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The Carotenoids of the Gonads of the Limpets *Patella vulgata* and *Patella depressa*

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The ripe gonads of the gastropods *Patella vulgata* and *P. depressa* are bright pink in the male and olive-green to chocolate-brown in the female. A preliminary examination by Lönnberg & Hellström (1931) indicated the presence of carotenoids.

The present paper is concerned chiefly with the identification of the constituent carotenoids. It has been shown, however, that the ovaries contain, in addition, an unidentified pigment which is not a carotenoid.

EXPERIMENTAL

Extraction of material. The bodies of the limpets were cut away from the shells, and the gonads carefully removed and ground to a fine powder with acid-washed silver sand and anhydrous Na_2SO_4 . The resulting powder was then extracted with successive portions of peroxide-free ether (obtained by distillation of commercial ether over reduced iron) until the final extract was colourless. The extracts were combined, the solvent removed *in vacuo* at room temperature and the residue saponified under N_2 at room temperature for 24 hr. with 20% (v/v) ethanolic KOH. The unsaponifiable material was extracted with ether using a conventional method (Goodwin & Morton, 1946). The ether was removed *in vacuo* and the residue dissolved in a small volume of methanol and cooled in solid CO_2 ; considerable amounts of precipitated steroids were filtered off using a Büchner funnel packed with solid CO_2 . The methanol was removed *in vacuo* at about 30° and the residue, dissolved in light petroleum (b.p. 40–60°), subjected to chromatographic analysis. About 2000 limpets were analysed in this way during the autumns of 1948 and 1949. Male and female gonads were examined separately, but apart from the presence of pigment Y (see below, p. 246) in the ovaries, the results were identical.

Chromatography was carried out using alumina (Spence, Grade O) deactivated by suspending it in methanol for about 30 min. After filtering off the methanol the alumina was allowed to dry at room temperature by spreading it out on

large pieces of filter paper. If the alumina thus obtained was too weak for the purpose in hand it was reactivated by heating at 30–60° for 3–4 hr.

Using deactivated alumina and developing the column with light petroleum (b.p. 40–60°) containing 1–2% (v/v) of ether, four main zones separated in the following order of increasing adsorptive power: (I) yellowish-orange band, (II) broad red band adsorbed very close to (III), a narrow zone, and (IV) a yellow-orange zone.

A very small fraction which rapidly washed through the column was generally encountered; this behaviour, together with the absorption spectrum of the fraction, suggested the presence of α -carotene, but sufficient was never obtained to make identification certain.

Pigment I. This pigment, which was epiphasic to 90% aqueous methanol, was purified by repeated chromatography, using 1% (v/v) of ether in light petroleum (b.p. 40–60°) as eluant, to separate the pigment from traces of other pigments. The chromatographically homogeneous material was dissolved in methanol and the greater part of the colourless impurities removed by cooling in solid CO_2 and filtering while cold. After this final purification the absorption spectrum of the pigment was determined in light petroleum (b.p. 40–60°), CS_2 , and pyridine; in each solvent the curve was identical with that of an authentic sample of crystalline β -carotene. On chromatographing a mixture of pigment I and β -carotene no separation was achieved, thus confirming the identification.

Pigment II. This pigment was purified by adsorption followed by elution with 2–3% (v/v) ether in light petroleum. The adsorption curve showed only one peak in the visible region indicating a keto-carotenoid (Heilbron & Lythgoe, 1936). The data available indicated that pigment II was most likely to be either echinone (Lederer, 1935) or aphanin (Tischer, 1938, 1939), the former isolated from the gonads of *Paracentrotus lividus* L. and the latter from the fresh-water blue-green alga, *Aphanizomenon flos-aquae*. Tischer's (1939) suggested formula for aphanin is the same as that of Lederer (1935) for echinone; this will be discussed later. It was not possible to obtain *A. flos-aquae* in

order to compare our pigment with aphanin nor to obtain a specimen of echininone from *Paracentrotus lividus*. A pigment was, however, isolated from *Echinus esculentus* L. gonads which had properties identical with those of Lederer's (1935) echininone; as echininone is widely distributed in sea-urchin gonads (see Goodwin, 1950) this pigment was accepted as being echininone. The absorption spectra of both pigment II and echininone in a number of solvents were identical and a mixture of the two could not be separated chromatographically; it was therefore concluded that pigment II is echininone. The visible absorption spectrum of echininone has not previously been recorded in full; therefore the curves obtained in various solvents are reproduced in Fig. 1.

Fig. 1. The absorption spectrum of echininone in various solvents. —, light petroleum (b.p. 40–60°); —●—, chloroform; - - - -, carbon disulphide.

Fig. 2. The absorption spectrum of echininone oxime in various solvents. —, light petroleum (b.p. 40–60°); —●—, chloroform; - - - -, carbon disulphide.

In order further to confirm the identity of pigment II and echininone the oximes were prepared in the following way: hydroxylamine hydrochloride (0.5 g.) in 25 ml. of ethanol was added to a pyridine solution of the pigment (100 mg.) and KOH added until the HCl was neutralized. The mixture was heated under reflux on a sand bath for 9 hr. in

the absence of light. After dilution with water the products were extracted with light petroleum (b.p. 40–60°) and the resulting solution, after drying over anhydrous Na_2SO_4 , was chromatographed on deactivated alumina to separate the oxime from any unchanged pigment; the purified product was then examined spectroscopically. The oximes from both pigments were spectroscopically identical and chromatographically inseparable. Echininone oxime exhibits in various solvents the spectra recorded in Fig. 2.

Reduction of echininone. The keto group of echininone was reduced with aluminium isopropoxide. A solution (25 ml.) of the pigment (80 mg.) in benzene was refluxed on a sand bath for 10 hr. in the dark with freshly prepared aluminium isopropoxide (0.5 g.) in isopropanol (10 ml.). After standing overnight under N_2 the excess aluminium isopropoxide was precipitated by addition of 10% (w/v) KOH (100 ml.), and the pigment mixture extracted with freshly redistilled ethyl ether; considerable destruction of pigment occurred during the reaction. The solvent was removed at room temperature *in vacuo*, and the very small residue dissolved in light petroleum (b.p. 40–60°) and chromatographed on deactivated alumina. Four zones were obtained (in decreasing order of adsorption): (i) dirty yellow, (ii) orange-yellow, (iii) violet, and (iv) orange-red. The major zone (ii) was separated from the rest and purified by repeated chromatography, and its spectrum measured in a number of solvents. The spectrum, which now showed three peaks, together with the chromatographic and partition behaviour of the pigment strongly suggested that it was a monohydroxy derivative of β -carotene, e.g. cryptoxanthin; insufficient material was, however, available to compare directly with cryptoxanthin.

Pigment III. The chromatographic purification of this pigment was carried out in the appropriate manner; it was extremely difficult to separate from echininone, but separation could be achieved by eluting with light petroleum containing between 5 and 10% (v/v) of ether according to the degree of activation of the column. The chromatographic behaviour, absorption spectrum and the fact that it was hypophasic to 95% (v/v) methanol but epiphase to 90% methanol all suggested that pigment III is cryptoxanthin. A chromatographically pure sample of cryptoxanthin was therefore obtained from crushed maize and it was found that pigment III and cryptoxanthin had identical spectra and could not be separated in a mixed chromatogram.

Pigment IV, which was very strongly adsorbed on alumina as a darkish yellow zone, was hypophasic to 90% methanol and exhibited an absorption spectrum similar to that recorded for zeaxanthin but not unequivocally different from some of the reported curves for lutein. Accordingly, authentic samples of lutein and zeaxanthin were prepared from nettles and maize respectively. Pigment IV was chromatographically easily separable from lutein, but could not be separated when mixed with zeaxanthin; further, the colour of lutein adsorbed on alumina was lemon-yellow, whilst that of pigment IV and zeaxanthin were identical and much darker. The identity of pigment IV with zeaxanthin was considered settled when its absorption spectrum was compared with that of the freshly prepared zeaxanthin; the two curves coincided almost exactly over the whole visible range of wavelengths.

Experiments on the pigment-protein complexes in limpet gonads. If the male gonads are made into a paste with kaolin and shaken with light petroleum all the carotenoids present in the tissues are easily extracted. This suggested

that the carotenoids were not present as protein complexes, and this was confirmed by the following experiment. The gonads were ground up with saline and the mixture centrifuged; the insoluble greyish protein collected at the bottom, the saline assumed a slight turbidity, but was not coloured, and the lipids, bright yellow, floated at the top. All the carotenoids were present in this lipid layer for none could be extracted from the saline extract or from the precipitate after denaturation of the protein with ethanol. If

Fig. 3. The visible absorption spectrum of pigment Y (the prosthetic group of the green chromoprotein). —, in ethanol; ----, in pyridine.

the green female gonads were treated similarly a major portion, but not all of the carotenoids were extractable from the kaolin-ovary paste with light petroleum and after this extraction the paste was still green. Further extractions with a mixture of ether and light petroleum (1:1) extracted the remainder of the carotenoids. As this fractionation could be repeated on all samples of ovaries investigated, it was concluded that the more difficultly extractable carotenoids were probably originally attached to a protein. Qualitatively the carotenoids in both fractions are identical.

Even after the removal of this final carotenoid fraction the gonad paste was still green; addition of acetone or ethanol completely decolorized it, and a pigment which did not appear to be a carotenoid was extracted. This pigment (pigment Y), which is a hydrocarbon, exhibits a visible spectrum containing a single sharp band with $\lambda_{\max}$ at 418 and 398 mμ. in pyridine and ethanol respectively.

A saline extract of the female gonads is green and after clarifying by shaking with kaolin, exhibited an absorption spectrum quite similar to that of ooverdin (Fig. 4) (Stern & Salomon, 1938; Goodwin & Srisukh, 1949) in having a maximum at 635 mμ., an inflexion at 520 mμ. and a large band at 390 mμ. As just noted, however, the prosthetic group of this pigment is not astaxanthin, but the, as yet

unidentified, pigment Y. In spite of the important contribution it makes to the colour of female gonads it is present in comparatively small amounts. In a chromatogram of an ether extract of Na_2SO_4 -dried ovaries it is very strongly adsorbed at the top of an alumina column as a small brownish band, which in early work was generally discarded as being the zone containing indeterminate oxidation products of carotenoids which is almost always encountered in this type of work.

Pigments from Patella depressa. These pigments were not given such a searching investigation as those from *P. vulgata* because of lack of material, but it became quite clear that the pigments of this species are the same as those found in *P. vulgata*.

Fig. 4. The absorption spectrum of the green chromoprotein extracted from limpet gonads (solvent, saline).

DISCUSSION

Of the four pigments identified in this study three are well known plant carotenoids and have structures which are generally accepted. It should be pointed out, however, that proof concerning the positions of the hydroxyl groups in cryptoxanthin and zeaxanthin is by no means rigid. The fourth pigment, echininone, is taken by Lederer (1935) to be 3-keto- β -carotene; we consider that 4-keto- β -carotene is more likely for the following reasons: β -ionone is fixed as one terminal residue because the pigment is a provitamin A (Lederer & Moore, 1936) and as $\lambda_{\max}$ for echininone is at 460 mμ. in light petroleum, whilst that for β -carotene is at 450 mμ. it is extremely probable that the keto group is in conjugation with the rest of the double bonds of the molecule, because in the cases of astaxanthin (Goodwin & Srisukh, 1949) and rhodoxanthin (Kuhn & Brockmann, 1933) which contain two conjugated keto groups, the maxima are displaced 18 and 12 mμ. respectively, in the direction of higher wavelengths compared with those of the parent pigments, β -carotene and dehydro- β -carotene (Karrer, Schopp & Morf, 1932). Our argument is based on data ob-

tained using light petroleum (b.p. 40–60°) as solvent; it applies equally well if results in other solvents are considered. The introduction of non-conjugated keto groups has little or no effect on the visible spectrum of a carotenoid [compare astacin with astaxanthin (Goodwin & Srisukh, 1949)]. Thus, spectroscopic evidence indicates that echininone is more likely to be 4-keto- β -carotene than 3-keto- β -carotene in which the keto group is isolated from the main conjugated polyene chain. This view is strengthened when oxime formation is considered. Oximation of a conjugated keto group of a carotenoid shifts $\lambda_{\max}$ to lower wavelengths (Kuhn & Brockmann, 1933; Heilbron & Lythgoe, 1936); we have found that $\lambda_{\max}$ of echininone oxime is 8 m μ . lower than that of the parent compound.

It has already been pointed out that the structures independently assigned to echininone and aphanin are identical; moreover, Lederer (1935) claims that echininone and myxoxanthin are also identical; this is considered improbable by Heilbron (quoted by Karrer & Jucker, 1948). It is important to consider

these claims in the light of new data and to clarify the following discussion all the known properties of these substances are assembled in Table 1.

Considering first echininone and aphanin, their properties with one exception are identical, and as this discrepancy suggests an inconsistency in Tischer's (1938, 1939) work, there seems little doubt that the two pigments are the same compound. Regarding the discrepancy, Tischer states that the maxima for aphanin and its oxime are the same, namely 460 m μ . These results cannot be reconciled with the suggested structure, 3-keto- β -carotene, for from the previous discussion it will be realized that if aphanin is 3-keto- β -carotene, its spectrum will not be altered on oximation, but as the keto group is not conjugated, the original $\lambda_{\max}$ should not be very different from that of β -carotene (450 m μ .); similarly, if the keto group is conjugated to give a band at 460 m μ ., the typical oximation shift should be encountered.

The spectroscopic data in three solvents for echininone and myxoxanthin are identical, but a slight

Table 1. *Comparison of the properties of aphanin, echininone and myxoxanthin*

(The data marked with an asterisk (*) are those obtained during the present study, otherwise those for aphanin are from Tischer (1938, 1939), those for echininone from Lederer (1935), and those for myxoxanthin from Heilbron & Lythgoe (1936).)

	Aphanin $C_{40}H_{54}O$	Echininone $C_{40}H_{52}\pm_2O$	Myxoxanthin $C_{40}H_{54}O$
Empirical formula	$C_{40}H_{54}O$	$C_{40}H_{52}\pm_2O$	$C_{40}H_{54}O$
Melting point (°)	176–180	178–179 and 192†	168–169
Absorption maxima (m μ .):			
CS ₂	(533.5)‡ 494	488; 494*	488
CHCl ₃	(504) 474	474–476*	473
Ethanol	—	—	470
Light petroleum (40–60°)	—	458–460*	465
Petroleum (70–80°)	(494) 460	458–460*	—
Benzene	(505) 472	472–474*	—
Methanol	(491.5) 457	—	—
Pyridine	(507.5) 477	478–480*	—
Colour reactions:			
Conc. H ₂ SO ₄ in CHCl ₃	Deep blue	Deep blue*	Deep blue
HNO ₃ in ether	No blue	No blue*	Green-blue
Partition test	Epiphasic with 90% methanol	Epiphasic	—
Vitamin A activity	+	+	+
Optical activity	Nil	—	—
Oxime			
Melting point (°)	208	—	195–196
Absorption maxima (m μ .):			
CS ₂	(530)‡ 492	484*	—
CHCl ₃	(504) 472	464*	463
C ₆ H ₆	(505) 472	466–468*	—
Petroleum (70–80°)	(494) 459	450–452*	—
Pyridine	(509) 471	466–468*	—
Methanol	(491) 457	—	—
Reduced pigment			
Melting point (°)	—	—	169–172
Absorption maxima (m μ .):			
CS ₂	—	—	529, 494, 464
CHCl ₃	Absorption spectrum of cryptoxanthin	—	508, 474, 441
Light petroleum	—	475, 450, 424*	495, 465, 431

† Lederer reported two melting points for echininone.

‡ Tischer found in his preparations of aphanin slight inflexions on the long-wave side of the main peak.

difference is recorded in a fourth (light petroleum); similarly, their oximes give identical spectra. The pigments also give identical colour tests and close but not identical melting points; thus, on these grounds, one has some justification for assuming that these pigments are identical. The main objections to this assumption are that myxoxanthin contains twelve double bonds and that myxoxanthol, prepared by reduction of myxoxanthin, has a very different spectrum from the reduced product of echininone.

On the basis of their findings Heilbron & Lythgoe (1936) and Karrer & Rutschmann (1944) have suggested the following structure I for myxoxanthin.

This structure is open to objection on spectrographic grounds for two reasons: (a) the opening of a β -ionone ring increases the wavelength of the absorption maximum of a compound (cf. β -carotene and γ -carotene with maxima at 450 and 462 m μ , respectively) so that this effect, enhanced by the presence of a further conjugated double bond should produce a spectrum with $\lambda_{\max}$, much higher than that of echininone, a prediction which is not sustained; (b) the data for the reduced product and the oxime are irreconcilable with structure I. Oximation of I should lead to a drop in $\lambda_{\max}$; this is observed; equally, reduction of the keto group in I should lead to a drop, probably greater than that noted on oximation; this is not observed.

It might be suggested that in order to accommodate twelve double bonds in the molecule, myxoxanthin could be 4'-keto- γ -carotene (II) in which the twelfth double bond is out of conjugation. This will still not fit in with the spectroscopic evidence. As $\lambda_{\max}$ for γ -carotene is 475 m μ in chloroform, that for the conjugated keto derivative should be higher, whereas that for myxoxanthin is lower (473 m μ). Similar considerations rule out the possibility that echininone has structure II.

Obviously there are good reasons for regarding myxoxanthin, echininone and aphanin as identical, but although the nature of the problem indicates that it will be difficult to make the identity certain, it will be equally difficult to disprove.

The compound formed by reduction of the keto group of echininone was very similar to cryptoxanthin, but because of lack of material complete identity could not be established. If the pigment we obtained was in fact cryptoxanthin it would suggest that cryptoxanthin is possibly 4-hydroxy- β -carotene and not 3-hydroxy- β -carotene (Kuhn & Grundmann, 1933). The only point we wish to emphasize here is that the structures of cryptoxanthin and most other xanthophylls are now too often accepted without question. The positions of the hydroxyl groups in these carotenoids are only fixed by analogy with lutein, 3,3'-dihydroxy- α -carotene. In this compound, however, it is only the position of one hydroxyl group (that in the α -ionone ring) which is rigidly fixed; the position of the other is again only fixed by analogy (see Karrer & Jucker, 1948).

It is very interesting to record the appearance of a fat-soluble pigment, pigment Y, which accumulates only in the ovaries and which, although forming a chromoprotein very similar to the carotenoid proteins frequently observed in marine invertebrates, does not appear to belong to this group of compounds. The specific accumulation of this chromoprotein in the female organs suggests that it may well be of importance in the reproductive processes of the limpet.

SUMMARY

1. The four carotenoid pigments of the gonads of the limpets, *Patella vulgata* and *P. depressa* have been identified as β -carotene, echininone, cryptoxanthin and zeaxanthin; traces of α -carotene are also occasionally encountered.
2. There is no sexual differentiation of carotenoids in the gonads.
3. The pink colour of the male gonads is due entirely to carotenoids unattached to protein; although a fraction of the ovarian carotenoids is attached to protein, these pigments play little part in the colour of these organs.
4. The green or greenish brown colour of the ovaries is in great part due to an unidentified pigment, Y, attached to a protein.
5. Arguments are advanced that echininone is 4-keto- β -carotene and not 3-keto- β -carotene as previously suggested.
6. The properties of echininone, aphanin and myxoxanthin point to the identity of these three pigments and certainly do not permit differentiation.

We are grateful to Mr J. R. Bruce, Marine Biological Station, Isle of Man, who brought to our notice the coloured gonads of limpets, and who kindly provided most of our material; to Prof. J. H. Orton, F.R.S., for interesting discussions concerning limpets; and to Prof. R. A. Morton, F.R.S., for his unfailing help and encouragement.

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Carotenoid Distribution in the Gonads of the Limpets *Patella vulgata* and *Patella depressa*

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Goodwin & Taha (1950) have shown that the four carotenoids, β -carotene, echininone, cryptoxanthin and zeaxanthin, occur in the male and female gonads of both *Patella vulgata* and *P. depressa*. The present report is concerned with the quantitative aspects of the distributions of these carotenoids as they are affected by sex, species and environment.

EXPERIMENTAL

P. vulgata, from the same region of the shore and of approximately equal size, were collected from Port St Mary, Isle of Man. They were delivered alive to the author. Samples from other sources, namely Plymouth, Trevone (Cornwall), Cullercoats (Northumberland), and Aberdeen were also occasionally obtained. Samples of *P. depressa* were obtained only from Plymouth.

The gonadal carotenoids were extracted and separated chromatographically by the previously described method (Goodwin & Taha, 1950). The various fractions were dissolved in a known volume of light petroleum (b.p. 40–60°), and the amount of each pigment present was determined spectroscopically using a Beckman spectrophotometer (see Goodwin, 1949). When the pigments were not separated chromatographically the total pigment concentration was determined by measuring the extinction of the test solution at 450 m μ . and basing the subsequent calculation on the assumption that all the colour was due to β -carotene. This is an approximation leading only to a very small error (about 5% at most) which, in any case, is constant throughout the series.

RESULTS AND DISCUSSION

A considerable mass of data has been condensed and collected in Tables 1–4.

Relative amounts of carotenoids in male and female gonads. The results recorded in Table 1 clearly indicate that no significant differences are observed between the relative distribution of the four component carotenoids in male and female gonads; especially interesting is the fact that no differences occur in this direction between the olive-green and the brown types of ovaries. Comment must be made on the very high standard deviation for the values of echininone and cryptoxanthin, especially in ovaries; this is undoubtedly due to the technical difficulties in effecting a complete separation of these two pigments in ovary extracts owing to the presence of large amounts of steroids which interfere with the normal development of the chromatogram.

The amount of pigments in male and female gonads. In Table 2 are collected the data obtained for the gonads of limpets of approximately the same age (size) collected from the same shore region at Port St Mary over a period of a month. Calculations according to the 't' test indicate that the concentration in the brown ovaries is significantly higher ($P < 0.01$) than is the concentration in green ovaries, but that there is no difference in the amount of carotenoids present in the two coloured types of ovaries; brown ovaries are therefore significantly smaller than the green ovaries. It is also to be noted that the brown samples varied between themselves much more than did the green samples.

When male and female gonads are compared it is found that their concentrations are not significantly different, but that the amount per limpet is greater in the male than in the female; this reflects the fact