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Chromatographic Separation of two Vitamin A_2 Isomers

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The presence of two vitamin A_2 isomers in perch and pike liver extract has been demonstrated, and the spectroscopic properties of the substances have been given.

By the action of iodine on natural vitamin A_2 a vitamin A_2 mixture is obtained which may be separated by chromatography into two substances, *a* and *b*, the ratio of *a* to *b*, expressed by $E_{351m\mu}$, being about 1 to 2. Substance *a* seems to be identical with the neovitamin A_2 occurring in the liver extract. There are some doubts as to whether substance *b* and the vitamin A_2 occurring in Nature are identical. Even though substance *b* and natural vitamin A_2 exhibit a number of similarities both spectroscopically and chromatographically, certain discrepancies as regards spectroscopic properties have made it impossible to ascertain whether *b* is a single substance or a mixture of vitamin A_2 and an irrelevant substance with light absorption in the wavelength range below 310 mμ.

When an isomerized mixture of vitamin A_1 and vitamin A_2 was chromatographed on dicalcium phosphate, the four substances present were eluted in the following order: neovitamin A_1 — substance *a* (neovitamin A_2) — all-*trans* vitamin A_1 — substance *b* (natural vitamin A_2?).

In view of the close relationship between vitamin A_1 and vitamin A_2 (= dehydrovitamin A_1) it is a reasonable assumption that vitamin A_2 may also occur in more than one of the theoretically possible stereoisomeric forms. In their determinations of the content of vitamin A_1 and vitamin A_2 in certain Nile fish Abdullah, Morcos and Salah [1] have assumed the existence of two different geometrical isomers of vitamin A_2 (neovitamin A_2 and vitamin A_2) and have determined the ratio of neovitamin A_2 to vitamin A_2, assuming that under identical experimental conditions the reactions of the two vitamin A_2 isomers with maleic acid anhydride both qualitatively and quantitatively are identical with the corresponding reactions of neovitamin A_1 and all-*trans*-vitamin A_1, respectively.

Since it has been shown to be possible to separate neovitamin A_1 and all-*trans* vitamin A_1 by chromatography (Bro-Rasmussen, Hjarde and Porotnikoff[2]), it would be reasonable to ascertain whether by applying a similar method to a supposed mixture of vitamin A_2 isomers it would be possible to obtain a

separation, or at any rate a partial separation, of the isomers, enabling a further examination of the course of their absorption curves. In the above-mentioned publication from this laboratory it has been suggested that vitamin A_2 may be present in cod liver oil in two stereoisomeric forms, which differ with regard to the readiness with which they are eluted when chromatographed on dicalcium phosphate.

However, preliminary investigations show fresh liver from various freshwater fish to contain almost exclusively one of the vitamin A_2 forms. On chromatography, the greater part of the light-absorbing substances with absorption maximum at about 350 mμ is found to be present in the form of one fraction with an absorption curve which is in good agreement with the one given in the above publication [2]. It was therefore necessary to perform an isomerization of the isolated vitamin A_2 fraction followed by chromatography to separate the isomeric substances.

In the following an account will be given of these experiments and the results obtained.

EXPERIMENTAL

The technique used is, with the exception of the isomerization process, described in the above publication. A few modifications will be referred to in connection with the individual experiments. The isomerization process will be described in section II below. All the chromatographic separations mentioned in the following have been performed at room temperature (22–23°C) and protected against daylight. The spectrophotometric measurements were made with a Beckman spectrophotometer, type D.U., slit-width about 0.15–0.08 mm within the measuring range used in the present investigation.

I,1. ISOLATION OF VITAMIN A_2 FROM PIKE LIVER

A dicalcium phosphate column, 86 cm long, 1.8 cm diam., was used for chromatography of the unsaponifiable fraction from 5.95 g of pike liver, the absorption curve of which when measured in light petroleum displayed three maxima (277 mμ, 287 mμ and 348—352 mμ, $E^{1\%}_{1cm,\ 350\ m\mu} = 0.920$ measured in relation to the liver weight) and $\frac{E_{287\ m\mu}}{E_{350\ m\mu}} = 0.513$. The eluent used was a mixture of light petroleum and diethylether (86:14 v/v). Eluate was collected in 4.7 ml fractions by means of an automatic fraction collector. The fractionation curve is illustrated in Fig. 1. The fractions collected were measured spectrophotometrically (after dilution with light petroleum, if necessary) at the wavelengths 325 mμ and 351 mμ. The value of the ratio $\frac{E_{351\ m\mu}}{E_{325\ m\mu}}$ for each individual fraction is also given in Fig. 1.

The peak fraction (No. 30) and the immediately subsequent fractions all displayed the same value of the ratio $\frac{E_{351\ m\mu}}{E_{325\ m\mu}}$. The vitamin A_2 fraction (No. 31) following the peak fraction with $E^{1cm}_{351\ m\mu} = 6.01$ was evaporated to dryness in a stream of carbon dioxide and redissolved in absolute ethanol. The resulting absorption curve (Fig. 2, curve 1) displayed three maxima (276.5 mμ, 287 mμ

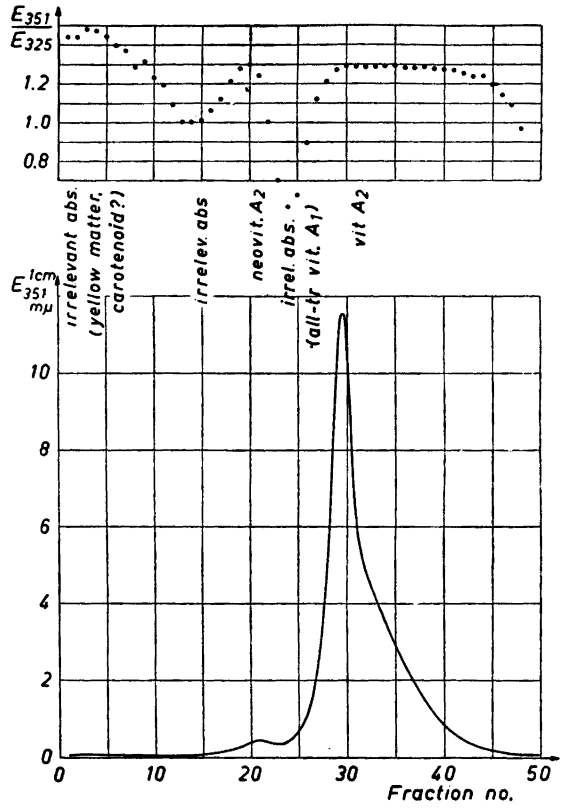

Fig. 1. Fractionation curve for the unsaponifiable fraction from pike liver with a column 86 cm long, diam. 1.8 cm. The upper part of the figure illustrates the ratio $\frac{E_{351\ m\mu}}{E_{325\ m\mu}}$ *for the individual fractions.*

and 351.5 mμ) and two minima (280 mμ and 297 mμ) and the following ratios were found:

$$\frac{E_{276.5}}{E_{351.5}} = 0.388, \quad \frac{E_{280}}{E_{351.5}} = 0.369, \quad \frac{E_{287}}{E_{351.5}} = 0.493, \quad \frac{E_{297}}{E_{351.5}} = 0.298 \quad \frac{E_{325}}{E_{351.5}} = 0.735$$

I,2. ISOLATION OF VITAMIN A_2 FROM PERCH LIVER

A dicalcium phosphate column, 70 cm long, 1.8 cm diam., was used for chromatography of the unsaponifiable fraction from 15.76 g of perch liver ($E^{1\%}_{1cm,\ 350\ m\mu} = 0.085$ measured in light petroleum and calculated in relation to the liver weight). Eluents used: light petroleum-ether mixtures, 300 ml (92:8 v/v), 300 ml (90:10 v/v). Eluate was collected in 3.2 ml fractions. The fractionation curve is illustrated in Fig. 3.

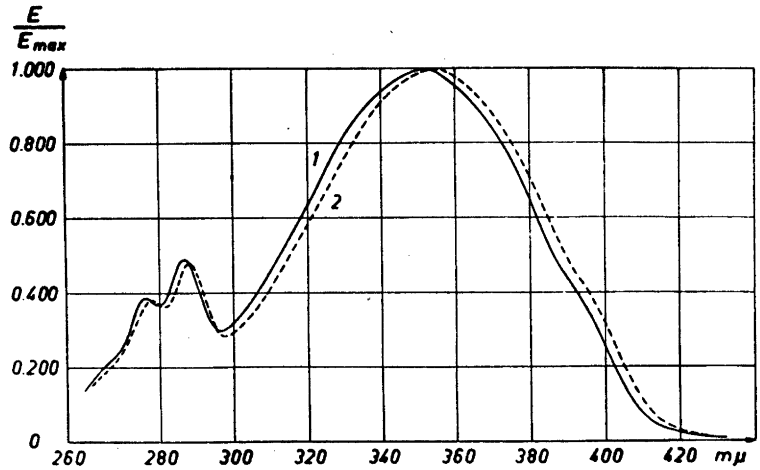

Fig. 2. Light absorption curves for natural vitamin A_2 (curve 1) and the substance a (curve 2). Solvent absolute ethanol.

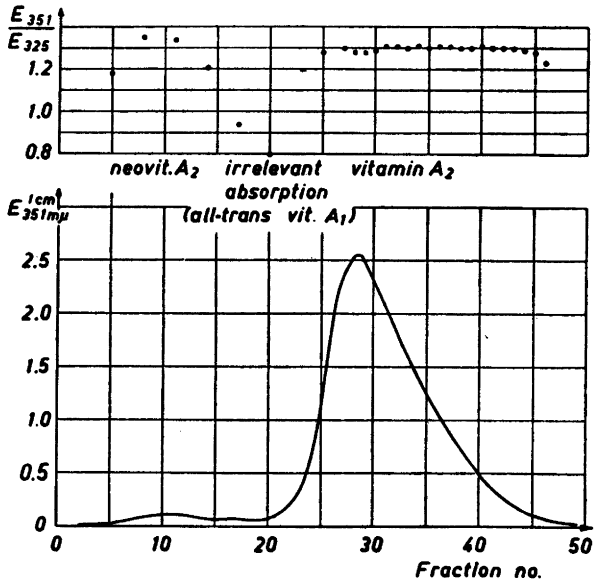

Fig. 3. Fractionation curve for the unsaponifiable fraction from perch liver with a column 70 cm long, diam. 1.8 cm. The upper part of the figure illustrates the ratio $\frac{E_{351\ m\mu}}{E_{325\ m\mu}}$ *for the individual fractions.*

The peak fraction with $E^{1\,cm}_{351.5} = 2.52$ displayed, when dissolved in ethanol, three maxima and two minima at the wavelengths mentioned under I,1. The following ratios were found:

$$\frac{E_{276.5}}{E_{351.5}} = 0.384, \quad \frac{E_{280}}{E_{351.5}} = 0.369, \quad \frac{E_{287}}{E_{351.5}} = 0.490, \quad \frac{E_{297}}{E_{351.5}} = 0.297, \quad \frac{E_{325}}{E_{351.5}} = 0.734.$$

In a subsequent chromatographic isolation on a dicalcium phosphate column, 82 cm long, 1.8 cm diam., of vitamin A_2 from 65 g perch liver (a solution of the unsaponifiable fraction in light petroleum gave $E^{1\,\%}_{1\,cm\ 351\ m\mu} = 0.236$, calculated in relation to the liver weight) all the vitamin A_2-containing fractions for which the ratio $\frac{E_{351.5}}{E_{325}}$ had the same value (1.29—1.31) were combined and again subjected to chromatography on a dicalcium phosphate column, 65 cm long, 1.8 cm diam. Previous to the chromatography, but after activation (*cf.* Brö-Rasmussen, Hjarde and Porotnikoff [2]) this dicalcium phosphate had been washed with ether * on a glass Büchner filter (1.5 l pure, peroxide free ether per 200 g $CaHPO_4$). The washed dicalcium phosphate was transferred into a 500 ml round-bottomed flask and exhausted for half an hour at room temperature. Finally the flask was filled with nitrogen, exhausted and again filled with nitrogen, the process being repeated altogether four times, following which the dicalcium phosphate powder was suspended in light petroleum. According to Schlabach [3], the light petroleum (b.p. 40—60°C) used for the chromatography had been filtered through a column of silica gel (70 cm long, 5.3 cm diam., grain size 100—200 mesh) and subsequently distilled to remove all substances which absorb light within a wavelength range from 240 mμ to 400 mμ when measured against distilled water.

The vitamin A_2 thus isolated by repeated chromatography was dissolved in ethanol and showed an absorption curve with characteristic $\frac{E}{E_{351.5}}$ values in good agreement with those previously given.

The fractionation curves illustrated in Figs. 1 and 3 both display 2 maxima of which the highest correspond to the vitamin A_2 isomer just mentioned. The peak fraction corresponding to the lower maximum of Fig. 1 displayed, when dissolved in absolute ethanol, three maxima, at 277.5 mμ, 287.5 —288 mμ and 353 mμ, and two minima, at 281 mμ and 298 mμ. The following ratios were found:

$$\frac{E_{277.5}}{E_{353}} = 0.46, \quad \frac{E_{281}}{E_{353}} = 0.44, \quad \frac{E_{288}}{E_{353}} = 0.54, \quad \frac{E_{298}}{E_{353}} = 0.36, \quad \frac{E_{325}}{E_{353}} = 0.74$$

The peak fraction corresponding to the lower maximum in the fractionation curve Fig. 3 also displayed three maxima (277 mμ, 287.5 mμ and 353.5 mμ) and two minima (283 mμ and 299.5 mμ), and the following ratios were found (in absolute ethanol):

$$\frac{E_{277}}{E_{353}} = 0.71, \quad \frac{E_{283}}{E_{353}} = 0.63, \quad \frac{E_{287.5}}{E_{353}} = 0.68, \quad \frac{E_{299.5}}{E_{353}} = 0.39, \quad \frac{E_{325}}{E_{353}} = 0.70$$

* The residue obtained by evaporation of the first 400 ml ether filtrate showed after solution in 5 ml absolute ethanol a certain light absorption within the wavelength range 275—325 mμ.

It must be assumed that the substances found in the first fractions by chromatography of pike liver and perch liver are identical, although mixed with different amounts of other light-absorbing substances. The substance which is presumably a vitamin A_2 isomer might be denoted neovitamin A_2 by analogy with neovitamin A_1 which, when chromatographed on a dicalcium phosphate column under corresponding circumstances, leaves the column immediately before all-*trans* vitamin A_1. As the content of this isomer in the liver extracts used is very small (about 1.5 % of the extinction at 351 mμ deriving from the total quantity of eluted neovitamin A_2 + vitamin A_2 is due to neovitamin A_2) and as, moreover, its location in the dicalcium phosphate column is in the transition range between the bands representing the small quantities of the neovitamin A_1 and all-*trans* vitamin A_1 deriving from the liver extract, the fractions containing this neovitamin A_2 cannot, of course, be expected to be pure.

II. ISOMERIZATION

Vitamin A_2 which has been isolated by chromatography according to the method described in section I was dissolved in light petroleum and isomerized by addition of an iodine solution (10 mg iodine per 100 ml light petroleum). To 9 ml of vitamin A_2 solution was added 1 ml iodine solution and after shaking for 30 s the mixture was poured over 0.5 g pulverized sodium thiosulphate ($Na_2S_2O_3$, $5H_2O$) in a 100 ml Erlenmeyer flask, and the flask was shaken until the violet iodine colour disappeared, the sodium thiosulphate precipitate being subsequently removed by filtration through a folded filter. The isomerization process was performed at 23°C, protected against daylight. Three experiments were made, in which the vitamin A_2 solution used had the following values of $E^{1\,cm}_{351\,m\mu}$: 0.980, 7,22 and 34,5 and consequently 0.882, 6.50, and 31.0 at the moment of admixture. After the isomerization $E_{351\,m\mu}$ had dropped by 6—10 % in all three cases. The ratio $\frac{E_{287}}{E_{351}}$ (measured in light petroleum) changed from 0.50 to 0.53—0.55. There was no measurable alteration in the location of the maxima and minima.

Finally an isomerization experiment was made in which 9 ml of a solution of vitamin A_2 in light petroleum, with $E^{1\,cm}_{351\,m\mu} = 0.895$, was mixed with 1 ml of the iodine solution previously mentioned, the iodine being removed after shaking for 100 s as already described. $E^{1\,cm}_{351\,m\mu}$, which at the moment of admixture was 0.805, had after isomerization dropped by about 15 % and $\frac{E_{287}}{E_{351}}$ had altered from 0.495 to 0.629.

III. CHROMATOGRAPHY OF ISOMERIZED VITAMIN A_2

III,1. A sample of 40 ml of a solution of vitamin A_2 in light petroleum which had been isomerized as described in section II (the iodine being allowed to act for a period of 30 s) and with $E^{1\,cm}_{351\,m\mu} = 0.882$ was used for this experiment. For the chromatography a dicalcium phosphate column, length 78 cm, dia-

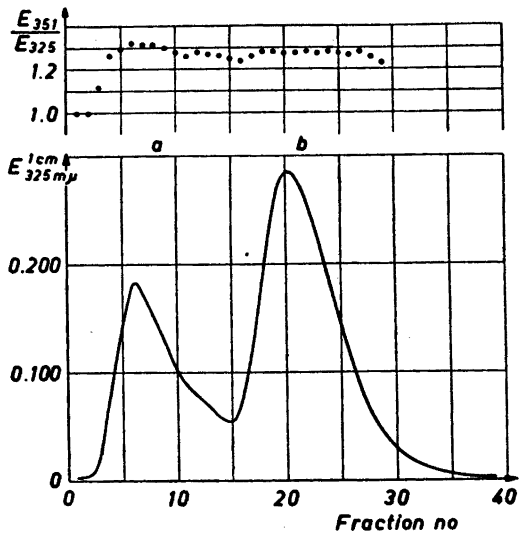

Fig. 4. Fractionation curve for isomerized vitamin A_2 chromatographed on a 78 cm column, diameter 1.8 cm.

meter 1.8 cm, was used. The eluent was a mixture of light petroleum and ether (85:15 v/v). Eluate was collected in 4.7 ml fractions.

The fractionation curve is given in Fig. 4 and shows the presence of two substances with absorption maximum at about 350 mμ. The peak fractions from each of the two curve sections *a* and *b* display the following spectrophotometric properties in absolute ethanol:

Substance a: three maxima: 277.5 mμ, 288—288.5 mμ, and 353.5 mμ
two minima: 281.5 mμ and 298 mμ

$$\frac{E_{277.5}}{E_{353.5}} = 0.432, \quad \frac{E_{281.5}}{E_{353.5}} = 0.415, \quad \frac{E_{288}}{E_{353.5}} = 0.530, \quad \frac{E_{298}}{E_{353.5}} = 0.344, \quad \frac{E_{325}}{E_{353.5}} = 0.724$$

Substance b: three maxima: 276.5 mμ, 287 mμ and 351.5 mμ
two minima: 280 mμ and 297 mμ

$$\frac{E_{276.5}}{E_{351.5}} = 0.472, \quad \frac{E_{280}}{E_{351.5}} = 0.453, \quad \frac{E_{287}}{E_{351.5}} = 0.574, \quad \frac{E_{297}}{E_{351.5}} = 0.339, \quad \frac{E_{325}}{E_{351.5}} = 0.735$$

The ratio of the two substances *a* and *b* may be calculated from the fractionation curve. Ratio *a* to *b* expressed by E_{351} measured directly in the eluate was found to be 34.5/65.5.

III,2. Larger quantities of the two substances *a* and *b* were prepared as follows: The unsaponifiable fraction from 157.6 g of perch liver was subjected to chromatography twice on alumina columns, 18 × 1.8 cm (standardized according to Brockmann). Half of the quantity of purified vitamin A_2 obtained, which in 180 ml light petroleum had an $E^{1\,cm}_{351\,m\mu} = 7.22$ and $\frac{E_{287}}{E_{351}} = 0.50$, was

isomerized as described in section II. After filtration and evaporation, chromatography on a 48×3.0 cm dicalcium phosphate column was applied. The eluents used were various mixtures of diethylether and light petroleum, in the following order:

100	ml	light	petroleum	—	ether	mixture (95: 5 v/v)
100	»	»	»		»	» (92: 8 v/v)
100	»	»	»		»	» (90: 10 v/v)
100	»	»	»		»	» (88: 12 v/v)
300	»	»	»		»	» (86: 14 v/v)
200	»	»	»		»	» (75: 25 v/v)

The eluate was collected in 4.7 ml fractions. The fractionation curve is illustrated in Fig. 5.

It should be mentioned at this point that while it is possible when chromatographing a mixture of neovitamin A_1 and all-*trans* vitamin A_1 on the dicalcium phosphate column to observe a fairly distinct difference in the fluorescence of neovitamin A_1 (yellowish green) and all-*trans* vitamin A_1(more bluish green), the two vitamin A_2 bands displayed only a slight difference in ultraviolet light. Both vitamin A_2 bands displayed a brown fluorescence, that of substance *a* having, however, a slightly reddish tinge while that of substance *b* was of a more violet-brown colour.

The ratio of the quantities in which the two substances *a* and *b* are present is 32: 68, when expressed by E_{351} measured directly on the eluate.

The three peak fractions corresponding to substance *a* which showed the same value of ratios $\frac{E_{288}}{E_{351}}$ and $\frac{E_{351}}{E_{325}}$ were combined, evaporated and rechromatographed on a dicalcium phosphate column, 28×0.8 cm (purified with ether as previously described) ,the eluent used was light petroleum (which had been purified by means of silica gel) mixed with diethylether (86: 14 v/v). Eluate was collected in 2 ml fractions. The fractionation curve showed the presence of one substance only with absorption maximum at about 351 mμ. The values of the ratios $\frac{E_{288}}{E_{351}}$ and $\frac{E_{351}}{E_{325}}$ for the peak fraction and the subsequent fractions were the same. When the peak fraction was dissolved in ethanol, the corresponding curve displayed three maxima and two minima at the wavelengths previously determined (III,1 *a*). The following ratios were found:

$$\frac{E_{277.5}}{E_{353.5}} = 0.383, \quad \frac{E_{281.5}}{E_{353.5}} = 0.364, \quad \frac{E_{288}}{E_{353.5}} = 0.480, \quad \frac{E_{298}}{E_{353.5}} = 0.286, \quad \frac{E_{325}}{E_{353.5}} = 0.674.$$

The absorption curve is illustrated in Fig. 2, (curve 2).

The peak fractions corresponding to substance *b*, for which the value of $\frac{E_{287}}{E_{351}}$ (= 0.55, measured in absolute ethanol) and of $\frac{E_{351}}{E_{325}}$ (= 1.36 = $\frac{1}{0.735}$, measured in absolute ethanol) were found to be the same, were given a corresponding treatment: rechromatography on a 28×0.8 cm column of dicalcium phosphate (purified by means of ether) and elution with a (85: 15 v/v) mixture of light petroleum (purified by means of silica gel) and diethylether.

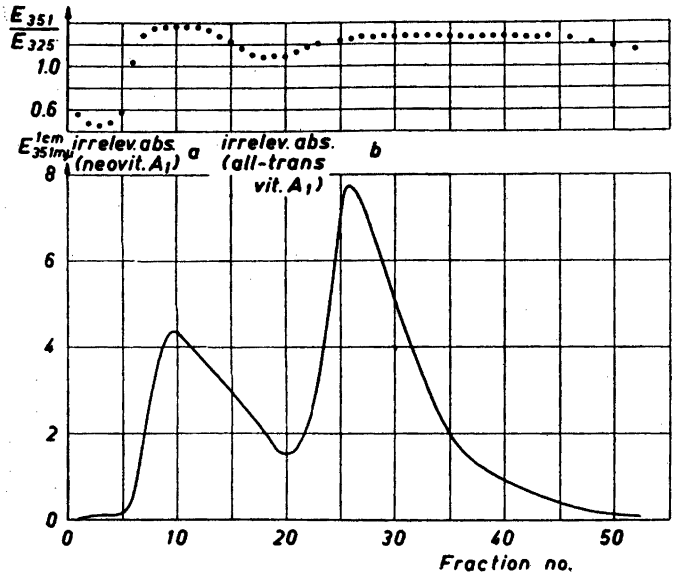

Fig. 5. Fractionation curve for isomerized vitamin A_2 with a column 48 cm long, diameter 3.0 cm.

Eluate was collected in 2 ml fractions. The fractionation curve showed the presence of one substance only with absorption maximum at 351 mμ. When dissolved in ethanol, the peak fraction gave a curve with three maxima and two minima at the wavelengths determined in section III,1 *b*, and the following ratios were found:

$$\frac{R_{276.5}}{R_{351.5}} = 0.445, \quad \frac{E_{280}}{E_{351.5}} = 0.426, \quad \frac{E_{287}}{E_{351.5}} = 0.555, \quad \frac{E_{297}}{E_{351.5}} = 0.321, \quad \frac{E_{325}}{E_{351.5}} = 0.738$$

The peak fractions from the last-mentioned chromatographic separation were combined and rechromatographed on a 58 × 1.0 cm dicalcium phosphate (ether-purified) column, elution with light petroleum (silica gel purified)-diethylether mixtures (90: 10 v/v increasing to 65: 35 v/v). Eluate was collected in 2 ml fractions. The peak fraction dissolved in absolute ethanol gave the same values of the five $\frac{E}{E_{351.5}}$ ratios as the material before it was rechromatographed.

It may be mentioned that after the isomerization experiment described in section II, in which the action of iodine on vitamin A_2 was prolonged for a period of 100 s, the subsequent chromatography resulted in a ratio of *a* to *b* equal to 33.4: 66.6 (expressed by E_{351} measured directly on the collected fractions).

IV. CHROMATOGRAPHY OF A MIXTURE OF VITAMIN A_2 AND SUBSTANCE *b*

A mixture was prepared consisting of 100 ml solution in light petroleum of natural vitamin A_2 purified by chromatography on dicalcium phosphate as described in section I, with $E^{1\,cm}_{351\,m\mu} = 0.466$ and $\frac{E_{287}}{E_{351}} = 0.495$, and 100 ml solution in light petroleum of substance *b*, with $E^{1\,cm}_{351\,m\mu} = 0.428$ an $\frac{E_{287}}{E_{351}} =$ $= 0.555$. The mixture was evaporated and chromatographed on a 78×1.0 cm column of (ether-purified) dicalcium phosphate. Elution by means of mixtures of light petroleum (silical gel purified) and diethylether in the following order:

20	ml	light	petroleum	—	ether	mixture (95: 5 v/v)
20	»	»	»		»	(92: 8 v/v)
40	»	»	»		»	(88: 12 v/v)
40	»	»	»		»	(85: 15 v/v)
20	»	»	»		»	(82: 18 v/v)
100	»	»	»		»	(79: 21 v/v)

Eluate was collected in 2 ml fractions.

The fractionation curve showed the presence of one substance only with absorption maximum at 351 mμ. Direct spectrophotometric measurements on the collected fractions gave $\frac{E_{287}}{E_{351}} = 0.52$—$0.55$. The variation of the ratio throughout the series of fractions did not, however, suggest any pronounced tendency towards separation into two substances. A subsequent, corresponding experiment gave the same result.

V. CHROMATOGRAPHY OF AN ISOMERIZED MIXTURE OF VITAMIN A_1 AND VITAMIN A_2

A mixture was prepared consisting of 15 ml solution of vitamin A_2 in light petroleum ($E^{1\,cm}_{351\,m\mu} = 2.00$) and 15 ml solution in light petroleum of (all-*trans*) vitamin A_1 ($E^{1\,cm}_{325\,m\mu} = 1.920$), and the mixture was isomerized by means of iodine in the manner described in section II. After filtration and evaporation to a volume of about 5 ml, the mixture was chromatographed on a 76×1.0 cm dicalcium phosphate column. Elution with light petroleum-ether mixtures as follows:

100	ml	light	petroleum	—	ether	mixture (90: 10 v/v)
200	»	»	»		»	(86: 14 v/v)

Eluate was collected in 4.7 ml fractions.

The fractionation curve (measurement of $E^{1\,cm}_{335\,m\mu}$ of the eluted fractions) is illustrated in Fig. 6.

Even if a complete separation of the four substances present has not been obtained, it appears clearly from the course of the $\frac{E_{351}}{E_{325}}$ curve that the four substances are eluted in the following order: neovitamin A_1, substance *a* (= neovitamin A_2), all-*trans* vitamin A_1 and finally substance *b* (= all-*trans* vitamin A_2?).

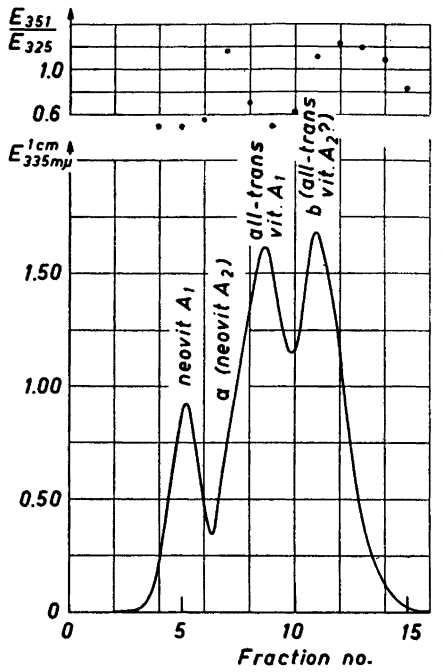

Fig. 6. Fractionation curve for an isomerized mixture of vitamin A_1 and vitamin A_2 with a column 76 cm long, diameter 1.0 cm.

DISCUSSION

Experiments III,1 and III,2 show that it is possible after treatment with iodine of vitamin A_2 prepared by chromatography to isolate from the isomerization product two different substances, *a* and *b*, the absorption curves of which show great similarity, although differing clearly with regard to the location of maxima and minima. In the case of substance *a* the wavelengths of the maxima and minima exceed by about 1—2 mμ those of substance *b* and of natural vitamin A_2, the isolation of which is described in experiments I,1 and I,2.

When bearing in mind that by chromatography on dicalcium phosphate substance *a* is eluted slightly before *b*, it will be seen that there is analogy between substances *a*, *b* and neovitamin A_1, all-*trans* vitamin A_1 — neovitamin A_1 being eluted slightly more readily from dicalcium phosphate than all-*trans* vitamin A_1. It is therefore a reasonable assumption that also as regards geometrical configuration the relationship of the two substances *a* and *b* is as that of neovitamin A_1 and all-*trans* vitamin A_1. In this connection it should also be mentioned that Robeson and Baxter [4] after isomerization for 2 h in daylight of all-*trans* vitamin A_1 anthraquinone-β-carboxylate by the action of iodine dissolved in benzene ascertained that the isomerization product contained 31 % neo-isomer. In the present isomerization experiments on free vitamin A_2 alcohol dissolved in light petroleum, with a reaction period of 30—100 s, the ratio of substances *a* to *b* — expressed by E_{351} — was about 1 to 2.

Keeping in mind the analogy *a*, *b* to neovitamin A_1, all-*trans* vitamin A_1, *a* and *b* might be expected to be identical with neovitamin A_2 and vitamin A_2, respectively, the two vitamin A_2 isomers which occur in the eluate from the liver extracts.

As the amounts of the supposed neovitamin A_2 fractions isolated from the liver extracts were insufficient to allow for further chromatographic purification, additional experiments to confirm the identity of the neovitamin A_2 occurring in the liver extracts and substance *a* have not been made. The location of the maxima and minima for substance *a* and the impure neovitamin A_2 isolated from the liver extracts show fairly good agreement, particularly with regard to the main maximum; at this wavelength (353.5 mμ) less interference from irrelevant substances may also be expected than at lower wavelengths. The difference between $\frac{E}{E_{353.5}}$ for the impure neovitamin A_2 of pike liver extract — which is obviously purer than the neovitamin A_2 isolated from perch liver — and for substance *a*, purified by chromatography, is greatest at the lower wavelengths (270—300 mμ) and small at wavelengths above 350 mμ, which seems to indicate that the difference is due solely to irrelevant light absorbing substances in the neovitamin A_2 isolated directly from liver.

When comparing the spectrophotometric properties of substance *b* and the vitamin A_2 isolated directly from liver, it would be reasonable to establish comparisons with previous investigations on the spectrophotometric properties of vitamin A_2 (Schantz [5], Cama and Morton [6], Farrar, Hamlet, Henbest and Jones [7]).

In Table 1 the experimental results are represented by a series of ratios between the extinctions at the maxima and minima. The table also lists the results of measurements made in this laboratory on chromatographically (dicalcium phosphate) purified synthetic all-*trans* vitamin A_1 and neovitamin A_1 prepared by chromatography after iodine isomerization of synthetic all-*trans* vitamin A_1.

As it will be seen from this table, there is good agreement between the location of the maxima and minima of the natural vitamin A_2 isolated in the present investigation and the synthetic and natural vitamin A_2 preparations described in the literature [5,6,7] although Schantz [5] and Farrar *et al.*[7] do not mention the existence of a maximum at 276.5 mμ, while Cama and Morton [6] mention the presence of an inflexion at 277 mμ. The ratios $\frac{E}{E_{max}}$ and $\frac{E}{E_{min}}$ calculated for the characteristic maxima and minima on the other hand show a certain deviation, since for the range 275—325 mμ the ratio $\frac{E}{E_{351.5}}$ in case of the vitamin A_2 isolated in the present investigation is about 5—20 % below and the ratio $\frac{E_{287}}{E_{297}}$ 9—15 % above the corresponding ratios given in the table for earlier vitamin A_2 preparations [5,6,7]. Maximum difference is observed in the case of the minimum at 297 mμ. The differences seem to suggest a greater purity of the vitamin A_2 prepared in the course of the present investigation.

Table 1. Spectroscopic properties of vitamins A_2 (abs. ethanol solution).

Maxima mμ	Minima mμ	$\frac{E_{276.5}}{E_{280}}$	$\frac{E_{287}}{E_{297}}$	$\frac{E_{297}}{E_{351.5}}$	$\frac{E_{276.5}}{E_{351.5}}$	$\frac{E_{287}}{E_{351.5}}$	$\frac{E_{325}}{E_{351.5}}$		
—, 286, 351	—, 297	(<1)	1.44	0.392*	—	0.561*	0.769	vitamin A_2	(A)
277 (infl), 286, 351	—, 295—97	(ca. 1)	1.38	0.393*	0.435**	0.544	0.810	»	(B)
—, 288, 352	—, 297,5	(<1)	1.45	0.365***	—	0.530†	—	»	(C)
276.5, 287, 351.5	280, 297	1.05	1.66	0.298	0.388	0.493	0.735	»	(D)
276.5, 287, 351.5	280, 297	1.04	1.73	0.321	0.445	0.555	0.738	substance *b*	(D)
277.5, 288, 353.5	281.5, 298	1.01	1.62	0.291	0.373	0.469	0.679	substance *a*	(D)
328	—	0.865	0.625	0.937	0.377	0.585	1.89	neovitamin A_1	(D)
324.5	—	0.860	0.651	1.30	0.524	0.845	2.38	all-*trans* vit. A_1	(D)

1) Schantz [5]
3) Cama and Morton [6]
7) Farrar, Hamlet, Henbest and Jones [7]
D) Barnholdt and Hjarde, work described here.

* $\frac{E}{E_{351}}$ ** $\frac{E_{275}}{E_{351}}$ *** $\frac{E_{297.5}}{E_{352}}$ † $\frac{E_{287}}{E_{352}}$

As already mentioned, substance *b* might be expected to be identical with natural vitamin A_2. With regard to the location of the characteristic maxima and minima the two substances show fairly good agreement, while the ratios $\frac{E}{E_{351.5}}$ at wavelengths below 310 mμ are somewhat higher in the case of substance *b* than in the case of the natural vitamin A_2 isolated according to section I, although at wavelengths above 310 mμ the differences between the ratios are only of a magnitude of 0—0.010. The object of the experiments made with respect to substance *b* has therefore been to find out whether it represented a single substance — a third vitamin A_2 isomer — or a mixture of substances, of which at least one might be expected to be a vitamin A_2 isomer, presumably the vitamin A_2 occurring in nature.

The assumption that substance *b* is identical with natural vitamin A_2, although in a state of incomplete purity, is supported by the agreement existing between the location of the maxima and minima of the absorption curves of the two substances and also by the fact that is has not hitherto been possible

to separate the two substances by chromatography on dicalcium phosphate — as appears from experiment IV.

On the other hand the rechromatographic experiments described in section III suggest that *b* is a single, pure substance; if this is the case, we are thus faced with a vitamin A_2 isomer which cannot, by chromatography on dicalcium phosphate, be separated from the vitamin A_2 occurring in nature, and which has maxima and minima at the same wavelengths as the latter and which only with regard to the ratio $\frac{E}{E_{351.5}}$ at wavelengths below 310 mμ deviates spectrophotometrically from the vitamin A_2 occurring in nature.

Consequently it is not possible on the basis of the results of the present investigation to decide whether substance *b* is a mixture of the vitamin A_2 isolated from pike and perch liver and an impurity deriving from the isomerization process, or a single substance, a third vitamin A_2 isomer, which are not readily separated by chromatography on dicalcium phosphate.

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