J. mar. biol. Ass. U.K. (1959) 38, 455–460
Printed in Great Britain

NAPHTHAQUINONE PIGMENTS IN *PSAMMECHINUS MILIARIS* (GMELIN)

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(Text-figs. 1–3)

Since MacMunn (1885), the nature and distribution of naphthaquinone pigments in sea urchins have been widely surveyed, and the main findings have been summarized in reviews by Lederer (1952), Fox (1953) and Thomson (1957). Among spinochromes and echinochromes, the most unusual one is the Spinochrome E discovered by Lederer (1952), which is insoluble in ether.

Recently, the author has examined the napthaquinone pigment in the echinoid, *Psammechinus miliaris* (Gmel.), and has found a similar pigment to Spinochrome E together with other common spinochromes.

SPINOCHROME E

Methods

Tests and spines were digested with concentrated HCl and extracted with re-distilled diethyl ether. The spectral characteristics were measured on Unicam SP 500. The crude ether phase showed absorption maxima at 269, from 315 to 318 and at 480 mμ with a hump at 360 mμ (Fig. 2*a*). When this was left for a short time at room temperature, needle-like crystals occasionally appeared, which, when dissolved in methanol, showed maxima at 267, between 359 and 360 and at 476 mμ (Fig. 2*b*).

A considerable amount of orange red pigment remained in the acid phase ($\lambda_{max.}$; 261, 330 and 465 mμ in HCl), which on adding a few drops of pyridine turned purple. Dilution with methanol produced an amorphous purple precipitate, which re-dissolved in methanol on acidification with a drop of concentrated HCl. From this solution, fine needle crystals separated out at room temperature. These, when dissolved in methanol, gave $\lambda_{max.}$ at 267, 358 and 476 mμ and the absorption curve (Fig. 1*b*) was exactly the same as that from the ether phase.

A more effective method of crystallization was as follows. Tests and spines were digested with a small amount of concentrated HCl and the solution was diluted at once with methanol, which gave a solution with the absorption spectrum shown in Fig. 1*d*. On adding a small amount of pyridine, the purple pigment precipitated in quantity. This was washed several times with methanol, dried and re-dissolved in acidified methanol or acetone. Crystals separated out at room temperature, which after washing with distilled water and drying, were re-crystallized from acidified methanol.

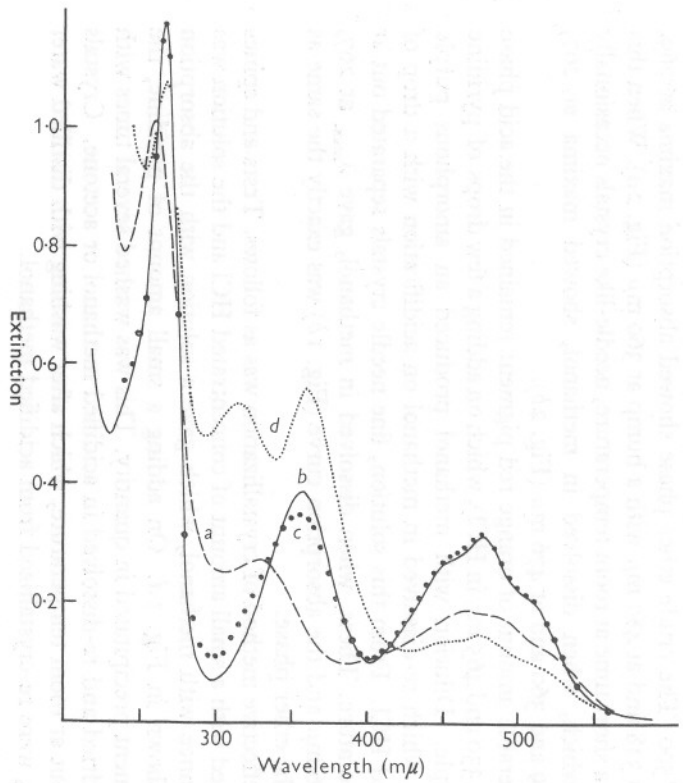

Fig. 1. Absorption spectra of pigment extracted from *Psammechinus miliaris*. HCl phase. *a* (broken line), crude extract in HCl; *b* (continuous line), crystallized pigment in methanol; *c* (dots), samples of Spinochrome E, furnished by Dr Lederer; *d* (dotted line), crude extract made with HCl methanol.

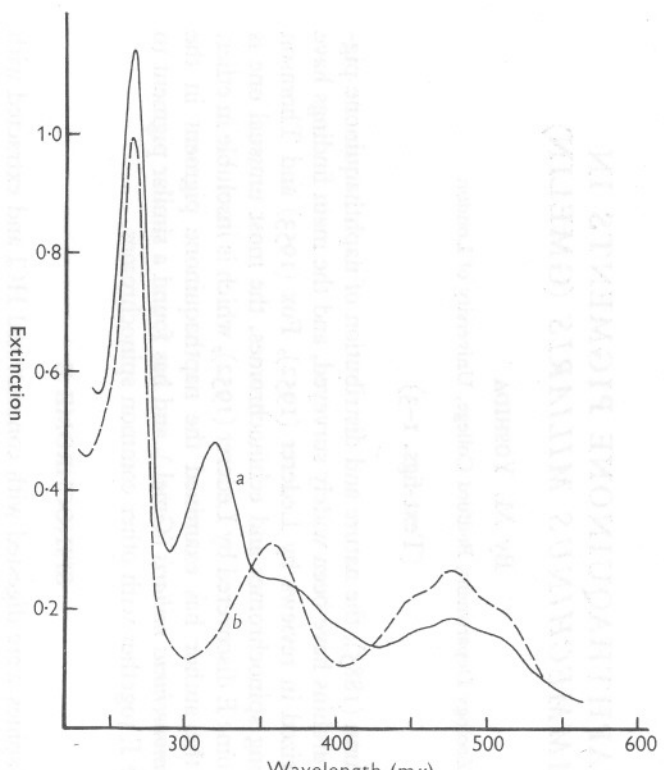

Fig. 2. Absorption spectra of pigment extracted from *Psammechinus miliaris*. Ether phase. *a* (continuous line), crude extract in ether, *b* (broken line), Spinochrome E in methanol.

Properties

The properties of this pigment resemble very closely those of Spinochrome E. On adding sodium hydrosulphite, the solution turns colourless but the colour soon comes back on contact with air. The pigment forms a slightly reddish yellow sodium salt when sodium bicarbonate is added to a methanolic solution. On acidifying with HCl, the spectral character of the original solution is restored.

The spectral absorption of a sample of Spinochrome E, kindly sent to me by Dr E. Lederer, was found to be almost identical with that of the above pigment, showing only a slight difference in the relative extinction at the vicinity of the middle peak (Fig. 1*c*).

The two pigments were compared by paper-chromatography, by running them in parallel with 2N-HCl/methanol (1:5) as developer. The R_F was found to be 0·39–0·41 for Lederer's and 0·38–0·41 for my samples of pigment. None of the pigments moved from the origin when the chromatogram was developed by butanol–water–1% formic acid. This solvent system, devised by Dr Barbier, was recommended to me privately by Dr E. Lederer.

The crystals from *Psammechinus* do not melt below 320° C, the highest temperature tested, and sublime at 280° C (according to Lederer (1952) his pigment did not melt below 350° C).

Both pigments were found to be insoluble in ether, carbon disulphide, chloroform and benzene, but very soluble in acetone, methanol and ethanol.

Thus the two pigments are closely similar in solubility, absorption spectra, melting-points and in their behaviour on paper chromatograms. The pigment found in *Psammechinus*, therefore, appears to be Spinochrome E.

An elementary analysis of the pigment after two successive re-crystallizations showed C = 41·66%, H = 3·82% and O (by difference) = 54·52%. The empirical formula which corresponds most closely to this is therefore $C_{10}H_{11}O_{10}$.

On addition of weak sodium hydroxide, the optical density in the visible relative to that in the ultraviolet range decreases, with an enhancement of the hump at 455 mμ, as well as a shift of the first peak to 270 mμ. If 0·5 c.c. of 20% NaOH is added to 5 c.c. of methanolic solution, the second peak (at 358 mμ) and the third (476 mμ) disappear completely, leaving a peak at 278 mμ and a hump at 300 mμ.

It is noteworthy that the pigment from *Psammechinus* shows a change during extraction. Thus after it has crystallized out of a crude extract made with either ether or HCl, it will not re-dissolve in either solvent.

Distribution

The pigment was found not only in tests and spines but also in the coelomic fluid. It was not detectable, however, in extracts of guts and gonads.

Discussion

In general, though the properties described above are well known and characteristic of hydroxynaphthaquinones, certain reservations must be made. First, the pigment is remarkably unstable. Although the crystallized pigment is so stable that it gives exactly the same absorption curve after several months storage, crude extracts go black overnight even when acid. Again, when a drop of 2% hydrogen peroxide is added to 10 c.c. of pigment solution, the colour disappears almost instantaneously. This may explain the fact that, if a crude HCl solution is shaken with ordinary ether, which usually contains small quantities of peroxide, the red colour fades within 30 min. Therefore, only pure re-distilled ether should be used during the course of extraction. Again, the absorption spectrum of the pigment eluted from alumina columns by acid methanol is altered greatly.

Secondly, there is the question of the elementary composition, which does not correspond closely to that expected for a hydroxynaphthaquinone, the value for O being too high.

Nevertheless, the three absorption peaks are characteristic of a hydroxynaphthaquinone (Spruit, 1949), as is the behaviour of the pigment towards sodium hydrosulphite, sodium bicarbonate and sodium hydroxide. Thus the shift of the peak towards the longer wavelength in alkali (see p. 457) is more or less in harmony with Spruit's ideas. The infra-red spectrum reveals nothing unusual for a hydroxynaphthaquinone.

The change in solubility occurring during the course of extraction is noteworthy and may indicate that the naturally occurring pigment differs somewhat from that which is crystallized from extracts. A similar change occurs when pyridine is used as described on p. 455, for the crystals which are deposited are insoluble in HCl, although the original pigment was extracted with it. Spinochrome E cannot therefore be accepted unreservedly as a naturally occurring pigment.

However, the spectral absorption of the pigment is consistent. Crude extracts with both HCl-methanol (Fig. 1*d*) and ether (Fig. 2*a*) show either a small peak or a hump at the vicinity of 360 mμ in addition to a peak at 320 mμ; the latter is clearly due to other spinochromes (see p. 459). Thus the peaks in the crystallized pigment at 267, 358 and 476 mμ correspond to those at 268, 360 and 478 mμ in the crude extract.

OTHER PIGMENTS

Other pigments in the ether phase were also studied by the following means. Tests and spines were digested by 2N-HCl and extracted with re-distilled ether. The ethereal solution was washed with distilled water and dried over anhydrous Na_2CO_3. The concentrated pigment solution was chromatographed on $CaCO_3$, which was activated for 3 h at 180° C before use.

By developing with ether, the pigment can be separated into three fractions on the column: (1) a red pigment which is only weakly adsorbed, (2) a blue (sometimes green) zone, which descends slowly, and (3) a violet top zone. The

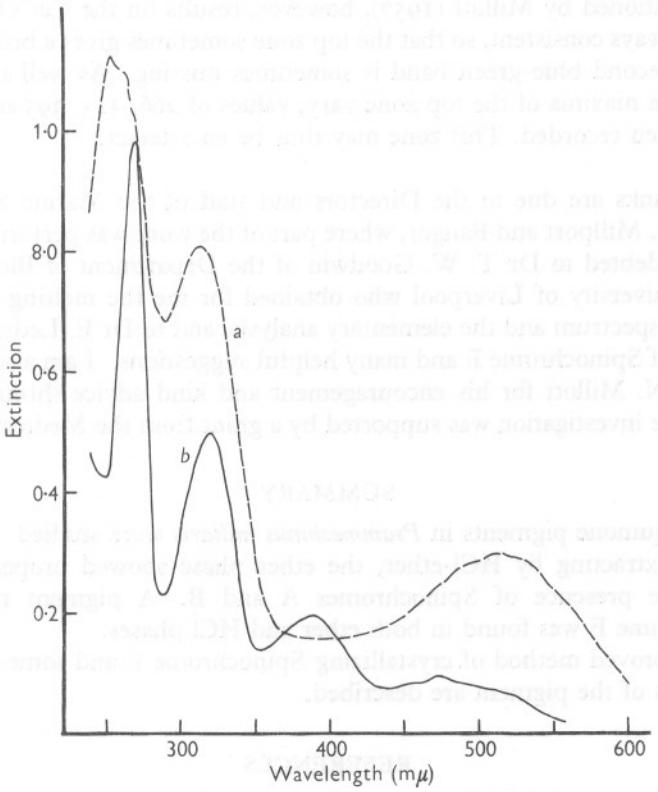

Fig. 3. Absorption spectra of pigment extracted from *Psammechinus miliaris*. Ether phase. *a* (broken line), Spinochrome A in ether; *b* (continuous line), Spinochrome B in ether.

second and the third zones were separated by dissecting the column, dissolving each part in 2N-HCl and taking up the pigment liberated into diethyl ether.

The absorption maxima of the three eluates were as follows and the curves are shown in Fig. 3:

Zone	Solvent	$\lambda_{max.}$ (mμ)			
Red	Ether	269	320	390	470
Blue or green	Ether (Fig. 3*b*)	272	320/2	388	470
	Methanol	270	318	—	480
Violet	Ether (Fig. 3*a*)	255	315	—	510
	Chloroform	255	318	—	515 535

Although no further tests were performed, the behaviour of these pigments on the chromatogram, their spectral absorption and their coloration on calcium columns, suggest that the violet and blue-green zones are Spinochromes A and B respectively (Goodwin & Srisukh, 1950; Lederer, 1952).

As mentioned by Millott (1957), however, results on the $CaCO_3$ columns are not always consistent, so that the top zone sometimes gives a brown colour and the second blue-green band is sometimes missing. As well as this, the absorption maxima of the top zone vary, values of 266, 315, 395 and 480 mμ having been recorded. This zone may thus be an artefact.

My thanks are due to the Directors and staff of the Marine Stations at Plymouth, Millport and Bangor, where part of the work was performed. I am deeply indebted to Dr T. W. Goodwin of the Department of Biochemistry at the University of Liverpool who obtained for me the melting-point, the infra-red spectrum and the elementary analysis, and to Dr E. Lederer for the samples of Spinochrome E and many helpful suggestions. I am also indebted to Prof. N. Millott for his encouragement and kind advice throughout the work. The investigation was supported by a grant from the Medical Research Council.

SUMMARY

Naphthaquinone pigments in *Psammechinus miliaris* were studied.

After extracting by HCl-ether, the ether phase showed properties indicating the presence of Spinochromes A and B. A pigment resembling Spinochrome E was found in both ether and HCl phases.

An improved method of crystallizing Spinochrome E and some additional properties of the pigment are described.

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