The Fluorescence Properties of Vitamin A and their Changes during Photodecomposition

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The influence of instrumental factors on the fluorescence properties of all-*trans* vitamin A_1 alcohol and acetate in cyclohexane has been studied, and the corrected excitation and emission spectra, and the relative quantum efficiency have been estimated.

A strict proportionality was found to exist between concentration and fluorescence intensity within the range of 0.003—6 μmole/l.

The fluorescence intensity was found to vary considerably with different solvents. The highest fluorescence intensity and the lowest blank fluorescence were obtained with cyclohexane.

Exposure of the vitamin to ultraviolet irradiation was found to decrease the fluorescence intensity. The rate of decrease was found to vary with different solvents. In ethanolic solutions, irradiation of vitamin A esters induced a transient rise in the fluorescence intensity associated with development of new excitation maxima.

Thin-layer chromatography, difference absorption spectrum of irradiated and unirradiated vitamin A, and the colour reactions with $SbCl_3$ indicated formation of anhydrovitamin A and epoxy compounds during photodecomposition.

The extent of photodecomposition during measurement of the fluorescence intensity was found to be negligible.

In connection with studies on the fluorimetric assay of vitamin A in biological samples,[1,2] it was considered necessary to study systematically the influence of instrumental factors, solvents, and concentration on the fluorescence properties of the vitamin. Changes in the fluorescence properties during photodecomposition of the vitamin were also included in this study since only a few, and in part contradictory, reports are available on this subject.[3-5]

MATERIAL AND METHODS

The chemicals used were of A.R. quality, obtained from E. Merck AG., Darmstadt, Germany, unless otherwise stated, and described previously.[1] In addition, isopropanol was obtained from May & Baker Ltd., London, and ethyl ether of the purity corresponding to the specifications of the Pharmacopoeia Suecia Ed. XI from AB Syntes, Stockholm. Crystalline vitamin A palmitate was obtained from Nutritional Biochemical Corp.,

Cleveland, Ohio, and crystalline 13-*cis* vitamin A_1 alcohol from Distillation Products Industries Ltd., Rochester, N.Y.

Instrumentation. Fluorescence intensity was measured with a Zeiss spectrofluorimeter supplied with an Osram XBO 450-W xenon-arc lamp and with an RCA 1P28 photomultiplier tube. The wavelength scale of the monochromators was checked with a medium-pressure mercury lamp. Variation in instrumental setting was corrected as described previously.[1] The amplified output of the photomultiplier tube was coupled to a Honeywell-Brown Elektronik 153X18 strip chart ratio-recorder. Suprasil cells of 1 cm light path and a sample volume of 4 ml were used.

The ordinates of the recorded spectra were measured in arbitrary units at intervals of 5 mμ. Corrections were introduced for solvent blank, for the variation of the intensity of exciting radiation at different wavelengths, and for the response characteristics of the detector system (see below).

Irradiation of samples. Samples were irradiated in the cell compartment of the fluorimeter using a medium-pressure mercury lamp (St 41, Quarzlampen G.m.b.H., Haunau, Germany) and a "monochromatic filter (365 mμ)", both supplied with the instrument. The distance from the light source to the cell surface was 12 cm. The irradiated cell surface was 1.56 cm^2. The radiation dose received by the sample was approximately 6 einstein/min $\times$ 10^7 as measured by the ferrioxalate chemical actinometer.[6]

Correction of excitation spectra. Potassium ferrioxalate was prepared according to Parker.[6] Three volumes of 1.5 M potassium oxalate were mixed with 1 volume of 1.5 M ferric chloride. The precipitated complex salt was recrystallized 3 times from deionised warm water and dried in a current of air of 45°C.

Three ml samples of 0.006 M potassium ferrioxalate in 0.1 N sulfuric acid were irradiated in the cell compartment of the fluorimeter for 15 min. The samples were stirred during irradiation by a current of oxygen-free nitrogen. After irradiation, the samples were analysed for the amount of ferrous iron formed. Irradiation and analysis were carried out in a dark room illuminated by a red safe light. The quantity of ferrous iron formed was corrected for the blank value of the unirradiated sample as well as for variation in the absorption and quantum yield at different wavelengths.[7]

The data thus obtained for the intensity of exciting radiation at different wavelengths were found to agree well with the average correction curve of the instrument for excitation spectra.[8] A further check was made by comparing the corrected excitation spectra of some fluorescence standard substances with their absorption spectra. In all cases a satisfactory agreement was found.

Correction of emission spectra. The response characteristics of detector system were estimated by recording the photomultiplier response at different wavelengths as a function of the radiant energy emerging from the cell compartment. The correction data thus obtained were compared with the average correction curve of the instrument for emission spectra [8] and also with the average response curve of the photomultiplier tube. This later was further corrected for variation in the band width during scanning with constant slit and for average light loss within the monochromator.[9] The validity of the obtained correction factors was tested by comparing the corrected emission spectra of some fluorescence standard compounds with those earlier reported.[10-13] A reasonably good agreement was found.

The relative quantum efficiency was estimated according to Parker and Rees [13] by comparing the integrated area under the corrected emission spectrum of vitamin A and that of quinine bisulphate obtained under identical conditions of excitation.

Vitamin A. The procedures used for the determination of vitamin A were described previously.[1]

cis-*Isomers of vitamin A* were determined according to Robeson and Baxter.[14] Equal volumes of all-*trans* vitamin A_1 acetate in benzene and 10 % (w/v) maleic anhydride in benzene were mixed and allowed to stand in darkness at 20°C for 16 h. The addition product of vitamin A acetate with maleic anhydride was estimated by measuring the difference between the intensities of the blue colour developed in the $SbCl_3$ reaction before and after addition of maleic anhydride. The relative amount of 13-*cis* and 9,13-di-*cis* isomers was calculated according to Robeson and Baxter.[14]

Iron was determined as described by Parker.[6] To 1 ml aliquots of the irradiated photolyte were added 1.5 ml 0.15 % (w/v) o-phenantroline and 0.5 ml sodium acetate-sulfuric acid buffer (600 ml N sodium acetate and 360 ml N sulfuric acid diluted to 1 l). After

mixing, the samples were allowed to stand for at least 30 min and the extinction was measured at 510 mμ.

The *thin-layer chromatographic techniques* used in this study were described previously.[2]

RESULTS

Fluorescence spectra. Fig. 1 shows the absorption and fluorescence spectra of all-*trans* vitamin A_1 alcohol in cyclohexane. As this figure shows there is a considerable difference between the recorded and corrected fluorescence spectra. Insertion of secondary filters, or reduction of the slit width from 2.0 to 0.1 mm, changed only the intensity of the fluorescence but not the resolution of the spectra. The overlap between the excitation and emission spectra is seen to be negligible.

The corrected excitation spectrum shows a good agreement with the absorption spectrum within the range of 270—370 mμ. The recorded and corrected fluorescence maxima found for all-*trans* vitamin A_1 alcohol and acetate in cyclohexane are given in Table 1.
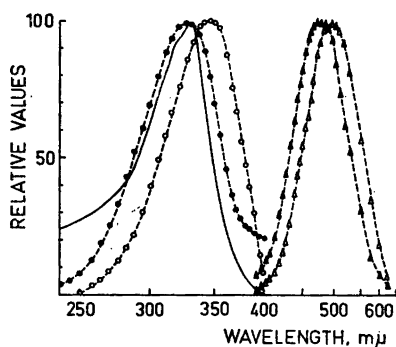

Fig. 1. Absorption and fluorescence spectra of all-*trans* vitamin A_1 alcohol in cyclohexane. Absorption was measured in a Beckman DK-2 spectrophotometer and fluorescence intensity in a Zeiss spectrofluorimeter. Fluorescence spectra were obtained by setting one of the monochromators to the optimum wavelength (excitation at 345 mμ, emission at 490 mμ) and scanning with the other one. Ordinate = optical density units, arbitrary fluorescence intensity units, and quanta per unit wavelength interval. ——— = absorption spectrum, O————O = excitation spectrum, ●————● = excitation spectrum corrected for variation in the intensity of exciting radiation at different wavelength, △————△ = emission spectrum, ▲————▲ = emission spectrum corrected for the response characteristics of the detector system.
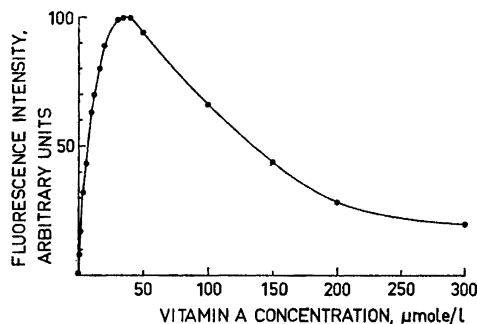
Fig. 2. Effect of concentration on the fluorescence intensity of all-*trans* vitamin A_1 alcohol in cyclohexane. Fluorescence intensity was measured in a Zeiss spectrofluorimeter at 345 mμ (exc.) and 490 mμ (fluor.) with slit widths of 2 mm.

Table 1. Ultraviolet absorption and fluorescence maxima of all-*trans* vitamin A_1 alcohol and acetate in cyclohexane. Absorption maximum was estimated in a Beckman DK-2 spectrophotometer and fluorescence intensity in a Zeiss spectrofluorimeter. All values were corrected for solvent blank, the corrected excitation maximum also for variation in the intensity of exciting radiation at different wavelengths, and the corrected emission maximum also for the response characteristics of the detector system.

	Vitamin A alcohol λ_{max} mμ	Vitamin A acetate λ_{max} mμ
Ultraviolet absorption	326	328
Excitation	347	345
Excitation, corrected	325	323
Emission	490	495
Emission, corrected	470	475

The quantum efficiency of all-*trans* vitamin A_1 alcohol and acetate, relative to the reported value of 0.55 for quinine bisulphate,[13] was found to be 0.67 and 0.68.

Effect of solvent. Table 2 shows the variation in the absorbancy and in the fluorescence intensity of all-*trans* vitamin A_1 alcohol and acetate with different solvents. The solvent effect on the fluorescence intensity is considerable. The fluorescence is most intense in cyclohexane and this solvent also shows the most favourable ratio between fluorescence intensity and blank reading. The fluorescence and absorption maxima varied only slightly with different solvents.

Effect of concentration. Fig. 2 shows the plot of the fluorescence intensity of all-*trans* vitamin A_1 alcohol in cyclohexane as a function of concentration. Within the range of 0.003—6 μmole/l, the fluorescence intensity was strictly proportional to the concentration of vitamin A. At concentrations exceeding

Table 2. Relative absorbancy and fluorescence intensity of all-*trans* vitamin A_1 alcohol and acetate in different solvents. Optical density was measured in a Beckman DU spectrophotometer at the absorption maximum, and fluorescence intensity in a Zeiss spectrofluorimeter at 345 mμ (exc.) and 490 mμ (fluor.). All values were corrected for solvent blanks. Results are means of 12 replicate determinations.

Solvent	Relative absorbancy		Relative fluorescence intensity	
	Vitamin A alcohol M $\pm$ ε (M)	Vitamin A acetate M $\pm$ ε (M)	Vitamin A alcohol M $\pm$ ε (M)	Vitamin A acetate M $\pm$ ε (M)
Cyclohexane	100.0 $\pm$ 0.9	100.0 $\pm$ 0.8	100.0 $\pm$ 0.6	100.0 $\pm$ 0.9
Heptane	100.5 $\pm$ 1.8	103.2 $\pm$ 0.9	85.3 $\pm$ 0.5	94.0 $\pm$ 2.3
Ethyl ether	119.2 $\pm$ 0.7	104.2 $\pm$ 0.5	83.6 $\pm$ 1.0	70.4 $\pm$ 1.0
Toluene	104.5 $\pm$ 0.9	92.3 $\pm$ 0.4	71.2 $\pm$ 1.1	70.8 $\pm$ 1.2
Ethanol	104.6 $\pm$ 0.9	104.0 $\pm$ 0.9	58.8 $\pm$ 3.9	57.5 $\pm$ 2.2
Isopropanol	103.2 $\pm$ 0.5	104.0 $\pm$ 0.8	52.8 $\pm$ 0.6	67.2 $\pm$ 1.6
Chloroform	93.2 $\pm$ 1.2	92.8 $\pm$ 0.8	45.8 $\pm$ 0.6	60.3 $\pm$ 0.5

30 μmole/l, considerable quenching was observed. The quenching also caused a successively increasing shift in the excitation maximum toward the longer wavelength region. At a concentration of 200 μmole/l, the uncorrected excitation maximum was found to be at about 390 mμ.

Random error. The fluorescence intensity of replicate samples of all-*trans* vitamin A_1 alcohol in cyclohexane was estimated at the highest sensitivity setting of the instrument, and the variation of a single determination was calculated. Measurements were made under identical experimental conditions using 2 different light sources. In a series of 30 samples, the mean fluorescence intensity was found to be 78.0 $\pm$ 1.4 arbitrary units using the xenon-arc lamp, the random error of a single determination being 1.7 % of the mean. Using the mercury lamp, the mean fluorescence intensity was 79.1 $\pm$ 0.5 arbitrary units, the random error being 0.6 % of the mean.

Effect of ultraviolet irradiation. Samples of all-*trans* vitamin A_1 acetate, containing 15 μmole/l in cyclohexane, were exposed to ultraviolet irradiation for varying periods of time. Table 3 shows the rate of photodecomposition as measured by 3 different assay methods. The results reveal a systematic discrepancy between the methods. The apparent rate of photodecomposition was about the same when estimated by fluorimetry or ultraviolet absorption but considerably slower when measured by the $SbCl_3$ reaction.

In another series of experiments, the fluorescence intensity of all-*trans* vitamin A_1 acetate in cyclohexane was continuously recorded during exposure to irradiation. Two different light sources were employed. The fluorescence intensity was measured 2 and 3 min after starting irradiation, and the rate of photodecomposition was estimated by calculating the difference between measurements. In a series of 30 samples, the mean extent of photodecomposition thus estimated was found to be 2.6 $\pm$ 0.6 % with the xenon-arc lamp, and 6.2 $\pm$ 0.6 % with the mercury lamp.

Fig. 3 shows continuous recordings of the fluorescence intensity of all-*trans* vitamin A_1 acetate on exposure to ultraviolet irradiation in different solvents. In non-ethanolic solvents, a steady decrease in the fluorescence intensity was observed, and the rate was roughly proportional to the initial fluores-

Table 3. Photodecomposition of all-*trans* vitamin A_1 acetate in cyclohexane as measured by different assay methods. Concentration: 15 μmole/l. Irradiation with monochromatic light (365 mμ). Radiation dose received by the sample = 6 einstein/min $\times$ 10^7. The apparent vitamin A content is given as per cent of the initial value. All values are means of 3 experiments.

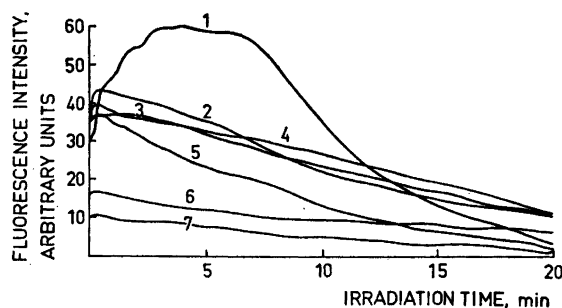| Irradiation time (min)	Apparent vitamin A content estimated by		
	Fluorimetry	Ultraviolet absorption	$SbCl_3$ reaction
5	88	78	93
.15	62	44	84
60	6	5	38
90	3	2	22

Fig. 3. Changes in the fluorescence intensity of all-*trans* vitamin A_1 acetate on exposure to ultraviolet irradiation. Concentration: 5 μmole/l. Irradiation with monochromatic light (365 mμ). Radiation dose received by the sample = 6 einstein/min × 10^7. The fluorescence intensity emitted at 490 mμ was continuously recorded. 1 = ethanol, 2 = cyclohexane, 3 = ethyl ether, 4 = heptane, 5 = toluene, 6 = isopropanol, 7 = chloroform.

cence intensity. In ethanol, all-*trans* vitamin A_1 acetate showed a steep initial rise in the fluorescence intensity.

Further experiments indicated that this phenomenon could only be observed for ethanolic solutions of the ester forms of the vitamin at concentrations below 15 μmole/l. In solvent mixtures, containing ethanol, the initial rise in the fluorescence intensity was proportional to the concentration of ethanol. The minimum effective concentration was about 40 % ethanol in the mixture.

Fig. 4 shows experiments where solutions of all-*trans* vitamin A_1 acetate were exposed to intermittent irradiation. It is seen that in ethanolic solutions each irradiation period caused a new rise in fluorescence intensity.

Fig. 5 shows the changes in the excitation spectrum of all-*trans* vitamin A_1 acetate induced by exposure to ultraviolet irradiation. In non-ethanolic solvents, there was a progressive decrease in the fluorescence intensity with a broadening of the curves (Fig. 5 A). In ethanol, on the other hand, the
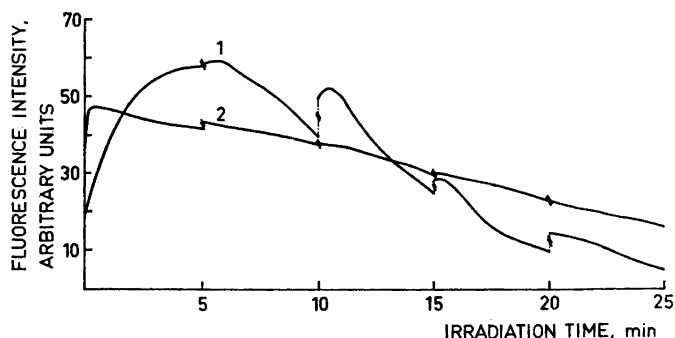

Fig. 4. Changes in the fluorescence intensity of all-*trans* vitamin A_1 acetate on exposure to intermittent ultraviolet irradiation. Concentration: 5 μmole/l. Irradiation with monochromatic light (365 mμ). At 5 min intervals the irradiation was interrupted for 5 min. Radiation dose received by the sample = 6 einstein/min × 10^7. The fluorescence intensity emitted at 490 mμ was continuously recorded. 1 = ethanol, 2 = cyclohexane.
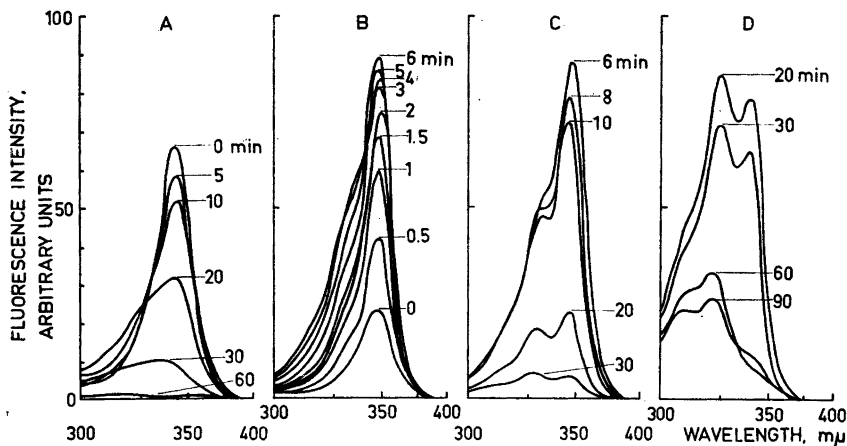
Fig. 5. Changes in the excitation spectrum of all-*trans* vitamin A_1 acetate on exposure to ultraviolet irradiation. Concentration: 10 μmole/l. Irradiation with monochromatic light (365 mμ) for varying periods of time as indicated in the figure (time in min). Radiation dose received by the sample = 6 einstein/min × 10^7. Emission measured at 490 mμ. A = cyclohexane, B—D = ethanol. The spectra in D were recorded at higher amplification.

fluorescence intensity of the main band increased during the first 6 min of irradiation (Fig. 5 B) and later decreased (Fig. 5 C, D). During irradiation, subsidiary bands also appeared and prolonged irradiation caused a shift in the excitation maximum from 345 mμ to 325 mμ and later to about 287 mμ. In solvent mixtures containing ethanol, the extent of changes was proportional to the concentration of ethanol.

The irradiation products were further characterized by recording the difference absorption spectrum of irradiated and unirradiated all-*trans* vitamin A_1 acetate (Fig. 6). After 5 min irradiation in non-ethanolic solvents, there were two broad bands in the 280 and 326 mμ regions. In ethanol, there were a triple-peak at 345, 368 and 388 mμ, a broad band in the near ultraviolet region (maxima at 270 and 285 mμ) and two additional minor maxima at 310 and 326 mμ.

The reaction product of the unirradiated all-*trans* vitamin A_1 acetate with $SbCl_3$ showed a single absorption peak at 620 mμ with an inflection in the 585 mμ region. The chromogen of the irradiated compound with $SbCl_3$ gave a number of additional bands at 470, 520, 575, and 680 mμ.

All-*trans* vitamin A_1 acetate before and after irradiation showed no difference in the rate of formation of additional products with maleic anhydride.

Thin-layer chromatograms of irradiated all-*trans* vitamin A_1 acetate revealed a progressively increasing reduction of the original compound with the appearance of 3 new components (Fig. 7). The absorption spectra of the eluted fractions showed a reduction in the absorption at 326 mμ and a corresponding increase in the 240 and 290 mμ regions. The extent of these changes was proportional to the duration of irradiation. One of the new components formed a yellow-pink reaction product with $SbCl_3$.
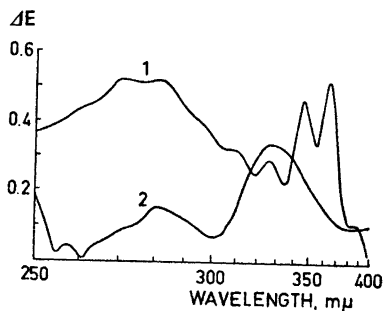
Fig. 6. Difference absorption spectrum of unirradiated and irradiated all-*trans* vitamin A_1 acetate. Concentration: 15 μmole/l. Irradiation with monochromatic light (365 mμ) for 5 min. Radiation dose received by the sample = 6 einstein/min $\times$ 10^7. 1 = ethanol, 2 = cyclohexane.
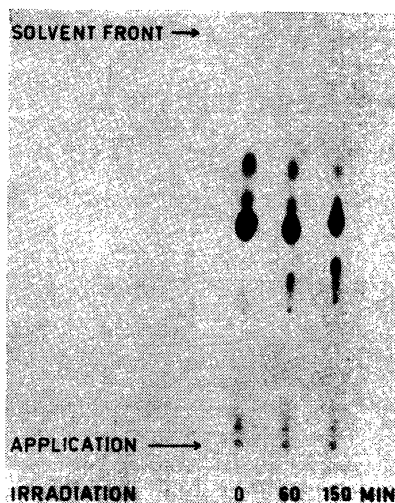
Fig. 7. Thin-layer chromatographic pattern of irradiated all-*trans* vitamin A_1 acetate. All-*trans* vitamin A_1 acetate in cyclohexane (10 mmole/l) was irradiated with monochromatic light (365 mμ). Radiation dose received by the sample = 6 einstein/min $\times$ 10^7. 10 μl of the sample was chromatographed on silica gel G, the chromatogram developed with cyclohexane — ethanol (97:3) and the spots were detected by spraying with $SbCl_3$ and with $SbCl_5$.

COMMENTS

Instrumental factors are known to influence greatly both the excitation and emission spectra of fluorescent compounds. The corrected fluorescence maxima of vitamin A, shown in Table 1, are in good agreement with previously reported data.[15] The corrected excitation spectrum was found to agree fairly well with the absorption spectrum (Fig. 1).

The optical density at the absorption maximum of vitamin A has been reported to vary only slightly with different solvents.[16-18] This could be confirmed in the present investigation. On the other hand, the intensity of the fluorescence was found to vary considerably with different solvents (Table 2).

During irradiation of vitamin A esters in ethanol, a series of changes in the absorption spectrum [19-22] and an initial rise in the fluorescence intensity [4,5] has been reported to occur. Similar observations were made in the present study. It was further shown that these changes were associated with the development of new excitation maxima (Fig. 6). In solvent mixtures containing ethanol, the extent of the observed changes was proportional to the ethanol concentration. In the absence of ethanol, only a steady decrease in the fluorescence intensity could be observed (Fig. 3).

The increase in the fluorescence intensity during irradiation of ethanolic solutions of vitamin A esters has been suggested to depend on the formation of isoanhydrovitamin A or on some other compound closely related to anhydrovitamin A.[20] Recently, evidence has been obtained for the formation of anhydrovitamin A both during irradiation of pure compounds [23] and during deterioration of pharmaceutical preparations.[24] The shape of the difference absorption spectrum in the visible region of irradiated and unirradiated vitamin A, shown in Fig. 6, also indicates the formation of anhydrovitamin A.

The extinction coefficient of anhydrovitamin A at 326 mμ is lower than that of vitamin A, whereas anhydrovitamin A shows a higher colour intensity in the $SbCl_3$ reaction.[25] Formation of anhydrovitamin A may therefore well explain the differences in the apparent rate of photodecomposition observed using different methods for determination (Table 3).

Photoisomerisation of vitamin A has been suggested to occur on the absorption of irradiation energy [26] and has been observed during deterioration of pharmaceutical preparations.[27] The present studies on the rate of formation of addition product of vitamin A with maleic anhydride failed to demonstrate appreciable accumulation of 13-*cis* and 9,13-di-*cis* isomers during irradiation.

Formation of other decomposition products, mainly epoxy compounds, has been reported to occur in autooxidized samples,[21, 28-30] during chromatographic separations,[31-33] and by the action of different chemical agents.[34-38] The shape in the ultraviolet region of the difference absorption spectrum of irradiated and unirradiated vitamin A, shown in Fig. 6, also indicates the presence of such compounds. These new compounds could be separated by thin-layer chromatography (Fig. 7). The new components showed colour reactions with $SbCl_3$ and, after elution, absorption spectra characteristic of epoxy compounds.

Although the fluorescence properties of vitamin A are influenced by a number of factors, it is possible to obtain highly reproducible results by using standardized experimental conditions. Within the concentration range of 0.003—6 μmole/l (Fig. 2), the random error of a single determination was thus found to be below 2 %. The experiments presented here also show that with suitable equipment and a short measuring time it is possible to maintain the error due to photodecomposition of the samples during measurements within the limits of this experimental error.

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