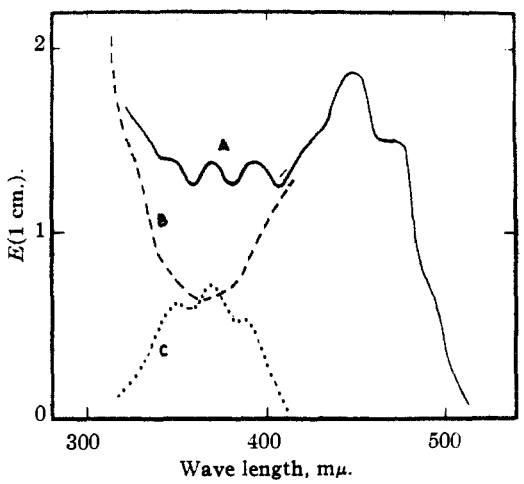

Fig. 4.—Ultraviolet absorption spectra of (A) blood plasma extract after treatment with anhydrous hydrogen chloride, (B) absorption curve of vitamin A-free plasma extract, and (C) resulting curve typical of anhydro vitamin A (curve A minus curve B).

(Fig. 3C). The saponified extract was dehydrated by treatment with $N/30$ alcoholic hydrochloric acid, giving rise to material whose absorption curve is shown in Fig. 4A. This material should not contain vitamin A, but may contain anhydro vitamin A. When the absorption curve for vitamin A-free plasma extract (4B) is subtracted from curve 4A, the resulting curve 4C is obtained. This shows the typical anhydro vitamin A bands and indicates that the original plasma extract must have contained vitamin A. As the value of $E(1\%, 1$ cm.) for the peak near 371 mμ of a crude anhydro vitamin A preparation usually is about 140–150% that of $E(1\%, 1$ cm.) (328 mμ) of the original vitamin A preparation, it is interesting to note that the values for $E(1\%, 1$ cm.) (368 mμ) on curve 4C and for $E(1\%, 1$ cm.) (328 mμ) for curve 3B are 0.00049 and 0.00035, respectively.

Summary

Anhydro (cyclized) vitamin A may be made from vitamin A by treatment with hydrochloric acid or antimony trichloride. Vitamin A in the form of its fatty acid esters is partially transformed into anhydro vitamin A by refluxing in alcohol. Crystalline anhydro vitamin A melts at 76-77° and has a value of 3650 for $E(1\%, 1$ cm.) (371 mμ). It is an unstable hydrocarbon, weakly adsorbed on most chromatographic adsorption agents and it probably has 6 double bonds and the formula $C_{20}H_{28}$. It has some biological vitamin A activity.

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[COMMUNICATION NO. 34 FROM LABORATORIES OF DISTILLATION PRODUCTS, INC.]

A Possible New Member of the Vitamins A_1 and A_2 Group[1]

BY NORRIS D. EMBREE AND EDGAR M. SHANTZ

The unsaponifiable fraction of fish liver oil contains several substances other than vitamin A which, like vitamin A, have absorption bands in the ultraviolet region and give blue colored products with antimony trichloride. The examination of the materials related to vitamin A in the unsaponifiable fraction of fish liver oil has most often been carried out by chromatographic adsorption of a petroleum ether solution of the material on a Tswett column of alumina or calcium hydroxide. The fractions with their properties that have been usually recognized are shown in Table I. The absorption maxima in the ultraviolet region (below 400 mμ) are for solutions in ethyl alcohol, while those in the visible regions are for the product obtained by the reaction of a chloroform solution of the material with a chloroform solution of antimony trichloride.

The fact that vitamins A_1 and A_2 are not readily separable by chromatographic adsorption is not necessarily a bar to the determinations of the relative proportions of each present, since, as we have already shown,[4] the "cyclized" or anhydro vitamins are readily separated chromatographically. The vitamins are usually dehydrated by treating them, in alcoholic solution, with $N/30$ dry hydrogen chloride.[5] Anhydro vitamin A_2 is quite strongly held by alumina in a Tswett column while anhydro vitamin A_1 is readily washed through with petroleum ether. Anhydro vitamins A_1 and A_2 have almost identically the same ultraviolet absorption spectrum.[4] This

(1) Presented before the Chicago meeting of the American Society of Biological Chemists, April, 1941.

TABLE I

Nature of zones in a chromatogram of the unsaponifiable fraction of a fish liver oil. Only those materials which give a blue color with antimony trichloride are listed.

Zone I. (Top.) Often colored yellow or orange; contains 580 chromogen of Eekelen, *et al.*[2] Presumed to be oxidation products of vitamin A.[3] Absorption maxima: ultraviolet, 270 to 280 and sometimes 328 mμ; $SbCl_3$ product, 580 and sometimes 620 mμ.

Zone II. Large, light yellow band. Vitamin A_1 and, if present, vitamin A_2.[4] The absorption maxima for vitamins A_1 and A_2 are, respectively, located at 328 and 350 mμ; $SbCl_3$ product, 620 and 695 mμ.

Zone III. Strongly colored red material. Vitamin A and presumably polymerization products.[3,5] Absorption maxima: U.V. 328 mμ with absorption continuing into the visible region with an inflection at 430 mμ; $SbCl_3$ product, 620 and 650 mμ.

Zone IV. A small amount of material containing "α-vitamin A"[6] which may be oxidation products of vitamin A.[3] Absorption maxima: U.V. 270 to 280 mμ; $SbCl_3$ product, 580 mμ.

Zone V. Yellow. Anhydro vitamin A.[5,7] Absorption maxima: U.V. 351, 371 and 392 mμ; $SbCl_3$ product, 623 mμ.

spectrum consists of three absorption bands located at 351, 371 and 392 mμ, the center band being the most prominent.

When a careful examination of the chromatographed fractions of the unsaponifiable fraction of shark liver oil was made by dehydration, a new substance was noted. It had an ultraviolet absorption spectrum very much like that of anhydro vitamin A_1 except that the three absorption bands were located at 332, 348 and 367 mμ. Apparently, this substance has a chromophoric group like that of anhydro vitamin A but with one less double bond in conjugation. Since this substance could not be detected in the concentrate before dehydration, it must have been formed by the dehydration of some progenitor present in the concentrate. For convenience in discussion, this progenitor has been temporarily designated by us as "subvitamin A," and its dehydrated product, "anhydro subvitamin A."

(2) M. Eekelen, A. Emmerie, H. W. Julius and L. K. Wolff, *Nature*, **132**, 171 (1933).

(3) D. C. Castle, A. E. Gillam, I. M. Heilbron and H. W. Thompson, *Biochem. J.*, **28**, 1702 (1934).

(4) N. D. Embree and E. M. Shantz, *J. Biol. Chem.*, **132**, 619 (1940); A. E. Gillam, I. M. Heilbron, W. E. Jones, E. Lederer, J. W. Batty and J. H. Beynon, *Biochem. J.*, **32**, 405 (1938).

(5) E. M. Shantz, J. D. Cawley and N. D. Embree, THIS JOURNAL, **65**, 901 (1943).

(6) P. Karrer, O. Walker, K. Schöpp and R. Morf, *Nature*, **132**, 26 (1933).

(7) N. D. Embree, *J. Biol. Chem.*, **128**, 187 (1939).

Concentrating Subvitamin A.—Eight hundred grams of shark[8] liver oil having a potency of 40,000 units of vitamin A per gram were saponified and extracted with ethyl ether in the usual manner. After removal of the solvent under nitrogen and reduced pressure, the non-saponifiable extract was placed in a laboratory cyclic molecular still and all the material distilling below 150° was collected in one fraction. The higher boiling material was discarded.

Since previous experiments had shown that subvitamin A was extremely soluble in aqueous alcohol, the distillate was dissolved in 83% ethyl alcohol and extracted seven times with equal volumes of light petroleum ether. The petroleum ether extracts were combined, the solvent removed, and the seven-fold extraction repeated twice more. The three aqueous alcohol extracts were combined and extracted with seven more portions of petroleum ether. From their respective solvent distribution ratios it was calculated that this series of extractions removed over 99.9% of the vitamin A alcohol while leaving about 65% of the subvitamin A in the 83% ethanol.

The aqueous alcohol extract was then diluted with two volumes of water and extracted five times with ethyl ether. The ethyl ether extracts were combined, washed with water and dried over anhydrous sodium sulfate. After removal of the solvent, the residue was taken up in 50 ml. of benzene for chromatographing.

The benzene solution was passed into a chromatograph column containing aluminum oxide (after Brockmann). The bands were developed with a 10% solution of ethyl ether in petroleum ether. It was found that this solvent mixture did not elute the subvitamin A but did wash out some highly colored material which gave a negligible blue color with antimony trichloride.

The subvitamin A was found to be contained in a yellow band immediately below a light brown band[9] at the very top of the column. The yellow band was removed and the material eluted from it by several washings with hot 90% ethyl alcohol. Upon removal of the solvent it was found to contain 0.20 g. of red oil which had an absorption maximum at 290 mμ in the ultraviolet with $E(1\%, 1 \text{ cm.}) = 150$, and had an apparent vitamin A potency of 250,000 units of vitamin A per gram as measured by the antimony trichloride blue color.[10]

Assuming that equal extinction coefficients at the peaks of the ultraviolet absorption curves correspond to equal amounts of vitamin A_1 and subvitamin A, we have estimated that about one part of subvitamin A occurs with about 500 parts of vitamin A_1 in the shark liver oils that we have examined.

Properties of Subvitamin A

Chromatographic Adsorption.—Subvitamin A is more strongly adsorbed by alumina than is vitamin A_1. When the unsaponifiable fraction of shark liver oil is chromatographed on a Tswett column of activated alumina (after Brockmann),

(8) Commercial product made in California. Samples of oils from several different sources gave substantially the same results.

(9) This band contains the 580 mμ chromogen of Eekelen.[2]

(10) An Evelyn photoelectric colorimeter was used with a calibration curve made from a study of commercially distilled vitamin A concentrates.

subvitamin A is found between the oxidized products and the main vitamin A band, that is, between zones I and II as described in Table I.

Anhydro subvitamin A is much more strongly adsorbed by alumina than is anhydro vitamin A_1, and the two may be separated readily by chromatographic adsorption. Anhydro vitamin A_2 is only slightly less strongly adsorbed than is anhydro subvitamin A, but the two substances may be separated by chromatography.

Ultraviolet Light Absorption.—Subvitamin A has a single absorption band in the ultraviolet region with a maximum absorption at 290 mμ (Fig. 1). The most potent preparation that has been made by us had a value of 150 for $E(1\%, 1\text{ cm.})$ (290 mμ).[11]

Anhydro subvitamin A has three absorption bands in the ultraviolet region, located at 332, 348 and 367 mμ (Fig. 1). The most potent preparation that has been made by us had values of 250, 255 and 180 for $E(1\%, 1\text{ cm.})$ (332 mμ), (348 mμ), and (369 mμ), respectively. The maximum extinction coefficient of the dehydrated product has a greater value than that of the subvitamin A. The absorption spectrum of anhydro subvitamin A differs from that of anhydro vitamin A, in that the two lower wave length bands

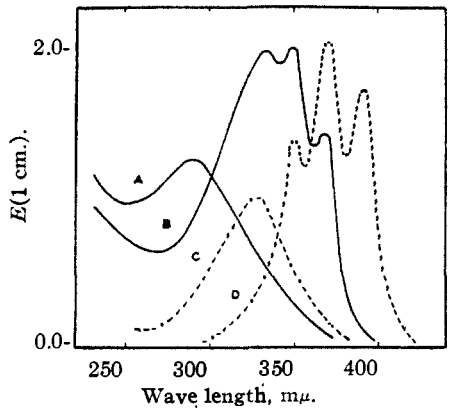

Fig. 1.—Absorption spectra in ethanol of: (A) subvitamin A, 0.008%; (B) anhydrosubvitamin A, 0.008%; (C) vitamin A, 0.00054%; (D) anhydrovitamin A, 0.00054%.

(11) The ultraviolet absorption spectra used in this work were determined with a Hilger Medium quartz spectrograph equipped with a Spekker photometer. Absolute alcohol was used as the solvent. The extinction coefficient, or as it is sometimes called, the percentage extinction coefficient, is represented here by the symbol $E(1\%, 1\text{ cm.})$, although it is often represented as $E^{1\%}_{1\text{ cm.}}$, or E. It is defined by the equation $E(1\%, 1\text{ cm.}) = (1/cd) \log I/I_0$, where I_0 is intensity of the light (at the indicated wave length) entering the solution, I is intensity of the light leaving the solution, c is concentration of solution in grams per 100 ml., and d is depth in centimeters of the absorption cell.

are always of approximately equal height, while in anhydro vitamin A, the center band is always stronger than the other two.

The Antimony Trichloride Reaction Product.—When a chloroform solution of subvitamin A reacts with a chloroform solution of antimony trichloride, a blue solution is formed which resembles the antimony trichloride reaction product of vitamin A_1. The visual absorption spectrum[12] shows an absorption maximum at 617 mμ and an inflection point at 580 mμ. The concentrate of subvitamin A mentioned above had a value of 310 for $E(1\%, 1\text{ cm.})$ (617 mμ).

The absorption spectrum of the antimony trichloride reaction product of anhydro subvitamin A is quite like that of subvitamin A. The concentrate of the anhydro vitamin described above had a value of 300 for $E(1\%, 1\text{ cm.})$ at 617 mμ.

Distribution between Petroleum Ether and 83% Alcohol.—When a solution of subvitamin A in petroleum ether (Skelly solve F) is shaken with an equal volume of 83% ethyl alcohol, 95% of the subvitamin A goes into the alcohol layer. When vitamins A_1 and A_2 are distributed between the two solvents in a similar manner, it is found that 55 and 59%, respectively, of the vitamin goes into the alcohol layer.

The fraction of anhydro subvitamin A leaving the petroleum ether solution to go into the 83% alcohol layer is 50%. The corresponding fractions for anhydro vitamins A_1 and A_2 are 3 and 10%, respectively. The corresponding fraction for isoanhydro vitamin A is 5%. This last substance is made by the prolonged treatment of vitamin A by $N/30$ alcoholic hydrochloric acid.[5] It may easily be confused with anhydro subvitamin A since it also has ultraviolet absorption bands near 330, 350, 370 mμ, and its antimony trichloride reaction product has an absorption band at 620 mμ.

Elimination Maxima of Subvitamin A and its Dehydrated Product.—By analytical molecular distillation using glyceride carrier oils and the technique of Hickman[13] as modified by Gray and Cawley,[14] the elimination maximum of sub-

(12) The visible absorption spectra used in this work were measured with a Hardy recording spectrophotometer built by the General Electric Co. The "blue colors" were made by adding within two seconds, 10 ml. of a saturated (at 20°) solution of antimony trichloride in chloroform to 1 ml. of a chloroform solution of the chromogenic material in the optical absorption cell.[7] The spectrophotometer was operated by E. E. Richardson of the Kodak Research Laboratories.

(13) K. C. D. Hickman, *Ind. Eng. Chem.*, **29**, 968 (1937).

(14) E. LeB. Gray and J. D. Cawley, *J. Biol. Chem.*, **134**, 397 (1940).

vitamin A was found to be 15° above that of celanthrene red dye (123°), while the elimination maxima of both vitamins A_1 and A_2 are essentially the same[15] as that of the dye. Anhydro subvitamin A was found to have an elimination maximum the same as that of the celanthrene red dye while those of anhydro vitamins A_1 and A_2 are 19 and 1°, respectively, below that of the pilot dye.

Biological Assay of Subvitamin A.—An oil solution of subvitamin A which had an apparent vitamin A potency of 3000 units of vitamin A per gram by antimony trichloride blue color was bioassayed using the U. S. Pharmacopoeia method with four rats at a level of 17.0 mg. or 51 apparent units per day. Two of the four animals died and the other two had lost 15 and 16 g. by the end of twenty-eight days. This confirmed the results obtained in feeding experiments on previous samples which indicated that the subvitamin A possessed no appreciable growth-promoting power. The bioassays were carried out by Philip L. Harris of these Laboratories.

Discussion

The properties of subvitamin A given above are summarized and compared with those of vitamins A_1 and A_2 in Table II.

TABLE II

PROPERTIES OF SUBVITAMIN A COMPARED WITH THOSE OF VITAMINS A_1 AND A_2

	Subvitamin A	Vitamin A_1	Vitamin A_2
Ultraviolet absorption maximum, mμ	290	328	345
of anhydro products	332 348 367	351 371 392	350 370 390
$SbCl_3$ product, absorption max., mμ	617	620	690
of anhydro product	617	622	690
Petroleum ether, 83% alcohol distribution ratio	14/86	45/55	41/59
of anhydro product	50/50	97/3	90/10
Degree of adsorption on alumina	Strongest	Strong	Strong
of anhydro product	Strongest	Weak	Strong
Elimination maximum,[a] °C.	138	123	123
of anhydro product	123	104	122

[a] With glyceride carrier oils under conditions giving an elimination maximum of 123° for celanthrene red.

Since subvitamin A and its anhydro product have so many properties similar to, but not the same as, those of the two vitamins A and their anhydro products, it is evident that the three compounds are quite closely related chemically.

(15) E. LeB. Gray, *J. Biol. Chem.*, **131**, 317 (1939); from a study of Atlantic salmon liver oil reported that vitamin A distilled 3° below vitamin A_2 and celanthrene red. We feel the elimination curves (his Fig. 6) used to determine this are unsatisfactory and call attention to his Fig. 5, concerning the distillation of wall-eyed pike liver oil, which shows that vitamins A and A_2 distill substantially together.

Members of a vinylogous series of organic compounds containing a series of conjugated double bonds, each member differing from the next lower one by having an additional (—CH=CH—) group, will have similar absorption spectra.[16] The addition of each new —CH=CH— group will cause the absorption bands to shift toward the longer wave length region by 20 to 40 mμ. Usually the derivatives of members of this series will also have similar absorption spectra, and the absorption band of the higher derivatives will have longer wave lengths.

Subvitamin A would appear to be a lower vinylog of vitamin A_2 due to the relative positions of the light absorption bands of the two substances, their antimony trichloride reaction products, their anhydro derivatives, and the antimony trichloride reaction product of their anhydro derivatives. The light absorption data, in themselves, would preclude the possibility that subvitamin A and vitamin A_1 are vinylogs since the absorption bands of the antimony trichloride products of the substances and their anhydro derivatives are located at the same wave length. Similarly, vitamins A_1 and A_2 are indicated not to be vinylogs since the absorption spectra of their anhydro derivatives are substantially identical.

The greater polarity of subvitamin A and anhydro subvitamin A shown by the distribution between petroleum ether and 83% alcohol, as well as the relative position of the adsorption bands on the Tswett column, suggests that subvitamin A may be an oxygenated derivative of vitamin A_1 or of vitamin A_2. This supposition is supported by the molecular distillation data which indicated that anhydro subvitamin A has a higher molecular weight than anhydro vitamins A_1 and A_2.

Subvitamin A has not yet been found by us to be formed by oxidizing vitamin A. If it is oxygenated vitamin A_1 or vitamin A_2, its structure is probably related to vitamin A_1 or vitamin A_2 as one of the oxygen containing carotenoids is to β-carotene.

Summary

A substance, tentatively called "subvitamin A," which occurs in traces in shark liver oil, has chemical properties related to those of vitamins A_1 and A_2. It has very little or no growth promoting power. It possibly is an oxygenated derivative of either vitamin A_1 or vitamin A_2.

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(16) K. Dimroth, *Angew Chem.*, **52**, 545 (1939).