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VITAMIN A AND CAROTENOIDS IN CERTAIN INVERTEBRATES

IV. MOLLUSCA: LORICATA, LAMELLIBRANCHIATA, AND GASTROPODA

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Introduction

Apart from a general account (Kon, 1954) of our work on vitamin A and carotenoids in invertebrates, our publications so far (Kon & Thompson, 1949*a*, *b*; Batham, Fisher, Henry, Kon & Thompson, 1951; Fisher, Kon & Thompson, 1951, 1952, 1953, 1954, 1955) have been concerned only with marine Crustacea. We have also studied, during our investigation of the metabolism of vitamin A and its possible precursors in the sea, numerous species from most other phyla of marine invertebrates. Except for the nematode worm, *Anisakis physeteris* Baylis, taken from the stomach of a sperm whale, the only two phyla of invertebrate animals in which we have so far found vitamin A are the Arthropoda and the Mollusca. The vitamin was present in at least some species from each of the molluscan classes, Loricata, Gastropoda, Lamellibranchiata and Cephalopoda, but we have no information yet about the Solenogastres or the Scaphopoda of which we have analysed no representatives. So far as our studies were concerned, the cephalopods differed considerably from the other molluscs examined, and the relatively large amount of information they have provided will be more conveniently presented in a subsequent paper. The account which follows, therefore, deals only with species from the first three classes just listed.

To the best of our knowledge, Jameson, Drummond & Coward (1922) were the first to report vitamin A activity in molluscs, but they did not name the species they had tested. Since that date all the published work on vitamin A known to us has been concerned with the food value of clams (Jones, Nelson, Murphy & Devine, 1928) and oysters, which were analysed in the United States by Jones & Murphy (1926), Jones, Murphy & Nelson (1928) and Whipple (1935), in New Zealand by Malcolm (1926, 1927, 1928) and in Malaya by Leong (1939). All these reports gave values for vitamin A activity rather than for vitamin A itself, since they were based on biological tests of whole specimens without previous separation of the vitamin from its precursors. The presence of vitamin A in Loricata and in Gastropoda has not previously been reported.

The history of the investigation of molluscan carotenoids began with the work of Merejkowsky (1881, 1883) and MacMunn (1883*a*, *b*) who extracted a red fat-soluble pigment they called tetronerythrin from many marine invertebrates, including several species of molluscs. Tetronerythrin had the properties of carotenoids, and probably represented the sum total of these pigments in extracts. No further work on the carotenoids of molluscs was reported until the appearance of Lönnberg's papers (Lönnberg, 1931, 1934*a*, *b*; Lönnberg & Hellström, 1932), in which were given the absorption spectra of pigments extracted from many species of marine invertebrates including several molluscs. More specialized studies of carotenoid metabolism in molluscs have been made by Scheer (1940) on mussels, Brooks & Paulais (1939) on oysters, and Goodwin (1950) and Goodwin & Taha (1950, 1951) on limpets. Goodwin (1953) has also investigated the carotenoids of the freshwater mussel, *Anodonta cygnea*.

Our own work has been concerned primarily with the detection and measurement of vitamin A in molluscs, and carotenoids have only been identified and measured when they were separated in our normal technique of analysis.

Material Collected

Eighteen species of Gastropoda, nine species of Lamellibranchiata and one species of Loricata were collected, many of them by ourselves when visiting the shores of different parts of Great Britain, and during a visit by one of us (S. K. K.) to the Scripps Institution of Oceanography, at La Jolla, California, in May 1953. The staff of various marine biological and fisheries laboratories have kindly collected much material for us or allowed us facilities to do our own collecting.

From La Jolla we had the following species: *Cypraea spadicea* Swainson, *Haliotis fulgens* Philippi, *Megathura crenulata* (Sowerby), *Astrea undosa* (Wood) and *Stenoplax conspicua* (Carpenter). We obtained *Lima hians* (Gmelin) and *Pecten maximus* (Linnaeus) at Millport, *Mya arenaria* Linnaeus and *Cardium edule* Linnaeus from Fairlie Sands, and *Aporrhais pes-pelecani* (Linnaeus) and *Chlamys septemradiatus* Müller from Loch Fyne. More species were collected on various parts of the Essex coast; these included *Scrobicularia plana* (da Costa) taken from mud flats at Leigh-on-Sea, *Ostrea edulis* Linnaeus, *Gryphea angulata* Lamarck and *Crepidula fornicata* (Linnaeus) from the rivers Crouch and Roach near Burnham, and *Littorina littoralis* (Linnaeus), *L. littorea* (Linnaeus), *L. rudis* (Maton), *Patella vulgata* Linnaeus and *Mytilus edulis* Linnaeus from the breakwater at Blackman's Point, Harwich. Groups of *Buccinum undatum* Linnaeus were collected at both Millport and Burnham-on-Crouch. *Osilinus lineatus* (da Costa) was collected for us at Plymouth. *Aplysia depilans* Linnaeus and *Murex trunculus* Linnaeus were obtained from collections of marine animals received by the London Zoological Society's aquarium from Madeira. The specimens of

Clione limacina Phipps were taken during a cruise of F.R.S. *Scotia* to Iceland in August 1951, and those of *Limacina retroversa* (Fleming) from R.V. *Ernest Holt* in the Barents Sea in January 1955. *Helix aspersa* Linnaeus was collected from a garden in Shinfield and *Planorbis corneus* (Linnaeus) var. *rubra* Oldham was purchased from an aquarist in Reading. These pulmonates, although not marine, were included for comparative purposes. The red variety of *P. corneus* seemed to be especially interesting in view of its apparent carotenoid colouring.

Methods of Preservation

Apart from the two planktonic species, *Clione limacina* and *Limacina retroversa*, which were preserved in the same way as Crustacea, by immersion in boiling sea-water for 2 min. (Fisher *et al.*, 1952), all the material was preserved either whole, or various organs separately, in absolute alcohol. On receipt at the laboratory the boiled specimens were weighed, preserved in alcohol and stored in the deep-freeze. Where possible, specimens or organs preserved in alcohol were weighed first. If not, a known volume of absolute alcohol was added so that the weight of the animals could be determined by difference, that of the alcohol being calculated from its specific gravity. Where specimens were sent to us in an unknown volume of alcohol the net weight was roughly determined by draining off the alcohol and weighing the specimens. Weights given in the results and tables were determined after removal of the shells, except for *Stenoplax conspicua* and *Limacina retroversa*.

Analytical Methods

Basically the analytical technique was that outlined by Fisher *et al.* (1952). Some difficulty was experienced in the Carr-Price reaction owing to interference by xanthophylls which were eluted with vitamin A alcohol in the chromatography following saponification. Recently we have found that some of this pigment may be removed at an early stage by passing the extract in *n*-hexane solution through an alumina column weakened with 8% ethanol in *n*-hexane before the chromatography on a column not so treated as used in method (*b*) of Fisher *et al.* (1952). A large proportion of the other carotenoid pigments is eluted with the vitamin A ester and β-carotene, leaving vitamin A alcohol and a reduced amount of pigments on the column. These are eluted with 8% ethanol in *n*-hexane. The first fraction is then re-chromatographed on an untreated alumina column. Vitamin A ester and β-carotene are eluted by 2% acetone in *n*-hexane, leaving the remaining pigments adsorbed on to the column.

So far in our work vitamin A has been measured by the blue colour produced in the antimony-trichloride reaction and the values determined as described by Thompson (1949). Since pure crystalline vitamin A is now readily available the photoelectric spectrophotometer has been recalibrated with solutions in chloroform of the alcohol, acetate and palmitate forms of vitamin A.

We found that these pure preparations gave 20% less blue colour per unit of vitamin A than the oils originally used as standards. The probable presence in these original oils of about 25% of neo-vitamin A (Dalvi & Morton, 1951) may account in part, at least, for the difference. Pure vitamin A alcohol now gives, under our conditions, a value for $E\,{}^{1\%}_{1\,cm.}$ (620 mμ) of 5000, in sensible agreement with that of 5070 given by Cama, Collins & Morton (1951).

Since the method of measuring vitamin A is purely chemical it seems logical to express results in terms of micrograms rather than international units. This will be done in the present and future papers of this series except where a measurement is the result of a biological assay, when we shall continue to use international units.

To compensate for the recalibration of the spectrophotometer and to convert international units to micrograms the factor 0·39 should be used to multiply all values resulting from chemical measurements of vitamin A given in our previous papers (Thompson, Ganguly & Kon, 1949; Kon & Thompson, 1949*a*, *b*; Batham *et al.*, 1951; Kon, 1954; Fisher *et al.* 1951, 1952, 1953, 1954, 1955).

Our earlier work (Fisher *et al.*, 1952) on euphausiids showed a discrepancy between the vitamin A potencies measured by physico-chemical and by biological methods. We now know that this discrepancy is due to the presence of a vitamin A isomer (Kon, 1954). No biological tests have been done on vitamin A from the molluscs analysed, so that no information is available regarding the nature of the vitamin in them.

Results

Class Loricata

Stenoplax conspicua (Carpenter)

Ten specimens were collected on the shore at La Jolla on 20 May 1953. The shells were not removed before analysis and the results were as follows:

Average weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene
		μg/spec.	μg/g	μg/spec.	μg/g	μg/g
17	1·2	0·73	0·044	85	5·1	0·41

The vitamin A was all in the ester form. Their chromatographic behaviour indicated that the carotenoid pigments included both carotenes and xanthophylls and possibly astaxanthin or its esters. Chlorophyll was also present.

Class Lamellibranchiata

Order Filibranchiata

Mytilus edulis Linnaeus

Three groups of mussels were collected near the breakwater at Blackman's Point, Harwich. The analytical results are shown in Table 1. In the whole

animals only vitamin A ester was measured, since the presence of much pigment in the fraction that would have contained vitamin A alcohol prevented a satisfactory reading in the Carr-Price reaction in the first two groups of specimens.

The pigmented edge of the mantle is believed to be light-sensitive in some molluscs and, because of our finding that vitamin A is concentrated in the eyes of Crustacea (Fisher *et al.*, 1951), this mantle edge was separated from the rest of the mussel before analysis of the second group. It contained no vitamin A, and the concentration of fat and total carotenoids differed little from those of the rest of the mussel, which, however, contained vitamin A ester.

TABLE I. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *MYTILUS EDULIS*

(All specimens collected at Harwich.)

Date	Tissue	No.	Average weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene
					μg/spec.	μg/g	μg/spec.	μg/g	(μg/g)
11. ix. 50	Whole animal	29	1·3	1·6	0·66	0·51	49	37	1·3
29. i. 51	Mantle edge	40	0·1	1·1	0	0	4	34	0
	Visceral mass	40	1·4	1·5	0·25	0·18	43	32	0·77
	Total	40	1·5	1·5	0·25	0·16	47	32	0·70
23. iv. 51	Digestive gland	36	0·45	1·2	0·58	1·29	91	203	14
	Mantle, with eggs	36	0·55	1·1	0·04	0·08	16	29	0
	Foot	36	0·04	3·2	0	0	2·6	74	0
	Ctenidia, muscle and rest of visceral mass	36	0·77	0·9	0·04	0·05	27	34	1·1
	Total	36	1·81	1·1	0·66	0·37	137	76	4·2

The mussels of the third group were dissected into several parts before analysis and, as Table I shows, vitamin A was concentrated mainly in the digestive gland where it was in the alcohol form. The small amounts of vitamin A in other parts of the animal were present entirely as the ester. Apart from β-carotene, the carotenoids were xanthophylls, with no evidence of astaxanthin. There was a trace of chlorophyll in the digestive gland.

Pecten maximus (Linnaeus)

The analytical results for various parts of a group of seven specimens are shown in Table II. The carotenoids were mainly xanthophylls and no astaxanthin was detected.

Chlamys septemradiatus Müller

Table II shows results for a group of whole animals of this species. The only carotenoids present were xanthophylls, and considerable amounts of chlorophyll were also observed.

Lima hians (Gmelin)

Two groups were analysed, the first as whole animals and the second after dissection. The results are shown in Table III. Vitamin A was present in the first group, all in the ester form, but not in the second. The carotenoids were all xanthophyllic, apart from traces of a carotene in the digestive gland.

TABLE II. DISTRIBUTION OF OIL AND CAROTENOIDS IN TWO SPECIES OF PECTINIDAE

(Both species collected in Clyde Sea area.)

Date	Species and tissue	No. of specimens	Average weight (g)	Oil (%)	Total carotenoids		β-carotene (μg/g)
					μg/spec.	μg/g	
13. ii. 51	*Pecten maximus*	7	—	—	—	—	—
	Muscle		56	0·3	28	0·5	Trace
	Mantle edge		19	0·3	9·2	0·5	Trace
	Visceral mass		9·5	4·2	364	39	0·58
	Gonad and foot		13	1·9	186	14	Trace
	Ctenidia and mantle		7·4	0·5	62	8·4	0
	Total		105	0·9	649	6·2	0·05
13. iii. 52	*Chlamys septem-radiatus*	31	1·5	0·6	3·6	2·4	0

Vitamin A absent from both species.

TABLE III. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *LIMA HIANS*

(All specimens collected near Millport.)

Date	Tissue	No.	Average weight (g)	Oil (%)	Vitamin A		Carotenoids	
					μg/spec.	μg/g	μg/spec.	μg/g
21. x. 53	Whole animal	12	7·5	1·7	0·20	0·022	188	25
7. xii. 53	Foot	27	0·1	0·5	0	0	2·8	29
	Digestive gland	27	0·7	6·1	0	0	31	44
	Visceral mass	27	1·7	1·1	0	0	5·8	3·4
	Mantle	27	3·6	0·6	0	0	64	18
	Total	27	6·1	1·4	0	0	104	17

β-carotene absent from all specimens.

Ostrea edulis Linnaeus

The analytical results for three groups of edible oysters are given in Table IV. Only vitamin A ester was measured in the whole animals, owing to interference of carotenoids with the measurement of any vitamin A alcohol that might have been present. In the dissected groups satisfactory readings were obtained for all organs. The visceral masses contained vitamin A ester but no alcohol, and the carotenoids were mainly xanthophylls with small amounts of β-carotene. The carotenoids of the remainder of the body were all xanthophylls. An unidentified olive-green pigment was strongly adsorbed at the top of the alumina column in the extracts of whole oysters and visceral masses.

TABLE IV. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *OSTREA EDULIS*

(All specimens collected near Burnham-on-Crouch.)

Date	Tissue	No.	Average weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene (μg/g)
					μg/spec.	μg/g	μg/spec.	μg/g	
10. x. 50	Whole animal	4	9·2	2·3	2·4	0·26	181	20	1·7
11. xii. 50	Visceral mass	16	2·6	1·2	0·35	0·14	12	46	0·05
	Mantle edge	16	0·8	0·5	0	0	1·0	1·2	0
	Muscle	16	1·8	0·5	0	0	1·2	0·7	0
	Ctenidia	16	1·3	0·2	0	0	0·8	0·6	0
	Total	16	6·5	0·7	0·35	0·05	15	2·3	0·02
25. iii. 52	Digestive gland	16	0·5	1·7	0	0	4·8	9·8	0
	Mantle & ctenidia	16	2·5	0·7	0	0	2·5	1·2	0
	Visceral mass	16	2·2	1·8	0·43	0·20	27	12	0·02
	Muscle	16	1·1	0·5	0	0	0	0	0
	Total	16	6·3	1·1	0·43	0·07	34	5·4	0·007

TABLE V. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *GRYPHAEA ANGULATA*

(All specimens collected near Burnham-on-Crouch.)

Date	Tissue	No.	Average weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene (μg/g)
					μg/spec.	μg/g	μg/spec.	μg/g	
10. x. 50	Whole animal	2	12	2·4	1·9	0·16	180	15	1·1
11. xii. 50	Visceral mass	7	15	2·3	5·4	0·35	270	18	0·36
	Mantle edge	7	2·0	0·9	0	0	1·8	0·9	0
	Muscle	7	5·1	0·5	0	0	2·0	0·4	0
	Ctenidia & mantle	7	7·2	1·1	0	0	6·2	0·9	0
	Total	7	29	1·6	5·4	0·18	280	9·4	0·19
27. ii. 51	Labial palps	30	0·7	3·6	0·20	0·30	1·3	1·9	Trace
	Digestive gland	30	1·7	2·2	1·4	0·83	229	134	3·1
	Visceral mass	30	4·5	2·0	0·64	0·14	16	3·5	0·07
	Muscle, mantle & ctenidia	30	9·6	0·7	0·29	0·03	4·3	0·5	0
	Total	30	17	1·4	2·6	0·15	251	15	0·34
25. iii. 52	Mantle & ctenidia	15	2·5	1·0	0·14	0·06	4·4	1·8	0
	Visceral mass	15	3·2	2·0	2·1	0·66	68	21	0·11
	Muscle	15	1·0	0·4	0	0	0	0	0
	Digestive gland	15	0·78	2·6	0·82	1·1	39	51	0·49
	Total	15	7·5	1·5	3·1	0·41	111	15	0·06

Gryphaea angulata Lamarck

Table V shows the analytical results for four groups of Portuguese oysters. In the analysis of the whole specimens the chromatographic behaviour of the pigments was very similar to that in *Ostrea* and the concentrations of fat, vitamin A, total carotenoids and β-carotene were of the same order in both kinds of oyster. The green pigment found in *Ostrea* was also present in *Gryphaea*.

The results for the second group were very similar to those obtained for the group of *Ostrea edulis* collected at the same time and dissected in the same way, except that the vitamin A of the visceral mass comprised about 6 parts alcohol to 1 part ester, whereas in *Ostrea* it was all in the ester form. Analysis of various organs in the third group showed that this species differed from *Ostrea* in having most of the vitamin A in the digestive gland, where the ester-alcohol ratio was 1:5. In the rest of the visceral mass the ratio was 2:1, and in other parts of the body containing it the vitamin was entirely in the ester form. The relative amounts of the two forms of vitamin A in the various parts of the last group were similar to those in the first three, except that in the visceral mass there was over twice as much vitamin A alcohol as ester. Xanthophylls and carotenes were present in all the organs from all the groups, but no astaxanthin.

TABLE VI. DISTRIBUTION OF OIL AND CAROTENOIDS IN *SCROBICULARIA PLANA*

(All specimens collected at Leigh-on-Sea.)

Date	Tissue	No.	Average weight (g)	Oil (%)	Total carotenoids		β-carotene (μg/g)
					μg/spec.	μg/g	
16. x. 50	Whole animals	46	1·55	0·9	38	22	1·0
29. i. 51	Visceral mass	32	1·22	1·3	14	12	0·42
	Siphon	32	0·37	1·4	3·5	9·6	0
	Mantle edge	32	0·23	1·1	0·4	1·6	0
	Total	32	1·82	1·3	18	9·9	0·28

Vitamin A absent.

Order Eulamellibranchiata

Scrobicularia plana (da Costa)

As shown in Table VI two groups of this species were analysed. Carotenoids were mainly xanthophyllic with some carotenes in the visceral mass. The green pigment noticed in the oysters also appeared in this species.

Cardium edule Linnaeus

Of five groups of cockles analysed, only the first, as Table VII shows, contained any vitamin A. As in the previous species, the carotenoids were mostly xanthophylls. The gonads, separated in the second group, contained a large proportion of the total carotenoids.

Mya arenaria Linnaeus

Four groups of clams were analysed and the results are given in Table VIII. In the first group, analysed whole, only vitamin A ester was measured, because of interference of carotenoids in the determination of the alcohol fraction, but in the subsequent groups vitamin A alcohol was absent from all the organs.

TABLE VII. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *CARDIUM EDULE*

(All specimens collected from Fairlie Sands.)

Date	Tissue	No.	Average weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene (μg/g)
					μg/spec.	μg/g	μg/spec.	μg/g	
16. v. 51	Whole animal	20	4·0	0·8	0·66	0·16	47	12	0·34
4. ix. 51	Foot	22	0·25	0·8	0	0	1·8	7·5	0
	Gonad	22	1·86	1·2	0	0	15	7·9	0
	Mantle, muscles and ctenidia	22	1·71	0·2	0	0	3·0	1·8	0
	Visceral mass	22	0·36	0·5	0	0	4·7	13	0·38
	Total	22	4·2	0·7	0	0	24	5·8	0·03
16. x. 51	Mantle, muscles and ctenidia	30	2·12	0·8	0	0	6·9	3·3	0
	Digestive gland	30	0·40	1·1	0	0	9·4	23	0·70
	Visceral mass and foot	30	2·49	0·5	0	0	8·4	3·4	0
	Total	30	5·0	0·7	0	0	25	4·9	0·06
24. iv. 52	Crystalline style	25	0·02	0·5	0	0	0	0	0
	Digestive gland	25	0·48	0·9	0	0	5·4	11	0·22
	Visceral mass and foot	25	2·22	0·4	0	0	4·6	2·1	0
	Mantle, muscles, ctenidia, siphons	25	1·36	0·8	0	0	4·1	3·0	0
	Total	25	4·1	0·6	0	0	14	3·5	0·03
24. iv. 52	Whole animal	25	4·8	0·4	0	0	14	2·9	0·04

TABLE VIII. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *MYA ARENARIA*

(All specimens collected from Fairlie Sands.)

Date	Tissue	No.	Average weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene (μg/g)
					μg/spec.	μg/g	μg/spec.	μg/g	
16. v. 51	Whole animal	6	34	0·7	0·80	0·02	527	16	0·39
4. ix. 51	Visceral mass	12	5·9	1·5	0·81	0·14	36	6·0	0·12
	Siphons	12	14	0·4	0·18	0·01	27	1·9	0·03
	Mantle, muscle and ctenidia	12	14	0·6	0·35	0·03	88	6·4	0·25
	Digestive gland	12	1·7	1·7	0·15	0·09	130	75	9·5
	Foot	12	0·4	0·8	0	0	2·3	5·8	0
	Total	12	36	0·5	1·5	0·04	283	7·8	0·58
16. x. 51	Visceral mass and foot	9	2·5	1·4	0·74	0·11	86	13	0·18
	Siphons	9	14	0·2	0·16	0·01	25	1·8	Trace
	Mantle, muscle and ctenidia	9	11	0·4	0·19	0·02	26	2·3	0
	Digestive gland	9	6·8	2·0	0	0	161	64	2·5
	Total	9	34	0·7	1·1	0·03	298	8·8	0·22
12. ii. 52	Crystalline style	18	0·18	0·01	0	0	0	0	0
	Visceral mass	18	5·9	1·2	0·34	0·06	13	2·2	Trace
	Mantle, muscle, ctenidia, siphons	18	18	0·4	0·13	0·007	22	1·2	Trace
	Digestive gland	18	1·3	1·2	0	0	1·8	1·4	0
	Foot	18	0·33	1·0	0	0	1·1	3·2	0
	Total	18	26	0·6	0·47	0·02	38	1·5	Trace

Vitamin A was present in the digestive gland of the second group, but absent from it in the last two groups. The carotenoids were much as in other lamellibranchs, with xanthophylls predominating. Variations in concentration between different organs are shown in the table.

Class Gastropoda

Subclass Prosobranchiata

Order Diotocardia

Haliotis fulgens Philippi

Two specimens were analysed singly, with the results given in Table IX. No vitamin A ester was detected. The presence of much xanthophyllic pigment in the alcohol fractions interfered with the measurement of vitamin A alcohol. The fractions were therefore passed through a column of calcium carbonate, using light petroleum (b.p. 40–60° C) as a solvent and eluent in

TABLE IX. OIL, VITAMIN A AND CAROTENOIDS IN SOME PROSOBRANCHS

(First three species collected at La Jolla on 20. v. 53 and *Osilinus lineatus* at Plymouth on 9. vi. 52.)

Species	No.	Average weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene (μg/g)
				μg/spec.	μg/g	μg/spec.	μg/g	
Haliotis fulgens	1	268	0·6	11	0·04	1066	4·0	0·84
H. fulgens	1	122	0·7	4·6	0·04	1034	8·5	1·7
Megathura crenulata	1	179	0·4	9·2	0·05	1863	10	3·0
Astrea undosa	1	8·7	1·0	0	0	26	3·0	Trace
Osilinus lineatus	50	1·1	1·1	0·12	0·10	58	52	6·1

an attempt to remove the pigment. Some of it, however, was still eluted and the fractions were dissolved in *n*-hexane and their absorption spectra determined in the Beckman spectrophotometer. Both fractions showed maxima at about 327 mμ, indicating the presence of vitamin A alcohol. The values for vitamin A were calculated after appropriate corrections for irrelevant absorption by the procedure of Cama *et al.* (1951). The pigments were carotenes and xanthophylls, astaxanthin being absent. A noticeable feature was the high proportion (*ca.* 20%) of β-carotene in the total carotenoids.

Megathura crenulata (Sowerby)

One specimen was analysed with the results shown in Table IX. Vitamin A was all in the alcohol form which was measured spectrophotometrically, because of the interference by carotenoids with the antimony-trichloride reaction. The pigments were similar to those of *Haliotis*, but the percentage (30) of β-carotene was even higher in this species.

Astrea undosa (Wood)

The analytical results for a single specimen are given in Table IX. The pigments were mainly xanthophyllic.

Osilinus lineatus (da Costa)

A group of fifty whole specimens was analysed with the results shown in Table IX. Vitamin A was in the ester form and the carotenoids were xanthophylls and carotenes.

Patella vulgata Linnaeus

The analytical results for all the limpets examined are shown in Table X. Analysis of the first group established the presence of vitamin A, although only the ester was measured. Nearly a third of the total carotenoids in this group was β-carotene.

The second group was analysed with mantle edge and tentacles, as possible photoreceptors, separated from the rest of the animals, but all the vitamin was in the main part of the body.

The tentacles and mantle edge had lower concentrations of carotenoids than the rest of the animal and β-carotene was absent from the tentacles.

The distribution of vitamin A and carotenoids in the limpet was further investigated in the third group. These animals were dissected as shown in Table X. The highest concentration and largest amount of vitamin A were in the digestive gland and excretory organ and the ratio of ester to alcohol was 1:9. In the intestine the ratio was about 1:5.

Vitamin A in the gonads was all in the ester form, and the content and concentration in the testis were two or three times higher than in the ovary. The carotenoid concentration was also much higher in the testis than in the ovary despite the more darkly pigmented appearance of the latter. More than half of the ovarian carotenoids consisted of β-carotene, but in the testis this pigment accounted for little more than a quarter of the total.

By far the highest concentrations of carotenoids were in the digestive gland, excretory organ and intestine. Xanthophylls and carotenes were present in all the extracts.

The ratios of vitamin A alcohol to ester and the proportions of different carotenoids were very similar in the group collected on 24 March 1952 to those of the preceding group. Before the two groups obtained on 12 May 1952 were analysed they were kept alive for 24 hr. and the faeces were collected. Scrapings were also taken of the algae encrusting the rocks on which the limpets were attached and feeding. Neither faeces nor scrapings contained vitamin A and the concentrations of carotenoids were almost the same in both but that of β-carotene was higher in the scrapings than in the faeces.

The limpets from Hoy were still alive on arrival and were treated in the same way as the previous group, the faeces being analysed separately. The ovaries of the dissected animals contained no vitamin A and very little carotenoid pigment. The carotenoid concentrations of the rock-scrapings and faeces were very similar, and no conclusion can be drawn regarding possible preferential absorption of carotenoids by the limpet.

TABLE X. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *PATELLA VULGATA* AND ITS FOOD

(All Patella from Harwich, except those of 4. viii. 52 from near Berry Head, Hoy, Orkneys.)

Date	Tissue	No.	Average weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene (μg/g)
					μg/spec.	μg/g	μg/spec.	μg/g	
11. ix. 50	Whole animal	12	7·8	4·7	1·5	0·19	399	51	16
29. i. 51	Visceral mass	22	5·6	2·9	2·3	0·41	208	37	12
	Mantle edge	22	0·2	2·1	0	0	3·9	22	5·9
	Tentacles	22	0·01	5·3	0	0	0·2	21	0
	Total	22	5·8	2·8	2·3	0·40	212	37	11
23. iv. 51	Muscle, foot and mantle	36	2·8	1·2	0·99	0·35	64	23	2·4
	Head	36	0·2	0·9	0	0	3·8	17	4·3
	Digestive gland and kidney	36	1·1	5·7	2·5	2·4	239	226	35
	Intestine	36	0·2	4·9	0·40	2·1	28	142	24
	Ovary	19	1·4	8·5	0·14	0·11	27	19	9·7
	Testis	17	0·8	3·8	0·56	0·67	45	112	30
	Total	36	5·3	3·4	4·2	0·79	398	74	13
24. iii. 52	Head and foot	40	5·9	0·4	0·25	0·04	56	9·6	2·9
	Intestine	40	0·4	2·6	0·28	0·67	21	52	5·4
	Visceral mass	40	1·2	6·2	2·6	2·1	238	192	40
	Testis	18	1·3	0·7	0·40	0·32	76	62	21
	Ovary	22	1·7	4·4	0·11	0·07	25	15	5·0
	Total	40	9·0	1·7	3·4	0·37	364	41	9·5
12. v. 52	Whole animal	13	8·4	2·2	5·2	0·62	479	57	8·8
12. v. 52	Digestive gland	12	1·8	9·6	5·1	2·8	439	239	43
	Intestine	12	0·2	3·9	0·37	1·5	29	121	15
	Rest of body	12	6·3	0·4	0	0	63	10	0·24
	Total	12	8·3	2·5	5·5	0·66	531	64	10
12. v. 52	Faeces	—	2·4	0·7	—	0	—	84	1·9
12. v. 52	Scrapings from rocks	—	7·3	0·3	—	0	—	83	4·8
4. viii. 52	Intestine	20	0·2	6·0	0·06	0·35	25	139	35
	Digestive gland	20	1·0	14	2·5	2·6	272	278	73
	Ovary	10	0·4	3·8	0	0	2·3	5·7	0
	Testis	10	1·0	2·6	0·70	0·74	79	83	12
	Rest of body	20	4·5	0·6	0·45	0·10	59	14	2·3
	Total	20	6·3	3·1	3·1	0·50	395	63	15
4. viii. 52	Whole animal ♀	10	5·4	2·8	2·2	0·40	216	40	7·1
4. viii. 52	Whole animal ♂	10	6·7	2·4	4·4	0·66	383	58	10
4. viii. 52	Faeces	—	0·17	2·8	—	0	—	113	3·5
4. viii. 52	Scrapings from rocks	—	0·2	20	—	0	—	132	Trace

Order Monotocardia

Buccinum undatum Linnaeus

As Table XI shows, two groups of whelks and a group of eggs were analysed. These molluscs were devoid of vitamin A and poor in carotenoids.

Murex trunculus Linnaeus

A singel specimen was analysed with results shown in Table XI. The carotenoids were mainly xanthophylls, although there were traces of β-carotene and possibly astaxanthin.

TABLE XI. OIL AND CAROTENOIDS IN *BUCCINUM UNDATUM* AND *MUREX TRUNCULUS*

Date	Species	Locality	No.	Average weight (g)	Oil (%)	Total carotenoids μg/spec.	Total carotenoids μg/g	β-carotene (μg/g)
10. x. 50	*Buccinum undatum*	Burnham-on-Crouch	19	5·9	1·0	34	5·8	0·80
11. xii. 50	*B. undatum* (eggs)	Burnham-on-Crouch	—	3·81*	0·6	—	2·4	0
9. vii. 52	*B. undatum*	Loch Fyne	31	18	0·3	14	0·79	0·07
13. v. 54	*Murex trunculus*	Madeira	1	11	2·2	147	13	Trace

Vitamin A absent from all specimens.

* Total weight.

TABLE XII. OIL AND CAROTENOIDS IN *LITTORINA* SPP.

(All specimens collected at Harwich on 11. ix. 50.)

Species	No.	Average weight (mg)	Oil (%)	Total carotenoids μg/spec.	Total carotenoids μg/g	β-carotene (μg/g)
Littorina littoralis	72	210	1·7	9·2	44	14
L. littorea	98	810	4·2	59	73	19
L. rudis	23	61	12	6·1	100	13

Vitamin A absent from all species.

Littorina littoralis (Linnaeus)
Littorina littorea (Linnaeus)
Littorina rudis (Maton)

Results given in Table XII show that these three species all lacked vitamin A. The pigments were xanthophylls and carotenes, with some chlorophyll. In *L. littoralis* β-carotene formed nearly one-third of the total carotenoids. *L. rudis* was richer in fat and carotenoids than its two congeners.

Aporrhais pes-pelecani (Linnaeus)

Table XIII shows the analytical results. The carotenoids included both carotenes and xanthophylls.

Crepidula fornicata (Linnaeus)

Two groups were analysed as shown in Table XIII. They contained good concentrations of carotenoids, both carotenes and xanthophylls.

Cypraea spadicea Swainson

Results for this specimen appear in Table XIII. The carotenoids were mainly xanthophylls.

TABLE XIII. OIL AND CAROTENOIDS IN *APORRHAIS*, *CREPIDULA* AND *CYPRAEA*

Date	Species	Locality	No.	Average weight (g)	Oil (%)	Total carotenoids μg/spec.	Total carotenoids μg/g	β-carotene (μg/g)
13. iii. 52	*Aporrhais pes-pelecani*	Loch Fyne	24	1·13	1·4	2·6	2·3	0
10. x. 50	*Crepidula fornicata*	Burnham-on-Crouch	80	1·42	0·7	24	17	1·3
11. xii. 50	*C. fornicata*	Burnham-on-Crouch	60	1·21	0·7	36	30	1·8
20. v. 53	*Cypraea spadicea*	La Jolla	1	6·1	1·6	49	8·0	0·37

Vitamin A absent from all species.

TABLE XIV. OIL, VITAMIN A AND CAROTENOIDS IN SOME TECTIBRANCHS

Date	Species	Locality	No.	Average weight (g)	Oil (%)	Vitamin A μg/spec.	Vitamin A μg/g	Total carotenoids μg/spec.	Total carotenoids μg/g	β-carotene (μg/g)
13. v. 54	*Aplysia depilans*	Madeira	2	64	0·3	7·0	0·11	170	2·7	0·32
29. viii. 51	*Clione limacina*	63° 18′ N. 18° 39′ W.	99	0·034	7·6	0	0	3·4	102	1·8
30. i. 55	*Limacina retroversa*	Barents Sea	405	0·00087	1·3	0·008	9·2	0·005	6·0	0

Sub-class Opisthobranchia

Order Tectibranchia

Aplysia depilans Linnaeus

Results for a group of two specimens are given in Table XIV. The ratio of vitamin A ester to alcohol was about 1:5. Both carotene and xanthophylls were detected in the small concentration of carotenoids present.

Clione limacina Phipps

The results for a group of this species appear in Table XIV. The high concentration of carotenoids included both carotene and xanthophylls.

Limacina retroversa (Fleming)

Table XIV shows that this species of pteropod, which was analysed with its shell, was relatively rich in vitamin A, all in the ester form. What little there was of carotenoid pigment appeared from its chromatographic behaviour to be either xanthophyll or possibly astaxanthin ester.

Subclass Pulmonata

Order Stylommatophora

Helix aspersa Linnaeus

Table XV shows the results for a group of garden snails. The pigments were mainly xanthophylls.

Order Basommatophora

Planorbis corneus (Linnaeus) var. *rubra* Oldham.

Results for a dozen of these bright orange snails show (Table XV) that vitamin A was absent and that the concentration of carotenoids was not especially high, the pigment being mainly xanthophyllic, with only traces of carotene.

TABLE XV. OIL AND CAROTENOIDS IN SOME PULMONATES

Date	Species	Locality	No.	Average weight (g)	Oil (%)	Total carotenoids μg/spec.	Total carotenoids μg/g	β-carotene (μg/g)
11. x. 51	*Helix aspersa*	Garden at Shinfield	60	3·9	0·4	6·0	1·5	0·04
25. x. 54	*Planorbis corneus* var. *rubra*	Aquarium dealer	12	2·1	0·3	30	14	Trace

Vitamin A absent from both species.

DISCUSSION

In the lamellibranch species we have examined, vitamin A was found in the mussel, *Mytilus edulis*, the clam, *Mya arenaria*, and the oysters, *Ostrea edulis* and *Gryphaea angulata*, but it was absent from the scallops, *Pecten maximus* and *Chlamys septemradiatus*, and the deposit-feeding bivalve, *Scrobicularia plana*. It was occasionally present in the cockle, *Cardium edule*, and the file-shell, *Lima hians*. Thus in both the lamellibranch orders, the Filibranchiata and the Eulamellibranchiata, there were some representatives with and others without vitamin A and there was likewise no apparent relationship between the carotenoid constituents of the various species and their affinities. All species except *Mytilus edulis* and *Lima hians*, which were richer, had carotenoid concentrations between 1·5 and 20 μg/g, and all species except *Chlamys septemradiatus* contained measurable amounts of β-carotene.

Analyses of the photoreceptive tissues of some of the species showed that vitamin A was absent and so does not appear to be concerned with vision as it may be in Crustacea (Wald, 1941; Fisher *et al.*, 1952). In this connexion it is not possible to determine whether vitamin A in the chiton, *Stenoplax conspicua*, had any visual role, since the animals of this species were analysed whole.

The vitamin A and carotenoid contents of the lamellibranch species do not appear to be associated with the food or feeding mechanism since some ciliary and deposit feeders contained vitamin A and others did not. In those species with vitamin A, *Gryphaea angulata* and *Mytilus edulis* had their richest concentrations in the digestive gland rather than in the rest of the visceral mass, whereas in *Mya arenaria* vitamin A was not invariably present in the digestive gland, and was in all instances absent from that of *Ostrea edulis*. In *Mya* the concentrations of vitamin A were always higher in the rest of the visceral mass

than in the digestive gland. The gonads would be included with the rest of the visceral mass in these species and so may have contained the vitamin. This lack of uniformity in the distribution of vitamin A between species indicates no very obvious function for it in the metabolism of these molluscs, in which the vitamin is more probably a chance by-product of metabolism of carotenoids derived from the algae of the food and appearing in those species with suitable enzyme systems. A similar explanation probably applies to the distribution and nature of the carotenoids present.

It is difficult to determine, from their distribution in those lamellibranchs studied, any special functions of the carotenoids present. Scheer (1940) was able, by feeding experiments, to shed some light on the metabolism and role of carotenoids in *Mytilus californianus*. He found that this mussel selected from its food xanthophylls in preference to carotenes and that the gonads acted as storage organs for carotenoids but not for lipids. Our own finding of higher concentrations of carotenoids in the gonads of *Cardium edule* indicates a similar tendency and also a possible reproductive role. In support of his suggestion that carotenoids play a part in the metabolism of the mussel and are not just stored, Scheer observed a reduction in the reserves of carotenoid pigments following feeding on a carotenoid-free diet. It is surprising that he does not mention vitamin A in *Mytilus californianus* since we have repeatedly found it in *M. edulis*. In *M. edulis*, the vitamin A, like the carotenoids, was mainly in the digestive gland, but we do not know whether it is derived unchanged from the food or formed from a precursor. From Scheer's work, conversion seems to be more likely, although vitamin A may not be present in *M. californianus* or essential to its existence, since ZoBell & Landon (1937) have shown that this species can be maintained exclusively on a diet of bacteria and state that Kincaid had found the same to be true for oysters.

Brooks & Paulais (1939) studied the distribution of carotenoids in the oysters, *Ostrea edulis* and *Gryphaea angulata*, and found little difference in carotenoid concentration between the 'green' and 'white' varieties of each species. They showed that the visceral mass in both species contained about twenty times as much pigment as the gills and mantle. We obtained ratios of a similar order and found that most of the pigment was in the digestive gland.

Among the gastropods analysed, vitamin A was present in *Haliotis fulgens*, *Megathura crenulata*, *Osilinus lineatus*, *Patella vulgata*, *Aplysia depilans* and *Limacina retroversa*, but absent from *Astrea undosa*, *Buccinum undatum*, *Murex trunculus*, *Littorina littoralis*, *L. littorea*, *L. rudis*, *Aporrhais pespelecani*, *Crepidula fornicata*, *Cypraea spadicea*, *Clione limacina*, *Helix aspersa* and *Planorbis corneus* var. *rubra*. Of those gasteropods with vitamin A, *Haliotis*, *Megathura*, *Osilinus* and *Patella* are all diotocardian prosobranchs, and *Aplysia* and *Limacina* are opisthobranchs of the order Tectibranchia. *Buccinum*, *Murex*, *Littorina* spp., *Aporrhais*, *Crepidula* and *Cypraea* which all lacked vitamin A are monotocardian prosobranchs. Of the other species

without the vitamin, *Astrea* is a diotocardian, *Clione* a tectibranch, *Helix* a stylommatophorous pulmonate and *Planorbis* a basommatophorous pulmonate. Apart from the absence of vitamin A from the single specimen of *Astrea undosa*, all the Diotocardia analysed contained the vitamin and all the Monotocardia lacked it. The Monotocardia are regarded as being less primitive than the Diotocardia, and it may be that in their evolution they have developed an enzyme system capable of breaking down the carotenoids of the food beyond the stage of vitamin A.

The presence or absence of the vitamin does not appear to be related to the food in the prosobranchs since those with vitamin A are all algal feeders, whereas those without it vary from ciliary feeders like *Crepidula*, through algal feeders like the *Littorinas*, to carnivorous predators like *Buccinum* and *Murex*. *Crepidula* has a similar feeding mechanism to that of the oysters and competes with them for the same food in the same environment, and yet the bivalves contain vitamin A. Such a difference would best be explained by the presence of diverse enzyme systems. Two planktonic tectibranchs, the pteropods *Limacina retroversa* and *Clione limacina*, were respectively possessed and devoid of vitamin A, the former being as rich in the vitamin as many of the Euphausiacea, richer in vitamin A than any other invertebrates so far analysed (Fisher *et al.*, 1955). *Limacina* contributes to the diet of plankton-feeding fish and so may be important to them as a source of preformed vitamin A. *Clione*, on the other hand, contained more carotenoids (102 μg/g) than *Limacina* (6 μg/g). Further investigations of the vitamin A content of these and other species of pteropods will be made when material is available to us.

In addition to *Clione*, some species of gastropod, namely, *Osilinus*, *Patella*, *Littorina* spp. and *Crepidula* also had high concentrations of carotenoids, whereas the other species studied had much smaller reserves. Whether these pigments have any positive function cannot be determined from comparison of the different species. We have, however, studied more fully the distribution of both vitamin A and carotenoids in the limpet, *Patella vulgata*. In this species most of the vitamin A (as well as the carotenoids) was in the digestive gland and there was always an appreciable concentration in the intestine, indicating its food origin. Vitamin A was absent from the food, so that *Patella* appears capable of converting a precursor to the vitamin. Comparison of the β-carotene concentrations in the rock-scrapings and in the faeces for the specimens collected on 12 May 1952 (Table X) indicates a possible preferential absorption of this pigment, but the evidence from similar specimens taken on 4 August 1952 is inconclusive or even contradictory, and so judgement must be withheld.

The gonads of *Patella* were of particular interest. They have already been the subject of an intensive study in *P. vulgata* and *P. depressa* by Goodwin (1950) and Goodwin & Taha (1950). These workers found in both testis and ovary β-carotene, echinenone, cryptoxanthin and zeaxanthin in the ratio

5:2:2:2. We found a similar ratio of β-carotene to total carotenoids. Goodwin & Taha (1951) have shown that echinenone and myxoxanthin, an algal carotenoid, are probably identical. They found echinenone in the gonads of *Patella* but we did not observe this pigment in our chromatographs of *Patella* extracts, nor did we see myxoxanthin in the extracts from the rock-scrapings, so that the question of whether echinenone in limpets was obtained by direct passage of myxoxanthin from algae or by conversion was not answered. The much higher concentrations of vitamin A and carotenoids in the testis of *P. vulgata* than in the ovary indicate a difference in the metabolism of carotenoids in the two sexes and possibly a function of the vitamin or pigments in reproduction, although in both ovary and testis the vitamin A was all in the ester (storage) form.

The form of the vitamin A in those molluscs containing it was extremely variable. In *Stenoplax*, *Lima*, *Ostrea*, *Mya*, *Osilinus* and *Limacina* only vitamin A ester was detected, but in *Haliotis* and *Megathura* only the alcohol form was found. In *Mytilus*, *Gryphaea*, *Patella* and *Aplysia* both ester and alcohol were present, but the alcohol form was always in excess. The alcohol is the active form of vitamin A and, in contrast to the condition in Mollusca, vertebrates and Crustacea almost invariably contain an excess of vitamin A ester in the storage organs, although vitamin A alcohol may predominate in the circulatory system of vertebrates.

There seems to be no obvious explanation of the extreme variability in this respect that we have found in the molluscs in relation to their food, ecology or affinities.

We have shown that vitamin A is present in a species of Loricata, some Lamellibranchiata and some Gastropoda, and absent from other species of the second and third classes. It had not previously been reported in either Amphineura or Gastropoda. Those species used as human food contain little or no vitamin A.

We are thus faced with the puzzling finding that some molluscs appear to manage quite well in the absence of vitamin A and its precursors, and it seems difficult to postulate for these substances a function as fundamental in molluscs as in vertebrates. The evidence we present in the paper that follows convinces us that vitamin A is essential for cephalopods in which it may well function in a way similar to that in vertebrates. When it comes to other molluscs, all we can say now is that the presence or absence of vitamin A and its precursors may possibly indicate in them nothing more than the peculiarities of their enzyme equipment.

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Summary

1. Vitamin A, total carotenoids and β-carotene were measured, where present, in one species of Loricata, nine species of Lamellibranchiata and eighteen species of Gastropoda.

2. Vitamin A was found in *Stenoplax conspicua*, *Gryphaea angulata*, *Mya arenaria*, *Mytilus edulis*, *Ostrea edulis*, *Aplysia depilans*, *Haliotis fulgens*, *Megathura crenulata*, *Osilinus lineatus*, *Patella vulgata* and *Limacina retroversa*, but not in *Chlamys septemradiatus*, *Pecten maximus*, *Scrobicularia plana*, *Astrea undosa*, *Buccinum undatum*, *Clione limacina*, *Crepidula fornicata*, *Cypraea spadicea*, *Helix aspersa*, *Littorina littoralis*, *L. littorea*, *L. rudis*, *Murex trunculus*, *Planorbis corneus* var. *rubra* or *Aporrhais pes-pelecani*. The vitamin was found in one of five groups of *Cardium edule* and one of two groups of *Lima hians*. Vitamin A has not previously been reported in Loricata or Gastropoda.

3. In those species containing it, vitamin A was concentrated mainly in the digestive gland or in the visceral mass.

4. With *Patella vulgata* there was evidence that the vitamin A reserves are derived by conversion of carotenoid precursors in the algal diet. In this species the testis contained much higher concentrations of both vitamin A and carotenoids than the ovary.

5. Except in the gonads of *Patella*, there was little evidence that vitamin A has any special function in the molluscs studied. The concentrations of vitamin A were low, except in *Limacina retroversa*, and there was no consistency as between species in the ratios of active to stored forms of the vitamin. The vitamin A and carotenoid contents of any species may possibly reflect its enzymic capacity to break down the carotenoids of the food.

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