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VITAMIN A AND CAROTENOIDS IN CERTAIN INVERTEBRATES

V. MOLLUSCA: CEPHALOPODA

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(Text-fig. 1)

Introduction

In our previous paper (Fisher, Kon & Thompson, 1956) on Mollusca we pointed out that the Cephalopoda were so different from the other classes in vitamin A and carotenoid relationships that they would be more satisfactorily considered separately. We have so far analysed ten species of cephalopods and found vitamin A in all of them. The studies of previous investigators of vitamin A in these molluscs were confined to its function in the visual cycle and to its contribution to the vitamin A reserves of sperm whales feeding on cephalopods. The carotenoids of cephalopods have been given even less attention than vitamin A.

MacMunn (1883*a*, *b*) was probably the earliest worker to study the pigments of a cephalopod. He examined the alcohol- and ether-soluble pigments of *Octopus vulgaris* and reported that the chromatophores and eyes contained 'tetronerythrin', a substance which probably represented the sum total of carotenoids in the tissues. We have found no further record of similar work on cephalopods until that of Lönnberg (1935) on the carotenoids of *Sepiola scandica*, *Rossia macrosoma* and *Eledone cirrosa*. Extracts from the eyes of all the species contained a xanthophyll and gave a blue colour with antimony trichloride. An extract from the eggs of *Rossia* contained no pigment but gave a strongly blue reaction with antimony trichloride. The remaining tissues of *Sepiola* and *Rossia* contained very little pigment and so were not further investigated. The liver of *Eledone* contained large quantities of carotenoids and gave a greenish blue antimony-trichloride reaction.

In the following year, Verrier & Pannier (1936) first reported the presence of vitamin A in a cephalopod. They stated that they had extracted the vitamin from the visual pigment in the retinas of the eyes of *Eledone moschata*. Escher-Desrivières, Lédérer & Verrier (1938) subsequently examined the pigment from the edges of the pigmented epithelia of the retinas of *E. moschata*, *Sepia officinalis* and *Octopus vulgaris*, and found that it closely resembled vertebrate retinal purples, vitamins A_1 and A_2 as such being absent. Wald (1941) made a quantitative study of the retinas of the squid, *Loligo pealii*, in

which he found 1–2 μg of vitamin A_1 and three times this amount of retinene$_1$ (vitamin A aldehyde) per retina. He found no carotenoids in other tissues of this squid. The amount of vitamin A in the retinas was the same in light and darkness, indicating that the vitamin did not participate directly in the visual process. Wald suggested that squid visual purple was reversibly changed by light into retinene plus protein. Bliss (1948) obtained squid visual purple in an almost pure state, and showed that its properties were very similar to those of vertebrate visual purples.

Further observations on cephalopod carotenoids were reported by Wagner & Vermeulen (1939) and Leong (1939) who detected no carotenoids in cuttlefish, and by Fox & Crane (1942) who studied *Paroctopus bimaculatus* and *Loligo opalescens*. They found only traces of pigment in any of the squid organs but, in the octopus, the liver contained 3·5 mg of carotenoids per 100 g moist tissue and the ink 0·55–0·7 mg/100 g.

Cephalopods had long been considered the most likely source of the rich stores of vitamin A in the liver of the sperm whale, but experimental evidence was first produced by Brachi (1953) who analysed the liver from a 3 ft. long female specimen of *Moroteuthis ingens* taken from the stomach of a sperm whale during the 1951–52 antarctic whaling season. He experienced great difficulty in obtaining a squid free from the nematodes infesting the stomach of the whale, since they immediately attack the liver of any squid eaten. We have found vitamin A in these nematodes, *Anisakis physeteris* Baylis, taken from the stomach of a sperm whale being flensed at the whaling station of Scottish Whalers Ltd in the island of Harris, Outer Hebrides, in 1951. They are the only representatives so far of an invertebrate phylum other than the Arthropoda and Mollusca we have found to contain vitamin A. Brachi also analysed the unsaponifiable matter from squid, *Loligo forbesi*, and cuttlefish, *Sepia officinalis*, caught near Plymouth, but, by his technique, found no vitamin A in them.

Material and Methods of Preservation

Ten species of cephalopods were collected, some by ourselves and others by marine biologists on research cruises from Plymouth or Millport. They comprised four species of squid, five of cuttlefish and one octopus.

Alloteuthis media (Linnaeus), *Eledone cirrosa* (Lamarck), *Sepia officinalis* (Linnaeus), *Parasepia elegans* (d'Orbigny), *Todaropsis eblanae* (Ball) and some groups of *Loligo forbesi* Steenstrup were collected for us from one of the research ships of the Plymouth laboratory and brought back there alive where they were immediately dissected and the organs weighed and preserved in absolute alcohol.

Several groups of *Loligo forbesi* were collected by a research ship of the Torry Research Station at Aberdeen and dissected by the staff of that station. This material was also preserved in absolute alcohol.

Rossia macrosoma (Delle Chiaje) and *Sepietta oweniana* (d'Orbigny) were taken on various cruises in the Clyde Sea area by the M.V. *Calanus* from the Millport Laboratory. These were dissected and preserved, or preserved whole, after weighing, in absolute alcohol. A single specimen of *S. oweniana* was taken on the surface of Loch Hourn, Inverness-shire, in the bay at Arnisdale, having been attracted by a light lure. This squid was preserved in solid carbon dioxide until arrival at the laboratory at Shinfield, when it was dissected, the organs being weighed and preserved in alcohol.

A single specimen of *Sepiola* sp. was collected near Monaco on 2 February 1952.

Ommastrephes pteropus Steenstrup was caught with a hand-line at two stations of a north Atlantic cruise of the R.R.S. *Discovery II* in November 1954. Only the nidamental gland, eyes and livers were preserved, by immersion in boiling sea water followed by cold-storage. These specimens were weighed when they arrived at Shinfield, and preserved in alcohol.

All the alcohol-preserved specimens were kept in the deep-freeze at $-20°$ C until they were analysed.

Chemical and Physical Tests

Methods

The method of analysis for carotenoids and vitamin A was that described in the first paper of this series (Fisher, Kon & Thompson, 1952). Most cephalopod tissues were much richer in sterols than any material we had previously studied, and in the analyses of extracts from large quantities of tissue these sterols interfered with the Carr-Price reaction for vitamin A. It was necessary, therefore, to remove them. The separation was achieved by extracting the tissues with light petroleum, removing the solvent and dissolving the solids in methanol. The methanol solution was placed for an hour in the deep-freeze at $-20°$ C when most of the sterols precipitated. They were filtered off and washed several times with methanol at $-20°$ C. The methanol was evaporated off from the filtrate and the lipids dissolved in *n*-hexane for the first chromatography.

Since it is broken down during saponification, retinene (vitamin A aldehyde) must be separated from extracts of cephalopod eyes by an additional chromatography. The first chromatography was done as previously (Fisher *et al.*, 1952), but the alumina was weakened with 8% ethanol in *n*-hexane as for the second chromatography. This procedure separated vitamin A ester, which was eluted by 2% acetone in *n*-hexane, from vitamin A alcohol which was eluted by 8% ethanol in *n*-hexane. Retinene was eluted with the ester fraction. This fraction was evaporated down and the residue dissolved in light petroleum (b.p. 40–60° C) for the next chromatography done by the method of Ball, Goodwin & Morton (1948) on a column of Peter Spence activated alumina

type 'O' previously weakened by shaking with 10% (w/w) of distilled water. Vitamin A ester was eluted with light petroleum and then saponified and subjected to further chromatography by our usual technique. Elution of the column with a solution of 10% diethyl ether in light petroleum removed the retinene. This fraction was evaporated down, dissolved in *n*-hexane and the absorption spectrum examined in the Beckman quartz photoelectric spectrophotometer.

A sample of pure crystalline retinene, kindly supplied by Prof. R. A. Morton, F.R.S., of Liverpool University, gave in *n*-hexane $E^{1\%}_{1\,cm}$ 370 mμ = 1610, and this value was used for the calculation of the concentration of retinene from the absorption maximum, after the curves had been corrected for irrelevant absorption by the method of Morton & Stubbs (1946).

Subsequently the retinene solution was again evaporated and dissolved in chloroform for the Carr-Price reaction. The colour in this reaction was read at 664 mμ, 180 sec after addition of the antimony-trichloride reagent. The Thompson (1949) direct reading photoelectric spectrophotometer was also calibrated with the pure crystalline retinene. $E^{1\%}_{1\,cm}$ 664 mμ for this sample was 4150.

In our earlier analyses retinene was not separated from eye extracts, and where only vitamin A alcohol is reported in these extracts retinene might well have been present initially, but would have been destroyed during saponification.

Results

Order Decapoda

Alloteuthis media (Linnaeus)

A group of six specimens including five females with ripe ovaries, was dissected into the parts shown in Table I. Carotenoid pigments were not present in measurable amounts and vitamin A occurred only in the eyes and ovaries.

TABLE I. DISTRIBUTION OF OIL AND VITAMIN A IN SIX SPECIMENS OF *ALLOTEUTHIS MEDIA*

(Collected near Plymouth on 10. vi. 1952.)

Organ	Average weight (g)	Oil (%)	Vitamin A	
			μg/spec.	μg/g
Eyes (pairs)	0·38	0·9	0·25	0·61
Ovary*	0·44	0·7	0·31	0·71
Liver	0·15	2·3	0	0
Rest of body	7·95	1·2	0	0
Total	8·85	1·2	0·51	0·06

Carotenoids not present in measurable amounts.

* Average values for five animals, see text above.

Loligo forbesi Steenstrup

Five groups of squid were analysed, with the results shown in Table II. In the first group the ratio of vitamin A ester to alcohol in the liver and rest of the body was nearly 3:1, and in the eyes it was 1:11. In those parts with carotenoids, both carotenes and xanthophylls were observed, but no astaxanthin.

In the second group, which was much poorer in vitamin A than the first, the ester:alcohol ratios were, in the eyes, about 1:3, in the liver, about 1:4, and in the rest of the body 6:1. The small amounts of carotenoid pigment in

TABLE II. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *LOLIGO FORBESI*

Date	Locality	Organ	No.	Average weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene (μg/g)
						μg/spec.	μg/g	μg/spec.	μg/g	
7. vi. 51	Plymouth	Eyes (pairs)	9	7·8	0·9	3·3	0·43	4·1	0·5	0·033
		Liver	9	5·6	11	88	16	34	6·1	0·078
		Ink sac	9	0·6	1·9	0	0	0	0	0
		Rest of body	9	163	1·4	1·3	0·01	41	0·3	0·001
		Total	9	177	1·7	93	0·52	79	0·4	0·005
6. viii. 51	Aberdeen	Eyes (pairs)	30	5·0	1·2	0·37	0·07	5·0	0·5	Trace
		Liver	30	4·3	1·8	4·8	1·1	3·1	0·7	0
		Rest of body	30	107	2·0	4·9	0·05	17	0·2	0
		Total	30	116	1·9	10	0·09	25	0·2	Trace
27. xi. 51	Aberdeen	Eyes (pairs)	547	7·5	0·53	1·8	0·24	—	—	—
		Livers	547	7·3	1·1*	7·0	0·96	0·25	0·04	0
9. vi. 52	Plymouth	Ink sac	1	3·6	2·6	0	0	0	0	0
		Eyes (pairs)	1	17	1·3	1·0	0·06	20	1·1	0
		Testis	1	3·4	1·8	0	0	0	0	0
		Gills and hearts	1	20	2·0	0	0	0	0	0
		Alimentary canal	1	13	2·1	2·4	0·18	0	0	0
		Male reproductive ducts	1	16	0·7	0	0	0	0	0
		Liver	1	33	9·0	17	0·52	46	1·4	0
		Caecum	1	47	3·9	11	0·24	74	1·6	0
		Muscle, skin and tentacles	1	830	0·5	5·8	0·01	0	0	0
		Total	1	983	1·0	37	0·04	140	0·14	0
9. vi. 52	Plymouth	Ink sac	14	0·36	2·4	0	0	0	0	0
		Eyes (pairs)	14	5·3	1·1	0·76	0·14	3·5	0·66	0
		Alimentary and reproductive systems	14	3·1	2·7	0·15	0·05	0	0	0
		Caecum	14	1·4	2·8	0·19	0·12	0	0	0
		Gills and hearts	14	3·2	1·8	0	0	0	0	0
		Liver	14	3·6	2·8	0·94	0·26	0	0	0
		Muscle, skin and tentacles	14	100	1·3	0·50	0·005	0	0	0
		Ovaries†	2	31	3·3	13	0·42	0	0	0
		Testis†	1	0·88	0·7	0	0	0	0	0
		Nidamental glands†	2	20	2·4	0	0	9·9	0·50	Trace
		Total	14	117	1·4	4·4	0·04	3·5	0·03	Trace

* Including 1·03% precipitated as sterols.

† Only three animals with recognizable gonads.

the eyes included both carotenes and xanthophylls, but in the liver and rest of the body only xanthophylls and possibly traces of astaxanthin were observed.

The eyes and livers were dissected from the third group at Aberdeen, preserved separately in abolute alcohol and despatched to us. Portions of the extracts were analysed for vitamin A by physical and chemical methods. Carotenoids were measured in the liver extract, but, inadvertently, not in the eye extract. The bulks of both extracts were retained for biological assay of vitamin A, dealt with later in this paper. The results for vitamin A given in Table II are, as usual, those calculated from the Carr-Price reaction. The value for the eyes was intermediate between those for the two earlier groups and that for the livers was similar to that for the previous lot from Aberdeen. The liver carotenoids were lower in content and concentration than before.

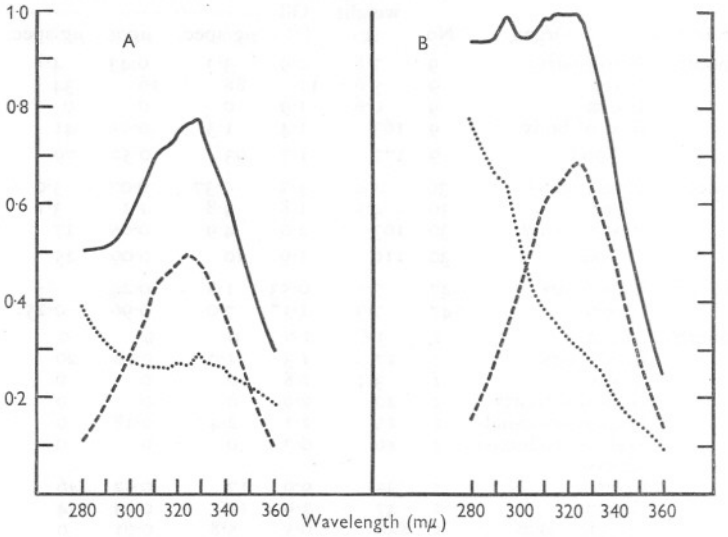

Fig. 1. Absorption spectra of fractions containing vitamin A from non-saponifiable residues of oils extracted from (A) eyes and (B) liver of *Loligo forbesi* (solvent, *n*-hexane). ——, *E* observed; – – – –, *E* corrected; . . . ., irrelevant absorption.

Vitamin A in these two extracts was also examined spectrophotometrically and absorption spectra for vitamin A in the eyes and livers are shown in Fig. 1, with curves corrected for irrelevant absorption by the method of Cama, Collins & Morton (1951). The values calculated from the absorption maximum in (spectroscopic) *n*-hexane at 324 mμ taking $E^{1\%}_{1\,cm}$ for pure vitamin A alcohol as 1800 were, after applying the correction procedure for irrelevant absorption, for the eyes 2·2 μg per pair or 0·30 μg per g, and, for the livers 5·1 μg per organ or 0·69 μg per g. The large irrelevant absorption, especially at the lower wavelengths, indicates that, despite efforts to remove sterols, a large amount of extraneous materials was still present in the final extracts.

Of the squid collected near Plymouth in June 1952, one was a large male, which weighed nearly 1 kg. It was dissected into the parts shown in Table II. Vitamin A was concentrated mainly in the liver, caecum and alimentary canal, but the amounts were smaller than those found in earlier specimens. The ester:alcohol ratio for the liver vitamin A was 4:1, and for that in the muscle-skin-tentacles extract 1:3. In the caecum the vitamin A was entirely in the ester form. The two components were not separated in the other organs containing the vitamin. The sparse amounts of carotenoids were mainly xanthophylls, although there may also have been a trace of astaxanthin in the liver.

TABLE III. VITAMIN A ESTER, ALCOHOL AND ALDEHYDE IN THE EYES OF *LOLIGO FORBESI* AND *OMMASTREPHES PTEROPUS*

						Vitamin A					
						Ester		Alcohol		Aldehyde	
Date	Locality	Species	No. of eyes	Average weight (g)	Oil (%)	μg/spec.	μg/g	μg/spec.	μg/g	μg/spec.	μg/g
14. xi. 53	Aberdeen	*L. forbesi*	100	3·1	1·6*	0·026	0·0086	0·088	0·029	0·77 (0·91)	0·25 (0·30)
10. xii. 53	Plymouth	*L. forbesi*	67	3·8	1·1†	0·019	0·0050	0·038	0·010	0·96 (0·93)	0·25 (0·25)
16. xi. 54 / 25. xi. 54	North Atlantic	*O. pteropus*	3	21	0·42	0·57	0·027	0·85	0·040	5·0 (4·1)	0·24 (0·19)

* Including 0·79 % subsequently precipitated as sterols.
† Including 0·52 % subsequently precipitated as sterols.
Figures in parentheses are values obtained from spectrophotometric measurements.

In the last group, the ovaries were dissected from two females and the testis from one male. The remaining animals were either immature or spent so that the gonads were not recognizable. Other organs were dissected and grouped for analysis in the way shown in Table II. The distribution of vitamin A was as in the male specimen, with concentrations again rather low; carotenoids were almost entirely absent. In the livers the ester:alcohol ratio for vitamin A was 2:9 and in the eyes it was 1:6. In the extracts from the alimentary and reproductive systems and from the muscle, skin and tentacles the vitamin A was all in the alcohol form, but in the caecum it was present only as the ester. The most striking feature of this group of analyses was the large amount of vitamin A, all as the alcohol, in the mature ova.

The possible presence of retinene in squid eyes was more fully investigated in two groups. The results in Table III show that, whereas the vitamin A ester and alcohol concentrations were higher in the eyes of the Aberdeen specimens than in the Plymouth ones, retinene was present in much greater amounts than either of the other two vitamin A components and the concentrations were the same in both groups.

Ommastrephes pteropus Steenstrup

Two specimens of this large squid, taken during a cruise of R.R.S. *Discovery II* in November 1954, were dissected and some organs preserved for us on the ship. The parts we received were the liver, one eye and the nidamental gland from one specimen, and both eyes and the liver from the other. For analytical purposes the two livers were treated separately, but the three eyes were extracted together. The results are shown in Table IV. The livers contained good concentrations of both vitamin A, of which about 75% was in the ester form, and carotenoids, which were mainly xanthophyllic although traces of astaxanthin may also have been present.

TABLE IV. OIL, VITAMIN A AND CAROTENOIDS IN SOME ORGANS OF *OMMASTREPHES PTEROPUS*

Date	Organ	No.	Average weight (g)	Oil (%)	Vitamin A		Carotenoids	
					μg/spec.	μg/g	μg/spec.	μg/g
16. xi. 54	Nidamental gland	1	15	2·0	0	0	21	1·4
16. xi. 54	Liver	1	20	9·4	134	6·6	273	13
25. xi. 54	Liver	1	32	11	474	15	1340	42
16. xi. 54 / 25. xi. 54	Eyes	3	21	0·42	1·4	0·07	31	1·4

β-carotene absent from all organs.

The concentration of vitamin A in the eyes was similar to that found in *Loligo forbesi*. Retinene was also separated from the extract, and the values for the various vitamin A components are shown in Table III. The close similarity in concentration of retinene in the eyes of *Ommastrephes* and in those of the two groups of *Loligo* is very striking.

Todaropsis eblanae (Ball)

Analytical results in Table V show that vitamin A was present only in the liver where the concentration was high; ester and alcohol were not separated in this analysis. The liver was also richest in carotenoids, which were xanthophylls with possibly a trace of astaxanthin.

Parasepia elegans (d'Orbigny)

Results in Table VI show that, as in the previous species, vitamin A was found, entirely in the ester form, only in the liver, but in *Parasepia* the concentration was much lower. Carotenoids were much more widespread in this species and were mainly xanthophyllic. No vitamin A was found in the eyes. It may have been present only as retinene and so destroyed during saponification by our routine analytical technique.

Rossia macrosoma (Della Chiaje)

A single specimen of *Rossia* was dissected into the parts shown in Table VII. The liver was richest in both vitamin A, which was not separated into ester and alcohol, and carotenoids. Small amounts of carotenoids were also found in all other parts.

TABLE V. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *TODAROPSIS EBLANAE*

(Specimen caught near Plymouth on 10. vi. 1952.)

Organ	Weight (g)	Oil (%)	Vitamin A		Carotenoids	
			μg/spec.	μg/g	μg/spec.	μg/g
Ink sac	0·29	2·5	0	0	0	0
Eyes (pair)	9·6	0·9	0	0	8·6	0·9
Alimentary canal	3·7	2·1	0	0	0	0
Male reproductive system	11	1·8	0	0	0	0
Gills and hearts	4·1	1·1	0	0	4·5	1·1
Liver	9·1	11	174	19	70	7·8
Rest of body	111	0·3	0	0	21	0·19
Total	149	1·2	174	1·2	104	0·70

β-carotene absent from all organs.

TABLE VI. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *PARASEPIA ELEGANS*

(Specimens caught near Plymouth on 10. vi. 1952.)

Organ	No.	Weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene (μg/g)
				μg/spec.	μg/g	μg/spec.	μg/g	
Ink sac	14	0·25	0·4	0	0	0·6	2·3	0
Eyes (pairs)	14	1·5	0·9	0	0	2·0	1·3	0
Alimentary canal	14	1·1	1·2	0	0	7·2	6·6	0
Liver	14	1·3	2·5	0·32	0·24	7·5	5·8	Trace
Nidamental glands	11	2·3	1·7	0	0	0	0	0
Ovary	11	2·8	2·6	0	0	2·8	1·0	0
Testis	3	1·1	0·5	0	0	2·0	1·8	0
Rest of body	14	19	0·3	0	0	1·9	0·10	0
Total	14	28	0·8	0·32	0·01	22	0·79	Trace

TABLE VII. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *ROSSIA MACROSOMA*

(Specimen caught in Firth of Clyde on 17. vi. 1954.)

Organ	Weight (g)	Oil (%)	Vitamin A		Carotenoids	
			μg/spec.	μg/g	μg/spec.	μg/g
Eyes (pair)	2·4	0·66	0	0	2·7	1·1
Liver	1·1	5·5	12	11	14	13
Rest of body	21	1·1	0	0	8·3	0·4
Eggs	0·8	21	0	0	3·2	4·0
Total	25	1·9	12	0·48	31	1·2

β-carotene absent from all organs.

Sepia officinalis (Linnaeus)

Three groups of the common cuttlefish, all collected near Plymouth, were analysed with results shown in Table VIII. As in most of the previous species, the liver was the richest in vitamin A and carotenoids, with vitamin A alcohol slightly in excess of vitamin A ester. The carotenoids included both carotenes and xanthophylls. The ovaries contained ripe ova, which were rich in vitamin A alcohol. Vitamin A was present in the alimentary canal as the ester and in the eyes and muscle, brain, skin and tentacles as the alcohol. Next to the liver, the ink sac had the highest concentration of carotenoids, these being mostly xanthophylls.

The eyes were dissected from fourteen much smaller cuttlefish, collected at the same time as the previous specimens, and the analytical results for these are shown in Table VIII. The vitamin A was all in the alcohol form.

As Table VIII shows, the results for specimens collected in November were similar to those taken in June, but the ratio of vitamin A ester to alcohol in the liver was 4:1. As before, the eyes contained only vitamin A alcohol

TABLE VIII. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *SEPIA OFFICINALIS*

(All specimens caught near Plymouth.)

Date	Organ	No.	Average weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene (μg/g)
					μg/spec.	μg/g	μg/spec.	μg/g	
7. vi. 51	Eyes (pairs)	2	16	0·67	1·3	0·08	11	0·68	0
	Gills and hearts	2	18	1·6	0	0	18	0·93	Trace
	Ink sac	2	6·6	0·33	0	0	29	4·4	0
	Liver	2	34	4·2	72	2·1	1255	37	2·6
	Female reproductive system	2	49	1·8	0	0	0	0	0
	Alimentary canal	2	19	0·91	3·3	0·18	31	1·6	Trace
	Muscle, brain, skin and tentacles	2	432	0·67	5·6	0·01	77	0·18	0·01
	Ovary	2	57	2·0	100	1·7	0	0	0
	Total	2	632	1·1	182	0·29	1421	2·3	0·15
7. vi. 51	Eyes (pairs)	14	4·4	0·47	0·20	0·05	3·6	0·82	0
16. xi. 51	Eyes (pairs)	2	14	0·60	4·4	0·32	2·2	0·16	0
	Liver	2	34	7·4	67	2·0	210	6·2	0·57
	Caecum	2	6·6	1·8	0	0	0	0	0
	Alimentary canal	2	12	1·3	0·57	0·05	9·1	0·78	0
	Ink sac	2	12	0·46	0	0	8·9	0·74	0
	Ovary	2	1·8	2·4	0	0	0	0	0
	Nidamental glands	2	6·9	1·9	0	0	0	0	0
	Gills and hearts	2	17	0·50	0	0	0	0	0
	Muscle, brain, skin and tentacles	2	406	0·51	0	0	24	0·06	0
	Total	2	510	1·0	72	0·14	254	0·50	0·04
10. vi. 52	Eyes (pairs)	9	2·5	1·1	0	0	3·5	1·4	0
	Ink sac	9	0·76	0·66	0	0	1·5	1·9	0
	Alimentary canal	9	3·5	0·94	0	0	6·4	1·8	0
	Liver	9	4·2	4·2	4·9	1·2	116	28	0·08
	Rest of body	9	39	1·0	0	0	0	0	0
	Total	9	50	1·3	4·9	0·10	127	2·5	0·007

and the alimentary canal only ester. The ovaries were immature and had no vitamin A. Carotenoid concentrations were lower than in the summer specimens.

The smaller specimens in the last group had vitamin A only in the liver, where the ratio of ester to alcohol was about 7:2. The carotenoid concentrations were of the same order as in the June specimens of the previous year.

Sepietta oweniana (d'Orbigny)

Analytical results for the groups and single specimens of this cuttlefish are shown in Table IX. Vitamin A was entirely in the ester form in the first group of specimens. The second group, consisting of three much smaller specimens, was analysed whole, and the results in Table IX show that they were even richer in vitamin A than the first group. Because of the small amount of material, vitamin A ester and alcohol were not estimated separately. In neither of these first two groups were carotenoids present in measurable amounts.

TABLE IX. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *SEPIETTA OWENIANA* AND *SEPIOLA* SP.

Date	Locality	Organ	No.	Average weight (g)	Oil (%)	Vitamin A		Carotenoids	
						μg/spec.	μg/g	μg/spec.	μg/g
		Sepietta oweniana							
18. x. 51	Loch Fyne	Whole animals	2	1·3	9·3	4·5	3·4	0	0
9. vii. 52	Loch Fyne	Whole animals	3	0·12	3·1	0·55	4·6	0	0
30. ix. 54	Clyde Main Channel	Whole animals	1	4·3	3·1	5·0	1·1	26	6·0
10. iii. 55	Clyde Main Channel	Whole animals	1	0·93	1·4	3·2	3·5	5·5	5·9
12. iii. 55	Loch Hourn	Eyes (pair)	1	0·32	0·25	0·57	1·8	1·4	4·3
		Liver	1	0·28	0·65	1·4	5·1	1·4	4·9
		Rest of body	1	2·4	0·52	0	0	2·9	1·2
		Total	1	3·0	0·47	2·0	0·66	5·7	1·9
		Sepiola sp.							
2. ii. 52	Monaco	Whole	1	1·4	0·76	0·77	0·54	6·7	4·7

β-carotene absent from all specimens.

The concentration of vitamin A in the large specimen, taken in September 1954, was lower than in the smaller ones but, as in the first group, the vitamin was entirely in the ester form. Measurable amounts of carotenoids were present, but no definite bands appeared on chromatography, so that they were not identified. Another specimen, weighing 0·93 g, was analysed whole. The results in Table IX show that the concentration of vitamin A, all in the ester form, was similar to that in the first two groups. There was again not enough carotenoid pigment for identification.

In the dissected specimen, the liver contained most of the vitamin A, with a smaller amount in the eyes and none in the rest of the body, but the concentration in the whole animal was less than in previous analyses. In the liver there were approximately equal quantities of vitamin A ester and alcohol, whereas in the eyes there was only vitamin A alcohol.

Sepiola sp.

A single specimen was analysed whole with the results shown in Table IX. The small amount of vitamin A was measured only in the ester fraction, since sterols interfered with measurements on the alcohol fraction. There was not enough pigment present in the chromatography for identification of the carotenoid.

Order Octopoda

Eledone cirrosa (Lamarck)

Analytical results for this specimen given in Table X show that, as in the decapods, the liver contained most of the vitamin A. Only the ester was determined. The alcohol fraction gave a red colour in the Carr-Price reaction, probably due to sterols which were not previously removed. These may have interfered in the reaction with any vitamin A alcohol that might have been present. Vitamin A alcohol was slightly in excess of the ester in the eyes. In the other parts of the body vitamin A was all in the ester form, although the presence of sterols may have interfered with the measurement of the alcohol.

TABLE X. DISTRIBUTION OF OIL, VITAMIN A AND CAROTENOIDS IN *ELEDONE CIRROSA*

(Specimen caught near Plymouth on 11. vi. 1952.)

Organ	Weight (g)	Oil (%)	Vitamin A		Total carotenoids		β-carotene
			μg/spec.	μg/g	μg/spec.	μg/g	(μg/g)
Eyes (pair)	2·9	0·6	0·59	0·20	5·8	2·0	0
Liver	24	16	64	2·7	340	14	0·20
Hearts, gills, alimentary and reproductive systems	20	0·8	0·54	0·03	46	2·3	0
Rest of body	281	0·5	4·6	0·02	43	0·15	0
Total	328	1·6	70	0·21	435	1·3	0·02

BIOLOGICAL TESTS

Methods

General

The biological activity of the vitamin A fractions from the eyes and livers of 547 squid, *Loligo forbesi* (see p. 68) was measured by rat-growth tests. These were done in the way described by Fisher *et al.* (1952). The standard used was the International Standard for vitamin A at 10,000 i.u./g in cotton-seed oil suitably diluted for the tests with arachis oil stabilized with hydroquinone. The material to be assayed was prepared from the non-saponifiable residue after removal of the sterols and extraction of the vitamin A fractions as described earlier in this paper. The residue, containing vitamin A in the alcohol form, was diluted with arachis oil for feeding to rats. During the tests the oils were kept under nitrogen in the refrigerator and their vitamin A content was checked periodically by the Carr-Price test.

Squid-eye oil

Groups of sixteen (all ♀) wholly vitamin A-deficient rats received twice weekly vitamin A standard at the nominal rate of 2 and 4 i.u. daily; a group of fourteen similar rats was dosed at a nominal daily rate of 2 i.u. and another group of thirteen rats at nominal rate of 4 i.u of the test preparation.

Squid-liver oil

As before, the test was done on female rats only. Three groups of eighteen were dosed twice a week with the standard and three with the liver extract at nominal rates of 1, 2 and 4 i.u. daily.

TABLE XI. CHEMICAL (ANTIMONY-TRICHLORIDE) AND BIOLOGICAL MEASUREMENTS OF VITAMIN A ACTIVITY IN SQUID OILS FED TO WHOLLY VITAMIN A-DEFICIENT RATS, IN I.U. PER GRAM OF ARACHIS OIL SOLUTION

Organ	Chemical Value	Biological	
		Value	True fiducial limits ($P=0{\cdot}95$)
Eye	195	212	157–279
Liver	210	210	184–241

Results

Table XI shows the results of the two tests. The values for chemical and biological measurements of vitamin A in both eyes and livers show good agreement, indicating that the vitamin was in the all-*trans* form.

DISCUSSION

Although some of the results are unsatisfactory owing to our failure in the early stages of the work to remove sterols from some of the more concentrated extracts and to separate retinene from eye extracts, we believe that they provide some useful information. The supply of specimens suitable for our analyses is so irregular and fortuitous that it may be a long time before we can rectify some of the earlier mistakes. We think it therefore advisable to publish our findings, pointing out results which may be doubtful owing to errors in technique.

We have found in all instances vitamin A in some part of each of the ten species of cephalopod we have studied. The concentration has varied between species, the average being of the same order as the highest found in *Patella*, the richest in vitamin A of other molluscs except *Limacina retroversa* (Fisher *et al.*, 1956).

Except in *Alloteuthis media*, vitamin A was always found in highest concentration in the liver. Usually most of the liver vitamin A was in the ester form, indicating that this organ was acting as a store for vitamin A, as in many vertebrates, but contrasting with the situation in other molluscs where

vitamin A in the digestive gland was in the alcohol form (Fisher *et al.*, 1956). In some specimens, mostly mature females bearing eggs, there was less vitamin A ester than alcohol. This fact, coupled with the presence of large concentrations of vitamin A alcohol in the eggs of, for example, *A. media*, *Sepia officinalis* and *Loligo forbesi*, indicates that the liver reserves of vitamin A are used up in the development of the eggs, possibly even to the point of absolute depletion, as in *Alloteuthis* (see Table I), where five of the six specimens contained ripe eggs and the other no gametes at all, probably being spent.

Vitamin A was found in the eyes of all the cephalopods studied, except *Parasepia elegans*, *Rossia macrosoma* and *Todaropsis eblanae*, and it was predominantly or entirely in the alcohol form, although the aldehyde (retinene) was found in those species, *Loligo forbesi* and *Ommastrephes pteropus*, for which the special technique for its separation was employed. When this technique was used for the eyes of *Loligo forbesi* the values we obtained for vitamin A alcohol were about one-tenth of those given by Wald (1941) for *L. pealii*, but the concentrations of retinene were of the same order in both species. It is difficult to reconcile our findings of vitamin A and carotenoids in other parts of *L. forbesi*, especially the liver, with their complete absence, except for the eyes, in *L. pealii*, but, in conversation, Prof. Wald has told us that his investigation, done some years ago, on those parts other than the eyes might well be repeated. According to Brachi (1953), vitamin A was absent from *L. forbesi* and *Sepia officinalis* at Plymouth, but our analytical technique was probably more sensitive than his, normally applied to the more massive quantities of vitamin A found in whale-liver oils.

In the eyes of *Ommastrephes pteropus* the values for both vitamin A and retinene were similar to those found by Wald (1941) in *Loligo pealii*. There are, thus, no qualitative differences between the vitamin A components in the visual cycles of squids living, like *Ommastrephes*, at great depths and those, like *Loligo*, in shallower waters. What is striking is that not only is there a remarkable resemblance between the structures of the cephalopod and vertebrate eyes, as Berrill (1951, p. 200) has pointed out at some length, but also that they function biochemically in a similar way. We have already noted the storage of vitamin A in the cephalopod liver as a vertebrate characteristic, found so far nowhere else among invertebrates, unless the storage of rich concentrations of vitamin A ester in the eyes of euphausiid Crustacea (Fisher *et al.*, 1952) be taken as similar in purpose.

Another interesting resemblance between the vitamin A metabolism of cephalopods and that of vertebrates is indicated by the presence of vitamin A alcohol in ripe eggs, just as found by Neff, Parrish, Hughes & Payne (1949) in hen eggs, where 73–93% of the total vitamin A was in the alcohol form. Parrish, Williams & Sanford (1951) subsequently showed that it was gradually converted to the ester and stored in the embryonic liver during incubation of

the chick. No similar observations on developing cephalopod eggs have yet been made. The absence of vitamin A from the ova of *Rossia macrosoma* and *Parasepia elegans* was presumably associated with their immaturity. Lönnberg (1935) reported a positive reaction with antimony trichloride which he described as 'surprisingly strong' for a colourless extract from ripe eggs of *Rossia macrosoma*, which would undoubtedly be due to vitamin A.

From our limited findings the cephalopod groups, squids, cuttlefishes or octopods appear to be fairly uniform in their vitamin A content. The concentrations of carotenoids were comparatively low in all species. In fact, Goodwin (1952) says of the cephalopods: 'The most outstanding fact concerning carotenoids in this group is their comparative absence'. Nevertheless, differences could be detected. The squids were, generally, poorer in carotenoids than cuttlefishes or *Eledone*. This difference may be because they are more active and feed, therefore, on pelagic Crustacea, which usually contain fewer carotenoid pigments than benthic species, very often only astaxanthin being present in the swimming Crustacea, whereas carotenes and xanthophylls occur in other species (Fisher *et al.*, 1952, 1953). We never found astaxanthin in cephalopods in more than faint traces, indicating that it is either little absorbed or is broken down quite rapidly.

Cephalopod livers contained the highest concentrations of carotenoids, but these were extremely variable even within a species. The carotenoids in the livers were usually carotenes and xanthophylls, but carotenes were not found in the livers of *Todaropsis* and *Rossia*. The livers of these two species, and of *Ommastrephes*, did, however, contain traces of astaxanthin.

In a study of the carotenoids of *Paroctopus bimaculatus*, Fox & Crane (1942) found xanthophylls and their esters in the ink. They suggested that this might be a unique method of excreting carotenoids. In some of the species we examined, the ink sacs and their contents were analysed, but not the ink separately. No carotenoids were found in the ink sacs of the squids, *Loligo forbesi* and *Todaropsis eblanae*, but xanthophylls or their esters were present in those of the cuttlefishes, *Parasepia elegans* and *Sepia officinalis*. The ink sacs of the other species studied, including the only octopod, *Eledone cirrosa*, were not analysed separately. We have already pointed out that the cuttlefishes and octopods are richer in carotenoids than the squids, which probably, therefore, do not require any special means of excreting the pigments.

The curves in Fig. 1 show that the absorption spectrum of cephalopod vitamin A is similar to that of all-*trans* vitamin A. The biological activity of squid-liver oil agreed with the potency determined by the Carr-Price test and there is no evidence of a discrepancy such as we found (Fisher *et al.*, 1952) in euphausiid Crustacea between the potencies determined physicochemically and biologically. The presence of all-*trans* vitamin A rather than other isomers shows another resemblance between cephalopods and vertebrates and a difference between them and other invertebrates.

Cephalopods feed mainly on Crustacea, most of which contain vitamin A. As we have already mentioned, the pelagic cephalopods probably feed on swimming Crustacea, among which, because of their swarming habits, the euphausiids must form an important part of the diet. Hjort & Ruud (1929) mention that euphausiids are important as food of the squid, *Gonatus fabricii*, which in its turn forms part of the diet of the bottle-nosed whale, *Hyperoodon rostratus*. Our work on vitamin A in Crustacea so far indicates that the euphausiids are the richest in vitamin A of all the Crustacea (Fisher *et al.*, 1955). They may thus be the main source of preformed vitamin A for the cephalopods which store it and pass it on to their predators, especially the toothed whales. It would be difficult to determine whether these whales eat sufficient cephalopods to obtain all their rich liver reserves of vitamin A preformed, since the feeding rates of whales are unknown. The enormous numbers of cephalopod beaks to be seen attached to the stomach lining of a sperm whale probably represent only a small proportion of the total intake, which may well be adequate.

The cephalopods are the most highly organized of the invertebrates and they exhibit several similarities to the vertebrates and few resemblances to other molluscs in their vitamin A metabolism (Fisher *et al.*, 1956). Perhaps it is more than coincidental that they, the richest in vitamin A of all the molluscs except perhaps certain pteropods, form the main food of the toothed whales just as the euphausiids, the richest in vitamin A of all the Crustacea, form the main food of the baleen whales. Furthermore, the euphausiids are probably one of the principal components of the diet of the cephalopods themselves.

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Summary

Ten species of cephalopods, namely, *Alloteuthis media*, *Loligo forbesi*, *Ommastrephes pteropus*, *Todaropsis eblanae*, *Sepia officinalis*, *Parasepia elegans*, *Rossia macrosoma*, *Sepietta oweniana*, *Sepiola* sp. and *Eledone cirrosa*, have

been analysed for fat, vitamin A and carotenoids. Vitamin A was present in all species. Carotenoids were found in most species but concentrations were low.

Vitamin A was found mainly as the ester, in largest amounts in the liver, but in females these reserves were depleted by developing eggs, which were rich in vitamin A alcohol. The vitamin was usually present in the eyes, mostly as the aldehyde (retinene) in those species, *Loligo forbesi* and *Ommastrephes pteropus*, in which it was separated, indicating a visual cycle biochemically resembling that of vertebrates.

Cuttlefishes and octopods were richer in carotenoids than squids, probably owing to the more varied and richer carotenoid content in the benthic Crustacea of their diet than in the pelagic Crustacea forming the food of squids. There is evidence that cuttlefishes and octopods excrete excess carotenoids in their ink, whereas squids do not.

Cephalopod vitamin A is probably all-*trans* vitamin A, and there was complete agreement between the potencies, determined chemically and biologically, of vitamin A from squid livers and eyes.

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