is the only chemical difference we have observed in addition to the difference in speed of reaction with maleic anhydride.

SUMMARY

1. Vitamin A ester concentrates can be enriched by chromatography on weakened alumina until practically free from glycerides, sterol esters and other substances.

2. Repeated chromatographic adsorptions permit the accumulation of neovitamin A esters in the strongly held fractions.

3. Ultraviolet absorption curves for neovitamin A esters closely resemble those of the all-*trans*

isomer except that on the long-wave side of 310 m μ , they are displaced by 2-3 m μ .

4. Neovitamin A alcohol yields neoretinene₁ on oxidation over manganese dioxide (light petroleum solution). The spectrum of neoretinene₁ is slightly displaced in the direction of shorter wavelengths as compared with retinene₁.

5. The occurrence of neovitamin A in fish-liver oils may result in over correction in spectroscopic assays if its presence is neglected.

The authors are indebted to the Medical Research Council for financial assistance and to Marine Oil Refiners of Africa Ltd., Simonstown, South Africa, for some of the materials used.

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Studies in Vitamin A

17. SPECTROSCOPIC PROPERTIES OF ALL-TRANS-VITAMIN A AND VITAMIN A ACETATE. ANALYSIS OF LIVER OILS

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(Received 7 February 1951)

The adoption by the World Health Organization (W.H.O.) (1950) of pure crystalline vitamin A acetate as the International Standard substance makes important the specification of criteria of purity. As a result of the widespread use of spectrophotometric methods for determining vitamin A in fish-liver oils and concentrates, spectroscopic properties are specially significant. The necessity for 'correcting' the absorption spectra of many natural products so as to allow for 'irrelevant' absorption adds to the importance of accuracy in the data for pure vitamin A. The advent of synthetic vitamin A as an article of commerce (conceivably contaminated by impurities differing from those found in natural products) raises new analytical problems. The existence of neovitamin A (Robeson & Baxter, 1945), a *cis*-isomer of the better known all-*trans*-vitamin A, and its occurrence in fish-liver oils

(Meunier & Jouanneteau, 1948) and some synthetic products (Schwarzkopf, Cahnmann, Lewis, Swidinsky & Wuest, 1949) raises issues which have not been fully studied. The simple correction procedure of Morton & Stubbs (1946, 1948) has been very widely used, sometimes with less caution than is required by the plainly stated assumptions on which it rests.

A simple analytical procedure applicable to all kinds of products is no doubt very desirable, but no such method has yet been described. The substances which can contribute 'irrelevant' absorption include: vitamin A₂, kitol, anhydrovitamins A₁ and A₂, oxidation products, carotenoids and unidentified coloured substances, polylene acids and in synthetic products small amounts of intermediates or artifacts. Other disturbing factors include neovitamin(s) A and the effect of variation in the proportion of esterified and free vitamin A.

Although it is not always necessary to aim at high accuracy, the degree of uncertainty in the results obtained by simplified procedures needs to be ascertained.

The present investigation is concerned first with the calibration of the spectrophotometer. This preliminary step is desirable for all accurate work on vitamin A and indispensable in drawing up a specification for the pure material. Secondly, the actual spectra are determined with care in various solvents. Finally, the use to which the spectra are put is illustrated by a full study of a few selected materials, and the whole problem of determining vitamin A is reviewed.

EXPERIMENTAL

Apparatus and materials

Spectroscopic instruments. A new Unicam photoelectric spectrophotometer was used both for determining ultra-violet absorption and for the study of the SbCl_3 colour test. A Hilger-Nutting visual spectrophotometer was also used in measuring the blue colour.

Potassium chromate and dichromate. These were used for testing the performance of the spectrophotometer and were of Analar quality.

Solvents. *cyclo*Hexane and ethanol were specially purified for spectroscopy. *iso*Propanol was used as purchased and CHCl_3 was left to stand over anhydrous CaCl_2 and filtered before use. Light petroleum (b.p. 40–60°) was of A.R. quality.

Vitamin A acetate. Recrystallized material prepared indirectly from fish-liver oils was kindly supplied through Dr J. Matet by Alimentation Equilibree, Commeny, France. Synthetic vitamin A acetate was kindly supplied through Dr O. Isler by Hoffmann La Roche, Basle, Switzerland.

Vitamin A alcohol. Dr Isler also supplied us with pure crystalline vitamin A alcohol (synthetic).

Vitamin containing oils. The following were used: (a) cod-liver oil compound, as supplied to antenatal clinics in England, (b) two rich fish-liver oils (species uncertain but probably Spanish tunny liver oils), (c) the International Standard preparation of vitamin A acetate issued by the Director of Biological Standards, National Institute for Medical Research, Mill Hill, London, N.W. 7.

Analytical methods

Antimony trichloride colour test. The reagent used was a practically saturated solution (near 15°) of anhydrous SbCl_3 in CHCl_3 which had been filtered after standing over anhydrous CaCl_2 to remove ethanol. The reagent must be filtered, quite clean and colourless, and acetic anhydride (approx. 1 ml./100 ml.) should be added before use. The blue colour produced with vitamin A fades rather quickly, and measurements must be made at a standard time after mixing. In our experiments the ratio of volume of SbCl_3 reagent to volume of vitamin A solution (in CHCl_3) was from 8:1 to 12:1. The measurements were made on the Unicam spectrophotometer and the total volume of the solution was usually between 3.0 and 3.5 ml. The volume of vitamin A solution was very accurately measured using an 'Agl'a' Syringe (Burroughs Wellcome and Co.).

It is essential to introduce the SbCl_3 reagent quickly from a good quality burette with a wide tip. The cells must be scrupulously clean and the CHCl_3 must not be allowed to evaporate from the vitamin A solution while repeated determinations are being made. The spectrophotometer is adjusted (wavelength, dark current, slit-width) after introducing the vitamin A solution into the bottom of the cell; the reagent is then quickly added, the cell holder placed in position and the extinction is read. The reading is made within 8–20 sec. of mixing (average 15 sec.). Under these conditions reproducibility at λ_{max} 620 m μ . is good and the colour is 'caught' very near its peak intensity. To plot the whole curve is laborious since a fresh solution must be taken for measurements at each wavelength every time (Fig. 1). The main difficulties are to prevent a change of concentration by loss of solvent and to keep the cells perfectly clean. To exclude such sources of error small quantities of solution should be poured from a stoppered flask into a stoppered weighing bottle as needed, and the cells should be frequently cleaned with conc. HCl.

Estimation of neovitamin A. Robeson & Baxter (1945) made use of the earlier observations of Kawakami (1935) and Hamano (1937) and others on the formation of maleic anhydride adducts. The method of Robeson & Baxter (1947) has been followed here. It is based upon the much more rapid formation of an adduct with all-*trans*-vitamin A than with neovitamin A.

Maleic anhydride (resublimed) was used as a 10% solution in benzene. A solution in light petroleum of the oil to be tested was prepared and a measured volume containing approx. 3000 i.u. of (total) vitamin A was carefully evaporated and made up to 10 ml. with benzene. A similar solution of crystalline all-*trans*-vitamin A acetate was prepared. 5 ml. portions of each solution were transferred to 10 ml. graduated flasks and made up to the mark with 10% maleic anhydride solution and left in the dark for 16 hr. at 25°. The remaining 5 ml. portions of benzene solution were retained as controls. After 16 hr. the solutions were tested with the Carr-Price reagent. The colour produced with all-*trans*-vitamin A acetate (after maleic anhydride treatment) was feeble and the solution did not require to be diluted, whereas the control solution needed to be suitably diluted. The maleic anhydride treatment reduced the colour intensity to 2% of the control all-*trans* solution (Robeson & Baxter recorded 5%). As no pure crystalline neovitamin A was available, Baxter & Robeson's figure of 90% for the corresponding result for the isomer was accepted. Dalvi & Morton (1951), in this laboratory, obtained essentially similar results at 15° using a neovitamin A ester concentrate. The percentage of neovitamin A in an oil was calculated from the equation $\frac{R - R_1}{R_2 - R_1} \times 100 = \text{percentage of total vitamin A occurring as the neo isomer}$. R is the recovery of vitamin A in the sample expressed as $E_{1\%}^{1\text{cm}}$ (620 m μ .) after maleic anhydride treatment, divided by $E_{1\%}^{1\text{cm}}$ (620 m μ .) for the control solution and multiplied by 100. [$R_1 = 2$ and $R_2 = 90$ (see above).] The degree of reproducibility in the estimates of the neo/all-*trans* ratio is probably a little less than that of the colour test itself. In the case of vitamin A alcohol the factors given by Baxter & Robeson were used, i.e. $R_1 = 3$ and $R_2 = 83$.

Estimation of vitamin A and A₂ by the colour test. If the total absorption at 620 m μ . is E_{620} and that at 693 m μ . is E_{693} and if x is the absorption at 620 m μ . due to vitamin A₁ and y is the absorption at 693 m μ . due to vitamin A₂, then

$$E_{620} = x + 0.328y,$$

and

$$E_{693} = 0.0136x + y.$$

The factor 0.328 is the ratio of E_{620}/E_{693} for pure vitamin A₂ as given by Shantz (1948). Similarly, the factor 0.0136 is the ratio E_{693}/E_{620} for pure vitamin A as shown in Table 7. Hence, solving the equations, we get

$$x = \frac{E_{620} - 0.328E_{693}}{0.996} \simeq E_{620} - 0.328E_{693}.$$

$$y = \frac{E_{693} - 0.0136E_{620}}{0.996} \simeq E_{693} - 0.0136E_{620}.$$

Chromatography. The adsorbent was activated alumina (P. Spence and Co., Grade 0) weakened with 8% water. The technique used has been described by Barua & Morton (1949).

Calibration of the spectrophotometer

Potassium chromate and dichromate are perhaps the best known solutes for calibration. The results of our tests compared with data from other laboratories are shown in Tables 1 and 2.

Absorption of vitamin A acetate and alcohol

The melting points of the material were as follows:

'Natural' acetate, 56–57° (uncorr.).

Synthetic acetate, 57–58° (uncorr.).

Mixed sample, 56.5–57.5° (uncorr.).

Synthetic vitamin A alcohol, 57–58° (uncorr.).

The melting points were determined on material from ampoules opened less than 30 min. previously. The recorded higher melting point (62–64°) for vitamin A alcohol is not disputed. We were primarily concerned to make weighings before any deterioration occurred.

Vitamin A acetate has been examined in four solvents. The results are summarized in Tables 3 and 4; Table 5 shows the intensities of absorption as fractions of $E_{\max}$. It is clear that $\lambda_{\max}$ is in the region 325–328 m μ . and $\epsilon_{\max}$ is near to 50,000. The absorption curves all show a very feeble second maximum near 250–253 m μ ., $\epsilon_{\max}$ approx. 550.

Synthetic vitamin A alcohol has been examined similarly (Tables 3–5); $\lambda_{\max}$ is from 324 to 327 m μ . and the values of $\epsilon_{\max}$ in the non-polar solvents agree very closely with those for the acetate. The weak secondary absorption peak near 250 m μ . is also shown by the free vitamin. These results are discussed later (p. 56).

Table 1. Comparison of results obtained by various observers for the maxima and minima in the absorption spectrum of K₂CrO₄ in 0.05N-KOH

(Light source is hydrogen tube unless otherwise stated.)

Reference	Wavelength (m μ .) (max.)	$E_{1\%}^{1\text{cm.}}$	Wavelength (m μ .) (min.)	$E_{1\%}^{1\text{cm.}}$	Wavelength (m μ .) (max.)	$E_{1\%}^{1\text{cm.}}$	Wavelength (m μ .) (min.)	$E_{1\%}^{1\text{cm.}}$
a	372	248.5	313	10.3	273	188.5	229	39.1
b	372.5	243.5	313	10.3	273	184.3	229	35
		248†				188.5		39.1
c	372	249	313	10.5	274	191	230	41.8
d	372	248.2	313	10.44	274	192.8	230	37.6
e	371.5	245.2	312.5	10.22	272.5	188.8	227	36.5
		248.8†						
f*	371.5	248.2	313.0	10.36	272.5	188.8	229	37.7

* Concn. (% w/v) 0.0025

0.05

0.0025

0.01

† Hg vapour lamp.

‡ Tungsten filament lamp.

a, Hogness, Zscheile & Sidwell (1937).

b, Zscheile (1947).

c, Gibson (1950).

d, Present authors, Beckman instrument (much used).

e, Riley (1950), new Unicam instrument.

f, Present authors, new Unicam instrument.

Table 2. Comparison of results on 0.005% K₂Cr₂O₇ solution (containing a little H₂SO₄)

Wavelength ... Reference	350 m μ . (max.) $E_{1\%}^{1\text{cm.}}$	313 m μ . (min.) $E_{1\%}^{1\text{cm.}}$	257 m μ . (max.) $E_{1\%}^{1\text{cm.}}$	235 m μ . (min.) $E_{1\%}^{1\text{cm.}}$
a	107.6	49.2	144.8	124.8
b*	107.8	48.6	144.9	124.2 _s
c	106.7	48.7	145.2	124.8

* Hydrogen source; reversing of cells made no difference except at 257 m μ ., where the result given is a mean value. At 313 m μ . the tungsten lamp led to a value of 47.8.

a, Riley (1950).

b, Present authors.

c, Selected data in collaborative tests arranged by the Photoelectric Spectrophotometry Group and analysed by Gridgeman (1950).

Table 3. *Absorption maxima of natural and synthetic all-trans-vitamin A*

Solvent	Source*	Vitamin A acetate				Vitamin A alcohol			
		$\lambda_{\max}$ (m μ .)	$\epsilon_{\max}$	f values†	$E_{1\text{ cm.}}^{1\%}$	$\lambda_{\max}$ (m μ .)	$\epsilon_{\max}$	f values	$E_{1\text{ cm.}}^{1\%}$
Light petroleum	N	325	52,200	1.42	{1590 1600}	324.5	52,300	1.40	1830
	S	325	52,500						
<i>cyclo</i> Hexane	N	327.5	49,700	1.35	{1515 1515}	326.5	49,800	1.33	1745
	S	328	49,700						
<i>iso</i> Propanol	N	326	50,500	1.34	{1540 1530}	325.5	52,400	1.38	1835
	S	326	50,100						
Ethanol	N	326	50,300	1.39	{1535 1560}	324	51,500	1.34	1800
	S	326	51,000						
Light petroleum	S_I	326	51,000	—	1558	325	51,800	—	1810
<i>cyclo</i> Hexane	N_M	328	49,200	—	1500	—	—	—	—
	S_I	{328 327.5}	{50,000 49,500}	—	{1525 1509}	{327 326.5}	{49,300 49,500}	—	{1725 1730}
<i>iso</i> Propanol	N_M	326	50,500	—	1540	—	—	—	—
	S_I	{326 326}	{50,200 49,700}	—	{1530 1515}	{325 324.5}	{52,000 51,800}	—	{1820 1810}
Ethanol	N_M	325.5	50,600	—	1544	—	—	—	—
	S_I	{326 325.5}	{50,800 50,100}	—	{1550 1527}	{325 324.5}	{52,000 51,500}	—	{1820 1800}

* N=natural; S=synthetic; S_I =data provided by Dr O. Isler; N_M =data provided by Dr J. Matet.

† f =oscillator strength = $4.31 \times 10^{-8} \int_{25,000 \text{ cm.}^{-1}}^{40,000 \text{ cm.}^{-1}} \epsilon d\nu$.

Table 4. $E_{1\text{ cm.}}^{1\%}$ values at the absorption maximum of vitamin A acetate and vitamin A alcohol and conversion factors

Solvent	$E_{1\text{ cm.}}^{1\%}$ (mean values)	Conversion factor*
Vitamin A acetate		
Light petroleum	1595	1825
<i>cyclo</i> Hexane	1515	1920
<i>iso</i> Propanol	1535	1895
Ethanol	1550	1880
Vitamin A alcohol		
Light petroleum	1830	1820
<i>cyclo</i> Hexane	1745	1910
<i>iso</i> Propanol	1835	1820
Ethanol	1800	1850

* Figures in this column represent defined potency (2.91×10^6 i.u./g. for acetate, 3.33×10^6 i.u./g. for alcohol) divided by observed $E_{1\text{ cm.}}^{1\%}$ value.

Correction for irrelevant absorption

The correction procedure of Morton & Stubbs (1946, 1948) is based upon the assumption that the irrelevant absorption is linear in the region 310–340 m μ . In its original form (1946) two wavelengths at which E for the pure vitamin A (alcohol or ester) was exactly six-sevenths of $E_{\max}$ were chosen. The present investigation on highly purified vitamin A provided an opportunity to check the three fixation wavelengths in different solvents.

Table 6 shows fixation points and the corresponding correction equations for various solvents. New fixation points (a) have been ascertained in which the ratio 6/7 is preserved. The old fixation wavelengths (b) (Morton & Stubbs, 1948) for ethanol and *cyclohexane* are also shown with the necessary formulae permitting correction based on

the new absorption curves. For each solvent a third set of fixation points (c) with equal wavelength intervals is now added with a suitable and simple correction equation.

Antimony trichloride colour test

It is important to know as precisely as possible the value of $E_{\max}$. The value of $E_{1\text{ cm.}}^{1\%}$ (620 m μ .) for the acetate was 4420; vitamin A alcohol gave a value of $E_{1\text{ cm.}}^{1\%}$ (620 m μ .) of 5070. The corresponding values of the molecular extinction coefficients did not differ significantly and these results were combined. The mean value of $\epsilon_{\max}$ (620 m μ .) was $144,900 \pm 870$ (for $P=0.95$; six determinations on the alcohol and nine on the acetate). Table 7 shows the intensity of absorption over the range 520–700 m μ .; there is no inflexion in the region of 580 m μ .

Tests on fish-liver oils

In order to test the applicability of the new spectroscopic data and the proposed new correction formulae, four oils (p. 49) have been examined. In Table 8 are given the experimental results on the first three oils and the results of applying the various correction formulae. The effects of maleic anhydride on the colour test and the calculated amounts of neovitamin A are shown in Table 9, which also shows the absorption at 620 and 693 m μ . and the calculated proportion of vitamins A_1 and A_2 (see Fig. 1).

The high-potency oil B was investigated in greater detail by means of chromatography, and these results are shown in Table 10 and Fig. 2.

International standard preparation

The standard preparation consists of a solution of vitamin A acetate in a selected cotton-seed oil. It is made up in sealed gelatin capsules, each meant to contain 0.250 g. of

Table 5. *Intensities of absorption expressed as fractions of $E_{\max}$.*

Vitamin A acetate					Vitamin A alcohol				
Wave-length (m μ .)	cyclo- Hexane	isoPro- panol	Ethanol	Light petroleum	Wave-length (m μ .)	cyclo- Hexane	isoPro- panol	Ethanol	Light petroleum
220	0.091	0.086	0.086	0.086	220	0.086	0.082	0.073	0.081
230	0.096	0.092	0.092	0.090	230	0.090	0.091	0.082	0.089
240	0.106	0.103	0.103	0.100	240	0.102	0.106	0.097	0.099
245	0.111	0.109	0.109	0.103	245	0.108	0.110	0.104	0.105
250	0.114	0.112	0.113	0.104	250	0.112	0.107	0.109	0.111
255	0.115	0.111	0.112	0.103	255	0.111	0.099	0.107	0.112
260	0.109	0.107	0.106	0.104	260	0.107	0.098	0.100	0.106
265	0.112	0.115	0.114	0.117	265	0.108	0.104	0.104	0.112
270	0.132	0.142	0.135	0.152	270	0.130	0.130	0.127	0.137
275	0.165	0.175	0.173	0.189	275	0.161	0.167	0.151	0.177
280	0.215	0.230	0.227	0.247	280	0.212	0.229	0.212	0.236
285	0.270	0.298	0.295	0.320	285	0.274	0.298	0.283	0.312
290	0.350	0.375	0.374	0.401	290	0.346	0.381	0.366	0.396
295	0.448	0.474	0.474	0.507	295	0.448	0.489	0.475	0.505
300	0.555	0.573	0.573	0.602	300	0.553	0.582	0.572	0.605
305	0.670	0.702	0.704	0.731	305	0.669	0.723	0.725	0.740
—	—	—	—	—	309	0.785	0.835	0.830	0.849
310	0.806	0.828	0.828	0.862	310	0.811	0.855	0.845	0.867
311	0.830	0.845	0.849	0.880	311	0.830	0.868	0.860	0.883
312	0.846	0.860	0.866	0.895	312	0.852	0.880	0.873	0.893
313	0.867	0.883	0.878	0.902	313	0.869	0.886	0.884	0.901
314	0.882	0.894	0.897	0.912	314	0.883	0.892	0.895	0.911
315	0.894	0.902	0.902	0.923	315	0.893	0.904	0.900	0.917
316	0.907	0.917	0.915	0.930	316	0.903	0.910	0.909	0.928
317	0.914	0.929	0.924	0.935	317	0.910	0.925	0.922	0.935
318	0.920	0.937	0.938	0.948	318	0.917	0.932	0.932	0.945
319	0.928	0.946	0.948	0.959	319	0.925	0.948	0.947	0.958
320	0.935	0.960	0.963	0.967	320	0.936	0.962	0.958	0.970
321	0.949	0.972	0.973	0.980	321	0.947	0.979	0.975	0.981
322	0.957	0.981	0.985	0.987	322	0.963	0.992	0.985	0.989
323	0.970	0.989	0.994	0.997	323	0.972	0.998	0.990	0.995
324	0.979	0.996	0.998	0.999	324	0.982	1.000	1.000	1.000
325	0.985	0.997	1.000	1.000	325	0.987	1.000	0.997	1.000
326	0.993	1.000	1.000	0.999	326	1.000	0.993	0.995	0.993
327	1.000	0.998	0.998	0.997	327	1.000	0.985	0.983	0.984
328	1.000	0.991	0.994	0.985	328	0.995	0.966	0.971	0.973
329	0.994	0.987	0.989	0.969	329	0.987	0.953	0.956	0.956
330	0.989	0.972	0.973	0.957	330	0.982	0.935	0.936	0.938
331	0.979	0.958	0.959	0.935	331	0.966	0.914	0.917	0.916
332	0.963	0.945	0.946	0.917	332	0.947	0.892	0.898	0.895
333	0.948	0.929	0.924	0.896	333	0.926	0.872	0.879	0.873
334	0.930	0.909	0.904	0.875	334	0.907	0.853	0.857	0.847
335	0.915	0.893	0.896	0.853	335	0.888	0.833	0.837	0.828
336	0.892	0.872	0.871	0.832	336	0.869	0.815	0.822	0.807
337	0.880	0.851	0.855	0.812	337	0.849	0.793	0.802	0.783
338	0.853	0.829	0.829	0.786	338	0.829	0.768	0.774	0.756
339	0.835	0.810	0.815	0.767	339	0.809	0.743	0.760	0.736
340	0.811	0.792	0.795	0.742	340	0.790	0.721	0.736	0.710
345	0.695	0.660	0.667	0.609	345	0.667	0.580	0.591	0.570
350	0.556	0.517	0.520	0.463	350	0.517	0.432	0.442	0.423
355	0.423	0.393	0.393	0.343	355	0.385	0.320	0.330	0.308
360	0.299	0.289	0.292	0.246	360	0.260	0.223	0.233	0.211
365	0.207	0.196	0.197	0.158	365	0.189	0.130	0.140	0.125
370	0.118	0.112	0.113	0.085	370	0.100	0.063	0.070	0.060
375	0.060	0.059	0.058	0.046	375	0.060	0.031	0.035	0.028
380	0.030	0.031	0.034	0.027	380	0.025	0.016	0.019	0.015
385	0.018	0.018	0.018	0.017	385	0.011	0.009	0.010	0.007
390	0.0105	0.011	0.010	0.012	390	0.008	0.005	0.007	0.005
400	0.004	0.003	0.003	0.005	400	0.004	0.001	0.001	0.001

Table 6. *Correction equations for eliminating irrelevant absorption*(For definition of a and c see text.)

Solvent		E_1	E_2	E_3	$E_{\text{corr.}} =$
(i) Vitamin A acetate (all- <i>trans</i>)					
cycloHexane	(a)	312.5	327.5	337.7	$7(E_2 - 0.405E_1 - 0.595E_3)$
	(b)*	313	328	338.5	$6.58(E_2 - 0.412E_1 - 0.588E_3)$
	(c)	316	328	340	$3.52(2E_2 - E_1 - E_3)$
Ethanol	(a)	311.5	326	337	$7(E_2 - 0.432E_1 - 0.568E_3)$
	(b)*	311.5	326.5	337.5	$6.62(E_2 - 0.423E_1 - 0.577E_3)$
	(c)	314	326	338	$3.65(2E_2 - E_1 - E_3)$
isoPropanol	(a)	311.7	326	336.9	$7(E_2 - 0.432E_1 - 0.567E_3)$
	(c)	314	326	338	$3.61(2E_2 - E_1 - E_3)$
Light petroleum	(a)	310	325	334.9	$7(E_2 - 0.399E_1 - 0.601E_3)$
	(c)	313	325	337	$3.5(2E_2 - E_1 - E_3)$
(ii) Vitamin A alcohol (all- <i>trans</i>)					
cycloHexane	(a)	312.5	326.5	336.7	$7(E_2 - 0.422E_1 - 0.578E_3)$
	(b)*	311	326	336	$6.82(E_2 - 0.4E_1 - 0.6E_3)$
	(c)	315	327	339	$3.36(2E_2 - E_1 - E_3)$
Ethanol	(a)	311	324.5	334	$7(E_2 - 0.416E_1 - 0.584E_3)$
	(b)*	311	325	335.5	$6.43(E_2 - 0.428E_1 - 0.572E_3)$
	(c)	312	324	336	$3.28(2E_2 - E_1 - E_3)$
isoPropanol	(a)	310	324.5	334	$7(E_2 - 0.396E_1 - 0.604E_3)$
	(c)	312	324	336	$3.28(2E_2 - E_1 - E_3)$
Light petroleum	(a)	309.5	324	333.6	$7(E_2 - 0.399E_1 - 0.601E_3)$
	(c)	312	324	336	$3.33(2E_2 - E_1 - E_3)$

* Old points (Morton & Stubbs, 1948).

Table 7. *Absorption data relating to the antimony trichloride colour test applied to all-trans-vitamin A acetate*

$\lambda_{m\mu.}$	$E_{1\text{ cm.}}^{1\%}$	$\epsilon_{\text{max.}}$
520	252	8,300
530	363	12,000
540	532	17,500
550	768	25,400
560	1,120	37,000
570	1,620	53,500
580	2,180	72,000
590	2,900	95,500
600	3,600	119,000
610	4,170	137,000
615	4,350	143,000
620*	$4,420 \pm 26$	$144,900 \pm 870$
625	3,900	129,000
630	3,220	106,000
640	1,740	57,300
650	1,010	33,300
664	325	10,700
670	191	6,300
680	74	2,400
685	47	1,550
693	21	690
700	15	495

$$\text{Oscillator strength} = 1.12 = 4.31 \times 10^{-8} \int_{14,500 \text{ cm.}^{-1}}^{20,000 \text{ cm.}^{-1}} \epsilon d\nu.$$

* $E_{1\text{ cm.}}^{1\%}$ 620 $m\mu.$ for vitamin A alcohol = 5070 ± 30 . Fig. 1. ----, absorption spectrum in the region 650–760 $m\mu.$ of the blue solution produced by the interaction of antimony trichloride and high-potency oil B in CHCl_3 . —, Vitamin A_1 solution free from A_2 . The difference between the two curves is due to the 693 $m\mu.$ absorption band shown by vitamin A_2 .

Table 8. *Spectroscopic data on fish-liver oils and application of correction procedures*

Material	Solvent	Gross	$E_{1\text{ cm.}}^{1\%}$ (at $\lambda_{\text{max.}}$ of vitamin A) corrected by					16 points M & S*
			(a)	(b)	(c)			
Cod liver oil compound (whole oil)	<i>cyclo</i> Hexane	0.69	0.537	0.526	0.491	—	—	0.560
	(unsaponifiable) <i>cyclo</i> Hexane	0.58	0.471	0.458	0.495	—	—	0.471
High-potency oil A (whole oil)	<i>cyclo</i> Hexane	132	116.4	113.3	112.7	116.2	—	116.4
	(unsaponifiable) <i>cyclo</i> Hexane	123.6	95.5	97.0	103.2	—	—	—
High-potency oil B (whole oil)	<i>cyclo</i> Hexane	166.5	142	141	141.5	150	—	150
	Light petroleum	177	147.7	—	150.5	—	—	—
	(169)†	(141)†	(144)†	—	—	—	—	—
	Ethanol	169	149	148.5	149.2	—	—	—
	(166)†	(146.5)†	(146.0)†	(146.7)†	—	—	—	—
	(whole oil)	168.2	146.4	—	142	—	—	—
	(166)†	(144.5)†	(140)†	—	—	—	—	—
(unsaponifiable)	<i>cyclo</i> Hexane	160	127	121.5	135.5	141	—	129

* Correction procedures (a), (b) and (c) explained in text (p. 51). For 16 points correction see Appendix. M & S stands for correction procedure of Morton & Stubbs (1946).

† Equivalent $E_{1\text{ cm.}}^{1\%}$ in *cyclo*hexane.

Table 9. *Spectroscopic data on fish-liver oils using the colour test showing proportions of neovitamin A and of vitamin A₂*

Material	$E_{1\text{ cm.}}^{1\%}$ (gross)			$E_{1\text{ cm.}}^{1\%}$ with maleic anhydride	Per-centage of neo-vitamin A	Calc. $E_{1\text{ cm.}}^{1\%}$	
	At 580 m μ .	At 620 m μ .	At 693 m μ .			At* 620 m μ . due to vitamin A ₁	At† 693 m μ . due to vitamin A ₂
Vitamin A acetate (all-trans)	2200	4400	21	67	—	—	—
Cod liver oil compound	0.671	1.31	0.155	0.323	24‡	1.26 (0.432)	0.137 (0.049)
High-potency oil A	—	357	23	84	25§	349.5 (120)	18.1 (6.47)
High-potency oil B	263	460	31.7	113	24§	449.5 (154)	25.5 (9.12)

* Calculated from $E_{620\text{ m}\mu} - 0.328 E_{693\text{ m}\mu}$: see text p. 50.

† Calculated from $E_{693\text{ m}\mu} - 0.0136 E_{620\text{ m}\mu}$: see text p. 50.

‡ Done on unsaponifiable fraction using the factors for the unesterified vitamin (see text p. 49).

§ Done on whole oil using the factors for the esters (see text p. 49).

|| Equivalent $E_{1\text{ cm.}}^{1\%}$ in *cyclo*hexane accepting the following data: vitamin A₁: $\epsilon_{\text{max.}}$ (620 m μ .)=145,000, $\epsilon_{\text{max.}}$ (328 m μ .)=49,700.

Vitamin A₂ (using the data given by Shantz, 1948): $\epsilon_{\text{max.}}$ (693 m μ .)=116,000 and $\epsilon_{\text{max.}}$ (351 m μ .)=41,500 (in ethanol), ϵ (328 m μ .)=32,750.

It is also assumed that $\epsilon_{\text{max.}}$ in *cyclo*hexane is the same as that in ethanol for vitamin A₂.

Table 10. *Spectroscopic properties of fractions obtained by chromatography of high-potency oil B on 8% weakened alumina*

Eluant	Fraction	$\lambda_{\text{max.}}$ (m μ .)	$E_{1\text{ cm.}}^{1\%}$ (325–328 m μ .) gross
Light petroleum	1	345, 365, 385	0.12 Anhydrovitamin A
Light petroleum	2	327.5	164* Vitamin A esters
Light petroleum	3	290	2.3 Kitol or epoxide?
5% Ether-light petroleum	4	None	1.07 —
10% Ether-light petroleum	5	319	4.3 Free vitamin A
Ether	6	None	1.43 —

* Corrected 148.5 equivalent to 144.4 in *cyclo*hexane.

Table 11. Spectroscopic data on standard vitamin A acetate preparation

(Solvent: cycloHexane, 1 cm. cell.)											
% (w/v)	Compensator	Extinction expressed as	At	At	At	At	At	At	At	At	At
			312.5 mμ.	313 mμ.	316 mμ.	327.5 mμ.	328 mμ.	337.7 mμ.	338.5 mμ.	340 mμ.	
0.1*	cycloHexane	E (obs.)	0.454†	0.460	0.477	0.522	0.520	0.446†	0.438	0.423	
0.1027	cycloHexane	E ₁ ¹ _{cm.}	4.66†	4.68	4.87	5.29	5.29	4.52†	4.43	4.27	
0.1027	0.1027% (w/v) Cotton-seed oil solution	E ₁ ¹ _{cm.}	4.38†	4.42	4.61	5.09	5.08	4.34†	4.24	4.09	
0.1*	cycloHexane	E/E _{max.}	0.870†	0.881	0.914	1.0	0.997	0.855†	0.839	0.811	
0.1027	0.1027% (w/v) Cotton-seed oil solution	E/E _{max.}	0.860†	0.868	0.905	1.0	0.998	0.852†	0.833	0.803	
Expected value for pure vitamin A acetate (see Table 5)			E/E _{max.}	0.867	0.907	1.0	1.0	0.858†	0.844	0.811	
% (w/v)	Compensator	Extinction expressed as	At	At	At	At	At	At	At	At	At
			250 mμ.	260 mμ.	270 mμ.	280 mμ.	290 mμ.	300 mμ.	310 mμ.	320 mμ.	330 mμ.
0.25*	Solution of cotton-seed oil with cyclohexane	E (obs.)	1.51	0.92	1.03	0.805	0.274	0.124	0.048	0.037	0.013
			0.020	0.012							

* Nominal concentration: contents of one capsule assumed to be 0.25 g. † Note that these differ. ‡ Note good agreement.

solution, the potency of which should be 10,000 i.u./g. Similar capsules containing the diluent oil only are also issued. The preparation has been treated as if it were a solution of unknown potency. The results are shown in Tables 11 and 12.

Table 12. E₁¹_{cm.} values for the International Standard Preparation in cyclohexane

	E ₁ ¹ _{cm.}	i.u./g. = (E ₁ ¹ _{cm.} × 1920)
Expected value	5.21	10,000
Found (compensator cyclohexane) (6 determinations)	5.18 ± 0.078 (P = 0.95)	9,950 ± 150
Found (compensator cotton-seed oil in cyclohexane) (5 determinations)	5.03 ± 0.134 (P = 0.95)	9,650 ± 260

Fig. 2. Absorption curves of fractions separated from high-potency oil B by chromatography on (8%) weakened alumina. Fraction 1, x-x-x, intensities are actual × 10²; fraction 2, —, main ester fraction; fraction 3, ●-●, 'kitol' (?) intensities are actual × 12.5; fraction 4, —, intensities are actual × 10; fraction 5,, free vitamin A intensities are actual × 8; fraction 6, - - - -, intensities are actual × 10.

DISCUSSION OF RESULTS

Performance of spectrophotometers

Well verified values of extinctions of simple and accessible solutes are extremely scarce, but potassium chromate and dichromate have been much studied and have the advantage of two well separated maxima and minima. If in either curve two out of the four wavelengths are 'correctly' recorded it is probable that the instrument is in good adjustment on the wavelength scale. Solute with rather broad flat maxima are not, however, ideal for revealing very small errors on the wavelength scale, and a substance with narrow absorption maxima would be

better. In the choice of a calibrating substance, however, it is in general more important to be able to determine intensities of absorption accurately, and in this respect a flatter peak tends to be less influenced by the 'age' of the light source and the consequent variability of slit widths as the output decreases. The two solutes we have used constitute together a severe test of the performance of the instrument.

The results in Tables 1 and 2 show good agreement on the wavelength scale and our own findings on the Unicam instrument appear to be as trustworthy as any. In considering our data on vitamin A, the performance of the spectrophotometer is to be judged by Tables 1 (*f*) and 2 (*b*).

Absorption spectra

Identity of 'natural' and 'synthetic' vitamin A acetate. The absorption curves (in four solvents) for recrystallized vitamin A acetate (all-*trans*) prepared either synthetically or from liver oil concentrates, are indistinguishable. Measurements communicated to us by Dr Isler and Dr Matet show, moreover, that our curves are quantitatively reproducible within very narrow limits of error over the whole spectral range (cf. Chatain & Debodard, 1950).

The behaviour of the two types of product in the antimony trichloride colour test, with and without pretreatment with maleic anhydride, was also identical, showing that both the 'natural' and 'synthetic' acetates were all-*trans* exclusively.

Effect of solvents. The range of $\lambda_{\max}$ (325–328 m μ .) is quite narrow, but the range of molecular extinction coefficients is wider, the highest value being shown for light petroleum, the least polar solvent. We do not recommend its use because it is so volatile that the solution of vitamin A may easily become slightly more concentrated in the course of actual measurements. In cyclohexane $\epsilon_{\max}$ is reproducibly and significantly lower than in isopropanol or ethanol (Table 3) (cf. Chilcote, Guerrant & Ellenberger, 1949). It is interesting that the oscillator strength (Table 3), which is a measure of the complete absorption curve from 250–400 m μ ., is, within the limits of error, the same for the acetate and the alcohol.

Vitamin A alcohol. Synthetic all-*trans*-vitamin A alcohol shows $\lambda_{\max}$ 324–327 m μ ., and in non-polar solvents $\epsilon_{\max}$ agrees extremely well with the corresponding figure for the acetate. In isopropanol and ethanol the free alcohol absorbs appreciably more than the acetate (Table 3). This is reproducible and in consequence cyclohexane is a better solvent for use with mixtures of free and esterified vitamin A.

Correction procedures. The samples of vitamin A and vitamin A acetate are undoubtedly a little purer than those formerly available, and the absorption curves are somewhat narrower than those

recorded by Morton & Stubbs (1948). The differences though very small, affect corrections for irrelevant absorption and it was necessary to redetermine the fixation points (Table 6). A new correction procedure making use of sixteen wavelengths (see Appendix) greatly reduces the error inherent in the use of three points only, at the cost of much labour. In principle all the correction equations apply to solutions of all-*trans*-vitamin A with superimposed linear irrelevant absorption. The difficulties will be discussed later.

Neovitamin A and the colour test

Once it becomes clear that the absorption spectrum of vitamin A in any given solvent is not entirely fixed and characteristic but will vary (even though only slightly) with the relative proportions of all-*trans*- and neo-vitamin A, the whole analytical problem is moved to a new level of complexity. The antimony trichloride colour test provides through maleic anhydride a means of determining the all-*trans*-/neo-vitamin A ratio. The literature shows very wide variations in the intensity of absorption in the direct colour test, depending partly on the instrument used and partly on the transient nature of the colour. We have therefore taken special care over the measurements recorded in Table 7.

It will be necessary to determine the spectroscopic properties of pure crystalline neovitamin A acetate as carefully as those of the all-*trans* modification, so as to arrive at correction formulae (with appropriate fixation points) applicable to known mixtures of all-*trans* and neovitamin A esters in natural oils. The work of Dalvi & Morton (1951) is a step in that direction.

Vitamin A₂

Vitamin A₂ is present to a greater or lesser extent in most fish-liver oils. This results in some distortion of the spectral absorption curves. The first step in studying vitamin A₂ is to determine $E_{1\text{cm}}^{1\%}$ (693 m μ .) in the colour test so that suitable corrections can be made (see p. 50).

Tests on natural products

Test on cod liver oil compound. The gross $E_{1\text{cm}}^{1\%}$ (328 m μ .) of 0.69 is too high for the vitamin A present. No anhydrovitamin A is detectable in the absorption, but the colour test shows the presence of vitamin A₂. Tetraene acids which may contribute to the irrelevant absorption are eliminated by saponification, so that the observed $E_{1\text{cm}}^{1\%}$ (326 m μ .) of 0.580 on the unsaponifiable fraction is nearer to the true value for the vitamin A₁ contribution. It still needs correction for the vitamin A₂ contribution at 326 m μ . The quantitative colour test shows that $E_{1\text{cm}}^{1\%}$ (693 m μ .) (corrected for the overlapping of A₁

and A_2 absorption) is 0.137 which in the direct ultraviolet absorption corresponds with 0.038 at 326 $m\mu$., so that the vitamin A_1 contribution (on the unsaponifiable fraction) is $0.580 - 0.038 = 0.542$. The remaining possible contributors to irrelevant absorption are oxidation products or kitol.

Using the three-point correction procedure on the oil, the average corrected figure is $E_{1\text{cm.}}^{1\%}$ (328 $m\mu$.) 0.52, and this should allow for all irrelevant absorption provided it is linear over the fixation range. The calculation assumes that the vitamin A_1 is entirely all-*trans*, but experiment shows that 24 % of it occurs in the neo form. In *cyclohexane* the presence of this proportion of neovitamin A will slightly displace the absorption curve, and it is necessary to multiply 0.520 (see below) by a factor of 1.04 to give 0.542. The fully corrected $E_{1\text{cm.}}^{1\%}$ (326 $m\mu$.) value is thus < 0.542 but > 0.520 .

In the unsaponifiable fraction it is clear that the irrelevant absorption is not strictly linear and application of the three-point correction procedure leads to erroneously low values.

High-potency oil A. The decrease from $E_{1\text{cm.}}^{1\%}$ 132 to 123.6 represents the 'saponifiable' contribution to irrelevant absorption. The colour test shows the presence of vitamins A_1 and A_2 (Table 9) and the A_1 effect ($E_{1\text{cm.}}^{1\%}$ (620 $m\mu$.) = 349.5) corresponds with $E_{1\text{cm.}}^{1\%}$ 120 at 328 $m\mu$. in the ultraviolet test. The A_2 effect in the colour test ($E_{1\text{cm.}}^{1\%}$ (693 $m\mu$.) = 18.1) corresponds with $E_{1\text{cm.}}^{1\%} = 5.1$ at 328 $m\mu$. Hence the difference $123.6 - 5.1 = 118.5$ represents the A_1 contribution at 326 $m\mu$. on the unsaponifiable fraction and agrees well with the value 120 deduced from the colour test. Application of the three-point correction procedure to the oil in *cyclohexane* leads to a mean value of 114.6. The A_2 contribution will have been eliminated, but the effect of the presence of 25 % of the total vitamin in the neo form will make this figure too low. Experience has shown (Dalvi & Morton, 1951) that the result needs to be multiplied by a factor 1.04, giving 119.

Since the three-point correction procedure applied to the unsaponifiable fraction gives results which are manifestly too low, the combination of variables has made untenable the linearity assumption for irrelevant absorption.

High-potency oil B. The decrease from $E_{1\text{cm.}}^{1\%} = 167$ to 160 is due to the elimination of saponifiable materials. The colour test shows that the A_1 contribution of $E_{1\text{cm.}}^{1\%}$ (620 $m\mu$.) = 449.5 (Table 9) corresponds with $E_{1\text{cm.}}^{1\%} = 154$ for the A_1 contribution at 328 $m\mu$. The colour test for A_2

$$(E_{1\text{cm.}}^{1\%} \text{ (693 } m\mu) = 25.5)$$

corresponds with A_2 absorption at 328 $m\mu$. of the value $E_{1\text{cm.}}^{1\%} = 7.2$ (see Fig. 1). Hence the unsaponifiable figure $E_{1\text{cm.}}^{1\%} = 160$ must be reduced to 152.8, when the A_2 contribution is eliminated. The colour

test and the ultraviolet absorption are not, therefore, discordant.

Application of the correction procedures to the whole oil in various solvents leads to a figure of 143.4 (reduced to *cyclohexane*). This eliminates all irrelevant absorption, but the presence of neovitamin A (24 %) makes it necessary to multiply by 1.04, leading to 149. Chromatography on the whole oil leads to 144.4 (*cyclohexane*) after correction eliminating vitamin A_2 , and this figure will need to be corrected for neovitamin A, leading to 150. The chromatographic separation disclosed the presence of a small free vitamin A fraction which must be added to 150. The chromatographic separation leads to a vitamin A_1 contribution of slightly over 150. The unsaponifiable figure of 152.8 has clearly not allowed for the presence of kitol and must be reduced to not more than 150.

It seems clear that each approach leads to a figure of about 149–150.

Summarizing, we have:

Method	Calculated $E_{1\text{cm.}}^{1\%}$ (328 $m\mu$.)	Remarks
Colour test	154	Too high, no correction made for oxidation products, kitol, etc.
Chromatograph	150	May be too high if separation incomplete, but needs addition for free vitamin A
Unsaponifiable fraction corrected for A_2	152.8	Not corrected for oxidation products or kitol
Geometric corrections on whole oil corrected for neovitamin A	149	—

Finally, application of geometrical correction procedures to the unsaponifiable fraction leads to erroneously low results and the basis of the correction must be invalid because of neovitamin A and the appearance of irrelevant absorption mainly on the short-wave side of the peak.

International standard preparation of vitamin A acetate

Examination of the standard preparation should be a relatively simple matter because the complications due to vitamin A_2 , neovitamin A and oxidation products do not arise. If the properties of pure vitamin A acetate given in Tables 3 and 4 are correct and the standard preparation contains pure all-*trans*-acetate and is stable, then it should be possible to find 10,000 i.u./g.

When the solvent is *cyclohexane* the expected intensity of absorption will be 10,000/1920 (Table 4) = $E_{1\text{cm.}}^{1\%}$ 5.21. The observed value using *cyclohexane*

only in the compensating cell is not significantly different from the expected value. The sounder method of compensation with the diluent oil gives a result which is significantly lower (Table 12). The assumption that each capsule contains exactly 0.250 g. of material may not necessarily be correct, but even when the oily solution was carefully weighed out, $E_{1\text{cm}}^{1\%}$ (328 m μ .) was low (5.09) with compensation using the same concentration of diluent oil. It is clear that the vitamin A acetate has not been appreciably oxidized because the fixation points are correctly recorded.

An interesting point emerges if the standard preparation is first saponified and the unsaponifiable fraction dissolved in isopropanol. It will be noted that in isopropanol ϵ_{max} for vitamin A acetate is near 50,300 and that of vitamin A alcohol is 52,400 (Table 3). The expected $E_{1\text{cm}}^{1\%}$ value for the international standard preparation would be 5.28 and that of an equivalent solution of vitamin A alcohol 5.49. To neglect the rise in E value would imply accepting as containing 10,000 i.u./g. a solution which actually contained 9620 i.u./g. This method of testing would fit the recommendation of the U.S. Pharmacopoeia (1950) and errors might pass undetected.

It seems very probable that the samples of vitamin A acetate used in the present work are a little purer than earlier ones including that used for the standard preparation.

CONCLUSIONS

The method of determining vitamin A in fish-liver oils and other products from the intensity of ultraviolet absorption is well tried and serviceable. The major obstacle in the way of accuracy is irrelevant absorption, but recent work shows that the co-existence of all-*trans*- and neo-vitamin A also gives rise to difficulty.

The work of Morton & Stubbs (1946, 1948) showed how, in favourable circumstances, the total irrelevant absorption could be allowed for. Some of the substances contributing to the irrelevant absorption, e.g. kitol and vitamin A epoxide can be removed by chromatography. In many cases (e.g. cod liver oils) preparation of unsaponifiable extracts removes much of the irrelevant absorption, but with high-potency materials the effect is much less noticeable and subsequent chromatography fails to remove vitamin A₂ and some other 'impurities'. The more elaborate the chemical manipulation the greater will be the risk of loss.

The spectra of all-*trans*- and neo-vitamin A are sufficiently different to cause appreciable distortion of the curve of either when mixtures are studied. The maleic anhydride procedure applied to the colour test permits determination of the relative proportion of all all-*trans*- and neo isomers, and

whenever the Morton-Stubbs or similar procedures suggest the need for a large correction for irrelevant absorption (particularly in high-potency oils) the proportion of neovitamin A should be determined.

It also seems necessary to look for vitamin A₂ as a routine. This means examining the solution by the antimony trichloride colour test for a band or an inflexion near 693 m μ . Indeed much is gained by determining $E_{1\text{cm}}^{1\%}$ at both 620 and 693 m μ . quantitatively and correcting for overlapping absorption (see p. 50). The ultraviolet absorption at 328 m μ ., due to vitamin A₂, must then be calculated and subtracted from the gross $E_{1\text{cm}}^{1\%}$ (326–328 m μ .) on the oil or the unsaponifiable fraction.

Application of the correction equations (Table 6) will be valid when the vitamin is entirely all-*trans*, and as soon as data for crystalline neovitamin A acetate comparable with those in Table 5 for the all-*trans* form are available, new equations to fit any ascertained mixture of the isomers can be deduced. An alternative is to correct for irrelevant absorption using the equations based on the all-*trans* form and afterwards to adjust for the presence of known proportions of neovitamin A as has been done in the present paper.

It is also theoretically possible, using four points, to construct an equation that would enable the amounts of all-*trans*- and of neo-vitamin A to be determined, provided all the remaining absorption in the region 310–340 m μ . is linear. The errors would, however, be large and if sixteen wavelengths were used, and a solvent in which the absorption of all-*trans*- and neo-vitamin A differed most (isopropanol?), then the errors should be no greater than those arising in the other methods available. The equation would be a simple extension of that given in the Appendix, but would involve a knowledge of the absorption spectrum of pure neovitamin A acetate. In a trial the use of four points led to 29% neovitamin A when the maleic anhydride procedure indicated 24%.

It is evident that the correction equations (Table 6) can be applied much more successfully to whole oils (in cyclohexane) than to unsaponifiable fractions. cycloHexane is a more suitable solvent than isopropanol because the difference between the spectra of the two isomers is least in the hydrocarbon solutions.

The three oils which have been studied in detail can be corrected for irrelevant absorption by different combinations of methods, all of which approach the same value in each case. The special difficulty with the unsaponifiable fractions arises from the fact that the main irrelevant absorption is not linear in the region 310–340 m μ .; it seems in fact to be considerable at 310 m μ . but not from 326 to 340 m μ . We have reason to suspect isomerization during saponification.

The outcome of this investigation is: (a) For low-potency oils of good quality the accepted use of un-

saponifiable extracts will give a 'normal' absorption curve the $E_{1\text{cm}}^{1\%}$ value of which needs to be corrected only for vitamin A₂; the 'corrected' figure on the oil will agree closely if adjusted for the presence of neovitamin A. (b) For higher potency oils, application of two or more of the correction formulae will give a result which, when adjusted for the presence of neovitamin A, will be practically the same as that resulting from the most laborious elimination one by one of interfering substances.

The analyst must at present treat vitamin A₂ as a contaminant, but a time may come when it will be legitimate to include its activity, apparently 40 % of that of an equal weight of vitamin A₁ (Shantz & Brinkman, 1950). That would depend on the unknown capacity of man to utilize vitamin A₂ and presumably on official recognition.

SUMMARY

1. The photoelectric spectrophotometer was calibrated with potassium chromate and dichromate solutions. The results provide a basis for testing the performance of other instruments.

2. Natural and synthetic vitamin A are indistinguishable. The values of $E_{1\text{cm}}^{1\%}$ and λ_{max} for vitamin A acetate and for vitamin A alcohol were obtained in cyclohexane, light petroleum, ethanol and isopropanol. The values of ϵ_{max} in cyclohexane and light petroleum were the same for the free acetate and for the alcohol. The values of ϵ_{max} in ethanol and isopropanol for the alcohol were, however, higher than for the ester.

3. All the absorption curves showed a second maximum at about 250–253 m μ ., ϵ_{max} approx. 550.

4. The value of the factor for converting $E_{1\text{cm}}^{1\%}$ to i.u./g. is not strictly constant, but varies with the solvent and the state of combination of the vitamin A.

5. The complete absorption curve for the reaction product of vitamin A and antimony trichloride has been obtained. The value of ϵ_{max} is $144,900 \pm 870$ at 620 m μ .

6. A cod liver oil preparation, two high-potency oils and the International Standard Preparation of vitamin A acetate have been studied in detail. The latter is perhaps not quite up to specification.

7. Although for a wide range of products the $E_{1\text{cm}}^{1\%}$ value at 325–328 m μ . on the oil or the 'unsaponifiable fraction' is a good guide to potency, it is necessary in aiming at an accurate assay to take full account of the presence of anhydrovitamins A, vitamin A₂, kitol, epoxides and other substances. Except with low-potency oils it is almost always safer to work on the whole oil because the correction procedure when applied to unsaponifiable materials tends to give low results. It is also necessary to determine the neo/all-trans ratio. This means that in addition to the main test based on ultraviolet absorption, a number of subsidiary tests are essential. The procedure is discussed fully and the results are compared with 'corrected' values using the equations described.

We are indebted to the Medical Research Council for a grant to the Department. One of us (F.D.C.) is the holder of an I.C.I. Fellowship. The gift of numerous samples of highly purified material from MM. Alimentation Equilibree, France, and Hoffmann La Roche, Switzerland, is gratefully acknowledged.

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