THE VITAMIN A OF THE LOBSTER

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Vitamin A appears to play much the same role in the vision of many invertebrates as it does in the vertebrate eye. In the squid, *Loligo pealii*, the retina contains vitamin A and a high concentration of retinene, the latter mostly bound in the visual pigment rhodopsin (Wald, 1941; Bliss, 1948; St. George and Wald, 1949). The eyes of the fresh water crayfish, *Cambarus virilis*, contain both vitamin A and retinene as well as astaxanthin. Those of marine decapod crustacea—green and fiddler crabs, lobsters—contain high concentrations of vitamin A in addition to astaxanthin and other carotenoids found in the integument (Wald, 1941, 1943, 1945–46). Recently Fisher, Kon, and Thompson (1952, 1954, 1955) have found particularly large amounts of vitamin A in the eyes of euphausiid crustacea.[1]

It is a remarkable fact that these animals contain little or no vitamin A in their bodies. In the squid no trace of vitamin A or other carotenoid could be found in the whole bodies less the eyes (Wald, 1941).[2] More than 90 per cent of the total vitamin A in euphausiid crustacea is found in their eyes. Frequently vitamin A could not be identified at all in other tissues, or in whole bodies less the eyes. The same was true of a number of decapods: lobsters, green crabs, and shrimp (Fisher *et al.*, 1952, 1955).[3]

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[1] Lönnberg (1935, 1936) has reported the presence of carotenoids in extracts of crustacean and octopus eyes. Positive antimony chloride tests were obtained from these extracts, but vitamin A was not explicitly identified.

[2] Fox and Crane (1942) found no carotenoids in the Pacific squid, *Loligo opalescens*; but did find them in the octopus, *Paroctopus*, confined to the hepatopancreas. Vitamin A was not investigated.

Recently Fisher, Kon, and Thompson (1956) have reported finding vitamin A in the livers of a European squid, *Loligo forbesi*, in higher concentrations and amounts than in the eyes. In both organs the vitamin A appears to be all-*trans* by both chemical and biological test.

[3] Neilands (1947) has reported finding vitamin A in the hepatopancreas of the American lobster. He gives the following measurements, made at the end of a nutri-

It should be noted also that no one has yet demonstrated symptoms of vitamin A deficiency in an invertebrate. Attempts to produce this condition in the cockroach and clothes moth failed (Crowell and McCay, 1937; McCay, 1938; Bowers and McCay, 1940); indeed a large body of work on insect nutrition has as yet failed to reveal any need of the fat-soluble vitamins A, D, or E (cf. Trager, 1953). "The preeminently *visual* role of vitamin A appears with great force in these animals; and indeed no evidence exists that in invertebrates it exercises other functions" (Wald, 1943).

In the vertebrate eye, vision involves a cycle of geometrical isomerization of vitamin A. The rod pigment, rhodopsin, is synthesized, over the intermediate aldehyde retinene, from a specific *cis* isomer of vitamin A called neo-*b*, and bleaches to yield all-*trans* vitamin A. This in turn—or the corresponding retinene—must be reisomerized to neo-*b* for vision to continue (Hubbard and Wald, 1952–53; Hubbard, 1955–56). The same isomerization cycle is associated with the cone pigment, iodopsin (Wald, Brown, and Smith, 1954–55).

Fisher *et al.* (1952) have made the surprising discovery that euphausiid vitamin A is only about half as potent as an equal amount of all-*trans* fish liver vitamin A, when fed to rats. It was difficult for a time to imagine what this could mean; but we know now that the various geometrical isomers of vitamin A have very different biological potencies (Ames, Swanson, and Harris, 1955). If the all-*trans* isomer is assigned the potency 100, neo-*a* (13-*cis*) has the potency 75.3, and the remaining natural isomers potencies of 22 to 24 (iso-*a* (9-*cis*); iso-*b* (9, 13-*dicis*); neo-*b* (11-*cis*)). It seemed possible therefore that crustacean vitamin A includes large proportions of low potency *cis* isomers.

We have begun by examining the vitamin A of the lobster, *Homarus americanus*. The vitamin A of the eye—and hence of the whole animal—is primarily the neo-*b* isomer. Indeed the properties of our preparations do not depart sufficiently from those of pure neo-*b* vitamin A to suggest that any other isomer is present.

This is an astonishing condition. Neo-*b* vitamin A is a sterically hindered *cis* form, the first to be found in nature (Wald *et al.*, 1955; Oroshnik, 1956; Oroshnik *et al.*, 1956). In true thermodynamic equilibrium with other isomers, it occurs in very small proportion (Hubbard, 1955–56). It is an intrinsically improbable structure, probably always requiring special energy for its formation, and special conditions for its retention. We have been unable so far to find it in any vertebrate tissue outside the eye; and in eye tissues it has never exceeded 25 to 40 per cent of the vitamin A present (P. H. Brown, N. I. Krinsky, unpublished observations). We do not yet understand how vertebrate tissues accumu-

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tional experiment lasting 48 days: vitamin A per gram, fresh weight: *Hepatopancreas*, (1) natural feeding, 83.9 ± 11.5 i.u.; (2) diet without carotene: 35.9 ± 11.0; (3) diet with added carotene: 53.3 ± 15.4. *Eyes*, (1) diet without carotene, 100.0 ± 5.6 i.u.; (2) diet with added carotene, 183.3 ± 58.1.

late and retain such high proportions of the neo-*b* isomer. That the lobster eye contains this isomer virtually alone is strange indeed. It shows that mechanisms exist which can circumvent all the apparent improbabilities of this situation, and can provide whatever neo-*b* vitamin A the tissues may require.

In this investigation we have encountered for the first time the problem of identifying and measuring the neo-*b* isomer in tissue extracts. For this reason we describe our procedures in detail.

EXPERIMENTS

Material.—All the experiments were performed in December and January, on Nova Scotia lobsters which had been kept in running sea water for 1 to 3 weeks. The eye stalks, on removal from the animals, were immediately frozen, and kept dark and frozen for 1 to 2 weeks before being used.

At the end of the eye stalk is the deep purple, almost black area containing the ommatidia, covered by the relatively soft corneal tissue (Fig. 1). This area was cut away from the remainder of the eye stalk with a razor blade. The cut was made far enough back on the stalk to include all the deeply pigmented tissue. This was collected from about 100 eyes at a time, on ice. The tissue was ground with an equal volume of anhydrous sodium sulfate until dry, then extracted repeatedly with petroleum ether until the extracts were colorless. Finally the residual tissue was extracted with acetone, which should have removed any adsorbed or protein-bound vitamin A. No vitamin A was ever detected in such acetone extracts; apparently all the vitamin A of the eye is free and extractable with petroleum ether.

The optic nerves were pulled out of the stumps of the eye stalks. This tissue was much larger in bulk than the retinas. It was treated in exactly the same way.

Hepatopancreases—four of wet weight 1.77 gm.—were ground with anhydrous sodium sulfate until dry and extracted similarly.[4]

Total Retinal Vitamin A and Carotenoids.—The retinal extract contains vitamin A, a large quantity of astaxanthin, and a small amount of carotene (apparently mainly β-).

Astaxanthin was determined by measuring the absorption spectrum of the total extract in pyridine. Almost the whole absorption in the visible region is caused by this pigment; only a small correction, of the order of 5 per cent, is required for the carotene present.

Carotene is measured after removing the astaxanthin by saponification. An aliquot of the total extract of retinas is brought into 6 per cent KOH in methanol, and incubated at 40°C. for 20 minutes. This process converts the astaxanthin to astacene. The saponification mixture is diluted with 40 per cent its volume of water, and extracted repeatedly with petroleum ether. The combined petroleum ether extracts are washed twice with 4 per cent KOH in 60 per cent methanol, to remove the last traces of astacene. They contain a yellow pigment, almost entirely epiphasic in partition with 90 per cent methanol, and possessing absorption bands in hexane at about

[4] The experiments on the hepatopancreas and the optic nerve were performed by Marilyn Thompson, whom we should like to thank for permission to cite her findings.

447 and 475 mμ (β-carotene in hexane: 450, 477 mμ). The concentration is estimated from the extinction at 450 mμ, on the basis that at this wave length β-carotene has *E* (1 per cent, 1 cm.) 2500.

Vitamin A is determined by the antimony chloride test in the non-saponifiable fraction. Too little carotene is present in this fraction to interfere with the measurement. The solution is transferred to dry chloroform, and 1 ml. mixed with 2.2 ml. of saturated antimony trichloride solution in chloroform. The spectrum is recorded immediately. A single sharp absorption band is obtained, with λ_{max} 618 mμ, characteristic of vitamin A. From its extinction the concentration of vitamin A is estimated, on the basis that *E* (1 per cent, 1 cm.) at 618 mμ is 4400 (Cawley *et al.*, 1948).

The results of such measurements, carried out with a batch of 155 eyes, are as follows: Astaxanthin, 3.0 μg. per eye; carotene, 0.144 μg. per eye; vitamin A, 1.18 μg. per eye.[5]

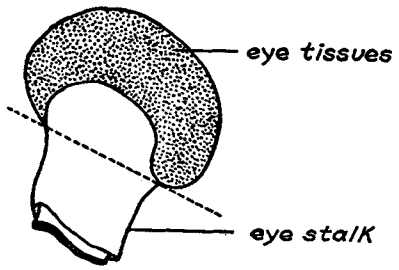

FIG. 1. Diagram of the lobster eye. The broken line shows roughly where the eye was divided so as to separate the retinal tissues, which are deeply pigmented, from the eye stalk containing the optic nerve.

Other Tissues.—No vitamin A could be detected in the optic nerve or in the combined petroleum ether and acetone extracts of the hepatopancreas.

Alcohol and Ester Fractions of Vitamin A.—The vitamin A extracted from the retina was separated into free alcohol and ester fractions by the chromatographic procedure of Ganguly *et al.* (1952). A 3 ml. aliquot of the total retinal extract in petroleum ether was adsorbed on a column of alumina (Merck, "suitable for chromatographic adsorption") which had been weakened by stirring with 5 per cent its weight of water. Then 25 ml. of 4 per cent acetone in petroleum ether were run through to elute vitamin A esters; followed by 25 ml. of 40 per cent acetone in petroleum ether to elute the free vitamin A alcohol. Each of these eluates includes portions of astaxanthin, adsorbed originally at the top of the column. To remove this, both fractions are saponified as described above, and then brought into solution in hexane. The absorption spectrum of each is measured; then each is isomerized by adding 5 μg. iodine in petroleum ether, and irradiating until no further changes occur (12 to 14 minutes)

[5] Fisher *et al.* (1954) report the following average measurements on extracts of eyes of the European lobster, *H. vulgaris*: vitamin A, 0.97 μg. per eye; carotenoids (predominantly astaxanthin with a little xanthophyll), 15 μg. per eye.

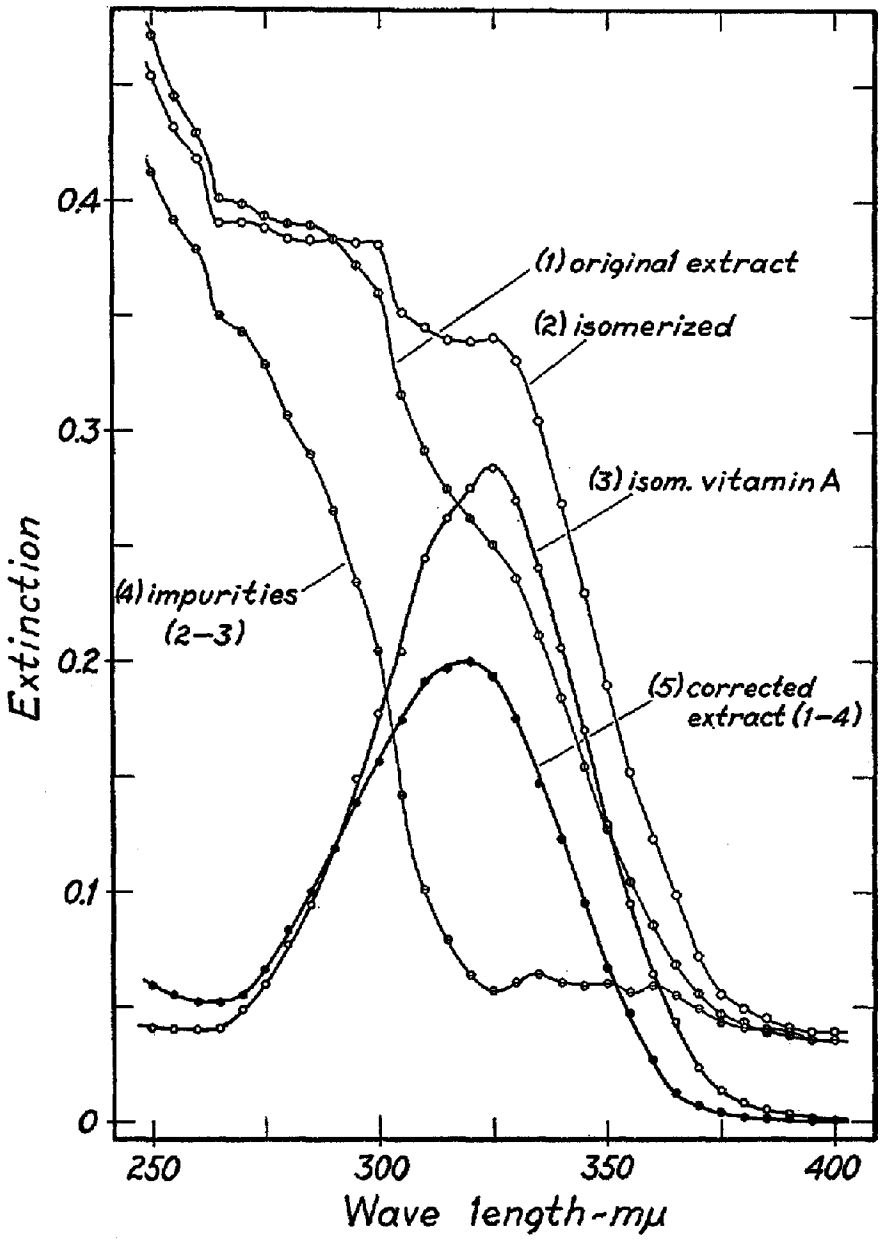

FIG. 2. Absorption spectra of lobster vitamin A and of the product of its isomerization. (1) Spectrum of an untreated extract of lobster eyes in hexane. (2) Spectrum remeasured after complete isomerization of the vitamin A with light in the presence of a trace of iodine. The concentration of vitamin A in this solution having been determined by the antimony chloride test, (3) shows the absorption spectrum of this concentration of pure, completely isomerized vitamin A. (4) The difference between this and (2), representing the spectrum of the impurities present. (5) The difference between (1) and (4), hence the absorption spectrum of the vitamin A present initially. This has the properties of neo-*b* vitamin A.

(see below). After remeasuring the absorption spectra of the isomerized solutions, they were brought into chloroform and their vitamin A content measured with antimony trichloride.

Of a sample containing originally 22.0 μg. vitamin A, 86 per cent was accounted for, of which 30 per cent was in the free alcohol fraction, 70 per cent in the ester fraction. Other experiments yielded similar results. Isomerization tests showed both fractions to consist equally of the neo-*b* isomer.

Isomerization and Derivation of Absorption Spectra.—Initially we worked with extracts and partly purified preparations which, though they yielded excellent antimony chloride tests for vitamin A, possessed very poor absorption spectra, owing to the presence of much extraneous material. On adding a trace of iodine to such solutions and irradiating with white light to isomerize the vitamin A present, the extinction in the vitamin A region rose considerably. This in itself suggested the presence of the neo-*b* isomer, which on isomerization exhibits by far the largest rise in extinction of all the natural isomers of vitamin A.

Eventually we worked out a procedure by which the spectra of the pure vitamin A initially present and of the product of its isomerization can be derived from even very crude extracts. This procedure is illustrated in Fig. 1 and Table I.

Curve (1) in Fig. 2 is the absorption spectrum of an untreated extract of lobster eyes in hexane. To 3 ml. of this solution, 2 drops of a stock iodine solution in petroleum ether, containing 0.15 μg. iodine, were added, and the solution was exposed for 2 to 3 minutes to the focussed white light of a 160 watt projection lamp passing through a neutral filter of density 1.3 (intensity *ca.* 30 foot-candles). Control experiments showed that this procedure produces complete isomerization.[6] The absorption spectrum had changed to curve (2) (Table I, columns (1) and (2)).

This solution was transferred quantitatively to chloroform and its content of vitamin A measured in the antimony chloride reaction. In this test all the geometric isomers of vitamin A yield the same result, a sharp absorption band maximal at 618 mμ with E (1 per cent, 1 cm.) 4400. This measurement tells how much vitamin A the original solution contained.

In a control experiment we have isomerized completely in the same way pure all-*trans* vitamin A, and measured the accompanying changes in absorption spectrum (Table II). All-*trans* vitamin A in hexane has its absorption peak at 325 mμ with E (1 per cent, 1 cm.) 1820. Isomerization lowers this maximum extinction to 0.94 its original height, or an E (1 per cent, 1 cm.) of 1711. The product of complete isomerization is the same regardless of the isomer or mixture of isomers used as starting material. Knowing the amount of vitamin A present, and the shape and height of its spectrum in the completely isomerized form, we can draw the latter as curve (3) in Fig. 2 (column (3) in Table I). The difference between it and curve (2) represents the absorption spectrum of the impurities present in the original solution (curve (4);

[6] No directions for complete isomerization are reliable in every instance. The presence of carotenoids and other substances which absorb iodine, including at times traces of aromatic hydrocarbons in the solvent itself, may slow the reaction. The only safe procedure is to follow the isomerization spectrophotometrically until no further changes occur.

TABLE I

Derivation of Absorption Spectrum of Pure Vitamin A from That of a Crude Extract

The spectrum of the extract in hexane is measured (1); then the vitamin A in the extract is isomerized (2). An antimony chloride test shows the extract to contain 1.62 μg. per ml. vitamin A. From Table II the spectrum of this concentration of pure isomerized vitamin A is obtained (3). Columns (2) minus (3) yield the spectrum of the impurities present (4). This subtracted from (1) is the spectrum of the vitamin A present initially (5).

Wave length	Extinctions				
	(1) Extract	(2) Isomerized extract	(3) Isomerized vitamin A (1.62 μg. per ml.)	(4) Impurities	(5) Vitamin A present initially
mμ					
400	0.036	0.038	0.001	0.037	−0.001
395	0.036	0.038	0.002	0.036	0.000
390	0.037	0.041	0.003	0.038	−0.001
385	0.039	0.045	0.005	0.040	−0.001
380	0.043	0.049	0.008	0.041	0.002
375	0.047	0.056	0.013	0.043	0.004
370	0.054	0.072	0.023	0.049	0.002
365	0.067	0.098	0.043	0.055	0.012
360	0.086	0.123	0.064	0.059	0.027
355	0.104	0.152	0.095	0.057	0.047
350	0.127	0.189	0.129	0.060	0.067
345	0.154	0.229	0.170	0.059	0.095
340	0.184	0.262	0.206	0.061	0.123
335	0.211	0.304	0.240	0.064	0.147
330	0.236	0.331	0.270	0.061	0.125
325	0.250	0.341	0.284	0.057	0.193
320	0.262	0.338	0.275	0.063	0.199
315	0.275	0.340	0.262	0.078	0.197
310	0.292	0.345	0.244	0.101	0.191
305	0.316	0.351	0.209	0.142	0.174
300	0.360	0.381	0.177	0.204	0.156
295	0.372	0.382	0.148	0.234	0.138
290	0.383	0.384	0.119	0.265	0.118
285	0.389	0.383	0.094	0.289	0.100
280	0.390	0.384	0.072	0.302	0.083
275	0.394	0.387	0.059	0.328	0.066
270	0.398	0.391	0.048	0.343	0.055
265	0.402	0.391	0.041	0.350	0.052
260	0.430	0.418	0.040	0.378	0.052
255	0.047	0.432	0.040	0.392	0.055
250	0.471	0.453	0.041	0.412	0.059

also column (4) of Table I). The latter subtracted from curve (1) yields the absorption spectrum of the pure vitamin A initially present (curve (5); also column (5) in Table I).

This procedure has yielded very good results, when applied to lobster preparations

as extracted and in various stages of purification. It has of course much broader application, since it can be used generally to derive pure vitamin A spectra from solutions however contaminated. It involves the assumption that on adding a trace of iodine and irradiating, the only spectral changes observed are caused by the isomeriza-

TABLE II

Isomerization of All-Trans Vitamin A

To a solution of about 10 μg. vitamin A in 3 ml. hexane, 2 drops of hexane solution containing 0.20 μg. iodine are added. This mixture is exposed for 2 minutes to white light of intensity about 30 foot-candles.

Wave length	*E* (1 per cent, 1 cm.) of vitamin A	
	All-*trans*	Isomerized
mμ		
400	0	0
395	5	4
390	8	7
385	19	18
380	34	31
375	66	63
370	137	134
365	261	256
360	420	412
355	585	575
350	793	781
345	1055	1034
340	1310	1273
335	1530	1472
330	1715	1652
325	1820	1711
320	1743	1641
315	1664	1578
310	1564	1460
305	1361	1250
300	1133	1073
295	952	903
290	765	737
285	623	610
280	507	508
275	446	443
270	414	417

tion of vitamin A. This is apparently not a difficult criterion to satisfy. Even though carotenoids were present in some of the extracts with which we worked, this procedure yielded accurate results, probably because the carotenoids were already in the all-*trans* condition and were changed only slightly by isomerization. In general, it is safer to exclude carotenoids from the preparations.

Curve (5) in Fig. 2 presents a reasonably accurate absorption spectrum of neo-*b*

vitamin A. A considerable number of parameters so identify it. For comparison, the absorption spectra of pure neo-*b* vitamin A and of the product of its isomerization are shown in Fig. 3. The absorption maximum of lobster vitamin A (Fig. 2, curve (5)) lies at 319 to 320 mμ, about 5 to 6 mμ toward shorter wave lengths from the all-*trans* position. Knowing the concentration of vitamin A which yielded this spectrum, we can calculate the E (1 per cent, 1 cm.). This, in three experiments, averages 1250; in pure neo-*b* vitamin A it averages 1200. On isomerization, λ_{max} shifts from 320 to

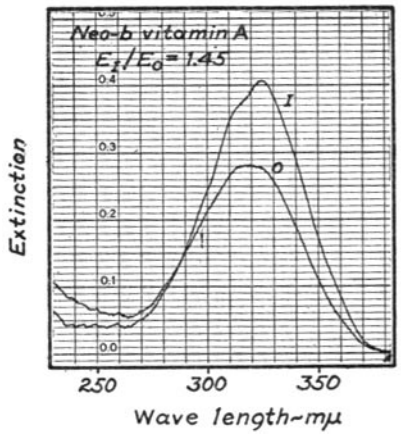

FIG. 3

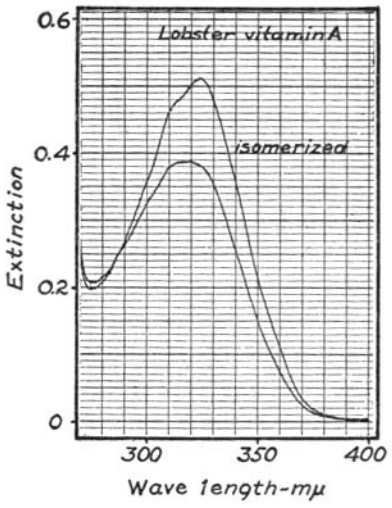

FIG. 4

FIG. 3. Absorption spectra of neo-*b* vitamin A in hexane (0) and of the product of its complete isomerization by light in the presence of a trace of iodine (I). In this figure the extinctions are for an arbitrary concentration; E (1 per cent, 1 cm.) = 1200, and is raised 1.45 $\pm$ 0.05 by isomerization, with characteristic changes in the shape of the absorption band. An isosbestic or common point appears at 287 mμ. All these changes are exhibited also by the derived spectra of lobster vitamin A (Fig. 2, curves 3 and 5), and by the directly measured spectra of a purified preparation (Fig. 4).

FIG. 4. Absorption spectra of a partly purified preparation of vitamin A from the lobster eye, dissolved in hexane, and of the product of its complete isomerization. The properties of the original preparation and its changes on isomerization are characteristic of neo-*b* vitamin A.

325 mμ, and the absorption band changes characteristically in shape, its E_{max} rising by a factor of 1.48 (average). The corresponding factor for pure neo-*b* vitamin A is 1.45 $\pm$ 0.05. As shown in Fig. 2, though the absorption rises on isomerization over most of the spectrum, it falls in the ultraviolet. A crossing point occurs at which the extinction does not change (common or isosbestic point). In the present experiments this lies at about 287 mμ, just as with pure neo-*b* vitamin A. These relationships are summarized in Table III. They show that the vitamin A of the lobster eye possesses very nearly the properties of the pure neo-*b* isomer.

Purification of Vitamin A.—An extract of lobster eyes was saponified by incubating in 6 per cent KOH at 40°C. for 20 minutes, converting all the astaxanthin present to astacene. The saponification mixture was diluted with 40 per cent its volume of water and extracted repeatedly with petroleum ether. The astacene remains behind in the

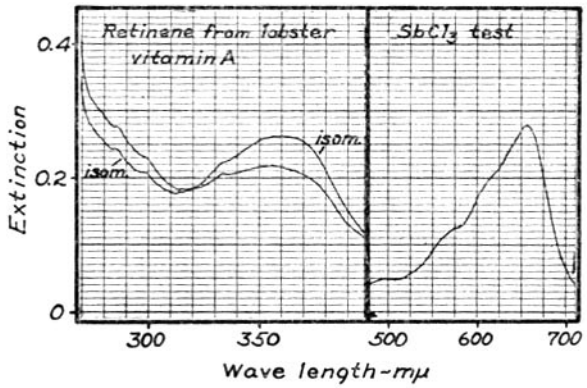

FIG. 5. *Left*, Absorption spectra of an impure solution of retinene in hexane, prepared by oxidizing lobster vitamin A, and of the product of its complete isomerization by light. The large rise in extinction of the retinene on isomerization is characteristic of the neo-*b* isomer. *Right*, Antimony chloride test with a sample of this material. The antimony chloride test is dominated by the retinene band at 666 mμ.

TABLE III

Properties of Vitamin A Extracted from the Lobster Eye, and of the Pure Neo-b Isomer

Experiments 2, 3, and 4 involve computations of the absorption properties of lobster vitamin A as described in the text. The next line shows the properties of a purified preparation of lobster vitamin A, measured directly. The last line shows the properties of pure neo-*b* vitamin A.

Material	λ_{max}	Rise of E_{max} on isomerization	E (1 per cent, 1 cm.) in hexane	Isosbestic point in isomerization
	mμ			mμ
Experiment 2	318	1.44	1290	287
Experiment 3	319	1.49	1230	286
Experiment 4	319	1.50	1220	—
Purified sample	319	1.38	1300	285
Neo-*b* vitamin A	318	1.45 ± 0.05	1200	287

saponification mixture. The combined petroleum ether layers were washed twice with fresh 6 per cent KOH in methanol diluted with 40 per cent its volume of water, to remove the last traces of astacene. The petroleum ether solution was evaporated to dryness under reduced pressure, and taken up in fresh solvent. It contained vitamin A and a small amount of carotene. It was chromatographed on a column of calcium carbonate, developed with further petroleum ether. The spectrum of each 2 ml. frac-

tion was measured as it emerged from the column. A fairly pure sample of vitamin A alcohol was collected just in front of the main carotene band. Its absorption spectrum is shown in Fig. 4, together with the spectrum following isomerization. It possessed the following properties: λ_{max} 319 to 320 mμ; E (1 per cent, 1 cm.) 1300; rise of E_{max} on isomerization, 1.38; isosbestic point on isomerization at 285 mμ. These properties are close to those of pure neo-*b* vitamin A (Table III), though departing sufficiently to suggest that slight isomerization may have occurred, perhaps caused by the saponification procedure.

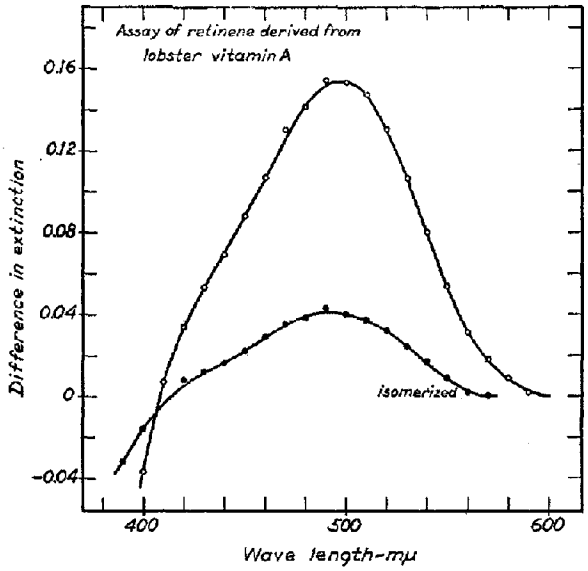

FIG. 6. Difference spectra of photosensitive pigments synthesized from retinene derived from lobster vitamin A. Of two identical samples of the retinene in digitonin solution, one was isomerized by exposure to light. Then both were mixed with excess cattle opsin, and incubated in the dark. At the end of incubation hydroxylamine was added to both solutions, and the difference spectra were measured—the difference in spectrum before and after bleaching. The unisomerized retinene had formed rhodopsin (λ_{max} 498 mμ). The isomerized sample had formed only about one-fourth as much photosensitive pigment, a mixture of rhodopsin and isorhodopsin (λ_{max} 487 mμ).

Oxidation to Retinene.—A microprocedure for this was worked out in preliminary experiments. To a sample of the purified lobster vitamin A containing 20 μg. of the vitamin in 2.5 ml. petroleum ether, 1 mg. of powdered manganese dioxide was added. This mixture was left in the dark at room temperature for 4 hours. It was centrifuged, and the clear supernatant poured off. The absorption spectrum is shown in Fig. 5. In addition to the band of retinene, it displays considerable extraneous absorption.

This preparation was isomerized with light in the presence of iodine. The absorption rose considerably, and λ_{max} shifted toward shorter wave lengths. Then 2 ml. of

this solution were transferred to 1 ml. chloroform, and 2.2 ml. antimony chloride reagent were added. The absorption spectrum of the resulting blue product is shown in Fig. 5 at the right. It displays the typical retinene band at 666 mμ, and a small inflection at about 620 mμ due to a small residue of vitamin A.

A calculation comparable with that illustrated in Fig. 2 can be performed with the data of Fig. 5. From the antimony chloride test at the right, on the basis that E (1 per cent, 1 cm., 666 mμ) = 3740, one can calculate that the retinene concentration at the left was 1.19 μg. per ml. Knowing the absorption spectrum of pure isomerized retinene, and that its E (1 per cent, 1 cm.) = 1570 (Brown and Wald, 1956), one can draw the absorption spectrum corresponding to this concentration. This, subtracted from the spectrum of the isomerized solution in Fig. 5, yields the spectrum of the impurities present; and this in turn, subtracted from the spectrum of the unisomerized solution, yields the spectrum of the original retinene.

The results of such a calculation show that the original retinene had λ_{max} 365 mμ. On isomerization, λ_{max} shifted to 367 mμ, and E_{max} rose 1.4 times. With pure neo-*b* retinene on isomerization λ_{max} shifts from 362.5 to 367 mμ, and E_{max} rises 1.7 times (Brown and Wald, 1956). It appears from this that our preparation contained about 69 per cent neo-*b* and 31 per cent all-*trans* retinene. Some isomerization had evidently occurred in the course of our procedures; yet clearly neo-*b* was still the major component of this retinene preparation.

Synthesis of Rhodopsin.—A sample of this retinene was dissolved in 2 per cent digitonin solution at pH 6.4. Two portions, each of 0.2 ml., were pipetted into microcells of capacity 0.6 ml. One portion was isomerized by irradiating for 3 minutes in the focussed light of a 160 watt projection lamp passing through Corning filters 3389 and 3962 to remove ultraviolet and heat radiation. Then 0.2 ml. of cattle opsin in 2 per cent digitonin was added to each cell, and also to 0.2 ml. of digitonin solution in a third cell to serve as blank. The opsin was in excess compared with the retinene. After 60 minutes' incubation in the dark at 23°C., 0.1 ml. of 1 M hydroxylamine was added to each cell, and the absorption spectrum was measured before and after bleaching. The difference spectra which resulted—the differences in absorption before and after bleaching—are shown in Fig. 6.

The retinene prepared from lobster vitamin A had formed rhodopsin, with λ_{max} 498 mμ. After isomerization, this retinene formed only about one fourth as much photosensitive pigment, with λ_{max} displaced to about 495 μ. Isomerization had converted the predominantly neo-*b* isomer to a mixture of isomers, predominantly all-*trans*, and containing about 25 per cent neo-*b* and iso-*a*, which formed a corresponding mixture of rhodopsin and isorhodopsin (λ_{max} 487 mμ). This is the usual result of isomerizing retinene in aqueous solution (Hubbard and Wald, 1952–53).

A calculation based on the data of Fig. 4 shows that before isomerization the retinene employed in this synthesis had E_{max} 0.121. It formed rhodopsin with E_{max} 0.152, a rise of 1.25 times. When pure neo-*b* retinene forms rhodopsin, E_{max} rises 1.5 to 1.7 times (Brown and Wald, 1956). Therefore the retinene in this preparation was 55 to 73 per cent neo-*b*. This is close to the neo-*b* content estimated from the isomerization experiment.

We can conclude that though the retinene prepared from lobster vitamin A consisted mainly of the neo-*b* isomer, some isomerization to all-*trans* had occurred in the course of our procedures. Control experiments with pure neo-*b* vitamin A show that these operations can be performed with much less isomerization; and we have no doubt that further work would show this to be true also of lobster preparations.

CONCLUSION

The vitamin A of the lobster eye has properties so close to those of pure neo-*b* vitamin A that one cannot be sure that any other isomer is present. It has already been remarked that this is a strange condition. Neo-*b* is a hindered *cis* form, normally expected to occur in very small amount. A careful examination in our laboratory of fish and cattle liver oils and cattle blood plasma has failed so far to reveal any of this isomer (P. H. Brown, unpublished observations). Direct measurement of the equilibrium established in aqueous solution in the dark between all-*trans* and neo-*b* retinene shows these isomers to be present in the proportion 95:5 (Hubbard, 1955–56). Clearly the lobster succeeds somehow in evading all the conditions that ordinarily make the neo-*b* isomer difficult to obtain and keep, and manages to store it in the eye virtually pure. One of the most puzzling problems in the chemistry of vertebrate vision is to understand how the eye obtains sufficient neo-*b* vitamin A to maintain rod and cone vision. Perhaps this will cease to be a problem, once we understand how the lobster performs the much greater feat of storing this isomer virtually alone.

Recently Dr. Kon sent us an oil extracted with petroleum ether from the eyes of the euphausiid crustacean, *Meganyctiphanes norvegica*. This was examined using the procedures described in the present paper (*cf.* also Brown and Wald, 1956). At least 90 per cent of the vitamin A was found to be in the form of the neo-*b* isomer. As in the present experiments, one could not be certain that any other isomer of vitamin A was present (Wald and Brown, 1956–57).[7]

It is possible therefore that Eucarid crustacea in general—decapods and euphausiids—store this isomer in the eye. This would explain in large part the observation of Fisher *et al.* (1952) that the vitamin A from euphausiid eyes has only about half the biological potency in rats expected of it on the basis of chemical and physical tests. Compared with all-*trans* vitamin A, the neo-*b* isomer has a biopotency of about 23 per cent (Ames *et al.*, 1955). When allowance is made for variations in bioassays performed in different laboratories, and for the possibility of small and varying amounts of other isomers of vitamin A in euphausiid oils, this discrepancy is perhaps not serious.

The hypothesis that crustacean vitamin A may in general be primarily the

[7] Kampa (1955) has reported the extraction from the eyes of *Euphausia pacifica* of a light-sensitive pigment, "euphausiopsin," with λ_{max} (difference spectrum) about 462 mμ. Following bleaching, these extracts contained vitamin A, but no retinene. Control extracts made prior to bleaching are not mentioned.

neo-*b* isomer accounts for the observation of Lambertsen and Braekkan (1955) that vitamin A extracted from the eyes of the deep sea prawn, *Pandalus borealis*, possesses an ultraviolet absorption band lying "to the left from the expected vitamin A_1 curve, $E_{310m\mu}/E_{325m\mu} = 0.926 \ldots$." In pure lobster vitamin A this ratio of extinctions in hexane is 0.96; in pure neo-*b* vitamin A, 0.97; in all-*trans* vitamin A, 0.86. Lambertsen and Braekkan also reacted their preparation with maleic anhydride, according to the procedure of Robeson and Baxter (1947). They recovered 70.6 per cent of the vitamin A. This is close to the result obtained by Robeson *et al.* (1955) with neo-*b* vitamin A (60 to 65 per cent recovery; neo-*a* 83 per cent; all-*trans*, 3 per cent). Lane (1950) has reported the extraction from the calanoid copepods *Temora turbinata* and *Centropages typicus* of a non-saponifiable fraction containing what appeared to be vitamin A, but with its absorption displaced toward shorter wave lengths than usual. Lane stated the absorption maximum to be 310 mμ; but inspection of his figure shows that though this is in fact the wave length at which he measured the highest extinction, the center of the absorption band as drawn is about 318 mμ, and the band as a whole resembles that of neo-*b* vitamin A.[8]

It should be noted finally that Fisher *et al.* (1952, 1953) have been unable thus far to identify vitamin A in the amphipods, isopods, or cladocerans they have examined, or in the copepod, *Calanus finmarchicus*. This may be due merely to low concentration, or to some as yet unrecognized factor that interferes with the measurement of vitamin A; yet it must be conceded to be as yet too early to assume that all crustacea possess vitamin A in any form, even in their eyes. It may be mentioned in this connection that attempts some years ago to extract vitamin A from the heads of insects—*Drosophila*, the grasshopper *Melanoplus*, the dragonfly *Sympetrum*—failed to yield a clear test for this substance though relatively large amounts of tissue (6 to 14 gm.) were employed (Wald, unpublished observations).

SUMMARY

In many crustacea, including the lobster, the bulk of the vitamin A of the whole animal is concentrated in the eyes. Recently Fisher, Kon, and Thompson found that vitamin A extracted from the eyes of euphausiid crustacea has only

[8] Fisher *et al.* (1955 *a*) have recently reported the extraction and partial purification from the eyes of the euphausiid, *Meganyctiphanes norvegica*, of what is apparently a *cis* isomer of vitamin A, yielding the typical antimony chloride test for vitamin A, but possessing initially λ_{max} in hexane at 311 to 312 mμ. On isomerization λ_{max} moves to 325 mμ, and the extinction rises about 36 per cent. This substance may account for only a few per cent of the total vitamin A of the eye (Dr. Kon, personal communication). Its properties are close to those of neo-*c* vitamin A (11, 13-*dicis*) (Wald *et al.*, 1955; Oroshnik *et al.*, 1956).

about one half the biological potency of the same amount of the all-*trans* acetate or fish liver vitamin A.

In the present experiments the vitamin A of the lobster eye is found to consist almost entirely of the hindered *cis* isomer, neo-*b*, the precursor in the vertebrate retina of the visual pigments rhodopsin and iodopsin. This isomer is known to have a low biological potency in the rat, only about one quarter that of all-*trans* vitamin A. In the lobster eye it is virtually all extractable with petroleum ether, about 30 per cent in the form of free alcohol, about 70 per cent in the form of esters. It was identified by its absorption spectrum, as derived from measurements on crude extracts, and measured directly in purified preparations; the changes in absorption which accompany isomerization; oxidation to the corresponding retinene; and synthesis from the latter of rhodopsin.

The examination of an extract of euphausiid eyes, provided by Dr. Kon, also revealed the presence of neo-*b* vitamin A virtually alone. This may be the characteristic condition in the eyes of Eucarid crustacea. It is peculiar in that the neo-*b* isomer, being a sterically hindered form, is ordinarily expected to be represented in any equilibrium mixture of geometric isomers in very small amount. Apparently certain crustacea have ways of circumventing the difficulties implicit in producing and retaining this isomer, and store it in the eye virtually alone.

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