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The Fluorescence of Vitamin A. II. Ultraviolet Absorption of Irradiated Vitamin A.[1]

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In a previous communication[2] it has been reported that alcoholic solutions of vitamin A esters show a steep rise of their greenish fluorescence during the first few minutes of ultraviolet irradiation; the rise is followed by a decrease of fluorescence during continued irradiation; this decrease is retarded in absence of oxygen. The observation of these phenomena is limited for optical reasons to concentrations below 5 μg./1 ml. Therefore, most of the usual methods of structural investigation cannot be applied to the study of the highly fluorescent intermediary product. It has, however, been possible to gather information regarding the nature of this compound by the study of ultraviolet absorption spectra of vitamin A solutions in the course of irradiation.

Experimental

Method.—The irradiation experiments and measurements of intensity of fluorescence were performed with the apparatus described in the previous communication.[2] All experiments discussed were carried out with solutions containing 3.3 μg. of crystalline vitamin A alcohol or the equivalent amount of crystalline vitamin A acetate in 1 ml. of absolute ethanol.

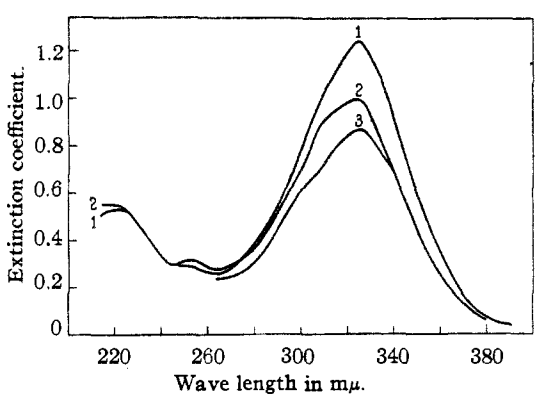

Fig. 1.—Ultraviolet absorption spectra of vitamin A alcohol in ethanol (3.33 μg./1 ml.) before irradiation (1) and after five minutes (2) and after forty minutes (3) of exposure to ultraviolet light.

The ultraviolet absorption data were obtained with a Beckman[3] quartz spectrophotometer in 1-cm. quartz cells. The short exposures and the narrow bands used in measurements with this instrument were of particular advantage because of the instability of the solutions to ultraviolet light. Control readings, especially at the critical wave lengths around 360 mμ, proved that the extinction coefficient at a given wave length, remeasured after more than 20 measurements at other wave lengths, always remained unchanged. The absorption data are given in terms of "percentage" extinction coefficients [E (1%, 1 cm.)], because the molecular concentration of the reaction product or products is unknown.

Material.—Crystalline vitamin A alcohol and vitamin A acetate[4] were used throughout these experiments. Anhydro vitamin A and isoanhydro vitamin A were prepared repeatedly according to Shantz, Cawley and Embree[5] by the action of anhydrous alcoholic hydrochloric acid on alcoholic solutions of crystalline vitamin A alcohol for shorter and longer periods. The resulting solutions showed practically no fluorescence when irradiated. The grade of absolute ethyl alcohol, used in this work, was practically non-fluorescent.

Absorption Spectra of Vitamin A Alcohol Solutions During Irradiation.—The course of fluorescence of vitamin A alcohol during ultraviolet irradiation shows a slow, steady abatement, as previously described and illustrated in Fig. 1, curve 6, of the first communication.[6] The alteration of the absorption spectrum of vitamin A alcohol in ethanol after five and after forty minutes of irradiation, is shown in Fig. 1. After five minutes of irradiation, the peak at 328 mμ has receded by one-fifth and the slight bulge at 310 mμ has become more pronounced; after forty minutes, the entire curve has further receded.

Absorption Spectra of Vitamin A Acetate During Irradiation.—Vitamin A acetate offers a strikingly different picture under the same conditions (Fig. 2). After five minutes of irradiation, when fluorescence has reached its maximum of intensity which is more than four times the original value, the absorption maximum at 325 mμ drops to less than one-half its original height. Two secondary maxima appear at wave lengths 346 and 364 mμ. When fluorescence after forty minutes has dropped to its final minimum, all three peaks have practically disappeared.

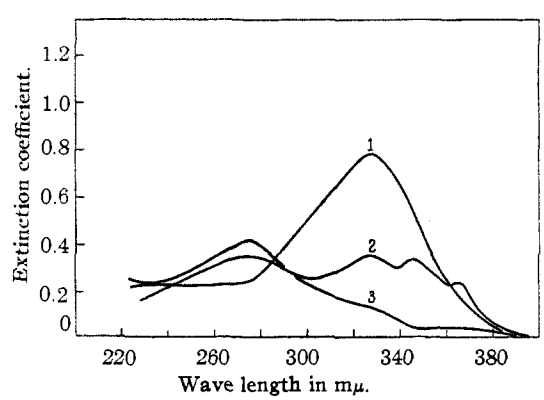

Fig. 2.—Ultraviolet absorption spectra of vitamin A acetate in ethanol (3.33 μg. in terms of vitamin A/1 ml.) before irradiation (1) and after five and forty minutes of exposure to ultraviolet light (2, 3).

(1) This paper was read before the Division of Biological Chemistry at the 106th Meeting of the American Chemical Society in Pittsburgh, Pa., Sept. 9, 1943. This investigation was supported by a Grant from Nutrition Foundation, Inc.

(2) H. Sobotka, S. Kann and E. Loewenstein, THIS JOURNAL, **65**, 1959 (1943); *cf.* H. Sobotka, S. Kann and W. Winternitz, *J. Biol. Chem.*, **152**, 635 (1944).

(3) H. H. Cary and A. D. Beckman, *J. Optical Soc. Am.*, **31**, 682 (1941).

(4) Obtained from Distillation Products, Inc.

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

(6) Correction of Fig. 1 in the preceding paper, ref. 2: The galvanometric deflections along the ordinate should be lowered by one step, so that "40" takes the place of "20" and so on.

Toward the left of the typical vitamin A peak, a new maximum has gradually arisen at 275 mμ. The absorption remains constant in the region about 285 mμ. The blue reaction with antimony trichloride decreases no more than 10% during the period of increasing fluorescence. The subsequent disappearance of fluorescence and of the three absorption maxima at 325, 346, and 364 mμ is accompanied by a decrease of the Carr–Price reaction to approximately one-half its original depth of color.

Influence of Irradiation Products on Fluorescence Phenomena.—It was furthermore investigated whether any of the photochemical reaction products inhibited the completion of the transformation of vitamin A on optical or chemical grounds. We learned from trials with various filters that the wave lengths, effective in the primary photoreaction, center around 360 mμ. Since the absorption bands of the primary reaction product fall within this range, we placed a test-tube with irradiated vitamin A solution in the path of the ultraviolet rays; no inhibition of the fluorescence phenomenon in a fresh solution, irradiated through this filter, was observed. Moreover, no change in the course of fluorescence was noticed when 10 ml. of an irradiated solution was used as diluent, instead of pure alcohol, in making up 12 ml. of a fresh solution to be irradiated.

Adsorption of Irradiation Products on Alumina.—The spectroscopic resemblance of our irradiation product with iso-anhydro vitamin A will be discussed below. As isoanhydro vitamin A passes rather easily through a column of alumina, the following experiment was devised: 24 ml. of vitamin A solution was irradiated to maximal fluorescence, the alcohol was evaporated and the residue redissolved in petroleum ether. After passing the solution through a column of chromatographic alumina, the filtrate was evaporated. The residue, taken up in chloroform, gave a Carr–Price reaction which could not be due to unchanged vitamin A. The absorption spectrum of the alcoholic solution of the residue resembled curve 3 in Fig. 2 and showed no inflections or maxima at wave lengths >300 mμ, indicating the absence of isoanhydro vitamin A as well as of vitamin A.

Discussion

Absorption Spectra of Irradiated Vitamin A Preparations.—The ultraviolet absorption of alcoholic solutions of vitamin A concentrates, containing the naturally occurring esters, during exposure to ultraviolet light has been studied by Chevallier and Dubouloz[7] without regard to the course of fluorescence. Representative samples of their curves are reproduced in Fig. 3. The identity of the absorption maxima at 328–9, 345, 363–4 mμ with ours after short irradiation, in regard to position and relative height, is evident. A report by H. H. Darby on the ultraviolet absorption of crystalline vitamin A[8] contains reference to the appearance of a new absorption band at 360 mμ "after some time of standing" in dilute ethanol solution. Darby also indicates the bulge at 310 mμ, referred to above.

Isoanhydro Vitamin A and Irradiation Product of Vitamin A.—A striking parallel obtains between the maxima of curve 2 in Fig. 2, and the data by Shantz, Cawley and Embree[5] for isoanhydro vitamin A whose absorption bands are situated at 330, 350 and 370 mμ. An absorption band at 392 mμ, such as is specific for the anhydro vitamin A of the same authors, is definitely absent from the spectrum of our irradiation product. This spectroscopic similarity of the irradiation product with isoanhydro vitamin A speaks for structural similarity of the two compounds. Their actual identity with each other is however excluded (1) by our finding that isoanhydro vitamin A is non-fluorescent under the given conditions, (2) because hydrochloric acid, used in the preparation of isoanhydro vitamin A, exerts an irreversible quenching effect on the fluorescence of the irradiation product, (3) by the fact that the irradiation product, in contrast to isoanhydro vitamin A, does not pass through an alumina column but is either adsorbed or destroyed in the column.

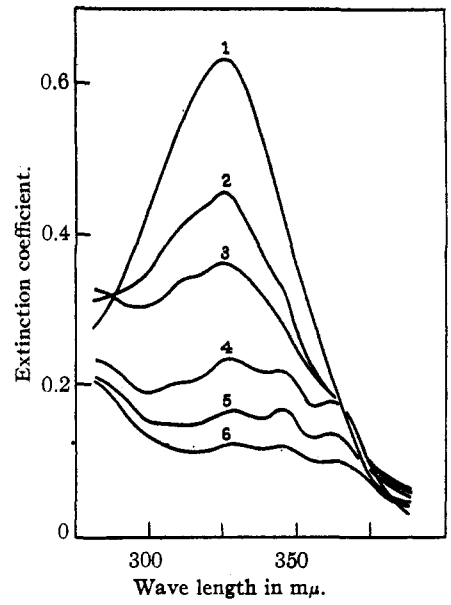

Fig. 3.—Ultraviolet absorption spectra of halibut liver oil, rich in vitamin A, in absolute alcohol before irradiation (1), after irradiation with light of 365 mμ (2, 3), and after varying periods of irradiation by a mercury arc (4, 5, 6), according to Chevallier and Dubouloz.[7]

The observations related in our first report[2] indicated that the highly fluorescent irradiation product of vitamin A could not consist of vitamin A in a reversibly excited state. The present spectroscopic evidence, however, suggests the possibility that one deals with an excited form of isoanhydro vitamin A. Comparison of the absorption data for isoanhydro vitamin A with the elevation of the hump at 350 mμ in curve 2 (Fig. 2) above the smooth slope, indicates that the amount of vitamin A converted into the hypothetical isoanhydro derivative may be as much as 15%. This calculation is based on the assumption that the absorption spectrum of this substance coincides with that of ordinary isoanhydro vitamin A. As stated above, the preparative isolation of the irradiation product in quantities necessary for chemical study, is impossible because of the ex-

(7) A. Chevallier and P. Dubouloz, *Bull. soc. chim. biol.*, **18**, 702 and 1115 (1936).

(8) H. N. Holmes and R. E. Corbet, THIS JOURNAL, **59**, 2042 (1937).

tremely low upper limit of concentration, compatible with irradiation effective in depth.

Secondary Reaction Products.—The study of the primary irradiation product is further complicated (1) by the formation of secondary reaction products, due to photo-oxidation of the primary product, and (2) also by the possible occurrence of other photochemical reactions of the starting material, running alongside the reaction mechanism responsible for the fluorescence phenomenon. The maximum at 275 mμ, which persists during irradiation of vitamin A esters, resembles the maximum of the band observed in deteriorated fish oils and especially in biological degradation products of the vitamin, such as has been described in blood and feces extracts by LePage and Pett.[9] Since the photochemical destruction of the highly fluorescent primary irradiation product seems to be in the nature of an oxidation, we believe that oxidative degradation of the side chain leads to a substance with three conjugated double bonds, and maximum absorption around 275 mμ.

Carr–Price Reaction of Irradiated Solutions.—The character of the irradiation mixture is too complex to permit one to draw conclusions from its behavior with antimony trichloride in chloroform solution. The Carr–Price reaction of the irradiated solution cannot be accounted for by unchanged vitamin A, but is presumably due to the presence of oxidation products such as the chromogenic compound with the absorption band at 275 mμ. The present evidence permits no decision of the question whether the original chromogen is the product of the secondary photo-oxidation or whether it is formed from vitamin A directly by an independent parallel reaction. This reaction is assumed to prevail in the irradiation of vitamin A alcohol, and also of vitamin A esters in non-alcoholic solvents.

The following composite picture may be derived from the reported phenomena: the irradiation of vitamin A esters in alcoholic solution induces the formation of a highly excited species of molecule with more than 5 conjugated double bonds. This irradiation product is not identical with isoanhydro vitamin A itself in spite of the spectrographic evidence. However, all other observations may be brought into accord with the assumption that the highly fluorescent irradiation product represents an activated form of isoanhydro vitamin A. The occurrence of this hypothetical reaction with esters, in contrast to the free vitamin A alcohol, and its specificity for alcoholic solutions would imply the removal of one molecule of fatty acid (acetic acid, etc.) and its subsequent reësterification with the solvent. The amount of ester so formed (*e. g.*, ethyl acetate) would be too minute for analytical detection.

As concerns the kinetics of the photo-reactions, the photo-oxidation of the highly fluorescent compounds widens in scope parallel with its accumulation until the two reactions balance each other and an equilibrium concentration in the order of magnitude of one-tenth of the original vitamin A concentration is attained. The resulting plateau of maximum fluorescence is prolonged and its height, which is proportional to the concentration of the substance, is increased when oxygen is excluded. In the quenching experiments,[2] a faster reaction of the activated molecule with carbon disulfide supersedes oxidation. The nature of the products of these secondary reactions with oxygen or with carbon disulfide is unknown; it is noteworthy that about one-half of the original Carr–Price chromogenicity persists, after fluorescence has been destroyed by prolonged irradiation or by quenching.

Summary

The highly fluorescent solution obtained by ultraviolet irradiation of vitamin A acetate, but not of the free vitamin A alcohol, in ethanol shows four absorption bands at wave lengths 275, 328, 345–346 and 364–365 mμ, the second of which is identical with that of vitamin A itself. This spectrum suggests the presence of more than five conjugated double bonds. The three longer wave lengths coincide with those of the absorption bands of isoanhydro vitamin A. The highly fluorescent irradiation product, while not identical with isoanhydro vitamin A, may constitute an excited form of the latter. The chromogenic power of irradiated vitamin A in the Carr–Price reaction is hardly impaired until a secondary oxidative photoreaction leads to decrease and eventual disappearance of fluorescence. The absorption band at 275 mμ is due to a chromogenic product of a less specific and possibly independent, oxidative degradation of vitamin A.

(9) G. A. LePage and L. B. Pett, *J. Biol. Chem.*, **141**, 753 (1941).

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