production of violet colour in the formaldehyde-preserved specimens is what one would expect from an indigo derivative, for the reducing action of the aldehyde would certainly produce colourless indigo-white derivatives, if indeed the whole molecule was not split up.

The recorded phenomena can, however, be explained if we suppose that in the healthy animal the pigment is present dissolved in the pigment cells in its reduced condition as a chromogen. Owing to its natural tendency to oxidation the animal by maintaining it reduced could use it as an oxygen carrier, and, since the only available oxygen is in the surrounding water, its presence would only be expected on the exterior of the colony, though the green-yellow chlorophyll-like pigment is present throughout its mass. As soon as the animal became moribund or unhealthy metabolic processes would change and oxidation would begin, with the consequent production of colour. In the dead animal oxidation would be complete, and the colourless body converted into the violet pigment. The same change would occur in alcohol, which by killing the animal would allow oxidation to proceed rapidly.

The colour results no doubt from the action of an oxydase, which in the formaldehyde-preserved specimens would be destroyed, and hence no colour would result. Until it is possible to experiment with living colonies one cannot express a definite view, but it appears more probable that some such series of changes as is outlined above takes place, rather than that the body in its fully oxidised condition is present in solution in the pigment cells of the live animal.

With only preserved colonies available it is not possible to prosecute further this enquiry as to these green and violet pigments or to express any opinion as to their possible relationship, as regards function in the organism, to those found in *Bonellia* and various Mollusca.

In conclusion my thanks are due to Prof. Herdman for providing several complete colonies of *Diazona* and the green alcoholic solutions obtained directly from the living organisms, and for suggesting to me that a chemical investigation might throw further light on the colour relations of the violet *Diazona violacea* and the green condition known as *Syntethys hebridicus*.



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