Biochemical Spectroscopy

by

Richard Alan Morton

Volume 2

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13. Vitamins and Coenzymes

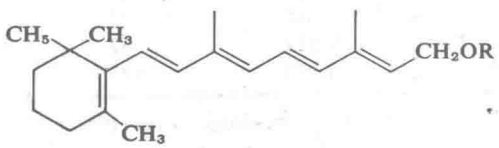
13.1 Provitamins and Vitamins A (Retinol and 3-Dehydroretinol)

The analytical chemistry of vitamin A has a history of progress by small steps, each valid in its immediate context but needing scrutiny as new variables and fresh complications emerged. It would not be appropriate here to deal with all aspects of the development despite the fact that spectroscopic methods had throughout a major role. Instead, the dominant factors are set out and the present state of knowledge summarized (cf. Morton, 1970).

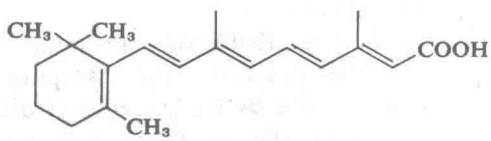
The initial aim was to find a physico-chemical 'label' for a then unidentified substance which gave rise to a roughly measurable biological effect. Attempts at isolation could then be monitored more easily and more rapidly than by biological assays. Two tests proved useful for that purpose: (*a*) measurement of ultraviolet absorbance at 325–328 nm and (*b*) measurement of the intensity of a blue colour ($\lambda_{max.}$ 606–618 nm) produced by the interaction of vitamin A and a solution of anhydrous antimony trichloride in chloroform. A number of fish liver oils were tested and the parallelism between biologically assessed potency, ultraviolet absorption and the colour test was very promising and the validity of spectroscopic criteria was confirmed when very rich concentrates were obtained. Eventually, pure vitamin A alcohol (retinol) and its esters (retinyl palmitate or acetate) were obtained from natural sources and by synthesis, and their properties were measured reproducibly. It is now not difficult to analyse commercial samples of synthetic vitamin A acetate. The ultraviolet absorption is very accurately known for solutions in several solvents; the colour test has been expressed quantitatively and formulations of the vitamin give the predicted absorbances.

In fact, however, the general problem of vitamin A determinations and the interpretation of the results remains quite complicated. Until synthetic vitamin A became an article of commerce the main medicinal sources were fish liver oils. For a time whale liver oil concentrates had an important part in the fortification of margarine. Students of nutrition were interested in the amount and nature of the liver stores of the vitamin in animals and birds, particularly the domesticated species. All fish liver oils are liable to contain vitamin A_2 (3-dehydroretinol) as well as vitamin A_1 (retinol) as esters; in fresh water species the proportion of the former may be high but is very variable, while in marine fishes the vitamin A_2 of liver oils may be one-sixth to one-twenty-fifth of the vitamin A_1 content. The presence of vitamin A_2 constitutes an analytical complication because its properties differ from those of retinol; moreover it has biological activity which under certain conditions is about 40% of that of an equal weight of retinol. The

liver stores of mammals and birds are devoid of vitamin A_2 except for those species which eat large amounts of freshwater fish. Whales also lack vitamin A_2 in their liver lipids but a substance *kitol* (a biologically inactive compound derived from two molecules of vitamin A) is present in sufficient amount to make the analysis somewhat difficult.



Vitamin A_1 (retinol) R = H
Retinyl acetate R = $COCH_3$



Retinoic acid

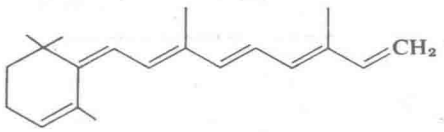
The vitamin A molecule is capable of *cis-trans* isomerism and six isomerides have been obtained. They differ significantly in respect of $\lambda_{max.}$ and $\epsilon_{max.}$ and biological potency. Fish liver oils contain all-*trans* vitamin A and a *cis* form neovitamin A in variable proportions.

Herbivorous animals ingest carotenoid provitamins A and the vitamin A stored in the liver is derived from the carotene by biosynthetic processes which do not include the formation of vitamin A_2 or kitol. Provitamin carotenoids are of significance in milk, butter and eggs and synthetic carotenoids are used to colour margarine.

It is clear that in the nutrition of animals (including man) the accurate assessment of vitamin A intake (actual and potential) is a formidable task. In fact it is necessary to make do with approximations and a measure of compromise is called for.

13.1.1 Ultraviolet Absorption and $SbCl_3$ Colour Test

A note on the colour test is necessary. The reagent is a nearly saturated solution of anhydrous antimony trichloride in alcohol-free chloroform and when it acts on retinol the first effect is to remove the elements of water to yield anhydrovitamin A which then reacts



Anhydroretinol

to form the coloured absorbing entity with $\lambda_{max.}$ 618–620 nm. The absorption spectrum shows a shoulder at 580–583 nm at about half the intensity of the main peak and some colour-test inhibitors (e.g. 7-methylindole) reduce the intensity of the 618 nm peak much more than the 583 nm peak. When cod liver oils are tested directly the colour test is partially inhibited and the peak is at 606 nm, with a shoulder at *ca.* 572 nm. The shift from 618 to 606 nm has not been fully explained. The normal procedure is to carry out the colour test on the unsaponifiable fraction for which inhibition largely ceases to occur and the peak is at 618 nm. Vitamin A_2 gives rise to a colour test with $\lambda_{max.}$ at 693 nm and the band overlaps little with the vitamin A_1 absorption. Under favourable conditions the ratio $E_{693}/E_{620\,nm}$ can be used to determine the relative proportions of the two vitamins. The colour test rests on the formation of an unstable product; the colour is transient and arrangements must be made to determine $E_{max.}$ quickly (e.g. within about 10 seconds) after mixing the reagents and the sample solution.

In carrying out ultraviolet absorption measurements the solvent preferred in England has been cyclohexane and in the U.S.A. isopropanol.

The potency of vitamin A is by definition $3{\cdot}33 \times 10^6$ international units per gram and the early samples of crystalline all-*trans* vitamin A possessed $E^{1\%}_{1cm}$ 325 nm 1750 in isopropanol, corresponding with a 'conversion factor' $3{\cdot}3 \times 10^6/1750 = 1905$ (rounded off to 1900). This agreed well with a collaborative study (23 laboratories, 1949) of a USP Reference Standard Cod Liver Oil.

It was later found that better preparations of all-*trans* retinol had $E^{1\%}_{1cm}$ 325 nm in isopropanol of 1840 instead of 1750. The crystalline vitamin A acetate preparations, however, with a theoretical potency of 2 907 000 i.u./g continued to show $E^{1\%}_{1cm} = 1530$, conversion factor 1900 (isopropanol). Table 13.1 summarizes the later measurements.

The retinoic acids have the interesting property of partially replacing vitamin A, i.e. in respect of the systemic biological properties but not in respect of visual processes. Retinol, acted upon by alcohol

Table 13.1 Ultraviolet absorption maxima of all-*trans* retinol and retinylacetate*

Solvent		Ethanol	Isopropanol	Cyclohexane	Light petroleum
Retinol	$\lambda_{max.}$	324	325·5	326·5	324·5
	$\epsilon_{max.}$	52 000	52 440	49 930	52 200
	$E^{1\%}_{1cm}$	1 816	1 830	1 741	1 822
Conversion factor†		1 835	1 822	1 915	1 827
Retinyl acetate	$\lambda_{max.}$	326	326	328	328
	$\epsilon_{max.}$	52 050	50 350	49 890	52 300
	$E^{1\%}_{1cm}$	1 555	1 532	1 517	1 592
Conversion factor		1 870	1 897	1 915	1 825

* Data obtained on specially purified materials by Cama *et al.* (1951) and by Boldingh *et al.* (1951); mean values given.
† Conversion factor = Potency ($3{\cdot}3 \times 10^6$ i.u./g for retinol, $2{\cdot}907 \times 10^6$ i.u./g for retinylacetate), divided by $E^{1\%}_{1cm}$ at $\lambda_{max.}$.

dehydrogenase, can be oxidized to retinaldehyde which serves as a precursor of visual pigments. The conversion of an aldehyde to the corresponding carboxylic acid is very much more likely than the reverse process, so that the failure of the acid to support vision is not surprising. All-*trans* retinoic acid has been much used in biological experiments; it permits normal growth when administered daily but as it is not stored, vitamin A deficiency symptoms appear abruptly after dosing is stopped. The absorption spectra of the retinoic acids (p. 391) allow biological tests to be monitored in respect of stability in diets, but the absence of storage in the animal organism sets limits to their usefulness. The retinoic acids are not known to occur naturally but this does not entirely rule out the possibility that they are metabolites of vitamins A or that they participate in vitamin A function.

The value 1830 for the conversion factor applicable to solutions of retinol in isopropanol has the support of the Vitamin Division of the International Union of Pure and Applied Chemistry (IUPAC) (Brunius, 1958).

13.1.2 Correction Procedures for Spectrophotometric Analyses

The advent of manually operated commercial photoelectric spectrophotometers paved the way to extended uses of ultraviolet absorption in analysis. The spectrographic method could only with great care be made trustworthy to within $\pm 1{\cdot}5\%$, whereas the photoelectric instruments (e.g. the pioneer commercial model brought out by Beckman during the 1939–1945 War) gave reproducible results to within $\pm 0{\cdot}1\%$ under good conditions.

Morton & Stubbs (1946) took advantage of the new situation. They noted that the absorption curve for a solute is modified by the presence of other absorbing entities, depending on the absorptive properties of the contaminants. The observed curve is a summation of the curves for the pure substance and those of the congeners. In analysing for the major absorbing constituent allowance must be made for extraneous or irrelevant absorption. By choosing three wavelengths not very far apart the assumption that the irrelevant absorption is linear over the given wavelength range is often found to be valid.

From the geometry (Fig. 13.1) algebra leads to the equation

$$E\lambda_1(\text{corrected}) = \frac{E_1k_1k_2(\lambda_3-\lambda_2)-E_2k_1k_2(\lambda_3-\lambda_1)-E_3k_1k_2(\lambda_1-\lambda_2)}{k_1(\lambda_2-\lambda_1)+k_1k_2(\lambda_3-\lambda_2)+k_2(\lambda_1-\lambda_3)}$$

Insertion of numerical values leads to

$$E\lambda_1(\text{corrected}) = AE_1 - BE_2 - CE_3$$

where A, B and C are constants for the problem under study. In practice the method works well when the absorption spectrum of the solute is accurately known. The selection of maxima and minima is often an advantage because the literature frequently contains these quantities only.

A derivation which is perhaps easier has been widely used. It uses fixation points on either side of $\lambda_{max.}$ such that the absorbance is 6/7 of the peak value.

Let ABC be the absorption curve for the substance to be estimated in the presence of irrelevant absorption DEF presumed to be linear between the wave-

Figure 13.1 Geometry of the correction procedure:

$$\frac{E_1-x_1}{E_2-x_2}=k_1,\quad \frac{E_1-x_1}{E_3-E_3}=k_2$$

lengths λ_1, λ_2 and λ_3; these wavelengths are chosen as that $E\lambda_2(BH)/E\lambda_1(CJ)=E\lambda_2(BH)/E\lambda_3(AG)=7/6$ (see Fig. 13.2).

The irrelevant absorption at λ_2 is made up of EM (x) due to the slope of the line DEF and MH (y) due to uniform absorption independent of wavelength.

From the triangles FEM and FDL $\lambda_3-\lambda_2$ and $\lambda_3-\lambda_1$ are known and the ratio $\lambda_3-\lambda_2/\lambda_3-\lambda_1$ is a numerical constant K and is equal to EM/LD so $x=LD\times K$.

The observed absorbances can be written

$E^1\lambda_1$, $E^1\lambda_2$ and $E^1\lambda_3$ and $E^1\lambda_1-E^1\lambda_3=LD=x/K$

$$\text{so } x=K(E^1\lambda_1-E^1\lambda_3)$$

and is numerically ascertained.

Now

$$E\lambda_2(\text{corr.})=E^1\lambda_2-x-y=\tfrac{7}{6}(E^1\lambda_3-y)$$

and

$$\tfrac{1}{6}y=\tfrac{7}{6}E^1\lambda_3-E^1\lambda_2+x$$
$$y=7E^1\lambda_3-6E^1\lambda_2+6x$$

y is thus numerically ascertained and generally

$$E\lambda_2(\text{corr.})=7E^1\lambda_2-7E^1\lambda_3(I-K)-7KE^1\lambda_1)$$

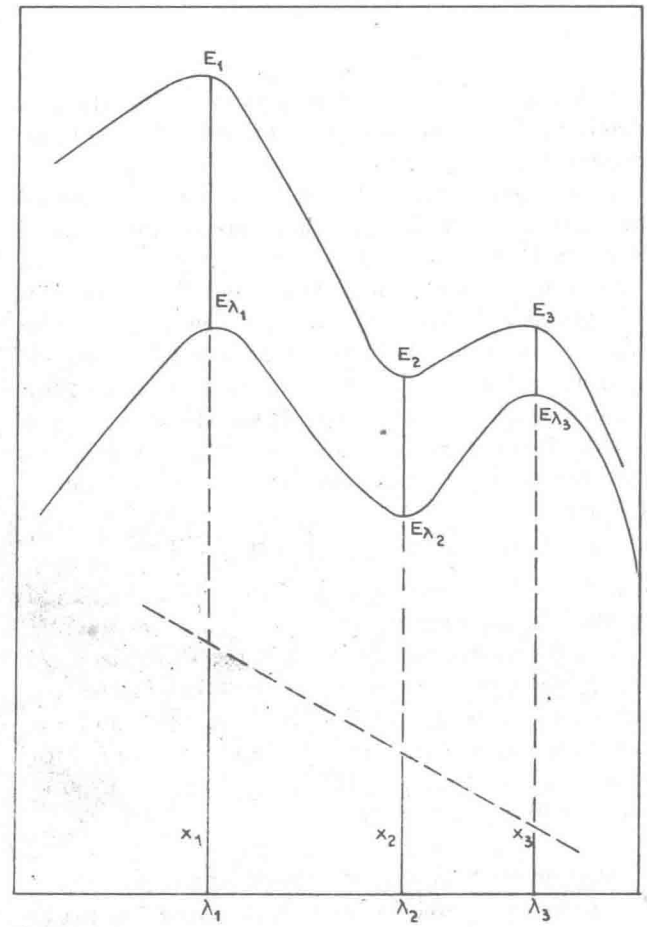
For some years the Morton–Stubbs correction procedure was applied to vitamin A, the fixation points being λ_1 310, λ_2 325, λ_3 334 nm.

$$\frac{\lambda_3-\lambda_2}{\lambda_3-\lambda_1}=\frac{9}{24}=0{\cdot}375=K$$

and

$$E\lambda_2(\text{corr.})=7E^1\lambda_2-7E^1\lambda_3(I-K)-7E^1\lambda_1K$$
$$=7E^1\lambda_2-4{\cdot}375E^1\lambda_3-2{\cdot}625E^1\lambda_1$$

Cama, Collins & Morton (1951) went into the analytical problem for vitamin A in some detail. The importance of a reference standard for the instrument was recognized and data for K_2CrO_4 in 0·05 N KOH were used (see p. 6). The intensities of absorption for vitamin A acetate and vitamin A alcohol were measured in four solvents and tabulated between 220 and 400 nm as fractions of $E_{max.}$ over the range 310–340 nm and readings were made every 1 nm. Revised correction equations were given in each case and in addition equations based on fixation points



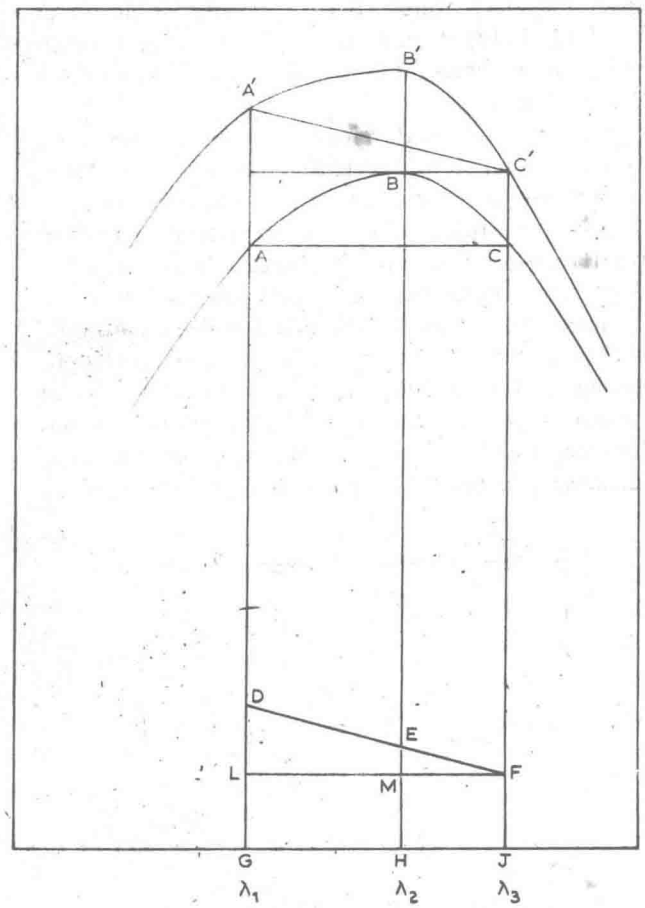


Figure 13.2 Geometry of the correction procedure using fixation points on either side of $\lambda_{max.}$

equidistant from $\lambda_{max.}$. Boldingh *et al.* (1951) also determined the spectroscopic properties of retinol and retinylacetate (Table 13.1).

It was argued in the U.S.A. that in the process of saponifying vitamin A ester preparations most analysts lose 1·2 to 1·5% of the vitamin A and a 'practical' conversion factor for saponified vitamin A was put at 1830 instead of 1810. A new collaborative study (34 laboratories, several countries) used a high potency solution of crystalline vitamin A acetate (in oil) which was saponified. It was decided that the standard equation gave results 2·6% too high so that the constants were multiplied by a fixed factor to give

$$E_{325}(\text{corr.}) = 6{\cdot}815E_{325} - 2{\cdot}555E_{310} - 4{\cdot}260E_{334}$$

This formula for retinol in isopropanol was approved by the Vitamin Division of IUPAC.

The *British Pharmacopoeia* and the *Pharmacopoeia Internationalis* approved for retinol in cyclohexane the fixation points 312·5, 326·5 and 336·5 and the equation $7(E_2 - 0{\cdot}422E_1 - 0{\cdot}578E_3)$ and (BP) for retinylacetate, 312·5, 327·5, 337·5 nm. $E_{corr.} = 7(E_2 - 0{\cdot}405E_1 - 0{\cdot}595E_3)$.

Correction for background absorption has been made by applying mathematically elaborated empirical 'base line' techniques (Beroza, 1950; Davidow & Woodard, 1949; Rotandaro, 1957). Allen & Rieman (1953) strengthened the theoretical basis but increased the complexity.

Jones *et al.* (1951) developed a method based on a variable reference or spectrophotometric 'matching' in which the concentration of the reference (known) solution is varied during the analytical operation until the absorption in the reference solution and that of the same compound in the unknown solution are equalized. The apparatus needed has been simplified by Wilkie (1963). The method has been applied to vitamin A (Wilkie, 1964) despite the fact that special problems arise in this connection. Work on ten pharmaceutical vitamin A samples is reported without indicating whether they represented synthetic vitamin

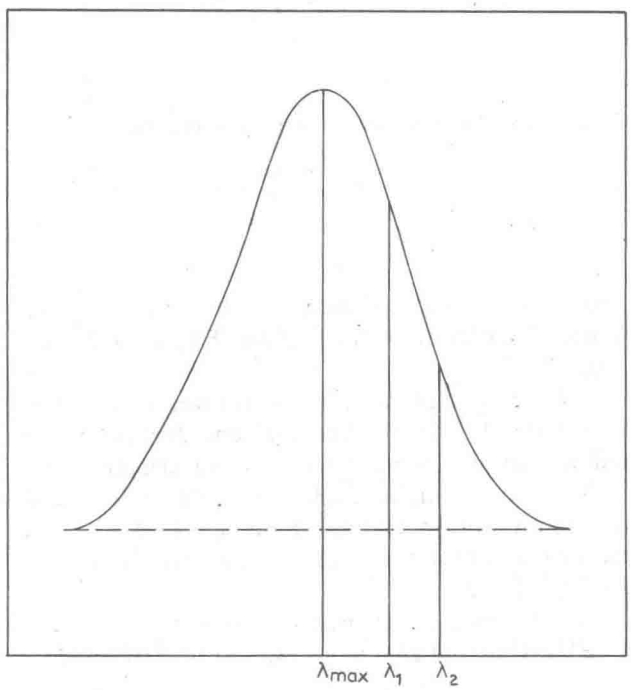


Figure 13.3

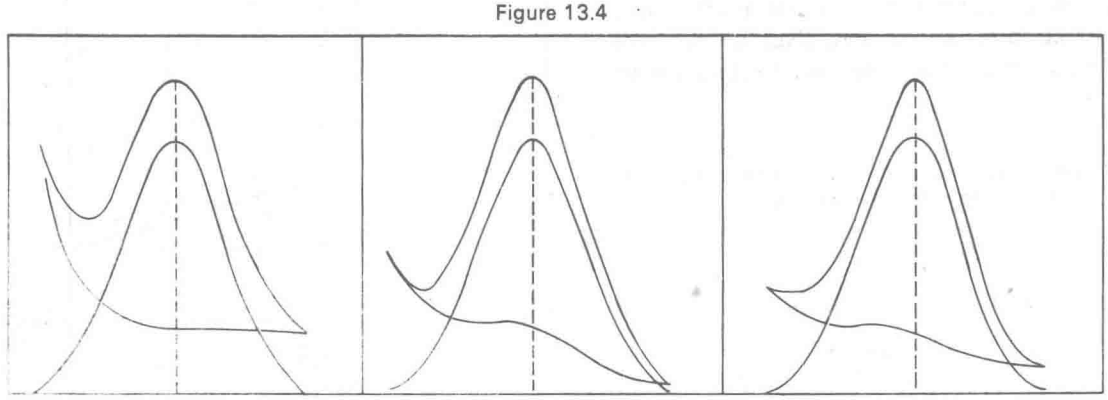
A or products derived from fish liver oils and containing unspecified amounts of vitamin A_1 isomers and vitamin A_2. Moreover attention was confined to wavelengths on the long wave side of 325 nm. The samples used showed extreme variability of absorbance values in the 310 nm region and this made them unsuitable for the USP or BP procedures based on the Morton–Stubbs correction.

Although Wilkie's (1964) paper regarded as a contribution to the determination of vitamin A is very incomplete, more general points made in it are important.

(i) If an absorption curve has a horizontal component of irrelevant absorption the difference $A_{\lambda_1} - A_{\lambda_2} = \Delta A^{\lambda_1}_{\lambda_2}$ (Fig. 13.3) will eliminate the horizontal irrelevant absorption from comparisons with a standard.

Given an absorption curve for the pure substance in which $\lambda_{max.}$ shows $A = 1{\cdot}0$ the curve will express A_λ as a fraction of $A_{max.}$ and $\Delta A^{\lambda_1}_{\lambda_2}$ values are readily obtained. It would then be easy to distinguish irrelevant absorption of the types in Fig. 13.4.

Figure 13.4



(ii) Wilkie showed that when two wavelengths were taken equidistant from a third ($\lambda_1 - \lambda_2 = \lambda_3 - \lambda_2$ for the pure substance) $\Delta A^{\lambda_1}_{\lambda_2} / \Delta A^{\lambda_1}_{\lambda_2} = X$ and is numerically ascertainable. When there is an angular irrelevant absorption

$$\Delta A^{\lambda_2}_{\lambda_3} - Y / \Delta A^{\lambda_1}_{\lambda_2} - Ky = X$$

In the case of vitamin A-bearing natural products the occurrence of vitamin A_2 in almost all fish liver oils makes the 'matching' or neutralization procedure inferior to a skilful use of the $SbCl_3$ (or similar) colour test which readily allows the two vitamins to be analysed in non-saponifiable fractions. In other problems the Wilkie technique may well be worth while.

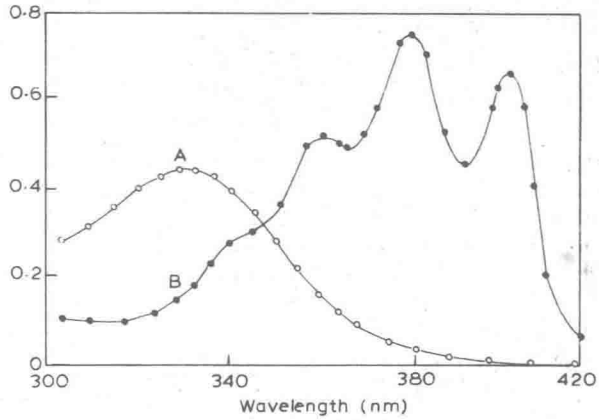


Figure 13.5 Absorption spectra of unsaponifiable matter from U.S.P. reference standard: (A) before dehydration; (B) after dehydration (from Budowski & Bondi, 1957)

13.1.3 Anhydroretinol

Budowski & Bondi (1951) made use of the ready conversion of vitamin A to anhydrovitamin A in the presence of mineral acid (Edisbury *et al.*, 1932; Shantz *et al.*, 1943). The appearance of the retro-structure and the lengthening of the conjugated chain by one double bond effects a bathochromic shift and an increased intensity of absorption accompanied by marked fine structure (Fig. 13.5). Budowski & Bondi (1957) carried out the formation of anhydrovitamin A in benzene solution at room temperature in the presence of toluene-*p*-sulphonic acid as a catalyst. This was more suitable than earlier procedures for the dehydration involving hydrogen chloride in ethanol. The vitamin A must be free (i.e. as retinol) and the procedure is applicable only to non-saponifiable fractions.

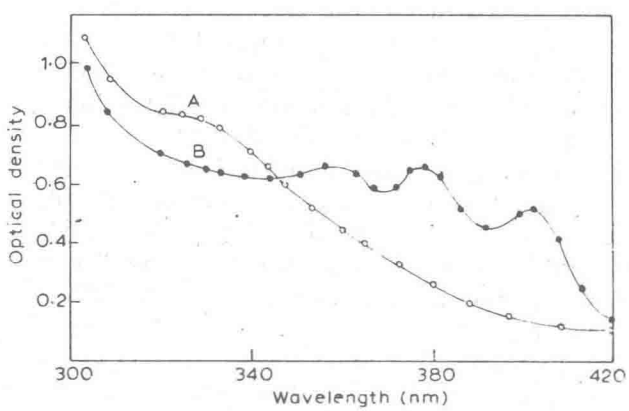
Retinol (in benzene) shows $\lambda_{max.}$ 331 nm and the anhydro peaks are at 358, 377 and 399 nm (minima at 364 and 389 nm) with an inflection at 340–345 nm (Fig. 13.5). In ethanol the maxima occur at *ca.* 351, 370 and 392 nm. The relative intensities are for pure anhydroretinol $E_{399}/E_{377} = 0{\cdot}868$ $E_{358}/E_{377} = 0{\cdot}692$ in agreement with the data of Shantz (1950) for ethanolic solutions. The molecular extinction coefficient at 377 nm is 1·59 times that of retinol at 331 nm, whereas comparison of the two pure substances indicates 2·0, so that the yield is about 80%. It is advisable for those who use the method to ascertain the correction by a calibration experiment.

The utility of the method is clear when as in Fig. 13.6 the unsaponifiable fraction shows only an inflection near 330 nm, whereas after dehydration three peaks are measurable. The difference spectra shown in Fig. 13.7 indicate how good an agreement can be obtained with a standard. (Today the standard would be obtained by saponifying vitamin A acetate rather than a reference cod liver oil.)

The method has been compared with the Carr–Price colour test as applied to vitamin A palmitate undergoing autoxidation. (For details see the original publication.)

Duggan *et al.* (1964) proposed the replacement of the Carr–Price $SbCl_3$ reagent for vitamin A by trifluoro-acetic acid. The nearly saturated solution of

Figure 13.6 Absorption spectra of an unsaponifiable fraction from a poultry mash: (A) before dehydration; (B) after dehydration (from Budowski & Bondi, 1957)



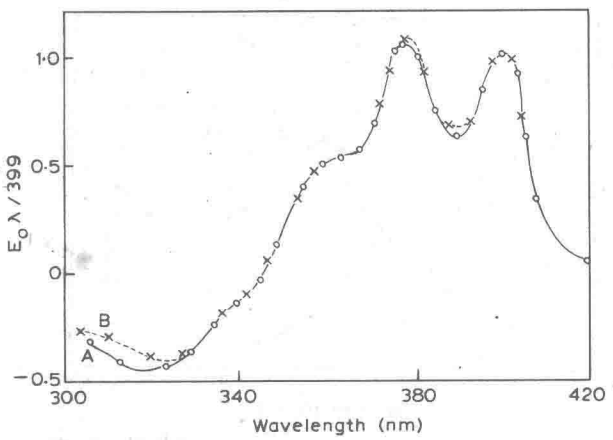


Figure 13.7 Difference spectra: (A) U.S.P. reference standard; (B) poultry mash (from Budowski & Bondi, 1957)

anhydrous antimony trichloride in ethanol-free chloroform has some disadvantages not shown by CF_3COOH.

Vitamin A solutions in chloroform ranging from 3×10^{-6} M to $1{\cdot}2\times10^{-5}$ M are used for calibration. An equal volume (e.g. 1·5 ml) of trifluoro-acetic acid (TFA) is added by syringe or quick delivery pipette with shaking. The blue colour is measured at 616 nm after 10 seconds. The position of $\lambda_{max.}$ is at 616 nm and $\epsilon_{max.}$ is 145 000 for retinol, retinylacetate and retinylpalmitate. Retinaldehyde shows $\lambda_{max.}$ 664 nm, $\epsilon_{max.}$ 95 000 and retinoic acid $\lambda_{max.}$ 574 nm, $\epsilon_{max.}$ 53 000. Stannic chloride $SnCl_4$, like TFA, gives the colour test with $\lambda_{max.}$ and $\epsilon_{max.}$ reproducing the results already well known for the $SbCl_3$ reagent. There is satisfactory evidence that the reagents form anhydrovitamin A and that the initial colour is due to interaction between that compound and the reagent (a 'Lewis' acid). The blue colour fades to a pink $\lambda_{max.}$ *ca.* 535 nm, $\epsilon_{max.}$ about 45 000. In the Carr–Price test optimum results are obtained when the ratio (v/v) of reagent to chloroform solution under test is 5:1. With TFA the ratio is 1:1 (v/v).

β-Carotene results in a transient band at 585 nm but a peak at 780 nm reaches its maximum intensity in 30 minutes. With a ratio of 5:1 or 10:1 TFA/chloroform solution of β-carotene a peak at 710 nm reaches its maximal absorption ($\epsilon_{max.}$ *ca.* 120 000) in 1–2 minutes.

Trifluoro-acetic anhydride (TFAA) also produced a blue colour with retinol and retinyl esters but failed to react with retinoic acid or β-carotene. Many Lewis acids were shown to produce the blue colour with retinol but TFA seems to be the most convenient. It is water-soluble and more stable than the Carr–Price reagent. As a reagent it excels over the latter in the detection of small amounts of vitamin A. So far as specificity is concerned, there is little to choose between TFA and the $SbCl_3$ reagent and non-saponifiable fractions from natural oils can be studied successfully.

13.1.4 Interpretation of Vitamin A_2 Spectrum

By virtue of its additional double bond, $\lambda_{max.}$ of vitamin A_2 is at 351 nm some 25 nm beyond the 325–326 nm peak of vitamin A. This shift is not very different from expectations but a drop in $\epsilon_{max.}$ from about 52 000 to 41 300 is surprising. Moreover two new peaks at 276 and 286 nm respectively appear with intensities of $\epsilon=15\,650$ and 20 200 for all-*trans* vitamin A_2.

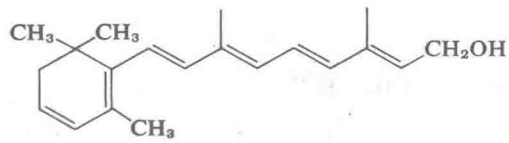
In simple polyenes $H(CH{=}CH)_nH$ when $n=3$ $\lambda_{max.}=268$ nm and when $n=4$ $\lambda_{max.}=304$ nm. For $n=5$ and 6 the main peaks are at 334 and 364 nm with $\epsilon_{max.}$ 121 000 and 138 000 respectively. In the series $CH_3(CH{=}CH)_nCOOH$ in ethanol the peaks for $n=2$, 3 and 4 are at 254, 294 and 327 nm, with ϵ values 24 800, 36 500 and 48 700 respectively.

Two conjugated double bonds in one ring occur in the cyclohexadienes, α-terpinene, α-pyronene, β-pyronene and related substances (O'Connor & Goldblatt, 1954). The chromophore shows $\lambda_{max.}$ 261–265 nm $\epsilon_{max.}$ 5700 to 6100. In the sterols however 5,7-diene chromophore gives absorption nearer the red:

7-dehydrocholesterol	$\lambda_{max.}$ 271, 282, 293·5 nm,	$\epsilon_{max.}$ 11 000
lumisterol	280	8 500
pyrocalciferol	274, 294	
isopyrocalciferol	262, 280	

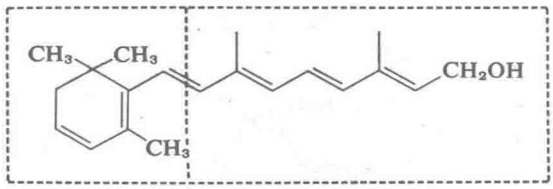
Previtamin D $\lambda_{max.}$ 262 nm and vitamin D $\lambda_{max.}$ 265 nm have peaks closer to those of the homoannular dienes.

The likeliest interpretation of the vitamin A_2 spectrum is that it is made up of two 'partials', (*a*) and (*b*),



(*a*)

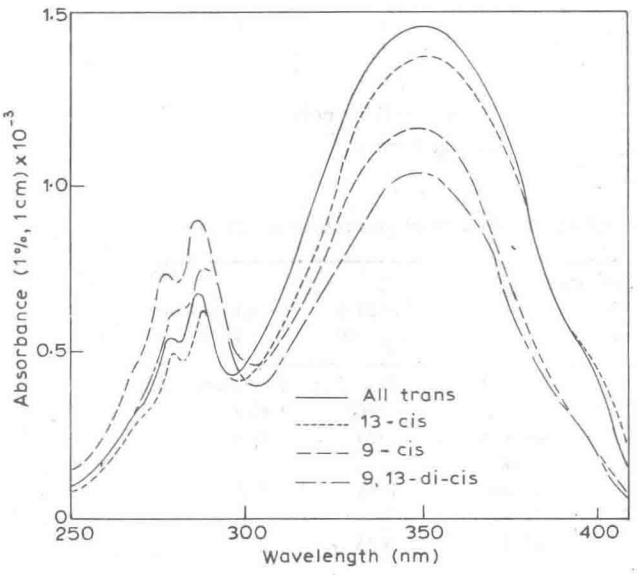
Vitamin A_2 3-dehydroretinol

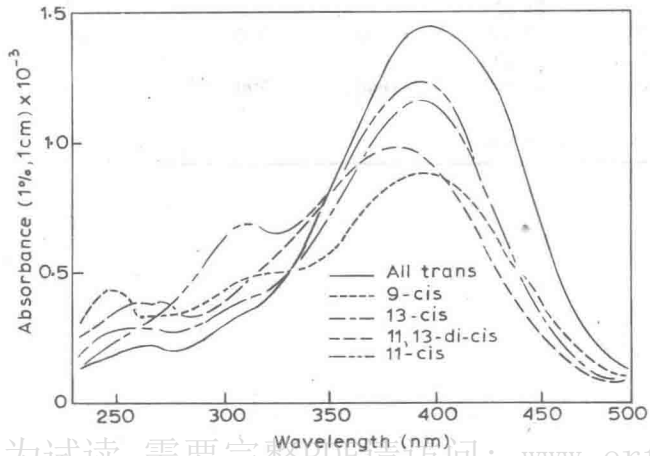


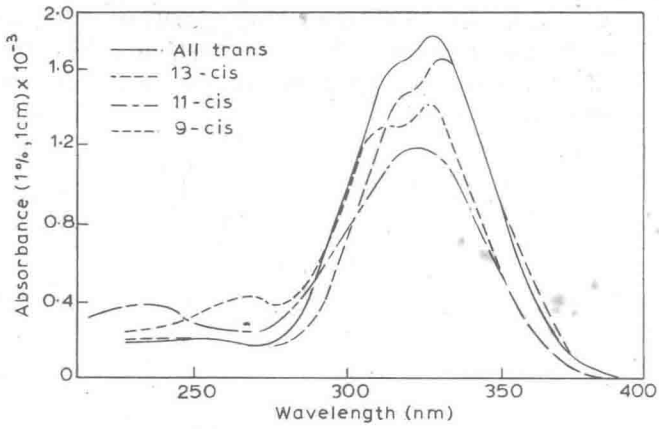
(*b*)

(*a*) being the complete conjugated system acting as the 351 nm chromophore and a smaller chromophore shown in a dotted square (*b*). Perhaps the remainder of the molecule should provide a second 'partial'. The hypothesis of partials fits the facts but it cannot easily be made quantitative.

The change from all-*trans* to 11,13-di-*cis* in retinol displaces $\lambda_{max.}$ from 325 to 311 nm and $\epsilon_{max.}$ is halved (52 480 to 26 000). In the 3-dehydroretinols the change from all-*trans* to 11,13-di-*cis* displaces







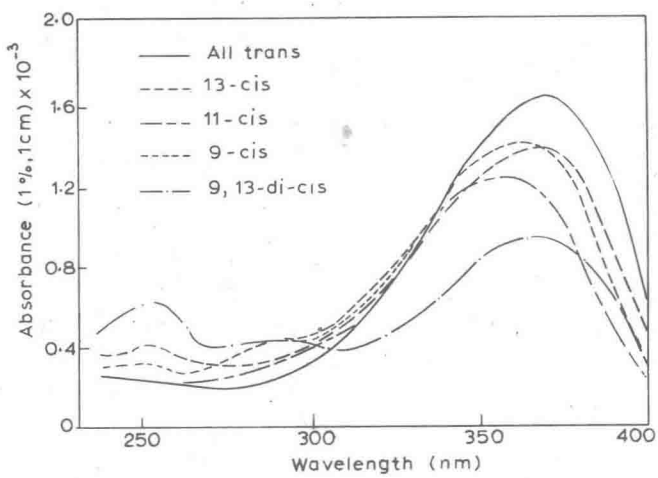
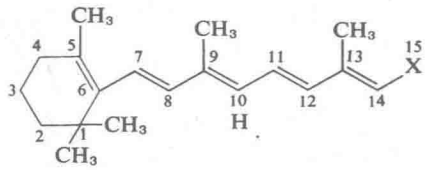


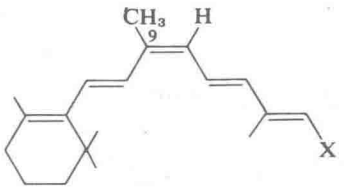
Figure 13.8 (*a*) Absorption spectra of four vitamin A_1 (retinol) stereoisomers in hexane. Note that the main bands of the unhindered isomers (all-*trans*, 13-*cis*, 9-*cis*) show fine structure while the main band of the hindered 11-*cis* isomer is symmetrical, but has a flat peak. Note also that the 9-*cis* isomer has a '*cis* peak' at 259 nm, and that the 11-*cis* isomer has a raised absorbance in the '*cis* peak' region and a subsidiary band at 233 nm. (*b*) Spectral absorbances of five $retinene_1$ (retinal) stereoisomers in hexane. Note that the 9-*cis* isomer has a '*cis* peak' (282·5 nm); the 11-*cis* isomer both a '*cis* peak' and a strong subsidiary band (251 nm); and the 13-*cis* isomer a raised absorbance in the '*cis* peak' region and a weak subsidiary band (252 nm) (from Hubbard, 1956)

Figure 13.9 (*a*) Spectral absorbances of four vitamin A_2 (3-dehydroretinol) stereoisomers in ethanol. (*b*) Spectral absorbances of five $retinene_2$ (3-dehydroretinal) stereoisomers in ethanol (after Von Planta *et al.*, 1962)

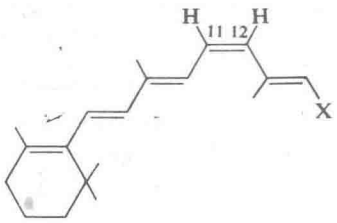
$\lambda_{max.}$ from 350 to 337 nm and $\epsilon_{max.}$ falls from 41 200 to 25 700. Both for retinol and 3-dehydroretinol the effect of *cis-trans* isomerism is least for the change all-*trans* to 13-*cis*.



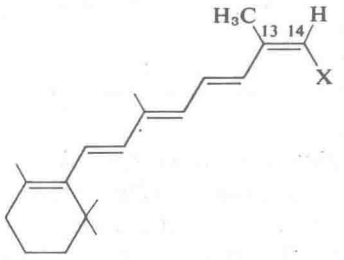
(*a*)



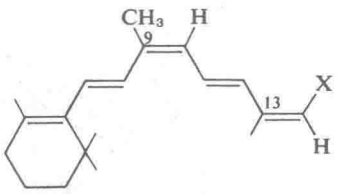
(*b*)



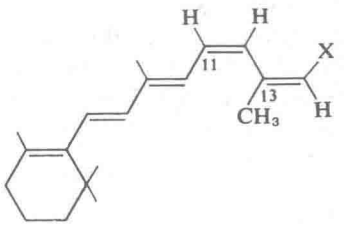
(*c*)



(*d*)



(*e*)



(*f*)

(*a*) all-*trans*
(*b*) 9-*cis*
(*c*) 11-*cis*
(*d*) 13-*cis* (neo-A)
(*e*) 9–13 di-*cis*
(*f*) 11–13 di-*cis*

X=CH_2OH retinols or CHO retinals

Table 13.2 Properties of *cis-trans* isomerides of vitamin A (retinol and retinyl acetate)

Substance			Ethanol $\lambda_{max.}$ nm	Ethanol $\epsilon_{max.}$	Potency i.u./g $\times 10^{-6}$	Conversion factor
All-*trans*		retinol	325	52 480*	3·3	1 830 approx.
		acetate	325·5	52 050	2·907	1 870
Neo-a	13-*cis*	retinol	328	48 300	1·25	740
		acetate	328	47 000		
Neo-b	11-*cis*	retinol	319	39 400	0·38	311
		acetate	320·5	31 960		
Neo-c	11,13-di-*cis*	retinol	311	26 000	0·25	275
		acetate	310·5	28 220		
Iso-a	9-*cis*	retinol	323	42 300	0·37	250
		acetate	323	39 400		
Iso-b	9,13-di-*cis*	retinol	324	39 500	0·40	290
		acetate	324	26 500		

* by definition

The intensity of the 351 nm peak for vitamin A_2 taking the whole system of conjugated double bonds as the chromophore might have been at least $\epsilon_{max.} = 60\,000$ but it does not exceed about two-thirds of the expected value. This suggests that perhaps two-thirds of the molecules act as the total chromophore, leaving perhaps one-third to form 'partials'. If the two partials each contain 3 conjugated double bonds the twin peaks at 276 and 288 nm might be accounted for. They do not seem to resemble the fine structure shown in the 5,7-dienes like 7-dehydrocholesterol. Superimposed on these possibilities are the effects of *cis-trans* isomerism and the evidence, incomplete as it is, suggests that the full chromophore may behave similarly to the retinol chromophore.

The principal forms of vitamin A in natural products and especially cod liver oils are retinol (all-*trans*), neovitamin A and vitamin A_2. There is no official recognition of vitamin A_2 as a nutrient. Morton & Bro-Rasmussen (1955) pushed the analytical

Table 13.3 Properties of *cis-trans* isomerides of vitamin A_2 (3-dehydroretinol)

Substance	$\lambda_{max.}$	$\epsilon_{max.}$	Potency i.u./g $\times 10^{-6}$	Conversion factor
3-Dehydroretinols	350	41 200	0·76	520
all *trans*	286	20 300		
	276	15 760		
13-*cis*	352	39 000	0·6	560
	288	18 430		
	277	14 000		
11-*cis*	344	28 100	0·25	250
	286	16 080		
	278	14 000		
11-13-di*cis*	337	25 700	0·23	255
	290	13 350		
	277	13 100		
9-*cis*	348	32 450	0·23	200
	287	26 100		
	277	21 800		
9-13-di*cis*	350	29 250	0·13	100
	288	21 600		
	280	18 600		
Anhydrovitamin A_2	391	81 740		
	370	92 980		
(3 alkoxyan-hydro retinol)	352	63 680		

Table 13.4 Absorption peaks of retinals and retinoic acids (in ethanol)

Isomer	Retinals			Retinoic acids	
	$\lambda_{max.}$ nm	$\epsilon_{max.} \times 10^{-4}$	Potency* i.u./g $\times 10^{-6}$	$\lambda_{max.}$ nm	$\epsilon_{max.} \times 10^{-4}$
Vitamin A_1 series					
all-*trans*	381	4·341	1·52	352	4·440
13-*cis*	375	3·57	1·53	359	3·83
	257	0·95	—	—	—
11-*cis*	377	2·49	0·80	—	—
	290	0·924	—	—	—
	254	1·744	—	—	—
9-*cis*	373	3·61	—	348	3·55
11,13-di-*cis*	373	1·99	0·32	—	—
9,13-di-*cis*	368	3·238	0·28	352	3·39
Vitamin A_2 series					
all-*trans*	401	4·15	—	370	4·15
	314	1·11	—	305	1·32
13-*cis*	395	3·33	—	372	3·875
	314	1·16	—	305	1·305
11-*cis*	393	2·48	—	—	—
	321	1·85	—	—	—
	252	1·28	—	—	—
11,13-di-*cis*	386	2·72	—	369	3·695
	269	1·1	—	300	1·675
	261	1·1	—	—	—
9-*cis*	391	3·41	—	366	3·3
	315	1·90	—	302	1·573

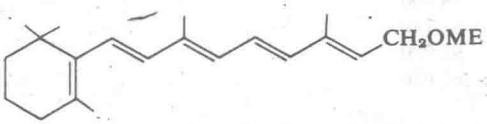
* depends on conversion to the corresponding retinol.

work as far as is perhaps worth while. All-*trans* vitamin A, neovitamin A and vitamin A_2 can be separated by chromatography (Bro-Rasmussen *et al.*, 1955) but the recovery is only 90%. Robeson & Baxter (1947) determined the proportions of all-*trans* and neovitamin A by a maleic anhydride titration which though probably nearly accurate is tedious.

In cod liver oils the proportions of the three forms are approximately A_1 (all-*trans*): A_1 (*cis* form(s)): A_2 70:24:6 and when corrected for the respective potencies 76, 21·6 and 2·4% represents an approximation to the contributions to a bio-assay. The measurement of absorption at 693 and 618 nm probably allows the best determination of vitamin A_2 and the results are rather higher than for the chromatographic separation. The geometrical correction procedure by means of the fixation points for all-*trans* vitamin A_1 over-corrects the neovitamin contribution to the total absorption but this is very nearly balanced by the somewhat lower biological activity of neovitamin A compared with the all-*trans* form. In fact three cod liver oils and two much richer oils when assayed by the normal correction procedure and by the chromatographic procedure of Bro-Rasmussen *et al.* (1955) gave much the same estimate of vitamin A_1 potency.

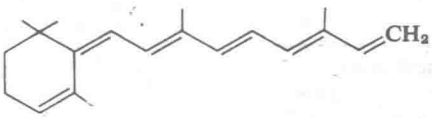
This study affords a good example of the need to pursue an analytical study until all the main variables have come under scrutiny; here it emerges that it is not necessary in routine work to use the most complicated method.

Retinol and related substances



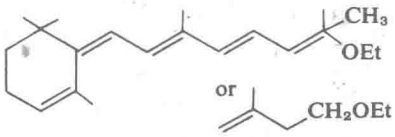
Vitamin A ether (retinol methyl ether)

$\lambda_{max.}$ nm 326 $\epsilon_{max.}$ 49 800



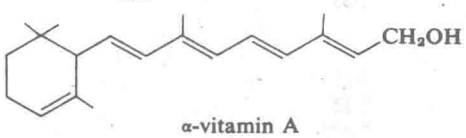
Anhydro vitamin A
$SbCl_3$ colour test
$\lambda_{max.}$ nm 620 $\epsilon_{max.}$ 147 000

$\lambda_{max.}$ nm 351, 371, 392



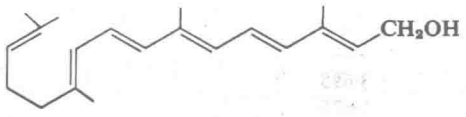
$SbCl_3$ colour test
$\lambda_{max.}$ nm 620

$\lambda_{max.}$ nm 332, 348, 366



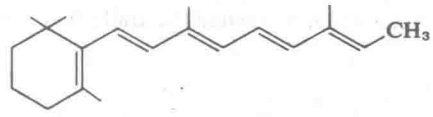
α-vitamin A
α-retinol

$\lambda_{max.}$ nm 298, 311, 325 $\epsilon_{max.}$ 34 900, 47 200, 32 900



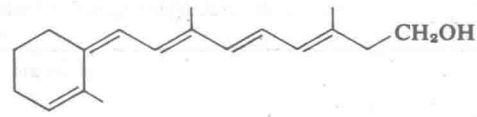
γ-vitamin A
γ-retinol

$\lambda_{max.}$ nm 324, 341, 359 $\epsilon_{max.}$ 55 770, 84 090, 78 650



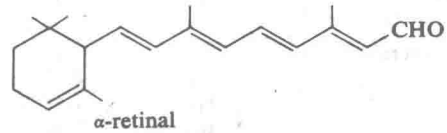
axerophthene

$\lambda_{max.}$ nm 326 $\epsilon_{max.}$ 49 950 (hexane)



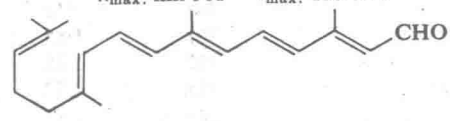
rehydroretinol
$SbCl_3$ colour test
$\lambda_{max.}$ nm 612

$\lambda_{max.}$ nm 330, 351, 369



α-retinal
$SbCl_3$ colour test
$\lambda_{max.}$ nm 561 $\epsilon_{max.}$ 125 530

$\lambda_{max.}$ nm 250, 368 $\epsilon_{max.}$ 8 470, 48 850



γ-retinal

$\lambda_{max.}$ nm 408 $\epsilon_{max.}$ 56 800

13.1.5 Low Temperature Spectroscopy

Quantitative spectrophotometry at very low temperatures requires the use of a solvent or solvent mixture that remains clear and the windows of the cell must be protected so that there is no clouding by condensation of moisture.

Vitamins A, retinals and carotenoids present little difficulty at temperatures above −78°C (attainable by the use of solid CO_2) since they are soluble in appropriate solvents, but between −78° and −196°C most such solvents freeze and the crystals scatter light freely. A mixed solvent EPA (ether, isopentane (or isohexane) and ethanol, 5:5:2 v/v) or EH amine (ether, isohexane and triethylamine 5:5:2 v/v; Jurkowitz, 1959) remains clear even at the temperature of liquid nitrogen. In the case of visual pigments obtained in the form of a micellar dispersion in a suitable detergent in water, addition of two volumes of glycerol gives a mixture that sets to a glass which remains clear to −100°C. Rapid cooling favours 'solidification' to a glass. The volume contracts by about 7·7% in 67% glycerol. If crazing cannot be avoided the use of opal glass to compensate for scattering has been recommended (see p. 822).

Many types of cryostats have been described, the latest designs being due to Yoshizawa & Horiuchi (1972).

Loeb *et al.* (1959) studied the effects of cooling on the spectra of retinol and retinal isomers.

The bathochromic effects of cooling will be noted and the roughly 10% increase in $\epsilon_{max.}$ except for the 11-*cis* forms of retinol and retinal, for which the increases in $\epsilon_{max.}$ are 45% and 63% respectively.

Loeb *et al.* (1969) observed the absorption spectra of β-carotene (all-*trans*) and its 11,11′-di-*cis* isomer at liquid nitrogen temperature in EPA. Figure 13.10

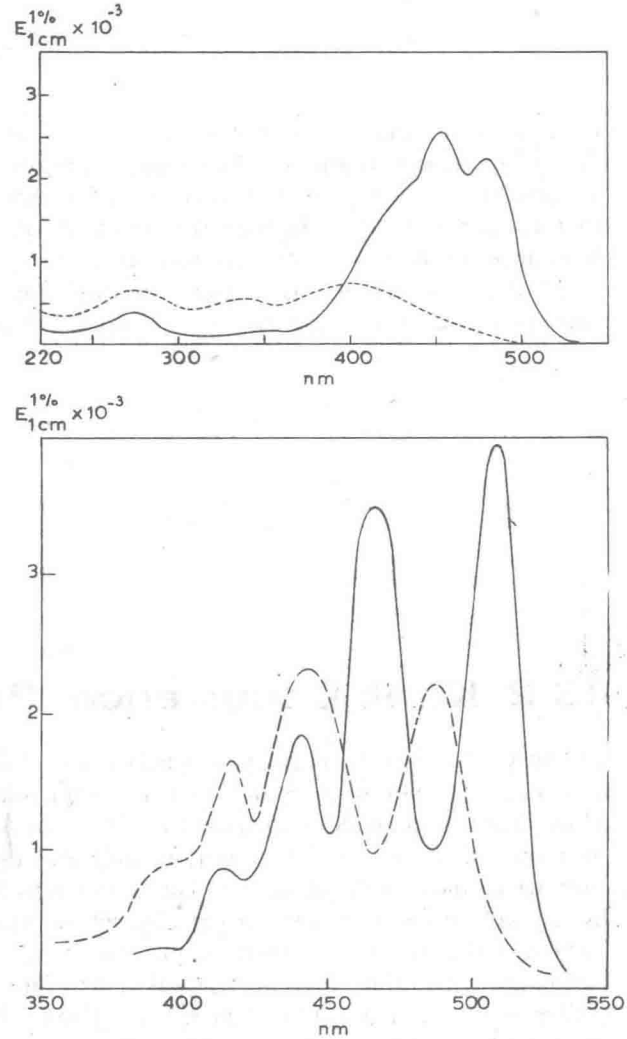


Figure 13.10 (*a*) Absorption spectra in light petroleum of: ———, all-*trans* β-carotene and — — —, 11,11′-di-*cis* β-carotene at room temperature (after Isler *et al.*, 1957) (*b*) Absorption spectra of: ———, all-*trans* β-carotene and — — — the hindered isomer, 11,11′-di-*cis* β-carotene at −190°C (after Loeb, Brown & Wald, 1959)

Table 13.5 Effects of cooling on the spectra of retinols and retinals

	Room temperature		−185 to −195°C	
	$\lambda_{max.}$ nm	$\epsilon_{max.} = 10^{-4}$	$\lambda_{max.}$ nm	$\epsilon_{max.} = 10^{-4}$
Retinol all-*trans*	324	5·21	333·5	5·42
Retinol 11-*cis*	318	3·42	332·5	4·87
Dehydroretinol acetate all-*trans*	349	3·95	360	4·50
Retinal all-*trans*	373	4·76	387	5·17
Retinal 9-*cis*	366	3·97	379	4·41
Retinal 13-*cis*	366	3·88	380	4·35
Retinal 9,13-di-*cis*	360	3·56	371	3·99
Retinal 11-*cis*	369	2·64	384·5	4·3
Dehydroretinal all-*trans*	392	4·20	409	4·55

indicates significant bathochromic effects and a very marked increase in resolution. The weaker unresolved absorption of the isomer with two *cis* bands shows that cooling tends to minimize the effect of steric hindrance. Theoretical studies on the electronic structures of retinal and its *cis* isomers and their interconversions have been carried out by Langley *et al.* (1969 *a*, *b*, 1970) and Sperling & Rafferty (1969) have shown spectroscopically the preferential torsion of the 10–11 link. The sterically hindered isomers are much more stable than was initially expected by Pauling (1939). Langley *et al.* refer to other work in this field.

13.2 Kitol: Occurrence, Properties, Structure

Edisbury *et al.* (1935) in studying whale liver oil as a source of vitamin A found that its ultraviolet absorption was anomalous compared with that shown by most fish liver oils. The unsaponifiable fraction from which most of the cholesterol had been removed by crystallization displayed strong absorption near 290 nm and gave an abnormal colour test with the antimony trichloride reagent. It was later found (Pritchard *et al.*, 1937) that when the unsaponifiable fraction was partitioned between 83% ethanol–water and light petroleum, the alcohol-soluble fraction showed $\lambda_{max.}$ 285–290 nm and in the colour test a strong band at 594 nm. Embree & Shantz (1943) subjected desterolated whale liver oil unsaponifiable matter to molecular distillation and obtained a fraction with $\lambda_{max.}$ 290 nm, which they called kitol. Clough *et al.* (1947) separated kitol palmitate, showing $E^{1\%}_{1cm}$ 379 at 290 nm (corresponding with $E^{1\%}_{1cm}$ 694 for free kitol). Molecular distillation of kitol palmitate resulted in vitamin A palmitate (*ca.* 0·7 mol/mol kitol palmitate). Various Japanese workers confirmed that pyrolysis under suitable conditions yielded vitamin A.

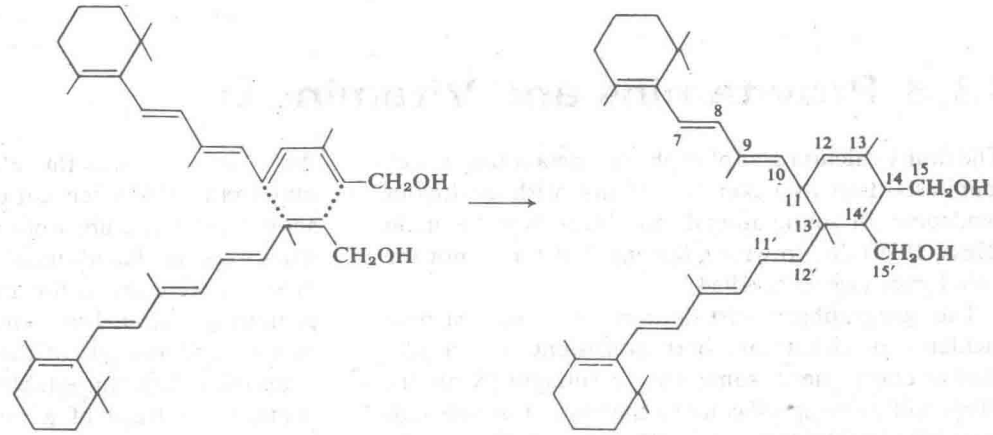
The work of Embree and Shantz established the molecular weight of kitol as about 575, which made it probably a dimeride of vitamin A (retinol). A crystalline preparation (m.p. 88–90°) showed $\lambda_{max.}$ 286 nm ($E^{1\%}_{1cm}$ 707, $\epsilon_{max.}$ 41 000). Kitol is present in whale liver oil as a mixture of esters (Barua & Morton, 1949) and it occurs in variable amounts in many fish liver oils and even in mammalian liver lipid. The natural distribution however merits further investigation.

A crystalline kitol diacetate, m.p. 149–150° (Chatain & Debodard, 1951, 1952), showed $\lambda_{max.}$ 290 $E^{1\%}_{1cm}$ 565 and it seems clear that for free kitol $E^{1\%}_{1cm}$ must be between 685 and 700.

Kaneko (1958, 1960) made the interesting discovery that retinyl palmitate dissolved in isopropanol undergoes photodimerization under the action of sunlight, the reaction requiring up to 40 days' exposure. Das (private communication) dissolved retinyl acetate in heptane and obtained in sunlight a good yield of kitol diacetate ($\lambda_{max.}$ 290 nm, $E^{1\%}_{1cm}$ 569). A substance of unknown constitution showing $\lambda_{max.}$ 310 nm is an intermediate.

Embree & Shantz (1945) studied some freshwater fishes and detected kitol$_2$ derived from vitamin A$_2$ (3-dehydroretinol). This substance had $\lambda_{max.}$ 310 nm and gave vitamin A$_2$ on pyrolysis. Kaneko *et al.* (1958, 1960) found that sunlight acting on 3-dehydroretinyl palmitate produced kitol$_2$-palmitate.

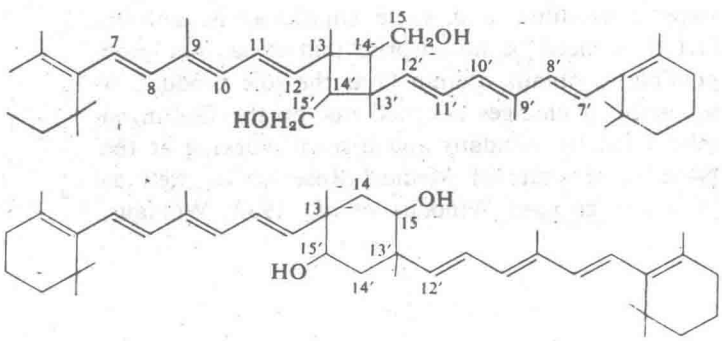
Das (private communication) has found that ethanolic hydrogen chloride acting on retinol can produce anhydroretinol and also retroretinol or kitol depending on the concentration of hydrogen chloride, the temperature and the duration of interaction. Thus 0·13 N hydrogen chloride and a temperature of 30–35° and a duration of 1·5 hours results in the best yield of retroretinol. Retroretinol (or rehydrovitamin A) is



produced to some extent *in vivo* by rehydration of anhydrovitamin A. It is an isomer of retinol, the double bonds being displaced one carbon atom away from the primary alcohol group. In fact, however, the best procedure for preparing retroretinyl acetate is to allow retinyl acetate to be in contact with concentrated hydrobromic acid at 0° for 30 seconds. This gives retroretinyl acetate ($\lambda_{max.}$ 332, 348, 467 nm, $E^{1\%}_{1cm}$ 1069, 1387, 1016 in light petroleum) in 86% yield. Retroretinol is not attacked readily by ethanolic hydrogen chloride. When retinyl acetate and 0·13 N anhydrous ethanolic hydrogen chloride are left to stand at 30–35° for 40 minutes kitol diacetate is formed in about 8% yield.

Numerous kitol esters have been prepared by standard methods and although *cis-trans* isomerism is a possibility, there is good agreement about spectroscopic properties for free kitol and its esters.

Kitol has 8 double bonds and the 290 nm absorption peak (328 nm for retinol) fits a chromophore containing 4 conjugated double bonds. The simplest structure consistent with this evidence was advanced by Kaneko (1958, 1960), namely a dimeric retinol in which the $C_{13,14}$ double bonds of two molecules form a cyclobutane ring. In this structure kitol contains two primary alcohol groups. Yano & Tomiyama (1963) considered that the reactivity of kitol was more consistent with the presence of two secondary alcohol groups and they advanced a structure based on a cyclohexane ring system.



Berger *et al.* (1965) also rejected the cyclobutane structure but advanced a new and unsymmetrical structure which revert to the presence of two —CH_2OH groups but reverted a six-membered ring. This type of structure was supported by Gianotti *et al.* (1966) and with a slight difference by Ganet *et al.* (1966). The case for an unsymmetrical structure depends very largely on the interpretation of nuclear magnetic resonance spectra. The results indicate one methyl group in a saturated environment (τ 8·87). The lack of symmetry is supported by τ 8·22 and 8·13 for the C9 and C9′ methyl protons and τ 4·06 and 4·02 for the 7,8 and 7′,8′ olefinic protons whilst the CH_2OH protons correspond with a multiplet τ 4·86. Two doublets τ 4·58 and 4·86 correspond with protons at C10 and C12.

The effect of heat on kitol diacetate (in the absence of oxygen) is very sharply dependent on temperature. Thus Das (private communication) heated 15 mg portions at 220°, 225° and 230° and obtained 4·8, 7·0 and 3·8 mg of retinyl acetate with 5·2, 2·5 and 1·5 mg respectively of unchanged kitol acetate.

From the biochemical standpoint kitol remains very puzzling. The amount present in some whale liver oils is very large and Hickman suggested that the whales might ingest so much vitamin A in their food as to make them liable to hypervitaminosis were it not for conversion to kitol which is biologically inactive. There are however numerous difficulties with this attractive hypothesis which certainly is difficult to square with the extremely large amounts of vitamin A found in the liver and intestinal lipids of many fish species. If kitol possesses any specific biological role it has escaped detection. There is no evidence as yet of any specific kitol-splitting enzyme. Finally there is little evidence that krill (*Euphausia superba*), the main food of the whale, contains a plausible precursor other than vitamin A.