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BILIPROTEIN COLORATION OF *PHYSALIA PHYSALIS*

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Abstract—1. The blue tentacular colour of *Physalia* is a biliprotein complex, the prosthetic group of which is a bilatriene.

2. Other bile pigments are probably responsible for most of the colours of the body of *Physalia*.

3. Ommatins are present in the tentacles.

INTRODUCTION

THE PHYSOPHORIDAN siphonophore *Physalia physalis*, the Portuguese man-of-war, is a large and brightly pigmented member of the pleustonic community of tropical and subtropical waters (Zaitsev, 1964; David, 1967) whose range occasionally extends into temperate areas. Much is now known about the toxic properties of the nematocysts of this extremely venomous animal (Lane, 1960) but the nature of its characteristic green–blue pigment has only been investigated by Ball & Cooper (1947). These workers made some preliminary observations to determine whether the toxicity was associated with the pigment. A rather more detailed investigation has been carried out in order to try to determine the relationship of the chemistry and function of the blue pigment of *Physalia* to those of the many blue-pigmented members of the warm water surface fauna (Herring, 1967).

MATERIALS, METHODS AND RESULTS

1. *Extraction and purification of the pigments*

Three adult animals (float lengths 10–12 cm) were caught in a hand-net from R.R.S. *Discovery* in February 1968 (Discovery Station 6628 latitude 20°47′ N longitude 17°47′ W) and deep-frozen at −20°C for subsequent analysis. The tentacles of these animals were a deep blue colour, whereas the body colours varied considerably. The float was a pale lavender blue, with a pink tinge to the crest, and the gonodendra and gastrozooids were a more greenish or purplish shade of blue. Tentacles were separated from the rest of the animals, homogenized in 0·1 M Na/K phosphate buffer, pH 7·0, and the very gelatinous suspension centrifuged. The red–purple precipitate (cf. Ball & Cooper, 1947) was retained for later analysis and the blue–green supernatant purified further. Ammonium sulphate was added to 15 per cent saturation and the colourless gelatinous precipitate removed by centrifuging. The coloured supernatant was taken to 50 per cent saturation with ammonium sulphate and the slightly coloured precipitate discarded.

Almost all the original colour remained in the supernatant and was finally brought out of solution as a blue precipitate by addition of further ammonium sulphate to 75 per cent saturation.

The blue pigment was redissolved in very dilute ($< 0{\cdot}005$ M) phosphate buffer, adsorbed on a column of DEAE cellulose (Whatman DE 11, 20×1 cm column) and eluted with 0·2 M buffer, leaving a trace of reddish material on the column. The eluate was diluted with distilled water to 0·05 M buffer and adsorbed at pH 7·0 on to freshly prepared calcium phosphate gel (Keilin & Hartree, 1938). The gel was washed successively with 0·1, 0·2 and 0·4 M phosphate buffer and with 0·2 M buffer containing 15 per cent by volume of a saturated solution of ammonium sulphate. Elution from the gel was finally achieved with 0·2 M buffer containing 30 per cent by volume of saturated ammonium sulphate. The resulting clear blue solution was dialysed against several changes of large volumes of distilled water at 4°C and most (but not all) of the blue pigment finally precipitated.

2. *The properties of the blue pigment*

The absorption spectrum of the purified pigment is shown in Fig. 1. It has absorption maxima at 587, 385 and 280 nm and a shoulder (whose prominence varied in different preparations) at about 610 nm. The ratio of the extinction at

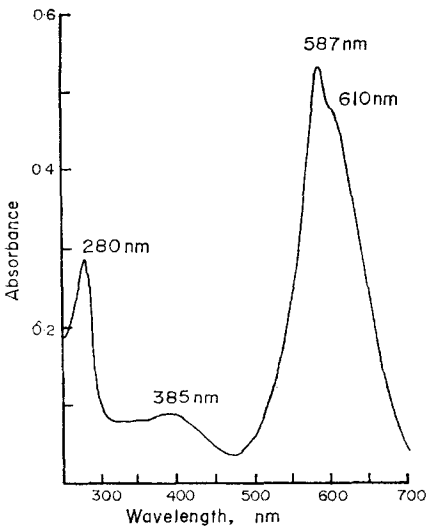

FIG. 1. Absorption spectrum in 0·2 M phosphate buffer of the tentacle pigment.

280 to that at 587 nm was 0·53. It is a protein or polypeptide and appeared homogeneous on cellulose acetate electrophoresis at pH 7·0 (phosphate buffer 0·05 M) and pH 8·6 ("Oxoid" barbitone acetate buffer), with a mobility similar to that of serum γ-globulins in the latter case (0·4 mA/cm, 2-hr run). It does not enter a 13 per cent starch gel pH 8·2 (borate buffer) with or without the addition of 6 M urea. The pigment does not stain with Schiff's reagent, indicating that it is not a

glycoprotein, nor does its appearance on the cellulose acetate strip suggest that it is a lipoprotein.

The pigment solution is either precipitated or is rapidly decolourized to a very pale blue–green solution (with 80–90 per cent loss in extinction at 587 nm) by the addition of protein denaturing agents such as acetone ethanol and dilute and concentrated HCl, but with concentrated H_2SO_4 gives a green colour and with glacial acetic acid a pale blue solution. It is rapidly bleached by sodium borohydride, and treatment with sodium nitrite does not restore the colour. Dilute NaOH or KOH also rapidly decolourizes the solution. Treatment with ammonia yields a solution with an absorption maximum at 610 nm, a small peak at 565 nm and increasing absorption above 680 nm. No colour could be extracted from the pigment solution by shaking with chloroform, but previous treatment with methanol containing 5% conc. HCl allows all the colour to be extracted into the chloroform, albeit at very much reduced intensity by comparison with the aqueous solution.

Addition of urea to 6 M concentration has little immediate effect upon the absorption spectrum but there is a very gradual reduction in the relative importance of the shoulder at 610 nm (Fig. 2). Aqueous solutions with slightly altered absorption spectra can be obtained by acetone precipitation of the pigment. Two different methods were employed. Addition of acetone, drop by drop, to a solution

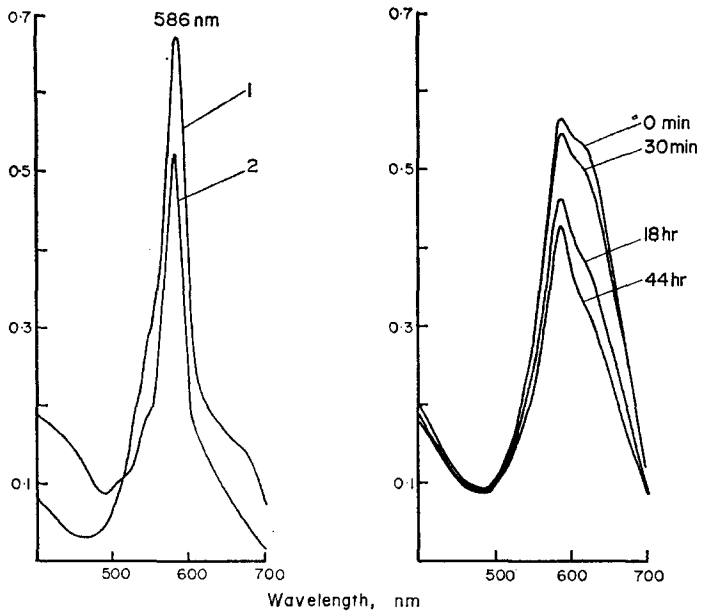

FIG. 2. Left: absorption spectrum of tentacle pigment after acetone precipitation of (1) purified pigment and (2) crude homogenate in 15 per cent saturated ammonium sulphate (see text). Right: absorption spectra of purified pigment in 0·2 M phosphate buffer containing 6 M urea, showing the gradual reduction in the shoulder at 610 nm after increasing time at room temperature.

of the purified material in 0·05 M phosphate buffer pH 7·0 produced a blue precipitate insoluble in 0·1 M buffer but which dissolved in 0·4 M buffer leaving a small amount of insoluble white material. The visible absorption spectrum has a much reduced shoulder but virtually no shift of the absorption maximum (Fig. 2). Similar treatment of a 0·05 M buffer solution that had been stored at 4°C for several months yielded a purple precipitate insoluble in dilute buffer but soluble in 0·5 M buffer to give an absorption spectrum completely lacking the shoulder at 610 nm. The second method was by acetone addition to the crude solution in 15 per cent saturated ammonium sulphate obtained after the first precipitation of the original tentacle homogenate. In this procedure a blue hypophase (in the ammonium sulphate) separates from a yellow epiphase (aqueous acetone) and the blue hypophasic pigment is soluble in 0·05 M buffer but its absorption spectrum has no shoulder (Fig. 2). It seems therefore that by partial denaturation of the pigment–protein to different degrees the shoulder at 610 nm in the absorption spectrum can be reduced or suppressed with concomitant changes in the solubility.

The molecular weight, as indicated by a single run on a Sephadex G-200 column (2×60 cm; standardized with α and β crustacyanin (Cheesman *et al.*, 1966) serum albumen, cytochrome c and blue dextran) is 245,000.

3. *The prosthetic group*

The characteristics of the blue protein pigment suggest a bile pigment prosthetic group, treatment with MeOH/HCl forming the chloroform soluble methyl ester(s) of the bile acid. If a methanol solution of the chloroform soluble material is treated with concentrated HNO_3 a sequence of purple, red and orange colours is obtained, similar to the Gmelin reaction of a typical bilatriene. Extraction of the protein solution with MeOH/5% HCl and chloroform takes almost all the pigment into the chloroform phase, but a small amount of a blue precipitate often remains, which goes reversibly purple on addition of alkali. Prolonged MeOH/HCl treatment of this material yields a purple solution with its absorption maximum at about 580 nm, a shoulder or minor peak at 500 nm and a shoulder at about 680 nm.

The blue–green chloroform extract (absorption maxima at 645 and 377 nm) when evaporated to dryness is readily soluble in neutral organic solvents. Addition of 5% of zinc acetate to a methanolic solution causes a marked change in the absorption spectrum (Fig. 3); an orange–red fluorescence in u.v. light also appears. If a trace of iodine is added to this solution the absorption maximum is enhanced and the fluorescence becomes a much more intense red (excess iodine quenches the fluorescence). A few drops of 2 N HCl added to the iodine-containing solution quench the fluorescence and produce a violet solution with absorption maximum at about 565 nm. Reduction of the chloroform-extracted pigment with sodium borohydride in methanol yields a yellow product, whereas reduction with zinc dust in glacial acetic acid yields a colourless solution.

4. *Pigments of tissues other than the tentacles*

Extracts of body tissue with 0·2 M phosphate buffer failed to yield any pigmented material, all the colour remaining in the extracted material, which was then re-extracted with MeOH/HCl, almost all the pigment going into solution.

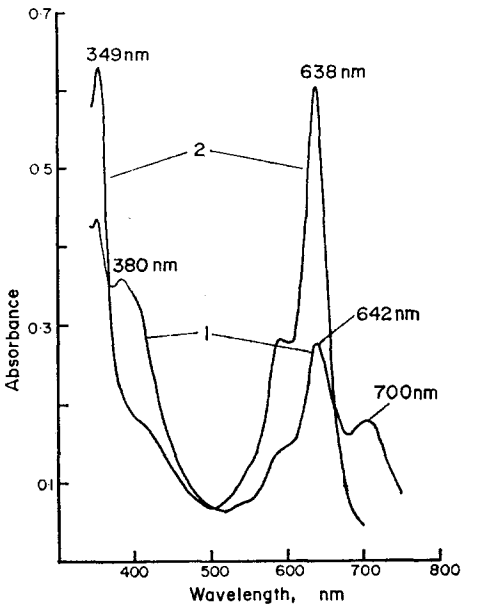

FIG. 3. Absorption spectra of MeOH/HCl extracted tentacle pigment in MeOH/5% zinc acetate before (1) and after (2) addition of iodine (not at the same concentrations).

The extracted pigments were taken into chloroform which was then washed with dilute Na_2CO_3, dried with anhydrous sodium sulphate and concentrated in a rotary evaporator at 40°C. The pigment concentrate was chromatographed on a 1-mm thick layer of silica gel G (Merck: "according to Stahl") developed with benzene : petroleum ether (120–160°C) : methanol (9 : 5 : 2). Six pigment bands separated under these conditions, numbered 1–6 in order of decreasing R_f, and coloured (1), yellow; (2), green-blue; (3), purple; (4, 5 and 6), green-blue. Band 2 comprised a visually estimated 90–95 per cent of the total extracted pigment, and in this system was chromatographically identical to the pigment extracted similarly from the tentacular pigment–protein complex. The very faint yellow band 1 was discarded and the other bands scraped off the plate and eluted separately with acetone. Their absorption spectra in chloroform, MeOH/HCl and MeOH containing 5% zinc acetate are shown in Fig. 4. In the latter solvent bands 2 and 5 had an orange–red fluorescence, the other three had a yellowish fluorescence. No further attempt at identification was made.

5. *The purple precipitate from the tentacular homogenate*

The precipitate was suspended in distilled water and centrifuged down in a fine granular form. It was readily extracted with MeOH/HCl to yield a yellow–orange solution whose colour could not be extracted into chloroform. Treatment of the precipitate with dilute NaOH turned it to a very pale yellow colour. Neutralization of the MeOH/HCl solution with NaOH reversibly produced a purple–brown gelatinous precipitate insoluble in MeOH but soluble in acetic acid. If

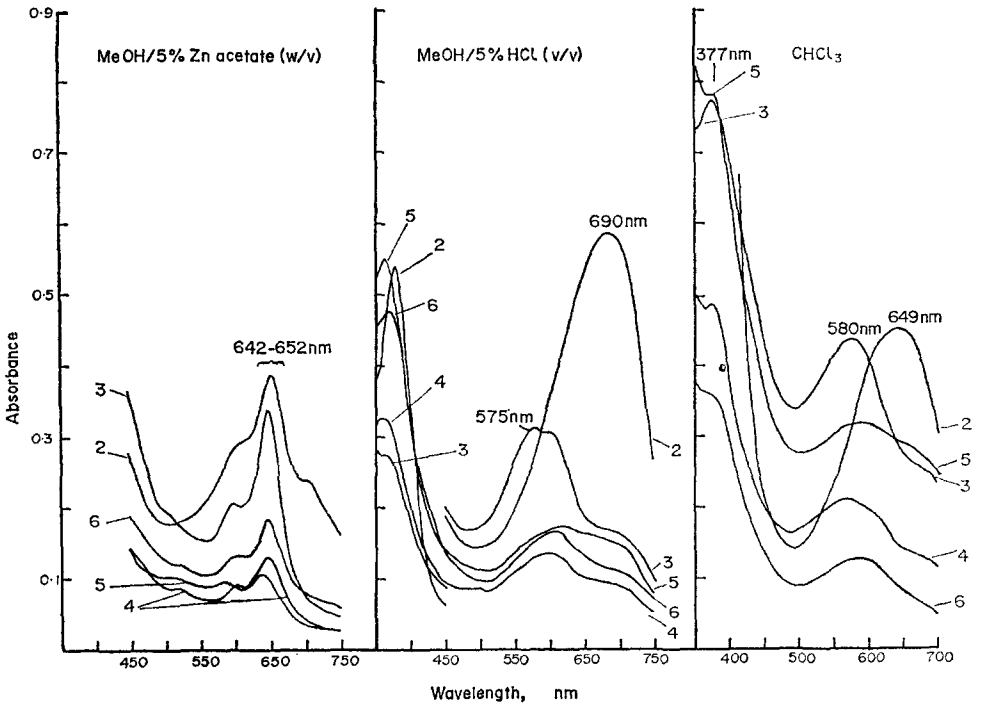

FIG. 4. Absorption spectra in different solvents of body pigments separated on TLC (bands 2–6).

concentrated H_2SO_4 was added to the MeOH/HCl solution a purple–violet solution could be obtained. Reduction with sodium borohydride yielded a pink solution changing back to yellow–orange on shaking with air. These are all reactions characteristic of ommochromes, particularly ommatins. Like other ommatins (and in contrast to ommins) the pigment solution proved to be completely dialysable.

DISCUSSION

From the results outlined above it is clear that the predominant blue pigment of *Physalia* is a bilin–protein complex. The reactions and absorption spectra of the esterified bilin and its compounds indicate that it is a bilatriene, but its absorption spectrum is clearly distinct from that of the methyl esters of biliverdin IXα

and IXγ (pterobilin) (Rudiger *et al.*, 1968). The fluorescence of its zinc salt is uncharacteristic of other bilatrienes, although after oxidation with iodine it has an absorption maximum at 638 nm similar to that of the proto β side chains of biliverdin. The pigment has some resemblance to the unidentified bilatriene of the blue coral *Heliopora coerulea* (Tixier, 1945) in its colours in strong acid solutions, and the fluorescence of its zinc complex before iodine oxidation; but determination of its precise structure must await the availability of larger quantities of material. Although the observed colour of the living animal is obviously due largely to the biliprotein complex it seems likely that the non-protein greens, purples and pinks of the float and other tissues may be caused by unconjugated bile pigments similar to those observed in thin layer chromatograms of whole body extracts.

The presence of ommatins in the tentacles is rather surprising, for in coelenterates these pigments have previously only been recorded from ocelli of the anthomedusan *Spirocodon* (Yoshida *et al.*, 1967) where they presumably have a visual role. Ommochromes do occur in quantity in crustacean members of the neuston such as the isopod *Idotea metallica* (Herring, 1969) and might perhaps be accumulated from any such animal eaten by *Physalia*, though Bieri (1970) reports that, whereas *Velella* and *Porpita* subsist primarily on crustacean food, *Physalia* is predominantly a fish-eater.

The source of the large amounts of bile pigment in the animal is probably exogenous; both preformed bile pigments and respiratory porphyrins are available in the tissues of the animal food, and their efficacy as a pigmentary system in relatively low concentrations is greatly enhanced by the intensification of colour achieved by conjugation as the protein complex, just as in *Crenilabrus* (Abolins & Rudiger, 1966). The function of the general blue pigmentation of the neuston has been discussed earlier (Herring, 1967); the totally different pigmentary system in *Physalia* that achieves results similar to those of the carotenoproteins of other species (even other siphonophores such as *Velella* and *Porpita*) suggests that it is the colour that is important to the animal, rather than the means by which it is achieved. This in turn lends weight to a camouflage hypothesis. The marked biochemical contrast between the superficially similar pigmentary systems of the chondrophores *Velella* and *Porpita* and the physophoridan *Physalia* may be related to their food preferences. If, as Bieri (1970) suggests, *Physalia* is almost entirely a fish-eater it might well be unable to accumulate sufficient dietary carotenoids to support a carotenoprotein pigmentary system, and has therefore evolved instead a biliprotein system. On the other hand, the crustacean diet of *Velella* and *Porpita* will supply large amounts of astaxanthin and other carotenoids, suitable for incorporation into a carotenoprotein pigmentary system.

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Key Word Index—Physalia physalis; Biliprotein; bilatriene; ommatins; colour in *Physalia*.