CHLOROPHYLL-DERIVED BILE PIGMENT IN BIOLUMINESCENT EUPHAUSIIDS

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1. Introduction

Most euphausiid shrimps are bioluminescent. The biochemical mechanism of luminescence, however, has been reported only for *Meganyctiphanes norvegica* [1,2]. In this euphausiid, light is emitted when a photoprotein is oxidized in the presence of a very unstable fluorescent substance, 'F'. The compound F ($M_r < 1000$, fluorescence emission maximum 476 nm) catalyzes the reaction and, at the same time, acts as the light-emitter of blue luminescence (λ_{max} 476 nm).

These results strongly suggest that F, obtained from *Meganyctiphanes norvegica* and *Euphausia pacifica*, is a bile pigment-type compound derived from a chlorophyll. Although quantitatively more chlorophyll is degraded than heme in nature, virtually all bile pigments known are structurally degradation products of heme and very little has been known about bile pigments derived from chlorophylls. Compound F is a rare example of naturally occurring bile pigment that is chemically closely related to chlorophylls.

Recently, the luciferin of luminous dinoflagellates was reported to be chemically similar to compound F [3].

2. Materials and methods

The specimens of *Meganyctiphanes norvegica* were obtained from the Millport Marine Biological Station, Scotland. *Euphausia pacifica* was collected by towing with CSS Vector in Saanich Inlet, BC, Canada, by Dr W. A. Heath. Specimens were frozen alive with dry-ice, then stored at −70°C until used. Compound F was extracted with 50% ethanol and purified as in [1]. Chlorophyll *a* was purchased from Sigma Chemical Co., St Louis, MO and pyrochlorophyll *a* was prepared by boiling a solution of chlorophyll *a* in pyridine for 24 h [4]. Bilirubin was obtained from Serva Chemicals, Heidelberg, and $^{18}O_2$ and $H_2{}^{18}O$ from Prochem., Summit, NJ. Thin-layer chromatography (TLC) was performed on ITLC sheets, Type SA (silicic acid) obtained from Gelman Instrument, Ann Arbor, MI.

Oxidation with CrO_3 was carried out essentially as in [5,6]: oxidation at pH 1.2 was done with 1% CrO_3 solution containing 1% $KHSO_4$ for 3 h (for F) or 5–15 h (for chlorophylls) at 25°C; oxidation in 2 N H_2SO_4 was done with 1% CrO_3 in 2 N H_2SO_4, at 25°C for 1 h, and, when hydrolysis of an ester group was needed, the mixture was subsequently heated at 90°C for 30 min. In the oxidation of chlorophylls, an equal volume of acetone was added to increase the solubility of chlorophylls, and the acetone was evaporated under reduced pressure at the end of the reaction. In the labeling of CrO_3-oxidation products with ^{18}O, CrO_3 and $KHSO_4$ (10 mg each) dissolved in 1 ml $H_2{}^{18}O$ (90 atom %) was heated at 90°C for 12 h to equilibrate the CrO_3 oxygens with ^{18}O, then cooled. F (0.5 mg) was oxidized with this CrO_3 solution (pH 1.2) in an atmosphere of $^{18}O_2$ gas (99 atom %) in a specially designed Y-shaped apparatus [7] for 3 h at 25°C.

On completion of the oxidation, the reaction mixture was extracted with ethyl ether, then the ether extract was concentrated and chromatographed by TLC with 2 solvent systems: CH_2Cl_2/ethyl acetate (10:1, v/v) and CH_2Cl_2/ethyl acetate/ethanol/acetic acid (200:10:5:0.5, by vol.). Maleimides and succinimides were detected with Cl_2 gas–tetramethylbenzidine, and pyrrole aldehydes were located with 2,4-dinitrophenylhydrazine, both basically as in [6]. Major bands were separately eluted with ether/methanol (10:1) and examined by ultraviolet absorp-

tion and mass spectrometry.

Methanolic NaOH-treatment of F to prepare a yellow compound (M_r 302) was carried out as follows. F (0.5 mg) in 1 ml 0.5 N NaOH made with 99% methanol (1% water) was heated at 65°C for 1 h. The solution (dark red) was acidified to pH 2 with 0.5 N HCl, and most of methanol was removed under reduced pressure, then the residual solution was extracted with ether. The yellow compound in the ether extract was purified by 2 steps of TLC: R_F 0.8 with CH_2Cl_2/methanol (95:5), and R_F 0.6 with CH_2Cl_2/acetone (100:20).

A blue compound was obtained by the following method: An ethanolic solution of F was diluted with 10 vol. acetone and slowly titrated with 0.1% iodine solution in ethanol, with frequent measurement of A_{630}. When the absorbance did not show any more increase, the solution was concentrated and a blue product having red fluorescence was purified by TLC with $CHCl_3$/methanol (94:6) (R_F 0.38).

3. Results and discussion

The fluorescent substance 'F' obtained from *Meganyctiphanes norvegica* and that obtained from *Euphausia pacifica* were indistinguishable in every aspect of properties described below, indicating that they are chemically identical.

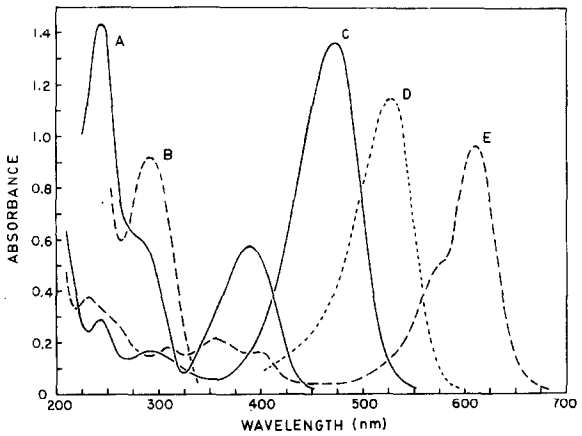

Fig.1. Absorption spectra of: F in 50% ethanol (A); compound II obtained by CrO_3 oxidation of F at pH 1.2, in 50% methanol (B); the yellow compound, IV, obtained by NaOH/methanol treatment of F, in methanol (C); IV in 0.01 N NaOH containing a trace of $Na_2S_2O_4$ (D); and the blue compound obtained by I_2 treatment of F, in methanol (E).

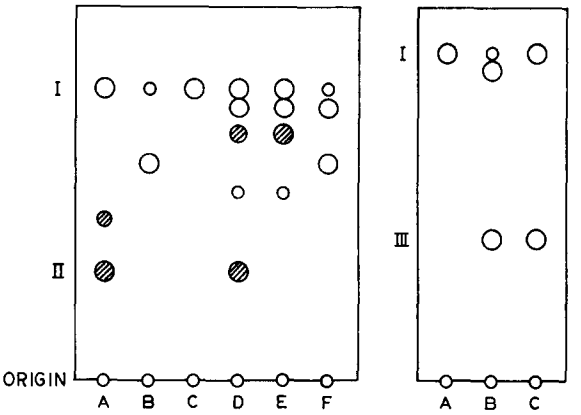

Fig.2. Thin-layer chromatogram of CrO_3 oxidation products: F oxidized at pH 1.2 (A), F oxidized in 2 N H_2SO_4 at 90°C (B), bilirubin oxidized at pH 1.2 (C), pyrochlorophyll *a* oxidized at pH 1.2 (D), chlorophyll *a* oxidized at pH 1.2 (E), and pyrochlorophyll *a* oxidized in 2 N H_2SO_4 at 90°C (F), developed with CH_2Cl_2/ethyl acetate (10:1, v/v) (left) and CH_2Cl_2/ethyl acetate/ethanol/acetic acid (200:10:5:0.5, by vol.) (right). Spots were detected with Cl_2–tetramethylbenzidine (open circles) or with 2,4-dinitrophenylhydrazine (hatched circles). A compound immediately below I, in D, E and F, is methylethylmaleimide. Other unmarked spots were not identified.

Compound F is light yellow ($\lambda_{max}^{50\%\ EtOH}$ 390 nm, fig.1) usually with a slight greenish tint due to a trace of oxidized compound. It is highly labile in air and in acidic solutions of pH 3 and below. The compound is soluble in water and alcohols, but not in most other organic solvents. Oxidation by O_2 and other oxidants under various conditions produced a number of products which included colored compounds of red (λ_{max} 510–520 nm) and blue (λ_{max} 610–640 nm). A blue compound (fig.1E) was prepared by treating F with iodine. The formation of these colored compounds, and the lack of the Soret band absorption, suggest a bile pigment-type structure for F.

The oxidation of F with CrO_3 at pH 1.2 yielded methylvinylmaleimide (I; fig.2,3) plus compound II which had M_r 177. Compound II was unstable in alkaline solutions as well as on dry TLC sheets, and gave an orange color with 2,4-dinitrophenylhydrazine, but did not give any maleimide or succinimide upon oxidation with CrO_3. When titrated with alkali, the A_{285} peak of this compound (fig.1) showed a bathochromic shift, which indicated pK_a 7–7.5. Main mass peaks and relative intensities (in parentheses) measured at 70 eV were: *m/e* 177 (100),

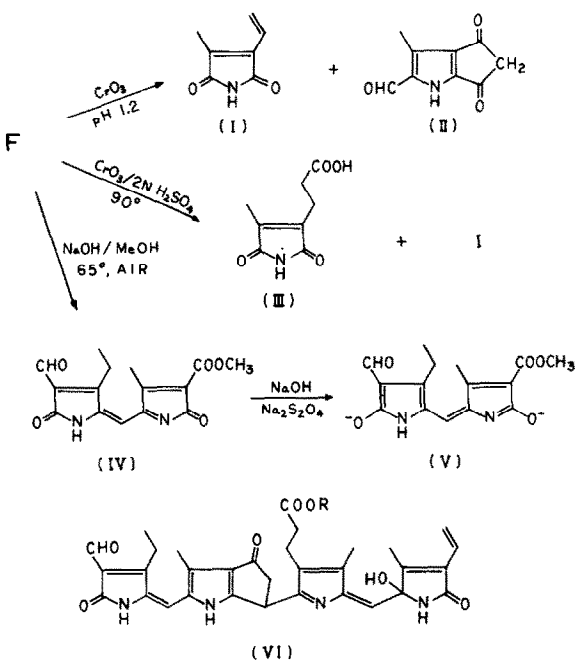

Fig.3. Structures of degradation products of F (I–V), and a possible partial structure of F (VI) which is closely related to pyrochlorophyll *b*.

149 (19), 121 (40), 107 (24), 91 (26), 81 (26), 69 (54). It was found, moreover, that pyrochlorophyll *a* gives the same compound by oxidation with CrO_3 at pH 1.2, but chlorophyll *a* does not (fig.2). Thus, the structure II (fig.3) was assigned for this compound. The formation of II from F, combined with other evidence described below, strongly suggest that the structure of F is related with that of a pyrochlorophyll.

Chromic acid oxidation of F in 2 N H_2SO_4 at 90°C afforded hematinic acid (III) in addition to I; the yield of I was relatively small due to decomposition of this compound under the conditions employed.

Treatment of F with methanolic NaOH yielded a yellow compound with A_{max} at 472 nm (fig.1). This compound gave a brown color by 2,4-dinitrophenylhydrazine, produced a purple color in the hydroxamic acid test for esters [8], and turned to red with alkali plus a trace of $Na_2S_2O_4$ (λ_{max} 528 nm, fig.1D) suggesting the formation of a pentdyopent [9]. Mass spectrum (fig.4) showed a molecular peak at *m/e* 302 and fragmentation peaks produced by the loss of CH_3, OCH_3 and, possibly, CHO from the molecular ion. Oxidation with CrO_3 did not give

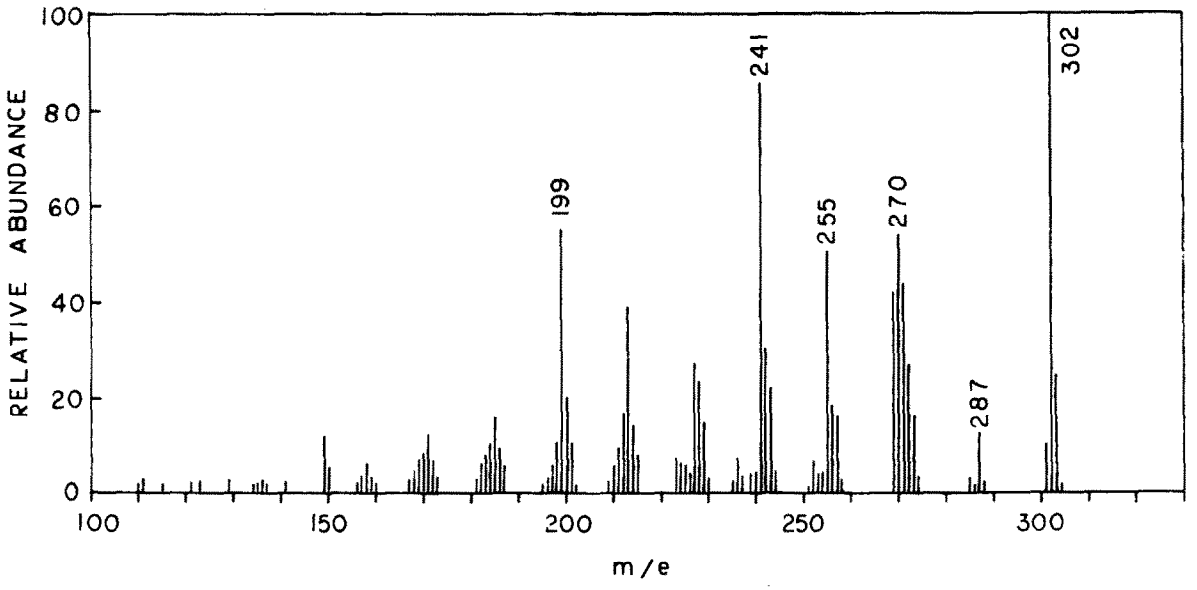

Fig.4. Mass spectrum (70 eV) of the yellow compound obtained by NaOH/methanol treatment of F.

any maleimide or succinimide. Reduction of the yellow compound with $NaBH_4$ in methanol gave a pale yellow compound (λ_{max}^{MeOH} 415 nm, M_r 304).

Based on the above evidence, the structures of the yellow compound and its pentdyopent are proposed to be, respectively, IV and V shown in fig.3. In these structures, the relative sequence of 4 substituents at β-positions were chosen on the assumption that F was derived from a chlorophyll.

Because both II and the right side ring of IV can derive from the same partial structure (i.e., ring III of pyrochlorophyll), it is considered that F contains 4 different pyrrole rings that yield, respectively, I, II, III, and the left side ring of IV.

To obtain information on the sequence of rings, F was oxidized with CrO_3 at pH 1.2, in $H_2{}^{18}O$ medium under $^{18}O_2$ gas. The product contained II that was labeled with two atoms of ^{18}O (M_r 181) and I that was not labeled. Thus, II must come from one of 2 inner rings and I from one of 2 outer rings.

If the two rings of IV consist the inner rings of F, the treatment of F with methanolic NaOH should produce some amounts of I and II in addition to IV. It was found, however, that the product of the methanolic NaOH treatment contained only a trace of I and none of III, even after treating the product mixture with 2 N H_2SO_4 at 90°C. On the other hand, the aqueous layer of ether extraction of the same product (see section 2) produced both I and III upon oxidation with CrO_3 in 2 N H_2SO_4 at 90°C, suggesting that the precursor rings of I and III, the latter in the form of an ester bound to a highly hydrophylic group, were adjacent in F.

Structure VI (fig.3) shows a possible partial structure of F deduced from the evidence described here. Studies on the structural details of F, including the nature of group R and the bond that is responsible for the highly acid-labile nature of F, are in progress.

Acknowledgements

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References

[1] Shimomura, O. and Johnson, F. H. (1967) Biochemistry 6, 2293–2306.
[2] Shimomura, O. and Johnson, F. H. (1968) Proc. Natl. Acad. Sci. USA 59, 475–477.
[3] Dunlap, J. C., Hastings, J. W. and Shimomura, O. (1980) Proc. Natl. Acad. Sci. USA 77, 1394–1397.
[4] Seely, G. R. (1966) in: The Chlorophylls (Vernon, L. P. and Seely, G. R. eds) pp. 67–109, Academic Press, New York.
[5] Rüdiger, W. (1968) in: Porphyrins and Related Compounds (Goodwin, T. W. ed) pp. 121–130, Academic Press, London.
[6] Rüdiger, W. (1969) Hoppe-Seyler's Z. Physiol. Chem. 350, 1291–1300.
[7] Shimomura, O., Goto, T. and Johnson, F. H. (1977) Proc. Natl. Acad. Sci. USA 74, 2799–2802.
[8] Feigle, F. (1960) in: Spot Test in Organic Analysis, 6th Engl. edn, pp. 249–253, Elsevier, Amsterdam, New York.
[9] Von Dobeneck, H. (1979) in: The Porphyrins (Dolphin, D. ed) vol. 6, pp. 651–662, Academic Press, New York.