THE VITAMIN A OF A EUPHAUSIID CRUSTACEAN

By GEORGE WALD AND PAUL K. BROWN*

(From the Biological Laboratories, Harvard University, Cambridge)

(Received for publication, November 12, 1956)

Among crustacea, the euphausiids appear to be particularly rich in vitamin A, almost all of which is concentrated in the eyes. In some species no vitamin A at all has been detected in other tissues, or in whole bodies less the eyes. The vitamin A is peculiar in possessing a very much lower nutritional potency in rats than is exhibited by all-*trans* or fish liver vitamin A (Batham *et al.*, 1950; Fisher, Kon, and Thompson, 1952, 1954, 1955).

The low biopotency of euphausiid vitamin A implies that it may consist largely of low potency *cis* isomers (*cf.* Kon's suggestion, 1954). The neo-*a* isomer (13-*cis*) has about 73 per cent the potency of all-*trans* vitamin A, the remaining natural *cis* isomers only 22 to 24 per cent (neo-*b*, iso-*a*, iso-*b*) (Ames *et al.*, 1955).

Recently it has been shown that lobster vitamin A, almost all of which is in the eyes, consists almost entirely of the neo-*b* isomer (11-*cis*) (Wald and Burg, 1955, 1956–57).

Dr. Kon has sent us for examination an oil extracted from the eyes of the euphausiid, *Meganyctiphanes norvegica*. 24 gm. of eyes, representing several thousand animals, were extracted with petroleum ether containing 23 per cent alcohol in a Waring blendor (*cf.* Fisher *et al.*, 1952). The solvent was evaporated off, and the oil sealed under nitrogen. It was in this form that we received it. It was examined by the same procedures as used with the lobster material (Wald and Burg, 1956–57).

A sample of the oil was saponified at room temperature (about 26°C.) for 2¾ hours in 6 per cent KOH in methanol. This mixture was diluted with 40 per cent its volume of water, and extracted repeatedly with petroleum ether. The absorption spectrum of the petroleum ether solution is shown in Fig. 1. It displayed a broad absorption maximum at about 317 mμ, which moved to 324 mμ on isomerization with light in the presence of a trace of iodine. Simultaneously E_{max} rose by a factor of 1.39, and the absorption band changed characteristically in shape. These observations with a relatively crude preparation already indicate that the bulk of this material is neo-*b* vitamin A (Brown and Wald, 1956).[1]

* This investigation was supported in part by funds from The Rockefeller Foundation and the Office of Naval Research.

[1] These isomerizations are slow compared with those reported previously (Brown and Wald, 1956; Wald and Burg, 1956–57). The reason is that while the earlier isom-

This preparation displayed absorption not due to vitamin A, the magnitude of which can be estimated by a procedure described earlier (Wald and Burg, 1956–57). This involves determining by means of an antimony chloride test the vitamin A concentration of the isomerized solution. The absorption spectrum of this concentration of isomerized vitamin A being known, the difference between it and the spectrum of the

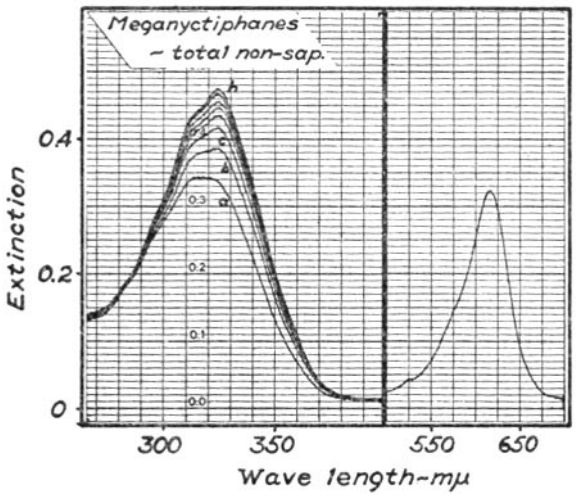

FIG. 1. The total non-saponifiable fraction of an extract of *Meganyctiphanes* eyes. *Left*, (*a*) absorption spectrum of the extract in 3 ml. petroleum ether. It displays the broad absorption band maximal at about 317 mμ characteristic of neo-*b* vitamin A. Iodine was added (2 μg. in a droplet of petroleum ether) and the solution was irradiated with white light of intensity about 30 foot-candles. Curve (*b*) was recorded after 2 minutes' irradiation, (*c*) after a total of 4, (*d*) 6, (*e*) 8, (*f*) 10, (*g*) 15, and (*h*) 25 minutes. The extinction rose, λ_{max} moving to 324 mμ, and the band became asymmetrical—all changes characteristic of the isomerization of neo-*b* vitamin A under these conditions. *Right*, 1 ml. of the isomerized solution after it had been transferred to 1 ml. chloroform was mixed with 2.2 ml. antimony chloride reagent, and the spectrum of the resulting blue product recorded at once, beginning at 700 mμ. It displays the absorption band at 618 mμ characteristic of vitamin A.

isomerized preparation represents the absorption caused by impurities. This subtracted from the spectrum of the unisomerized preparation yields the corrected spectrum of the unisomerized vitamin A.

As an example, this computation can be performed with the data of Fig. 1. 1 ml. of the isomerized preparation was transferred to 1 ml. chloroform and mixed with

---

erizations had been performed in purified hexane, the present ones were done in petroleum ether contaminated with aromatic hydrocarbons in amounts sufficient to bind much of the added iodine.

2.2 ml. antimony chloride reagent. The resulting blue product had E_{max} 0.322 (618 mμ). Since the E (1 per cent, 1 cm.) of vitamin A in this test is 4400, the concentration of vitamin A was (0.322/4400) × 10,000 = 0.733 μg. per ml. The 3.2 ml. of the test mixture therefore contained 3.2 × 0.733 = 2.34 μg. vitamin A. The vitamin A content of the solution in Fig. 1 was therefore 2.34 μg. per ml. Since completely isomerized vitamin A has E (1 per cent, 1 cm.) 1711 at 325 mμ, this concentration should have E_{max} (2.34/10,000) × 1711 = 0.400. However, the E_{max} of the isomerized solution in Fig. 1 is 0.474. The difference, 0.074, is the extinction at 325 mμ caused by impurities. In the same way, knowing the extinction of pure isomerized vitamin A at 318 mμ, one finds that the extinction caused by impurities at that wave length is 0.075, and that the E_{max} of the unisomerized vitamin A is 0.342 − 0.075 = 0.267. Therefore E_{max} rises on isomerization 0.400/0.267 = 1.50 times. Also since 2.34 μg. per ml. of the unisomerized vitamin A had E_{max} 0.267, its E (1 per cent, 1 cm.) was (10,000/2.34) × 0.267 = 1140.

TABLE I

Constants Associated with the Absorption Spectrum of Meganyctiphanes Vitamin A, and Its Changes on Isomerization, Compared with Corresponding Constants of Neo-b Vitamin A

The *Meganyctiphanes* preparations include the total non-saponifiable, and two partially purified fractions from a chromatographic adsorption on alumina. λ_{max} and λ_{sub} are respectively the maxima of the main and the subsidiary absorption bands.

Preparation	λ_{max}	λ_{sub}	Rise of E_{max} on isomerization	E (1 per cent 1 cm.)	Isosbestic point
					mμ
Total non-saponifiable	317	—	1.50	1140	—
1st fraction	317	234	1.44	1190	288
2nd fraction	318	234	1.41	1220	289
Averages			**1.45**	**1183**	
Neo-*b* vitamin A	318	233	1.45 ± 0.05	1200	287

In this way one can compute the λ_{max} and E (1 per cent, 1 cm.) before isomerization, and the rise of E_{max} on isomerization. A further constant, which can be read from the uncorrected data, is the crossing point of the curves for unisomerized and isomerized vitamin A; *i.e.*, the common or isosbestic point. In all these parameters, *Meganyctiphanes* vitamin A is identical, within the limits of error, with pure neo-*b* vitamin A (Table I). A marked change in the *shape* of the absorption band on isomerization, apparent in Figs. 1 and 2, also is characteristic of the neo-*b* isomer (*cf.* Brown and Wald, 1956).

A portion of the *Meganyctiphanes* vitamin A was partly purified by chromatographic adsorption. A large sample of the oil was saponified, the non-saponifiable fraction extracted with petroleum ether, and absorbed on a column of alumina (Merck reagent, "suitable for chromatographic analysis") weakened with 5 per cent its weight of water. The chromatogram was developed with 10 per cent acetone in petroleum ether. The first two fractions to run through the column contained the bulk of the vitamin A. Absorption spectra of the

first filtrate in hexane are shown in Fig. 2. This fraction accounted for half the vitamin A in the total eluate. It possessed initially λ_{max} 317 mμ, with a subsidiary band at 234 mμ. On isomerization, λ_{max} moved to 325 mμ, and E_{max} rose 1.38 times. Also the subsidiary band disappeared, the extinction at 234

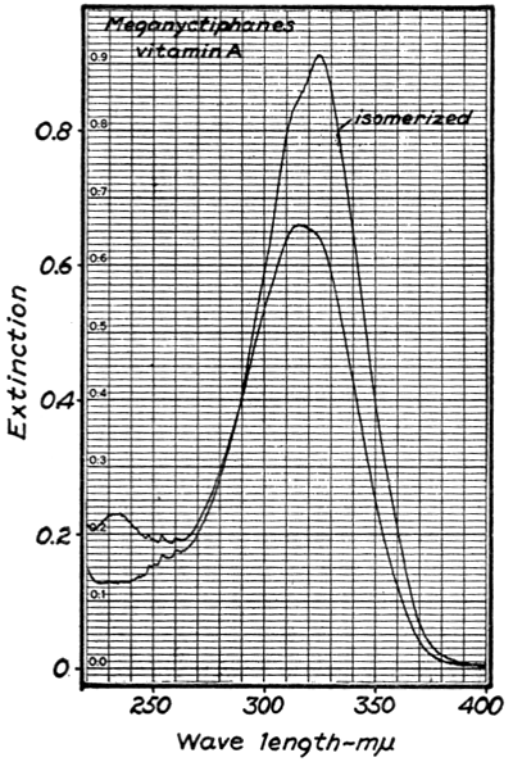

FIG. 2. Absorption spectra in hexane of an extract of *Meganyctiphanes* eyes, partly purified by saponification and chromatographic adsorption on alumina. The spectrum originally displays the low, broad absorption band maximal at 317 mμ and the subsidiary band at 234 mμ characteristic of neo-*b* vitamin A. On isomerization with light in the presence of iodine, the main band rises in height, moves to λ_{max} 324 mμ, and becomes sharper and highly asymmetrical, while the subsidiary peak at 234 mμ is eliminated—all changes characteristic of the isomerization of neo-*b* vitamin A.

mμ falling to 0.56 its former value; in pure neo-*b* vitamin A this factor is 0.55. The second filtrate fraction was less pure, but yielded essentially the same results. These two fractions accounted for 90 per cent of the total eluate, or 75 per cent of the vitamin A originally adsorbed. The data from both fractions were corrected for extraneous absorption as already described. The constants which emerged are summarized in Table I. Again, they are virtually identical with constants obtained with pure neo-*b* vitamin A.

Two samples of free *Meganyctiphanes* vitamin A alcohol, prepared by saponification, were oxidized to the corresponding retinene. The vitamin A in petroleum ether solution was let stand for 5 and 8 days in the refrigerator with 17 times its weight of manganese dioxide powder prepared according to Attenburrow *et al.* (1952). The absorption spectrum of one such product is shown in Fig. 3. It had λ_{max} in hexane at 362 mμ. On isomerization by light in the pres-

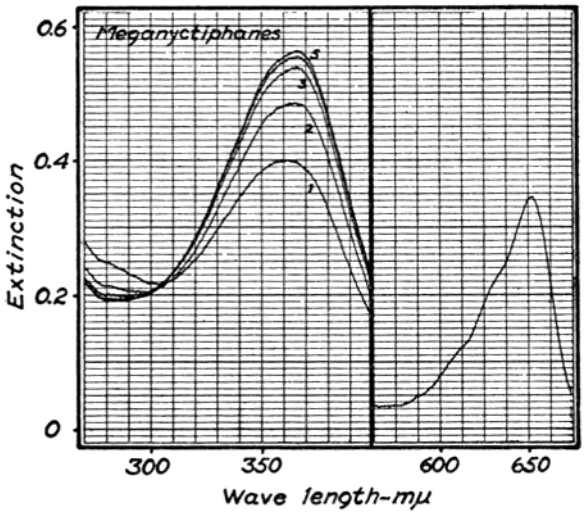

FIG. 3. Absorption spectra of retinene prepared by the oxidation of *Meganyctiphanes* vitamin A. *Left*, curve (1) is the spectrum of a petroleum ether solution which had to this point been protected from light. To 1 ml. of this solution, 0.15 μg. iodine was added in a droplet of petroleum ether, and the solution was irradiated with white light of intensity about 30 foot-candles (2) for 5 minutes, (3) for a total of 10, (4) 15, and (5) 20 minutes. The rise of extinction of the main band and displacement of λ_{max} to longer wave lengths are characteristic of the isomerization of neo-*b* retinene. *Right*, 1 ml. of the isomerized retinene was transferred to 1 ml. chloroform, 2.2 ml. of antimony chloride reagent were added, and the spectrum was recorded at once, beginning at 700 mμ. The resulting blue product had λ_{max} 656 mμ, characteristic of retinene when recorded so quickly.

ence of iodine, E_{max} rose 1.41 times, and λ_{max} shifted to 367 mμ.[1] The second preparation yielded similar results. Antimony chloride tests displayed the absorption band characteristic of retinene.[2] When the concentrations had

[2] The antimony chloride test with retinene, as usually recorded in the past in this laboratory, yields an absorption maximum at 664 to 666 mμ. In the present instance λ_{max} is about 656 mμ. The principal reason for this is more rapid recording. When such recording is started, as here, at 700 mμ, the absorption peak is reached within 7 to 8 seconds after mixing the reagents, and lies at about 656 mμ. In about the next 20 seconds the peak rises somewhat in height, and moves to 664 to 666 mμ.

been determined through the antimony chloride tests, these data could be corrected for the presence of extraneous absorption in the way already described for vitamin A, with the result that in these two preparations isomerization raised E_{max} 1.58 and 1.64 times. For pure neo-*b* retinene this factor is 1.7; so that following saponification and oxidation on manganese dioxide these preparations apparently contained about 84 and 92 per cent of the neo-*b* isomer.

One of these preparations was dissolved in digitonin, mixed with cattle opsin in excess, and incubated in the dark for 2.3 hours at room temperature. It formed rhodopsin, in 88 to 96 per cent the amount obtained in similar experiments with pure neo-*b* retinene.

To determine the proportions of *Meganyctiphanes* vitamin A present as free alcohol and as esters, a portion of the untreated oil was dissolved in petroleum ether, and adsorbed on a column of alumina weakened with 5 per cent its weight of water. 25 ml. of 4 per cent acetone in petroleum ether were run through the column to elute vitamin A esters, followed by 25 ml. of 40 per cent acetone in petroleum ether to elute the free alcohol (Ganguly *et al.*, 1952). The vitamin A in each fraction was determined through the antimony chloride test. The total recovery was 88 per cent, of which 90.7 per cent was in the ester fraction, 9.3 per cent free vitamin A alcohol.

DISCUSSION

It may be concluded that the vitamin A of *Meganyctiphanes* eyes, like that of the lobster, consists almost wholly of the neo-*b* isomer. Its properties after saponification do not depart sufficiently from those of pure neo-*b* vitamin A to indicate that any other isomer is present. The measurements by which this isomer was identified involve limits of variability such that at most 10 per cent of the vitamin A could have been in other forms. This information, which was communicated to Dr. Kon, has since been confirmed in his laboratory (Plack *et al.*, 1956).

Fisher *et al.* (1955 *b*) have described the isolation from *Meganyctiphanes* eyes of a vitamin A with λ_{max} 311 to 312 mμ in hexane. On isomerization with light in the presence of iodine, λ_{max} moved to 325 mμ, and E_{max} rose 1.36 times. These properties are close to those of neo-*c* vitamin A (11, 13-*dicis*), for which λ_{max} in hexane is 311 mμ, and E_{max} rises 1.36 times on isomerization (Wald *et al.*, 1955; Oroshnik *et al.*, 1956). This substance accounts for only a few per cent of the vitamin A of *Meganyctiphanes* eyes (Plack *et al.*, 1956). For this reason, and because of the fact that among the geometric isomers of vitamin A its properties most resemble those of neo-*b*, it can readily be accommodated within our measurements.

The only remaining discrepancy with our observations seemed for a time to involve bioassays. Fisher *et al.* (1952, 1955 *b*) had reported that vitamin A from *Meganyctiphanes* eyes possesses on the average about 60 per cent the

biological activity of all-*trans* vitamin A. Ames *et al.* (1955) assess the biopotency of neo-*b* vitamin A at about 23 per cent of all-*trans*. If, as we find, *Meganyctiphanes* vitamin A is at least 90 per cent neo-*b*, it should possess a considerably lower biopotency than had been observed.

Since this information was communicated to Dr. Kon, the bioassays have been reexamined in his laboratory, with the result that they have been very considerably revised. *Meganyctiphanes* vitamin A is now found to assay at 32 per cent of all-*trans*; while those components of it which react slowly with maleic anhydride, and which include the neo-*b* isomer, assay at 25 per cent of all-*trans* vitamin A (Plack *et al.*, 1956). Little if any discrepancy in this regard remains therefore with the present observations.

SUMMARY

The vitamin A of the euphausiid crustacean, *Meganyctiphanes norvegica*, consists almost wholly of the hindered *cis* isomer, neo-*b* (11-*cis*). In this animal vitamin A is concentrated almost entirely in the eyes; and its properties so closely resemble those of pure neo-*b* vitamin A as not in themselves to indicate that any other isomer is present. However, Fisher *et al.* (1955 *b*) have isolated a small fraction from this material which may be neo-*c* vitamin A (11, 13-*dicis*). The neo-*b* isomer was identified by its absolute absorption spectrum, the changes of absorption spectrum on isomerization, oxidation to neo-*b* retinene, and synthesis from the latter of rhodopsin. This identification is also in good accord with new, revised bioassays of *Meganyctiphanes* vitamin A by Plack *et al.* (1956).

REFERENCES

Ames, S. R., Swanson, W. J., and Harris, P. L., Biopotencies of geometric isomers of vitamin A acetate in the rat, *J. Am. Chem. Soc.*, 1955, **77**, 4134.

Attenburrow, J., Cameron, A. F. B., Chapman, J. H., Evans, R. M., Hems, B. A., Jansen, A. B. A., and Walker, T., A synthesis of vitamin A from cyclohexanone, *J. Chem. Soc.*, 1952, 1094.

Batham, E., Fisher, L. R., Henry, K. M., Kon, S. K., and Thompson, S. Y., Preformed vitamin A in marine crustacea, *Biochem. J.*, 1950, **48**, X.

Brown, P. K., and Wald, G., The neo-b isomer of vitamin A and retinene, *J. Biol. Chem.*, 1956, **222**, 865.

Fisher, L. R., Henry, K. M., Kon, S. K., Plack, P. A., and Thompson, S. Y., A vitamin A isomer in the eyes of *Meganyctiphanes norvegica*, *Biochem. J.*, 1955 *b*, **61**, vi.

Fisher, L. R., Kon, S. K., and Thompson, S. Y., Vitamin A and carotenoids in certain invertebrates, I. Marine crustacea, *J. Marine Biol. Assn. United Kingdom*, 1952, **31**, 229; Vitamin A and carotenoids in certain invertebrates. II. Studies of seasonal variations in some marine crustacea, *J. Marine Biol. Assn. United Kingdom*, 1954, **33**, 589; Vitamin A and carotenoids in certain invertebrates. III. Euphausiacea, *J. Marine Biol. Assn. United Kingdom*, 1955 *a*, **34**, 81.

Ganguly, J. Krinsky, N. I., Mehl, J. W., and Deuel, H. J., Jr., Studies of the distribution of vitamin A as ester and alcohol and of carotenoids in plasma proteins of several species, *Arch. Biochem. and Biophysic.*, 1952, **38,** 275.

Kon, S. K., Vitamin A des invertébrés marins et métabolisme des caroténoides du plancton, *Bull. Soc. chim. biol.*, 1954, **36,** 209.

Oroshnik, W., Brown, P. K., Hubbard, R., and Wald, G., Hindered *cis* isomers of vitamin A and retinene: the structure of the neo-b isomer, *Proc. Nat. Acad. Sc.*, 1956, **42,** 578.

Plack, P. A., Fisher, L. R., Henry, K. M., and Kon, S. K., *cis*-Isomers of vitamin A in some marine crustacea, *Biochem. J.*, 1956, **64,** pt. 1, 17 P.

Wald, G., Brown, P. K., Hubbard, R., and Oroshnik, W., Hindered cis isomers of vitamin A and retinene: the structure of the neo-b isomer, *Proc. Nat. Acad. Sc.*, 1955, **41,** 438.

Wald, G., and Burg, S. P., Crustacean vitamin A, *Fed. Proc.*, 1955, **14,** 300.

Wald, G., and Burg, S. P., The vitamin A of the lobster, *J. Gen. Physiol.*, 1956–57, **40,** 609.