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A Study of the Pigments of the Sea-urchins, *Echinus esculentus* L. and *Paracentrotus lividus* Lamarck

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Although the purple pigments of the test (shell) and spines of the common sea-urchin *Echinus esculentus* L. have attracted some attention they have not been unequivocally identified. In his pioneer investigations MacMunn (1885) noticed a red quinonoid pigment which he named echinochrome in the perivisceral fluid of a number of sea-urchins; it was first isolated in crystalline form by McClendon (1912) from the ovaries of *Arbacia pustulosa* Leske, but it was not until much later that its empirical formula was established as $C_{12}H_{10}O_7$ (Ball, 1936; Glaser & Lederer, 1939; Tyler, 1939). Kuhn & Wallenfels (1939, 1940) isolated the same pigment, together with small amounts of two similar pigments, from the gonads of *Paracentrotus lividus* Lamarck and decided that it was 2-ethyl-3:5:6:7:8-pentahydroxy-1:4-naphthaquinone (I). Confirmation of this structure was obtained when Wallenfels & Gauhe (1943) achieved a synthesis of the pigment. Meanwhile, Lederer & Glaser (1938) isolated a new quinonoid pigment (spinochrome, spinochrome A, spinochrome P) from the spines and tests of the purple variety of *P. lividus*; this was confirmed by Musajo & Minchilli (1940), who later isolated a further green pigment spinochrome P_1 from the spines of the green variety of *P. lividus*.

Apart from MacMunn's (1885) work, the only other reports on the pigments of *Echinus esculentus* indicated that reduced echinochrome exists in the elaeocytes and that echinochrome itself is the pigment of the shell, dermis and spines (Lederer, 1940).

It was because of the paucity of information regarding the pigments of *E. esculentus* that the present investigation was undertaken. As the investigation proceeded it became apparent that two pigments were present in the tests and spines and

that they were in fact spinochrome A (P) and spinochrome B (P_1); no evidence for the presence of echinochrome was ever obtained. Authentic samples of these two pigments were obtained from *Paracentrotus lividus* and the opportunity was taken not only of recording their properties more fully than had been done previously, but also of determining their distribution in the olive-green and violet varieties of this urchin.

Considerable confusion exists in the literature of sea-urchin pigments for two reasons: (i) various names have been given at different times to the same pigment, and (ii) authors have often been unaware of the synonymy existing in the systematic naming of sea-urchins. In order to clarify the position, Table 1, recording all the published information together with indications of synonymy in both chemical and systematic nomenclatures, has been compiled; names to be used in this paper are also indicated. The logical procedure has been adopted of identifying the pigments by adding the suffixes A, B, C, etc. after the general terms echinochrome and spinochrome, according to the order in which the pigments were isolated; this follows the procedure initiated by Kuhn & Wallenfels (1939). Much less difficulty arises in this way than in adding a suffix indicating the species from which the pigment was first isolated. In this way difficulties soon arise, e.g. spinochrome 'P' (*Paracentrotus*), has now been found in *Echinus esculentus*. The two group terms echinochrome and spinochrome have been accepted, for there appears to be good reason to believe that echinochromes occur only in the gonads and body fluid, whilst the spinochromes are characteristic of the spines and tests. The pigments obtained from Japanese sea-urchins (Kuroda & Oshima, 1940) are

Table 1. *Naphthoquinone pigments of sea-urchins*

Pigment		Sources		Organs in which pigment is found	M.p. of pigment (°)	Reference
Name used in this paper	Other reported names	Usual name	Common synonyms			
Echinochrome A	Echinochrome Arbaine	<i>Arbacia pustulosa</i> Leske	<i>A. asquiberculata</i> Blainville <i>A. livida</i> L.	Perivisceral fluid Eggs, test, elaeocytes Eggs, shell Eggs Ovaries	— — — 220.5-221 220	McMunn (1885) McClendon (1912) Cannan (1927) Vlés & Velling (1928) Ball (1936) Lederer & Glaser (1938), Glaser & Lederer (1939) Kuhn & Wallenfels (1939)
		<i>Echinus esculentus</i> L.	—	Ovaries	217	McMunn (1885)
		<i>Paracentrotus lividus</i> Lamarck	<i>Strongylocentrotus lividus</i> Lamarck, Brandt	Perivisceral fluid Dermis, shell* Spines* Spines*	— — — —	Lederer (1940) Glaser & Lederer (1939)
Echinochrome B	Echinochrome B	<i>Arbacia pustulosa</i>	As above	Ovaries	173-175	Kuhn & Wallenfels (1940)
Echinochrome C	Echinochrome C	<i>Arbacia pustulosa</i>	As above	Ovaries	—	Kuhn & Wallenfels (1940)
Echinochrome D	Isochinochrome	<i>Arbacia pustulosa</i>	As above	Ovaries	247	Glaser & Lederer (1939)
Spinochrome A	Spinochrome Spinochrome P	<i>Arbacia pustulosa</i> —	As above —	Spines and tests —	185 187-188	Glaser & Lederer (1939) Musaio & Minchilli (1940)
	Echinorubin	—	—	—	187-189	Tyler (1939)
		<i>Echinus esculentus</i>	—	Spines and tests	—	Stern, quoted by Moore (1937)
Spinochrome B	Spinochrome P ₁	<i>Arbacia pustulosa</i>	As above	Spines and tests	185 (sublimes)	The present work
		<i>Echinus esculentus</i>	—	Spines and tests	350-355	Musaio & Minchilli (1942)
Spinochrome AK _a	Spinochrome F	<i>Pseudocentrotus depressus</i> <i>Heterocentrotus mamillatus</i>	— —	Spines and tests Spines and tests	283 (decomp.) 285-295	The present work Kuroda & Oshima (1940)
Spinochrome M		<i>Anthadidaris crassispina</i>	—	Spines and tests	229	Kuroda & Oshima (1940)
			—	Spines and tests	193	Kuroda & Oshima (1940)

* Not confirmed in the present investigation.

not considered at the moment because of doubt concerning their possible identity with pigments previously described.

EXPERIMENTAL

Isolation of spinochromes A and B. A mixture of roughly broken tests and spines was first well washed with water, acetone and ethanol and then dissolved in 2N-HCl. The pigments were extracted into freshly redistilled ether which was then washed with small amounts of water until all the acid was removed. Care had to be taken not to overwash because, in the absence of acid, water extracts considerable amounts of pigment from the ether phase. The ethereal solution was dried over anhydrous Na_2SO_4 and the solvent removed *in vacuo* at room temperature. The residue was dissolved in a small volume of dry redistilled ether and the ruby-red solution chromatographed on a CaCO_3 (British Drug Houses Ltd., A.R.) column. On eluting with ether five fractions were obtained: (a) a violet zone (most strongly adsorbed), (b) a pink zone, (c) an olive-green zone, (d) a yellow zone and (e) a yellow filtrate. Similar chromatograms were obtained with both *Echinus esculentus* and *Paracentrotus*

lividus extracts although the relative amounts of (a) and (c) varied. The pigments (b), (d) and (e) were extremely minor constituents and were given only a cursory examination; little information could be obtained concerning (d) and (e), but (b) was characterized to some extent; (d) and (e) appear to be neither carotenoids nor naphthoquinones.

The developed column was extruded and the green, pink and violet zones separated and dissolved in N-HCl. The green and violet fractions were transferred to ether as previously described, and rechromatographed to remove final traces of impurities. After transferring the purified pigments to ether, the ether was removed and the pigments recrystallized three times from methanol. It should be noted that the olive-green pigment was obtained crystalline only from *P. lividus*; it occurs in *Echinus esculentus* only in traces.

The ruby-red pigment, which is violet on the column owing to the formation of a Ca salt, crystallized as fine dark violet rods, m.p. 185° (Found: C, 51.5; H, 3.5. Calc. for $\text{C}_{22}\text{H}_{10}\text{O}_8$: C, 51.1; H, 3.5%) and appears to be identical with the pigment isolated by Glaser & Lederer (1939), and by Musajo & Minchilli (1940), and termed spinochrome and spinochrome P respectively; as explained earlier, we consider spinochrome A a more appropriate name.

Table 2. *Properties of spinochromes A and B, and pigment X*

Reagents, etc.	Spinochrome A	Spinochrome B	Pigment X
Solubility			
Light petroleum (40–60°)	Insoluble	Insoluble	Insoluble
Ethyl ether	Very soluble	Very soluble	Very soluble
Ethanol	Very soluble	Very soluble	Very soluble
Methanol	Very soluble	Very soluble	Very soluble
Water	Very soluble	Soluble	Soluble
Chloroform	—	—	Soluble
Carbon disulphide	Very sparingly soluble (in hot)	Slightly soluble in warm, insoluble in cold	Soluble
<i>n</i> -Hexane	Very sparingly soluble (in hot)	Slightly soluble in warm, insoluble in cold	Soluble
Conc. H_2SO_4	Very soluble	Soluble	Soluble
NaOH (0.02N)	Very soluble soon decolorized	—	Black ppt.
Fluorescence	Nil in Et_2O solution	Nil in ethyl ether solution	—
Ferric chloride	Dirty green	Dirty green	Purplish
Methanolic lead acetate	Dark blue ppt. with blue solution	Brown ppt.	Black ppt.
Hydrogen peroxide	No action	No reaction	Brownish
Optical activity	None demonstrable with concn. of 0.014 and 0.03% using 10 cm. cells	—	Black ppt.
Mol. wt.	Too highly coloured in camphor for determination by Rast method	Insoluble in camphor	—

Table 3. *Absorption maxima of spinochromes A and B and pigment X in various solvents*

~ Denotes an inflexion.

Solvent	λ_{max} (m μ)		
	Spinochrome A	Spinochrome B	Pigment X
Ethanol	271, 310, 518	272, 320, 388, 480	267, 323, 468, ~520
Ethyl ether	254, 318, 525	272, 321, 388, 480	266, 323, 467, ~482, ~520
Chloroform	— — ~515, 530, ~572	— — 397, 474	— — 468, 488, ~522
Benzene	— — ~515, 540, ~582	— — 395, 478	— — 468, 488, ~524
Conc. H_2SO_4	262, 316, 495, 530, 565	276, 360, 445, 582	— — 440, 468, 500
<i>n</i> -Hexane	252, 316, 510, 543, 586	— — — —	252, 326, 460, 484, ~520
Carbon disulphide	— — 518, 550, 592	— — — —	— — 469, 484, ~527

Fig. 1.

Fig. 2.

Fig. 1. The absorption spectra of spinochromes A and B in ethanol. —, spinochrome A; - - - - -, spinochrome B.

Fig. 2. The visible absorption spectrum of spinochrome A in various solvents. —, in ether; - - - - -, in *n*-hexane; - · - · -, in carbon disulphide; · · · · ·, in conc. H_2SO_4 .

Fig. 3.

Fig. 4.

Fig. 3. The absorption spectra of spinochromes A and B in sat. $NaHCO_3$. —, spinochrome A; - - - - -, spinochrome B.

Fig. 4. The absorption spectra of reduced spinochromes A and B and of pigment X. —, spinochrome A; - - - - -, spinochrome B; - · - · -, pigment X.

The orange-red pigment, appearing on the column as an olive-green Ca salt, was isolated in crystalline form only from *Paracentrotus lividus*, but was unequivocally identified by spectroscopic and chemical tests in *Echinus esculentus*. The pigment (spinochrome B) crystallized as fine glistening thin red rods, m.p. 283° (decomp.) (Found: C, 51.3; H, 3.9. Calc. for $C_{12}H_{12}O_8$, C: 51.1; H, 3.9%). It is highly likely, from its absorption spectrum and general chemical properties, that it is identical with Musajo & Minchilli's (1942) spinochrome P_1 , although these authors reported a m.p. of 350–355° (decomp.).

The general properties of these two pigments are recorded in Table 2. Previously the absorption spectra of these compounds have been recorded only in the visible spectral region, and in the case of spinochrome B (P_1) the solvent used was not specified. The complete spectra of these two pigments in ether are recorded in Fig. 1 and the absorption maxima in a number of other solvents are collected in Table 3. As the shape as well as the position of the visible band of spinochrome P is altered according to the solvent, the spectrum of this band in a number of solvents is recorded in Fig. 2; spinochrome B shows no such differences in shape. The spectra of the two pigments in alkaline solution and when reduced are also recorded (Figs. 3, 4). Being quinonoid derivatives the pigments are easily reducible with dithionite and equally rapidly reoxidized in air. By working very quickly in ethereal solution it was, however, possible to measure the spectra of the reduced forms. The reduction of spinochrome A follows different paths depending on whether the reaction takes place in acid or alkaline solution, viz.

By contrast, a solution of spinochrome B in NaHCO₃ which is olive green is reduced to a light-yellow solution by Na₂S₂O₄ which, on acidification, is immediately oxidized to its original orange-red form.

The pigment from the pink zone (pigment X) was transferred to ether in the usual manner, but, although it was purified sufficiently to be completely free from any traces of the two other pigments, insufficient was obtained to make crystallization feasible. However, its spectrum was obtained in a number of solvents (Figs. 4, 5; Table 3), and its

general properties examined (Table 2). They were very similar to spinochrome A, and it is undoubtedly a naphthaquinone type of compound.

Fig. 5. The absorption spectrum of pigment X in ether.

Fig. 6. The absorption spectra of the leuco acetyl derivatives of spinochromes A and B. —, leuco acetyl derivative of spinochrome A; ----, leuco acetyl derivative of spinochrome B.

Reductive acetylation of the pigments. The pigments were reductively acetylated in a conventional manner: 10 mg. of crystalline pigment in 2 ml. of glacial acetic acid were refluxed for 1 hr. on a water bath with 1 g. of Zn dust and 0.5 g. of fused Na acetate. In the case of spinochrome A the colour changed from ruby red to yellow almost immediately

but no further colour changes took place; with spinochrome B, however, the orange-red colour was completely discharged. When the reaction was complete the resulting mixture was filtered into ice water and allowed to stand overnight, when a granular precipitate was obtained. In the case of spinochrome A efforts to recrystallize the small amount of material available were unsuccessful, but the leuco acetyl derivative of spinochrome B was crystallized from warm glacial acetic acid as colourless fine rod-like crystals which melted at 226–230° (decomp.); Musajo & Minchilli (1942) report m.p. 235°. The spectra of the two leuco acetyl derivatives are recorded in Fig. 6.

Determination of the distribution of spinochromes A and B. The amounts of these two pigments in various samples of test and spines were determined using a spectrophotometric method based on a procedure described by Goodwin & Morton (1946) for the determination of tyrosine and tryptophan in proteins; this consists of measuring the absorption at two selected wavelengths, one of which is the isosbestic point of the system. In the present case when ether is the solvent the isosbestic point is at 435 m μ . and $E_{1\text{ cm}}^{1\%}$ at this wavelength is 65 ($E_{1\text{ cm}}^{1\%}$ values can be used instead of ϵ values because the mol.wt. of the two pigments are almost identical); the other wavelength chosen was 525 m μ . at which the $E_{1\text{ cm}}^{1\%}$ values for spinochrome A and spinochrome B are 149 and 78 respectively. Thus, after determining the total pigment concentration from the $E_{435\text{ m}\mu}$ value, the composition of a mixture can be calculated using the equation $y = \frac{E - xB}{A - B}$, where y = concn. of spinochrome A, x = total pigment concn., $A = E_{1\text{ cm}}^{1\%}$ 525 m μ . value for pure spinochrome A, $B = E_{1\text{ cm}}^{1\%}$ 525 m μ . value for pure spinochrome B and E is the observed extinction at 525 m μ . The presence of the other pigments can have little or no effect on the results as they are present only in traces.

RESULTS AND DISCUSSION

The distribution of spinochromes A and B in *E. esculentus* and *Paracentrotus lividus* are recorded in Table 4.

Table 4. Amounts of spinochromes A and B in spines and tests of *Echinus esculentus* and *Paracentrotus lividus*

Material	Amount (mg./g. fresh wt.)	
	Spinochrome A	Spinochrome B
<i>E. esculentus</i>		
Spines (violet)	0.0019	Trace
Shell (violet)	0.023	Trace
<i>P. lividus</i>		
Spines (violet)	0.52 0.50	0.39
Spines (olive green)	0.17	1.07

This work has demonstrated by isolation of the material in crystalline form that spinochrome A is the major pigment occurring in the spines and tests of *Echinus esculentus*; this does not confirm Lederer's (1940) short report that the pigment is echinochrome,

but does verify Stern's unpublished observations (see Moore, 1937) that the pigment was slightly different from echinochrome. Spinochrome B, the orange-red component of the pigment system of *Paracentrotus lividus* spines was also identified in *Echinus esculentus*, but was not present in sufficient amounts for isolation.

The spinochrome A isolated from *Paracentrotus lividus* is identical with that isolated by Glaser & Lederer (1939) and Musajo & Minchilli (1940); spinochrome B is identical with spinochrome P₁ isolated from olive-green *P. lividus* by Musajo & Minchilli (1942) in all properties except melting point, but as the melting points of the leuco acetyl derivatives prepared by Musajo & Minchilli (1942) and ourselves are almost identical, it is reasonable to assume that the pigments are in fact identical.

The violet and olive-green colours encountered in the spines are due to the formation of salts between the pigments and the calcium of the test and spines, the normal colour of the pigments being ruby red and orange red respectively. The formation of such salts is readily seen on chromatography of the pigments on calcium carbonate when they separate into bright violet and olive-green bands; even brighter colours are produced on magnesium carbonate. It is important to correct an impression given by the work of Musajo & Minchilli (1940, 1942) that in the case of *P. lividus*, violet specimens contain only spinochrome B, whilst olive-green specimens contain both spinochromes A and B. The fact that both pigments exist in both variants is immediately apparent on chromatography of a crude extract, and is also apparent if the spines from the two varieties are examined closely. The spines of the olive-green animals always have a violet tip and in occasional cases this violet area can extend one-third of the way down the spine. In the violet animals the reverse is the case, small areas of olive-green pigments are always present at the base of the spines, while the rest is violet. The observation that the difference between the coloration of the violet and olive-green specimens is merely quantitative is well brought out in Table 4. There is apparently no clear-cut biological differentiation between the two colour variants of *P. lividus*, but considering the relative positions of the pigments in the species, it may well be that they represent different developmental stages. It is also important to realize that Glaser & Lederer's (1939) suggestion that the olive-green coloration is due to a mixture of echinochrome A and spinochrome A cannot be correct.

Structures of spinochromes A and B. Spinochrome A is very similar in its properties to echinochrome A (I) and must differ only slightly from it in structure. The analytical results of Lederer & Glaser (1938) and of ourselves suggest that it contains an additional hydroxyl group compared with echinochrome A;

1 mole of acetic acid is obtained on oxidizing 1 mole of the pigment (Lederer & Glaser, 1938; Musajo & Minchilli, 1940) which is consistent with a $-\text{CHOH}.\text{CH}_3$ side chain. The spectroscopic data are consistent with the replacement of ethyl by $-\text{CHOH}.\text{CH}_3$ in position 2, for considerable work (e.g. see Spruit, 1949) has indicated that the introduction of varying saturated side chains into naphthaquinones has little effect on their absorption spectra and the visible spectrum of echinochrome A

hydroxyl group is present or absent in position 3 (Spruit, 1949).

Musajo & Minchilli (1942) consider that (III) represents the structure of spinochrome B; there are a number of reasons why this cannot be accepted; viz. (a) our analytical data cannot be reconciled with this structure; (b) the introduction of an acetyl group into position 2 of a naphthaquinone has little if any effect on its spectrum (Spruit, 1949) and thus the spectrum of spinochrome B should be very

(I)

(II)

(III)

is, in position at least, very little different from that of spinochrome A; further, it should be noted that substitution of ethyl by methyl has no effect on the spectrum of echinochrome (Weygand, Vogelbach & Zimmerman, 1947) and that the complete spectrum of solanione (2-methyl-3-methoxy-6-(or 7-)-acetyl-5:8-dihydroxy-1:4-naphthaquinone) is very similar to that of spinochrome A (Weiss & Nord, 1949). According to Kuhn & Wallenfels (1941) the

similar to that of spinochrome A; (c) spinochrome B exhibits an absorption band at 388 $m\mu$. (not noted by Musajo & Minchilli (1942) who did not record its spectrum below 400 $m\mu$.) which is atypical of a simple hydroxy-substituted naphthaquinone (Spruit 1949); and (d) Musajo & Minchilli (1940, 1942) obtain very much less acetic acid on oxidation of the pigment than on oxidation of spinochrome A; according to Kuhn & Wallenfels's (1941) work on

(IV)

(V)

(VI)

(VII)

presence of an enolizable hydroxyl group in the side chain can account for the difference between spinochrome A and echinochrome. It seems reasonably certain then that the structure of spinochrome A (II) suggested by Kuhn & Wallenfels (1941) is correct and that the analytical data cannot be reconciled with Musajo & Minchilli's (1940) suggestion that no hydroxyl is present in position 3; it should be noted, however, that it is virtually impossible to decide from spectroscopic data alone whether an

spinon A (IV) the same amount of acetic acid should have been obtained in each case.

Any structure suggested for spinochrome B must attempt to explain, at least qualitatively, its atypical spectrum. The two most plausible structural types are (a) a hemiquinone and (b) a dihydroquinone. The pigment cannot be an ordinary hemiquinone because it ought to be easily oxidizable in air or by hydrogen peroxide to a pigment similar to spinochrome A; as this does not occur it is suggested that

the hydroxyl in position 4 may be methylated and thus the system is stabilized in a structure such as (V). Such a compound has been produced from benzoquinone by the action of Grignard reagent (e.g. see Karrer, 1938). Regarding the second possibility, Kuhn & Wallenfels (1942) produced by the action of silver oxide on echinochrome A, a substance dihydro-echinochrome (VI) which exhibits a band at 380–390 m μ . and an inflexion at about 420 m μ . The analytical data rule out such a structure for spinochrome B, but (VII) may well be correct. It should be emphasized that we are here mainly concerned with suggesting the type of structure spinochrome B may possess; considerably more data are required before a complete structure can be suggested; lack of material has limited our investigations along these lines. The spectrum of the reduced form of spinochrome B is also consistent with a structure of the type (V) or (VII) for it displays only one band at 290 m μ . (Fig. 3). This band can be ascribed to absorption by the hydroxybenzenoid nucleus and there is no visible absorption band due to naphthol absorption (Spruit, 1949) because no such residue exists. This is in contrast to the spectrum of reduced spinochrome A which exhibits absorption bands characteristic of both hydroxybenzenes and naphthols.

The pigment which forms a pink calcium salt (pigment X) must be a naphthaquinone pigment because it exhibits very similar properties to spinochromes A and B (see Table 2); its absorption spectrum indicates that it is more closely allied to spinochrome A than to spinochrome B. We have refrained from naming it because it has not been isolated crystalline.

SUMMARY

1. Five pigments have been detected in the sea-urchins *Echinus esculentus* and *Paracentrotus lividus*; of these three are naphthaquinone pigments and two are neither naphthaquinones nor carotenoids.

2. The two major pigments spinochromes A (P) and B (P₁) are naphthaquinone derivatives and have been isolated and their properties, especially spectral, studied in detail.

3. The properties of the third naphthaquinone pigment which has not been isolated are reported.

4. A spectroscopic method for the determination of the amounts of spinochromes A and B in a mixture of the two is described.

5. The distribution of the two major pigments in the spines and tests of *Echinus esculentus* and the olive-green and violet variants of *Paracentrotus lividus* has been examined.

6. The data obtained are consistent with the structure suggested by Kuhn & Wallenfels (1939) for spinochrome A, but not with that ascribed by Musajo & Minchilli (1942) to spinochrome B. Possible structures of spinochrome B are discussed.

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