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Some Observations on Astaxanthin Distribution in Marine Crustacea

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During an investigation into the carotenoids of two locust species (Goodwin & Srisukh, 1949) it became necessary to obtain an authentic specimen of astaxanthin for comparative purposes; such a sample was obtained from the lobster (*Homarus vulgaris* H. M. Edwards). As the isolation of this material proceeded it became apparent that the experience of Kuhn & Lederer (1933) on the distribution of free and esterified astaxanthin was not being reproduced; consequently the distribution was reinvestigated in some detail. A similar investigation was carried out on the prawn (*Nephrops norvegicus* L.). At the time the lobsters and prawns were being examined an opportunity arose to examine the pigment of the small red copepod, *Tigriopus fulvus* Fisch. (Fraser, 1936).

EXPERIMENTAL

The first sample of astaxanthin used was obtained from old dry carapaces of lobster with which was mixed a small amount of carapaces of crab (*Cancer pagarus*). The carapaces were softened by placing in approx. 0·2N-HCl for 24 hr. to dissolve the $CaCO_3$, filtered off, washed with water until free from acid, ground up in a mortar with acid-washed silver sand and anhydrous Na_2SO_4 and extracted 3 times with acetone at a temperature below 30°. The combined acetone extracts were concentrated at the pump, diluted with 2 vol. of water and extracted with light petroleum. When a small sample of the light petroleum extract was shaken with 90% (v/v) aqueous methanol the pigment was almost completely hypophasic; this indicated that the pigment was unesterified, for esterified astaxanthin is epiphasic in this test (Kuhn, Lederer & Deutsch, 1933). A small yellow residue in the epiphase showed no selective absorption in the region 400–450 mμ.

The light petroleum extract was then chromatographed using $CaCO_3$ (A.R.) as adsorbent. Development with light petroleum containing 4% (v/v) of ethanol resulted in the separation of three bands: (1) orange red—most strongly adsorbed, (2) reddish pink, and (3) yellow. The yellow fraction, which was only small, was discarded; it was the same pigment as that found in the epiphase in the partition test. Absorption spectrum measurements in four solvents indicated that the orange-red pigment was astacin and the reddish pink pigment astaxanthin. Although the astaxanthin thus obtained was pure and was identical with the locust pigment, it was considered advisable to compare locust astaxanthin with astaxanthin prepared from fresh material. It was also important to examine fresh material because all our tests indicated that in the carapaces astaxanthin was present in the free form, although Kuhn & Lederer (1933) had claimed that the carapace pigment was esterified. In this experiment the presence of astacin in considerable amounts indicated that oxidative changes must have taken place in the stored carapaces and there was always the possibility that hydrolysis of the original ester (if ester were present) had also occurred.

Three fresh lobsters were killed by immersion in fresh water and the hypodermis removed carefully and as completely as possible from the carapace; the eggs of the two females and the hepatopancreases were also removed for examination.

The hypodermis on extraction with acetone in the manner just described yielded a pigment which was chromatographically homogeneous and epiphasic in a methanol-light petroleum partition. It was the astaxanthin ester previously described by Kuhn and his co-workers; its spectral characteristics were recorded using a number of solvents.

The carapace was divided into two portions. One portion was extracted with acetone after it had first been softened by standing overnight in 2N-HCl. The pigment extracted was not homogeneous; it consisted of two components, one epiphasic and the other, the major component, hypophasic. Neither component could be further fractionated by chromatography on weakened alumina (Goodwin & Srisukh, 1949). This experiment strongly indicated that the hypophasic fraction was obtained from the carapace itself and that the presence of a small amount of epiphasic pigment was due to contamination with small amounts of hypodermis. In order to confirm this, the second portion of carapace was allowed to stand for 48 hr. in 0·02N-HCl. This treatment softened the carapace and extracted a small proportion of the astaxanthin-protein complex but not the free pigment. The resulting clear blue solution was filtered off and some

colourless interfering protein removed by addition of $(NH_4)_2SO_4$ to half saturation. The blue astaxanthin-protein complex was then broken down by the addition of ethanol (equal vol.); the solution immediately turned orange and the liberated pigment which remained dispersed in the solution was extracted with light petroleum. On shaking this petroleum solution with 90% (v/v) aqueous methanol the pigment was completely extracted by the methanol. This experiment clearly confirms that the carapace astaxanthin is unesterified.

Lobster eggs were ground up and allowed to stand for some days in contact with water. A green chromoprotein was extracted which exhibited an absorption spectrum with maxima at 476 and 660 mμ.; the spectrum was very similar in shape to that reported by Stern & Salomon (1938) who, however, recorded slightly different maxima, viz. 470 and 640 mμ. Denaturation of the chromoprotein by addition of acetone yielded unesterified astaxanthin, thus confirming the observations of Kuhn & Lederer (1933).

The hepatopancreases were extracted with acetone in the usual way and yielded only very small amounts of a pigment which was, as far as could be ascertained, β-carotene.

Three fresh prawns (*Nephrops norvegicus* L.) were examined in the same way as lobsters. The results obtained were the same, i.e. the hypodermis contained esterified astaxanthin, whilst the carapace contained free astaxanthin attached to a protein.

The carotenoids from *Tigriopus fulvus* were extracted by acetone after grinding with sharp sand. The acetone was removed *in vacuo* and the residue dissolved in light petroleum (b.p. 40–60°), partitioned, and the two fractions purified by chromatography on weakened alumina. Using the methods described previously in this paper, these two pigments were identified as esterified and free astaxanthin; the latter is the major portion of the mixture.

Quantitative measurements were made in order to determine the amount of astaxanthin contained in male and female *T. fulvus*. Using samples of 100 animals, values obtained for males were 5·74 and 4·66 μg./animal and for females 6·0 and 5·77 μg. Gravid females are conspicuous by their egg sacs; consequently two lots of 100 gravid females were collected, separated from their eggs and the two portions examined separately. It was found that whilst females from which sacs had been removed contained 2·93 and 2·00 μg./animal, the sacs themselves contained astaxanthin to the extent of 2·89 and 1·58 μg./sac. From these limited experiments it appears that male and female *T. fulvus* contain about the same amount of pigment, but that gravid females lose about half their store to their eggs.

RESULTS AND DISCUSSION

Whilst Kuhn & Lederer's (1933) claim that the hypodermis of the lobster and prawn and ova of the lobster contain esterified and free astaxanthin, respectively, was confirmed, it was never possible to detect more than traces of astaxanthin ester in the carapaces of either species; the major pigment was always free astaxanthin. It can only be suggested that previous workers had investigated carapaces to which some hypodermis was still adhering. As the pigment concentration in the hypodermis is very much greater than in the carapace, the pigment from small amounts of the former might well have masked that obtained from large amounts of the latter. It was found in the present investigation that complete removal of the hypodermis from the carapace was virtually impossible, and it is considered that the presence of small amounts of esterified pigment in our carapace extracts was due to traces of hypodermis. In order to eliminate the interference produced by the pigments of adhering hypodermis, air-dried carapaces were ground with water containing traces of hydrochloric acid (about 0·02 N) and allowed to stand. The astaxanthin-protein complex was thus extracted without denaturation and the unconjugated hypodermal pigment was not. The resulting pale blue solution was denatured by the addition of ethanol or acetone; the colour changed to orange and the astaxanthin could be extracted with light petroleum. This pigment was completely hypophasic in the phase test, and no esterified astaxanthin could be detected.

Further, the absorption spectrum of the greenish astaxanthin-protein complex (ovoverdin) which occurs in lobster eggs and which is soluble in water has been examined. The spectrum obtained agreed qualitatively with that previously recorded by Stern & Salomon (1938) in exhibiting a broad shallow band in the red region and a much sharper band in the blue-green region of the spectrum. It is in the position of these bands that the present observations differ slightly from those of Stern & Salomon; they record $\lambda\lambda_{max.}$ at 640 and 470 mμ., whilst we record them at 660 and 476 mμ.

Light absorption data on astaxanthin are much less numerous than are those for astacin; Wald (1943) and Karrer & Würgler (1943) record the spectrum in hexane, and Kuhn & Sörensen (1938) in pyridine. However, it is now apparent that the spectrum recorded by Kuhn & Sörensen is incorrect. In order to extend the data, the absorption maximum of astaxanthin in carbon disulphide, pyridine, glacial acetic acid and light petroleum (b.p. 40–60°) are recorded in Table 1. Similarly, only few data have been produced on the spectra of astaxanthin esters (Wald, 1943; Kuhn & Sörensen, 1938) and these indicate that they are very close to that of the parent

Table 1. *Absorption maxima of free and esterified astaxanthin in various solvents*

Solvent	Absorption maximum (mμ.) Free astaxanthin	Esterified astaxanthin
Hexane	470	467–468
Light petroleum (b.p. 40–60°)	470	—
Pyridine	490–491	488
Carbon disulphide	505–506	503
Glacial acetic acid	483–485	481–482
Acetone	475	—

compound. It has now been found that compared with free astaxanthin the esterified pigment occurring in lobster hypodermis shows an absorption band of exactly similar shape, but with its wavelength maximum shifted 1–2 mμ. to shorter wavelengths (Table 1). All the data were obtained using the Beckman photoelectric spectrophotometer.

SUMMARY

1. It has been confirmed that esterified astaxanthin occurs in the hypodermis of the lobster, *Homarus vulgaris* Edw. and the prawn, *Nephrops norvegicus* L., and that the unesterified pigment occurs in the eggs of the lobster.

2. It has not been possible to confirm the presence of esterified astaxanthin in the carapaces of these species; from the evidence presented it is considered that the pigment is in fact free astaxanthin.

3. The lobster hepatopancreas contains only traces of β-carotene.

4. Free and esterified astaxanthin, the former predominating, have been identified in the sea flea, *Tigriopus fulvus* Fisch. In gravid females about 50 % of the pigment is in their eggs.

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The Intermediary Metabolism of the Mammary Gland

2. RESPIRATION AND ACID PRODUCTION OF MAMMARY TISSUE DURING PREGNANCY, LACTATION AND INVOLUTION IN THE RAT

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In a previous paper (Folley & French, 1949*c*) we have shown that slices of lactating mammary tissue respire fairly actively in a medium containing glucose. In the rat the activity is less than that of nervous tissues and kidney, but is of the same order as that of liver; unlike that of liver, however, the respiration is markedly increased over endogenous values in the presence of glucose. The respiratory quotient (R.Q.) of lactating mammary tissue (in glucose) is well above unity in the mouse, rat, rabbit, and, to a lesser degree, the guinea pig, but below unity in ruminants (goat, cow).

Our results on the rat indicated that Q_{O_2}, and perhaps R.Q., is lower in early than in full lactation. This preliminary finding was in line with that of Kleiber, Smith & Levy (1943), who reported a higher respiration for lactating tissue than for tissue taken from pregnant rats, provided the results were calculated to a dry tissue basis. On a moist tissue basis, however, there was no difference in metabolic rate at the end of pregnancy and at the twenty-first day of lactation, because the dry-matter content of the gland was much higher in pregnancy than in lactation.

The relation between the functional activity of a tissue and its respiratory metabolism is of considerable interest, and the mammary gland readily lends itself to a study of this question. We have previously shown (Folley & French, 1949*c*) that, in the rat, experimental depression of lactation due to restriction of the food intake or to adrenalectomy, lowers Q_{O_2}, decreases the R.Q. to values near unity, and increases the aerobic glycolysis. Another approach to this question is to study the metabolism of mammary tissue at various stages of the lactational cycle, using the term in its widest sense to include late pregnancy and post-lactational involution. The present paper reports results of such a study.

METHODS

Animals. Hooded Norway rats undergoing their first lactations were used. The stock diet, fed *ad lib.*, was as described previously (Cowie & Folley, 1948) save that 10 of the parts of whole wheat were replaced by wheat germ. All