REVIEW ARTICLE

THE DETERMINATION OF VITAMIN A

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At certain stages in vitamin studies biological assays on animals are indispensable but as knowledge increases the analyst strives to become independent of them. This is not merely because good analytical procedures are often quicker, cheaper and more trustworthy than biological assays (except perhaps for some microbiological methods) but also because the two approaches are complementary. The potency of a vitamin preparation when assessed by animal tests is the resultant of the vitamin content and a complex pattern of absorption and utilisation. If, let it be supposed, a fish liver-oil has a precisely known vitamin A content and gives in experimental rats a biological response indistinguishable from that elicited by a standard preparation providing the same amount of vitamin per rat per day, the pattern of absorption and utilisation must be the same for the liver-oil and the standard preparation. If, however, the biological responses are significantly different, important information will have been gained which could not have been obtained by either alone. This principle is very important in the vitamin A field for the following reasons:—

(i) Numerous carotenoid precursors of vitamin A exist, equal quantities of which (administered as pure substances) give different "yields" of vitamin, e.g., α-, β- and γ-carotene and their stereo-isomers.

(ii) The absorption of a given precursor varies with the form in which it is ingested, e.g., β-carotene in vegetable oil is more efficiently utilised than the β-carotene present in cooked carrots[1].

(iii) The absorption and utilisation of vitamin A or its precursors may be influenced by the nature of the vehicle, e.g., by the anti-oxidants or the vitamin E present in the diluent oil.

(iv) The biological test is not strictly specific for vitamin A_1 ($C_{20}H_{29}OH$), e.g., vitamin A_2 ($C_{20}H_{27}OH$), vitamin A_1 aldehyde $C_{19}H_{27}CHO$, vitamin A_2 aldehyde $C_{18}H_{25}CHO$, vitamin A_1 acid $C_{19}H_{27}COOH$ and vitamin A_1 methyl ether $C_{19}H_{27}CH_2OCH_3$ are all known to be highly potent.

(v) The problems of stereo-isomerism in vitamins A have not been fully worked out (see p. 142).

From the foregoing it may be seen that animal experimentation in this field is by no means obsolete.

The purpose of the present article is to deal with the narrower problem of determining the vitamin A content of oils, pharmaceutical preparations and tissues. Although the problem is admittedly rather complicated, all the major difficulties have been overcome.

Historical Background

Vitamin A does not occur in vegetable products, but herbivorous animals possess considerable liver stores of vitamin A esters. Carotenoid

precursors (provitamins) such as the β-carotene present in green foodstuffs are converted to vitamin A in the gut wall

$$C_{40}H_{56} \rightarrow C_{20}H_{29}OH + ?$$

and transported (in esterified form) to the liver to be stored partly in the Kupffer cells and partly in the true liver cells[2]. The latter contain an esterase (apparently lacking the former) and help to maintain a fairly constant plasma level of the functional vitamin A alcohol.

The carotenoid provitamins show selective absorption in the region near 450 mμ and vitamin A_1 shows a well marked ultraviolet absorption maximum near 328mμ (vitamin A_2, 350 mμ). With the antimony trichloride reagent (a saturated solution of anhydrous antimony trichloride in pure chloroform) blue solutions are formed with maxima at the following wave-lengths:—β-carotene, 590mμ; vitamin A_1, 617mμ; vitamin A_2, 693mμ., the extinction coefficients for provitamins A in general being very much lower than those for the vitamins A.

In principle, quantitative determinations of intensities of absorption can be used to ascertain provitamin or vitamin A content either by the ultra-violet maximum or the indirect colour test. The notation is as follows:—

If I_0 is the intensity of light incident on an absorption cell and I is the intensity of the light emerging after passage through a layer d cm. long of a solution of molar concentration c (pure solute) then

$\log I_0/I = E = \epsilon\ cd$ where ϵ is the molecular extinction coefficient. For vitamin A, $\epsilon_{max.}$ (at 328mμ) is 50,000, and for β-carotene $\epsilon_{max.}$ (at 455mμ) is about 130,800 (in *cyclo*hexane). An alternative way of expressing intensity of absorption is to give the $E\,{}^{1\ \text{per cent.}}_{1\ \text{cm.}}$ value. For vitamin A, $E\,{}^{1\ \text{per cent.}}_{1\ \text{cm}}$ 328mμ = 1750. (In practice this means, for example, that with a 1 cm. cell and a 0·0004 per cent. solution or with a 2 mm. cell and a 0·002 per cent. solution $E = 0{\cdot}7$.) For a fish-liver oil (or other preparation to be tested) $E\,{}^{1\ \text{per cent.}}_{1\ \text{cm.}}$ is expressed in terms of the weight of material used. Thus, a fish-liver oil showing $E\,{}^{1\ \text{per cent.}}_{1\ \text{cm.}}$ 328mμ, 17·5 would contain 1 per cent. of vitamin A (subject to any correction).

The qualification *in principle* in the previous paragraph is necessary because the measured intensity of absorption at 328mμ is the sum of that due to vitamin A itself and a contribution due to other substances; in fish-liver oils the "irrelevant" absorption varies from sample to sample independently of the variation in vitamin A content. The gross $E\,{}^{1\ \text{per cent.}}_{1\ \text{cm.}}$ value affords a first approximation to vitamin content but a correct value (in which due allowance has been made for irrelevant absorption) is now regarded as essential. The antimony trichloride test depends upon determinations of intensity of absorption by rather transiently blue solutions; and measurements must be made quickly. Some materials, however, contain colour test inhibitors which make estimates of vitamin A content too low. Since 1931[3] the aim has been to express vitamin A content and vitamin A potency in International Units. Carotene was already known as a crystalline substance but vitamin A had only been obtained as an "oil," and the first definition of an International Unit was the potency of 1μg. of a specimen of "carotene." By 1934 it had become clear that α-carotene and β-carotene differed quan-

titatively in their power to prevent or cure avitaminosis A. The first specimen of "carotene" was a mixture and the International Unit was redefined as 0·6μg. of pure β-carotene[4].

A range of fish-liver oils had been examined spectroscopically and the gross $E_{1\text{ cm.}}^{1\text{ per cent.}}$ value at 328mμ determined. Each oil had been assayed biologically against β-carotene. It was found empirically that the $E_{1\text{ cm.}}^{1\text{ per cent}}$ values multiplied by 1600 gave estimates of potency agreeing with the biological values within the limits of error of the animal experiments. Subsequent work has shown that no better conversion factor (for fish-liver oils in general) could even to-day be chosen for expressing gross $E_{1\text{ cm.}}^{1\text{ per cent}}$ values in terms of International Units, but oils showing little irrelevant absorption would be somewhat under-valued and oils exhibiting more irrelevant absorption would be over-valued. It was widely agreed by 1934 that relatively low-potency oils such as cod-liver oils exhibited considerable irrelevant absorption, most of which could be eliminated with the saponifiable fraction[4]. It has since been usual for such oils, to determine the intensity of absorption at 325 to 328mμ on the unsaponifiable fraction, but to express the result in terms of the weight of the original oil. Use of the unsaponifiable fraction also reduces the error caused by inhibitors of the antimony trichloride colour.

For a wide range of more potent fish-liver oils substitution of the unsaponifiable fraction for the whole oil resulted in little reduction in $E_{1\text{ cm}}^{1\text{ per cent.}}$ values at 328mμ. and no enhancement of the colour test intensity. It is however now established that most liver oils contain materials which interfere with both tests and that many "impurities" (including vitamin A decomposition products) pass into the unsaponifiable fraction with the vitamin A.

The 1934 Conference recommended for adoption an International Unit for Vitamin A defined as the Vitamin A activity of 0·6 microgramme (0·6μg.) of pure β-carotene.

Further recommendations (1934) are worth quoting in full:—

"The Conference recommends that a sample of cod-liver oil, the potency of which has been accurately determined in terms of the International Standard Preparation of β-carotene shall be provided as a Subsidiary Standard of Reference.

"In view of the fact that the Reference Cod-liver Oil of the United States Pharmacopœia, which has been accurately assayed in terms of the provisional International Standard adopted in 1931, has been in effective use in the United States of America for some time, the Conference recommends that the Board of Trustees of the United States Pharmacopœia be approached and invited to place a quantity of their Reference Cod-Liver Oil at the disposal of the Health Organisation of the League of Nations with a view to its adoption for international use as a Subsidiary Standard for Vitamin A.

"In the event of the Reference Cod-liver Oil (of the U.S.P.) not being available for international adoption the Conference recommends that another sample of cod-liver oil be selected, its potency in terms of the International Standard Preparation of β-carotene accurately determined by biological comparison and independently by spectrophoto-

metric measurements, and that this selected sample be then adopted as a Subsidiary International Standard for Vitamin A.

"It has been found that, within certain defined conditions, measurements of the coefficient of absorption (E) at 328mμ., affords a reliable method for measuring the vitamin A content of liver oils and concentrates. As a means of converting values obtained for $E^{1\%}_{1\,cm.}$ 328mμ into a figure representing International Units of Vitamin A per gramme of the material examined the factor 1600 is recommended for adoption."[4]

In the U.S.A. the U.S.P. Reference Cod-liver Oil was much more freely employed than the β-carotene preparation and a conversion factor of 2000, deemed more appropriate than 1600, came into general use. (The U.S.P. authorities were, of course, under no obligation to conform to recommendations made by the Conference.)

The U.S.P. Reference Cod-liver Oil was not very stable. Two other U.S.P. Reference oils have since been issued and a considerable volume of work has appeared on their properties and stability. The upshot has been general agreement that $E^{1\%}_{1\,cm.}$ 328mμ values and the relative biological potencies of oils run very parallel. The conversion factor of 2000 applied to uncorrected E values received in the U.S.A. general and somewhat uncritical acceptance.

The β-carotene standard preparation could not be impugned on the score of instability, but it was impossible to remain permanently satisfied with a standard of vitamin A activity based on a substance other than the vitamin itself.

Experience with the U.S.P. Reference Oil tended to weaken initiative in providing a new Cod-liver Oil Subsidiary Standard for international use, and the outbreak of war temporarily put an end to the project.

For many purposes, however, it became tacitly accepted that the $E^{1\%}_{1\,cm.}$ value was the best criterion of vitamin A content, and that in translating such values into "units" 1 U.S.P. unit meant the same thing as 0·8 I.U.

The advent of pure crystalline vitamin A and vitamin A acetate changed the situation with regard to possible new standards, and developments in the production of photoelectric spectrophotometers increased the potential accuracy of the spectroscopic method and also of the antimony trichloride colour test.

The Present Position

The 1949 Conference (Expert Committee on Biological Standardisation, World Health Organisation) held in London made recommendations[5] (adopted in August, 1949) which constitute a real advance, in that they maintain essential continuity with the earlier international unit but provide for practical, and it is believed stable, International Standard Preparations acceptable everywhere. In the first place, the β-carotene Standard Preparation is retained, not as the Reference Standard for Vitamin A activity, but as the reference material for work on vitamin A precursors or, more specifically, carotenoid provitamins A.

By definition, then, 1 I.U. of provitamin A activity is 0·6μg. of

β-carotene, and pure β-carotene necessarily has a potency of $1{\cdot}66\dot{6} \times 10^6$ I.U./g.

In the second place, a new International Standard Preparation of Vitamin A acetate is now made available[6]. " The preparation is a solution in a suitable vegetable oil of such a strength that 0·1mg. of the solution contains 0·344μg. of vitamin A acetate. An oil suitable as solvent is one containing not less than 0·1 per cent. of tocopherol and not more than 32 parts per million of total peroxide oxygen."[7,8]

The International Unit of vitamin activity is defined as 0·344μg. of pure vitamin A acetate, corresponding stoichiometrically to 0·30μg. of vitamin A_1 alcohol. Necessarily, then, pure vitamin A_1 contains $3{\cdot}3\dot{3} \times 10^6$ I.U. per gramme. Spectroscopists are agreed (within very narrow limits of error) that the $E^{1\%}_{1\,cm.}$ value (at the ultraviolet maximum) for vitamin A_1 is 1750 and that of the acetate 1525 ($\varepsilon_{max.}$ 50,000). The " theoretical " or " true " conversion factor must, then, be near 1900 (i.e., $3{\cdot}3\dot{3} \times 10^6/1750$). The *E* values vary a little with the solvent.

In 1948 a new U.S.P. Vitamin A Reference Standard became official in the U.S.A.[9] It consisted of a solution of pure vitamin A_1 acetate in cottonseed oil (3·44mg. per gramme) and was labelled 10,000 U.S.P. units per gramme. Since the $E^{1\%}_{1\,cm.}$ value at 328mμ. for the preparation was stated, a conversion factor was implicit but " unofficial and given simply as information." The only official assay method (U.S.P. XIII p. 721) was biological, and it was the *Reference Standard Preparation* which became official on January 1, 1948, in the U.S.A. Of course, the Standard necessarily implied a " true " conversion factor near 1900, but as the U.S.P. had not recognised quantitative spectrophotometric assays it could ignore (officially) the difficulties attendant upon a change from 2,000 to 1,900 in the U.S.A. or a possible change from 1,600 to 1,900 elsewhere. This gave rise to some confusion[9]. The new U.S.P. Reference Standard was chosen and defined after very careful biological comparison of β-carotene and vitamin A_1 acetate, and the fact that the " unit " (0·3μg.) and the β-carotene " unit " (0·6μg.) are related as 1 : 2 is the result of experiment. After statistical examination of the protocols, the potency of the Standard Preparation (tested against β-carotene) is 9750 I.U./g. (between 9000 and 10,500 for P = 0·95). If $E^{1\%}_{1\,cm.}$ 328mμ is 5·23 the conversion factor will be 9,750/5·23 = 1,864 (limits 1,721 and 2,007).

Earlier co-operative tests carried out in England under the auspices of the vitamin A Sub-Committee of the Medical Research Council had led to the following results: —

Biological tests	*Conversion Factor*
Cod-liver oil against β-carotene (*E* values on unsaponifiable fraction)	1,820
Vitamin A naphthoate against β-carotene	1,770
Halibut-liver oil against β-carotene:	
Uncorrected *E* values	1,570
Corrected *E* values	1,824

The British experiments taken together thus led to a conversion factor of 1,805 (limits not narrower than 1,700—1,900) and the American experiments to 1,864 (limits 1,721—2,007). It was almost inevitable that the 1949 London Conference should decide that the responses to 0·6μg. of β-carotene and 0·3μg. of vitamin A were indistinguishable by the accepted methods of biological assay carried out on any practicable scale.

The new U.S.P. Standard had, moreover, acquired momentum and great care had been devoted to issuing it in a convenient and stable form. There was, therefore, a strong case for recommending an International Reference Standard with the same specification.

The 1949 Conference recognised, however, that for much research and testing the spectrophotometric method was preferable to the biological assay, and it grasped firmly the nettle of the conversion factor. It accepted that if by definition the potency of vitamin A alcohol is 3·33 $\times 10^6$ I.U. per gramme and the $E_{1\text{ cm.}}^{1\text{ per cent}}$ value is known to be 1,750, the conversion factor must be and can only be 1,900 within the limits of error of the spectrophotometric measurements. But the Conference also made it clear that the analyst must be satisfied that the effect of impurities in raising the $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 328mμ. above that due to the vitamin A content has been eliminated or allowed for before multiplying by 1,900. The Vitamin A acetate Reference Standard (dissolved in the solvent to be used for testing other materials, e.g., *cyclo*hexane, ethanol, *iso*propyl alcohol) can be examined by means of the analyst's own spectrophotometer, and the absorption spectrum may be plotted on a scale in which $E_{\text{max.}}$ is put at 1·00. The curve affords a criterion of "normality," i.e., of the absence of irrelevant absorption, especially if the compensating cell contains a solution of the diluent oil. If the absorption curve for a sample of oil, plotted in the same way, is appreciably distorted the $E_{1\text{ cm.}}^{1\text{ per cent.}}$ value at 328mμ must be corrected.

In effect, the true vitamin A content must be ascertained; the result could just as easily be expressed as mg. per g. or as percentage of vitamin A. The "potency in I.U. per g." is an alternative expression of the vitamin content, with the merit of preserving continuity with earlier work. The 1949 Conference broke new ground in recommending that the analyst as well as the bio-assayist should use the Reference Standard Preparation. It should be noted that spectrophotometers are best tested for adjustment by determining the absorption spectrum of a simple solution of potassium nitrate or chromate. The novel point in the use of a "biological" Standard is that the Reference Preparation will give the analyst an undistorted vitamin A absorption spectrum applicable to the performance of his own instrument. This is important because a definitive specification of the absorption spectrum of vitamin A determined under optimal conditions may well be less serviceable to the analyst than the curve he can himself obtain under ordinary working conditions. It is more important that he should be able to assess the degree of abnormality in an absorption curve than that he should be able to reproduce with extreme accuracy a "standard" absorption curve.

Bio-assays Compared with Spectrophotometric Assays

It is obviously necessary to know what can be expected of the two methods. Six recent biological assays of a fish liver-oil against the Vitamin A acetate Reference Standard[10] gave estimates of potency ranging between 46,290 and 86,350 I.U. per gramme (Coefficients of Variation ranging from 7·39 to 15·77 per cent). The weighted mean was 67,190 (C.V. 4·44 per cent.). Uncorrected $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 328mμ. values (31 laboratories) gave 39·06 (Standard Deviation 0·405, C.V. 1·03); overall conversion factor, 67,190/39·06 = 1,720.

From this it must be concluded that, whatever the difficulties of the spectrophotometric evaluation, biological assays are only capable of advantageous use in determining vitamin A content if the number of animals used and the statistical treatment applied, are both on a scale prohibitive for any but the most important work.

The Need for Correcting Gross $E_{1\text{ cm.}}^{1\text{ per cent.}}$ Values

(i) Recent work by an important American group has shown[10]:—

(a) that using the normal biological technique, and testing seven oils or concentrates against the Vitamin A acetate Reference Standard, the weighted means of 6 assays gave potencies with coefficients of variation between 3·73 and 6·3 per cent.

(b) that the gross (uncorrected) $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 328mμ values determined by photoelectric spectrophotometry gave C.V. values ranging from 1·03 to 2·25 per cent.

(c) that the apparent conversion factors (biological estimate in I.U./g. ÷ $E_{1\text{ cm.}}^{1\text{ per cent.}}$) were all less than 1,900 and ranged from 1,624 to 1,867.

This work makes it clear that to multiply all uncorrected $E_{1\text{ cm.}}^{1\text{ per cent.}}$ values at 328 mμ by 1,900 would result in an over-estimate of potency which would become progressively greater the worse the "spectroscopic" quality of the oil—a very discouraging situation to manufacturers able to avoid vitamin A decomposition.

(ii) Irving and Richards (at the Rowett Institute, Aberdeen) observed degenerative changes in the myelin substance of the central nervous system as a result of vitamin A deficiency. Coetzee[11] working under Irving at Cape Town has adapted a histological method (Marchi technique) to the quantitative determination of myelin degeneration for the purpose of a biological assay for vitamin A.

Comparison of the β-carotene Standard Preparation with a solution of crystalline vitamin A acetate gave a potency of 12,300 I.U./g. for $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 328 mμ, 6·85 (gross) 6·80 (corrected). This corresponds with a conversion factor near 1,800. A vitamin A distillate, $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 8·44 (gross) 8·15 (corrected) gave conversion factors of 1,957 and 2,028. For these two materials the ultra-violet absorption is little distorted and the average conversion factor is surprisingly near to 1,900.

On the other hand, the apparent conversion factors for 6 fish liver oils ranged between 1,411 and 1,817 (average 1,593, S.D., 134), using gross $E_{1\text{ cm.}}^{1\text{ per cent.}}$ values. Here, again, the need for correcting gross $E_{1\text{ cm.}}^{1\text{ per cent.}}$

values is manifest. By applying the correction procedure of Morton and Stubbs (see below) the average apparent conversion factor was revised to 1,768—a figure probably not significantly different here from 1,900.

The Effect of Irrelevant Absorption

The analyst's first aim is a true measure of the vitamin A contribution to the absorption at 328mμ and the determination of the whole absorption curve from say 250 to 380 mμ, is only necessary if it helps in correcting the gross $E^{1\ \text{per cent.}}_{1\ \text{cm.}}\lambda_{\text{max.}}$ value at 328mμ. Figure 1 shows the effect of irrelevant absorption; in a(i) the absorption curve is distorted on the short wave side of 328mμ and in a(ii) on the long wave side, but in neither case does the E value at 328mμ need correction; in b(i) the irrelevant absorption is the same over a range of wave-lengths, $\lambda_{\text{max.}}$ is not displaced from 328mμ but the E value needs to be corrected by the quantity corresponding to the double arrow, whilst in b(ii) and b(iii) $\lambda_{\text{max.}}$ is markedly displaced and the corrections needed are again shown by the arrows. The presence of irrelevant absorption is thus detectable by a displacement of the absorption maximum from 328mμ or a change in the ratio $E_{\text{max.}}/E_{\lambda_1}$ or $E_{\text{max.}}/E_{\lambda_3}$.

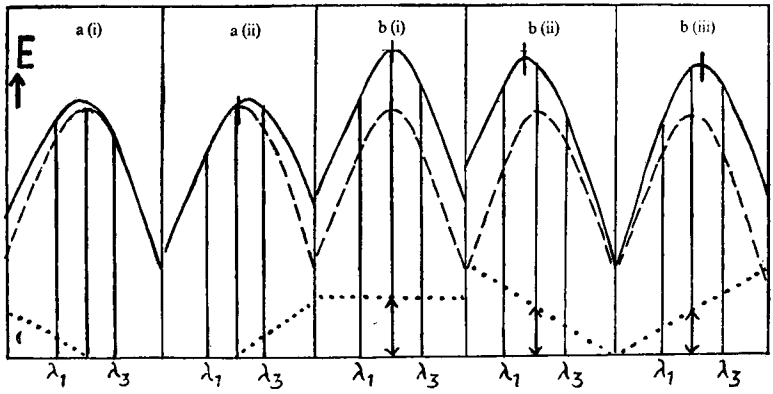

FIG. 1.—The effects of the presence of impurities exhibiting irrelevant absorption on the absorption spectrum of a substance showing selective absorption.
..... Irrelevant absorption.
— — — — — Absorption of a constant amount of selectively absorption material. e.g., Vitamin A.
———— Summation curves.

Correction For Irrelevant Absorption

Modern photoelectric spectrophotometers often permit considerably more accurate determinations of intensities of absorption than do the older photographic methods, and are particularly good for recording small differences over a restricted wave-length range.

Morton and Stubbs[12] discussed the general case where constituents other than the one which it is required to estimate make an unknown contribution to the measured absorption. Their general method of cor-

rection for unknown irrelevant absorption is based on (a) accurate measurements on the pure substance under study at three wave-lengths λ_1, λ_2 and λ_3 not very far apart and (b) similar measurements on the sample at the same wave-lengths. The assumption is made that the irrelevant absorption is linear over the narrow wave-length range chosen. Geometrical considerations lead to the formula

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

where E_1, E_2 and E_3 are readings at λ_1, λ_2 and λ_3 and A, B and C are constants calculated from the curve of the pure substance.

A much simpler method of approach is possible if the wave-lengths λ_2 and λ_3 can be chosen so that for the pure substance the extinctions at those wave-lengths are equal. The geometrical problem is illustrated in Figure 2 and it has been found convenient to choose λ_2 and λ_3 so that the intensities of absorption are 6/7 of that at λ_1 the absorption peak. [6/7 was chosen because the irrelevant absorption is only likely to be linear if λ_2 and λ_3 are not too far apart; if, on the other hand, λ_2 and λ_3 are very close together the differences in E values will be too small in

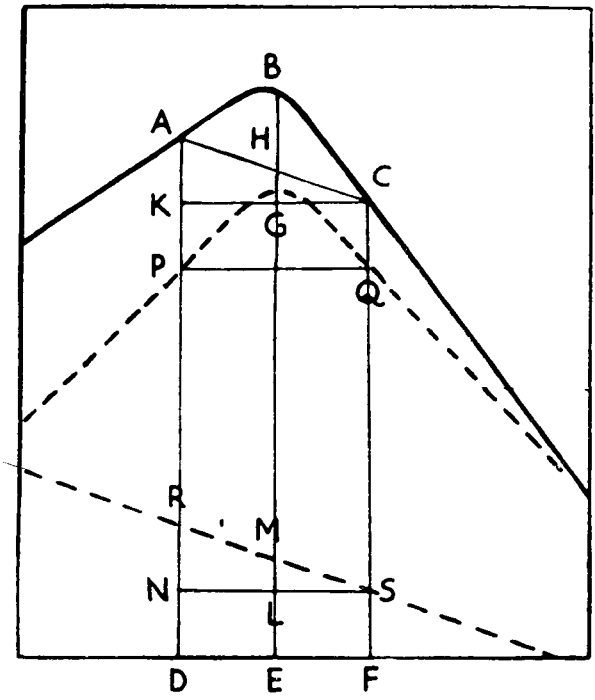

FIG. 2

relation to instrumental limitations.] The correction procedure then requires a knowledge of the absorption curve of pure vitamin A and the determination of ME in two steps ML and LE (Fig. 2).

The same authors have published[13] absorption measurements on the pure substances. It must be noted that the absorption curves for free

vitamin A and esterified vitamin A are not identical and the former curve is needed for studies on unsaponifiable fractions and the latter is suitable for work on most liver oils.

The intensities of absorption for vitamin A_1 (crystalline alcohol) and for pure vitamin A_1 acetate (cryst.) have been measured very carefully both in *cyclo*hexane and ethyl alcohol and E values at wave-lengths between 250 and 400 mμ are expressed as fraction of $E_{max.}$. Table I illustrates the most useful findings.

TABLE I

	Vitamin A Alcohol		Vitamin A Acetate	
Solvent	*cyclo* Hexane	Ethyl alcohol	*cyclo* Hexane	Ethyl alcohol
$\lambda_{max.}$ mμ	326	325	328	326·5
$\epsilon_{max.}$	48,310	51,100	48,460	50,020

Fixation points.

$E_{311m\mu} = E_{336m\mu}$ $E_{311m\mu} = E_{335\cdot5m\mu}$ $E_{313m\mu} = E_{338\cdot5m\mu}$ $E_{311\cdot5m\mu} = E_{337\cdot5m\mu}$

$\frac{E_{326m\mu}}{E_{311m\mu}} = \frac{7}{6}$ $\frac{E_{325m\mu}}{E_{311m\mu}} = \frac{7}{6}$ $\frac{E_{328m\mu}}{E_{313m\mu}} = \frac{7}{6}$ $\frac{E_{326.5m\mu}}{E_{311.5m\mu}} = \frac{7}{6}$

The usefulness of this kind of correction procedure is shown in a study of halibut liver oil[14].

In 1936 two halibut-liver oils were mixed and used by the Vitamin A Sub-Committee of the Medical Research Council in a large-scale co-operative study undertaken to ascertain afresh the conversion factor for expressing $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 328mμ in terms of International Units. The biological results were examined by statistical methods and a conversion factor of 1,570 emerged. A later co-operative experiment was carried out on vitamin A naphthoate (crystalline) and a conversion factor of 1,770 resulted. It was not easy to decide upon the significance of the difference.

Fortunately, however, samples of the two halibut-liver oils (in practically full, sealed, brown glass ampoules) had been kept at 0°C. from 1936 to 1946. The materials were re-examined in 1946 by photoelectric methods. One of them had kept perfectly, and the 1936 findings were reproduced almost exactly:—

		1936	1946	
$E_{1\text{ cm.}}^{1\text{ per cent}}$	617 mμ.	143	145	$SbCl_3$ colour test
	328 mμ.	47	46·2	ultra-violet test
			47·2	(two ampoules)

The corrected $E_{1\text{ cm.}}^{1\text{ per cent.}}$ value at 328 mμ was 40·1, so that if 1570 was the conversion factor applicable to the gross E value, the factor applicable to the corrected E value would be $\frac{1570 \times 46{\cdot}7}{40{\cdot}1} = 1828$. Thus the three co-operative tests organised by the vitamin A Sub-Committee were

brought into line (see p. 133) and it may fairly be concluded that the conversion factor resulting from the labours of the Vitamin A Sub-Committee is not significantly different from 1900.

Many molecular distillates, vitamin ester concentrates and some samples of rich liver oils give absorption curves which need hardly any correction for irrelevant absorption, but other oils need substantial correction.

Cod-liver oils in general require correction if the E value is determined on the whole oil. It has, however, been customary for the last 15 years to measure the absorption on the carefully prepared unsaponifiable fraction[4]. If the fixation points given in Table I for the free vitamin are used, most fresh cod-liver oils give " unsap." fractions which need little correction. That this applies to a particular sample should, however, be ascertained and not assumed.

The availability of the Vitamin A acetate Reference Preparation enables the shape of the absorption curve and the fixation points[13] for esterified vitamin A (Table I) to be checked at any time. If the results in the Table are reproduced it will probably be safer to accept the fixation points[13] for the free vitamin A rather than to saponify the Standard Preparation. The figures given in the Table are based on very pure crystalline vitamin A.

Detailed Procedure for the Examination of Most Samples of Fish-Liver Oil

It is assumed here that the solvent is *cyclo*hexane, but ethyl alcohol or *iso*propyl alcohol could be used given the curves for pure vitamin A acetate in those solvents. The solution should contain an amount of oil such that $E_{328m\mu}$ for a 1 cm. cell is between 0·4 and 0·9 or at a value appropriate to the performance of the spectrophotometer in use.

The absorption curve may be measured at 5mμ intervals between 250 and 370mμ and at 313mμ, 328mμ and 338·5mμ (E values at the 3 last-mentioned wave-lengths provide the minimum information needed). The actual value of $\lambda_{max.}$ should be checked. Then the value of ML (Fig. 2) should be calculated by similar triangles. If LE is put equal to x,

$$\frac{E_{328\ m\mu.} - ML - x}{{}^{*}E_{313\ m\mu} - x} = \frac{7}{6}$$

*or $E_{338.5\ m\mu}$ whichever is the lower.

The total correction ME (ML + x) is subtracted from $E_{328m\mu.}$ The corrected $E^{1\ per\ cent.}_{1\ cm.}$ 328mμ value is multiplied by 1,900 to give I.U. per g. Once $E^{1\ per\ cent.}_{1\ cm.}$ (corrected) at 328mμ has been ascertained, the entire curve for the vitamin A contribution can be reconstructed from the published data[13] on the pure substance. Subtraction of this curve from the observed curve will give a close approximation to the total impurity absorption curve. In some instances anhydrovitamin A is seen to be

the predominant source of irrelevant absorption. In others (e.g., ling cod-liver oils) vitamin A_2 with $\lambda_{max.}$ 350mμ is clearly revealed, and in some refined whale-liver oils the kitol spectrum is seen.

Determination of Vitamin A in Whale-Liver Oils

The geometrical correction procedure is based on the assumption that the irrelevant absorption is linear over a narrow range of the spectrum. As the correction becomes an increasing fraction of the total absorption, the validity of the method diminishes.

Whale-liver oil obtained by processing livers at sea is a very variable commodity, often containing phospholipids, free fatty acids, vitamin A decomposition products and kitol, a divitaminA,$C_{40}H_{60}O_2$, with $\lambda_{max.}$ at 286mμ). The kitol is biologically inactive and also interferes seriously both in the spectrophotometric test and in the antimony trichloride colour test. Until recently there has been for whale-liver oils no satisfactory alternative to the biological assay, itself of limited value since the fiducial limits of error (80 rats, P = 0·95) may often be no better than 70 to 140 per cent. of the estimated potency.

With many whale-liver oils the correction procedure is pushed beyond the limit of its usefulness, and may still be unsatisfactory even when it is applied to the unsaponifiable fractions which show less irrelevant absorption than the crude oils.

The first trustworthy satisfactory analytical procedure to be published[15] was based on a preliminary chromatographic treatment of the unsaponifiable fraction so as to obtain a vitamin A-alcohol fraction giving an absorption curve either needing no correction or amenable to correction by the geometrical procedure.

The following modification[16] gives good results with perhaps less risk of manipulative losses. The adsorbent is alumina (Spence, Grade O) weakened by thorough admixture with 10 per cent. by weight of water. The column (12 × 1 cm.) is made by pouring the alumina into a tube as a slurry with light petroleum. The sample (0·1 to 0·2 g. in 25 ml. of light petroleum) is poured on the column and the chromatogram is developed with light petroleum. Passage of solvent through the column is hastened by pressure from above and the percolate is collected in 5 ml. portions in small test tubes. The first runnings contain anhydrovitamin A, detected by treating 1 or 2 drops of eluate with the antimony trichloride reagent. Several 5 ml. portions of eluate will then be obtained which give no blue colour with the reagent. Continued development with light petroleum results in the appearance in the eluate of vitamin A esters. The intensity of the blue colour obtained with one drop of eluate will increase steadily and then decrease until finally no colour is given. Development is continued with light petroleum containing diethyl ether (4 per cent. by vol., 50 ml.; 8 per cent., 50 ml.; 12 per cent., 50 ml.). The percolate now gives a purple colour (with the antimony trichloride reagent) and shows $\lambda_{max.}$ at 285 to 290 mμ (kitol esters). Further development results in a small fraction which gives little or no colour with the

antimony trichloride reagent, but continued development with light petroleum containing 16 per cent. and 20 per cent. of ether results in more or less sharply defined small fractions containing free vitamin A and free kitol respectively. Thus an oil with $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 328 mμ 60·1 (gross) and a greatly distorted absorption curve gave a small anhydrovitamin A fraction $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 370 mμ 2·1, a vitamin A ester fraction $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 328 mμ 37·9 (no correction needed), a kitol ester fraction $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 286 mμ 24·35 and a free vitamin A fraction $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 326 mμ 2·2 (corrected). The total $E_{1\text{ cm.}}^{1\text{ per cent.}}$ value at 328 mμ is therefore 40 and the potency 40 × 1900 = 76,000 I.U./g. In the above example only 0·125 g. of oil was used, and it cannot too strongly be stressed that much depends on good sampling of whale-liver oil at all stages; thorough mixing of the thick dark oil is essential. The chromatography must be controlled by testing each 5 ml. portion of eluate with the antimony trichloride reagent and using the colour test to distinguish the various fractions. The original papers should be consulted for further details.

Alternative methods of eliminating kitol[16,17] from whale-liver oil unsaponifiable fractions depend upon extraction with 50 per cent. aqueous ethyl alcohol in which the free vitamin A is preferentially soluble.

Some materials exhibiting distorted ultra-violet absorption curves may be studied before and after photo-chemical destruction of vitamin A by ultra-violet light, the decrease in absorption at 326 to 328 mμ being attributed to the vitamin A.

Vitamin A_2. Certain fish-liver oils, particularly those from fresh-water fishes contain a substance which with the antimony trichloride reagent gives a blue colour with $\lambda_{max.}$ 693 mμ and showing an ultra-violet maximum near 350 mμ. Salmon-liver oils, snoek-, red cod-, black cod- and ling cod-liver oils all contain appreciable quantities of the 693 mμ chromogen (Vitamin A_2), but in nearly all the liver oils of commerce vitamin A_1 preponderates over vitamin A_2. Thus in ling cod-liver oil the ratio vitamin A_1/vitamin A_2 is 8:1, whilst in many halibut-liver oils it is perhaps 20:1. The biological potency of vitamin A_2 is of the same order as that of vitamin A_1.

The presence in an oil of an appreciable proportion of vitamin A_2 distorts the absorption curve, particularly on the long-wave side of the maximum, and application of the correction procedure practically eliminates the vitamin A_2 contribution[13]. Hitherto most commercial and official testing of fish-liver oils has ignored the existence of vitamin A_2 and only in rather rare cases is there likely to be any significant error as a result of continuing to do so. There is no ruling on the matter, probably because of a lack of knowledge concerning the extent to which vitamin A_2 can replace vitamin A in human nutrition.

Synthetic preparations. Vitamin A acetate (synthetic) is now an article of commerce and there is no evidence that it can be distinguished from the acetate prepared from vitamin A of natural origin. It is, however, quite impossible to foresee the analytical difficulties which may arise from synthetic preparations in general, and the safest rule in cases

of doubt would be to carry out a comparison with the International Standard preparation using both the colour test and the ultra-violet absorption test.

The position of the colour test in quatitative work. For many purposes the antimony trichloride colour test is still very valuable, particularly in the determination of plasma vitamin A. An alternative colour test using activated glycerol dichlorhydrin has some advantages[18]. There are too many ways of measuring the colour intensity to justify giving details here but one general comment is necessary.

The International Standard Preparation of vitamin A acetate (or a secondary laboratory standard derived from it by comparison of $E_{328m\mu}$ values) should be used to calibrate the instrument for measuring the blue colour. This will lead to trustworthy results in testing unsaponifiable fractions, but the colour test is not recommended for unsaponified low-potency fish-liver oils because of the presence of the colour test inhibitors already referred to.

Provitamins A. The determination of carotenoid precursors of vitamin A depends upon preliminary chromatographic separation followed by spectrophotometry. The chromatography is used mainly to separate carotenes from "xanthophylls," but any accurate estimate requires a quantitative separation, identification and estimation of all the carotenoids present. As there are several provitamins A, each of which may occur as *cis-trans* isomerides the problem is sometimes very complicated.

As Zscheile and Porter[19] have said, "Different plants, plant parts or products made from plants require individual treatment and study, as extraction methods, identity of pigments and interpretation of both chromatographic columns (or other fractionation procedures) and characteristic absorption curves cannot be carried over arbitrarily from one material to another." Although perhaps over-cautious the above warning is salutary. The outstanding practical problem of this kind is the determination of β-carotene in dried grass—a matter of veterinary and agricultural importance, and it has a literature of its own which is outside the scope of this review. Figures giving the provitamin content of foodstuffs should be used with reserve and the temptation to equate or to treat as additive "provitamin" units and vitamin "units" should be firmly resisted.

Neovitamin A. Recent work tends to show that the vitamin A present in fish-liver oils consists mainly of all-*trans* vitamin A and *neo*vitamin A (a *cis*-form). Dr. P. D. Dalvi working in the writer's laboratory has isolated a very rich *neo*vitamin A ester fraction, the spectral absorption curve of which differs very little from that of vitamin A acetate. Although it is possible that further research may bring surprises there is so far little to indicate that any serious error in the determination of vitamin A will arise from neglecting the existence of *neo*vitamin A.

The satisfactory outcome of the 1949 Conference reflects consideration of great and sustained effort in several countries and the various experts

brought to the task knowledge of work done during the War when scientific communication had broken down. A full bibliography would now be very unwieldy.

Much excellent work, particularly the large American contribution, cannot be referred to here, but tribute must be paid to the labours of those who engaged in the various co-operative assays in academic institutions and industrial laboratories. In this country the brunt of the work fell on the Vitamin A sub-committee of the Medical Research Council. The present position is well summarised in the following quotations[5]:—

"The new conversion factor of 1900 cannot be applied indiscriminately because few of the materials commonly tested are free from irrelevant absorption in the ultra-violet region of the spectrum, including the significant region 325 to 328 mμ. It is therefore necessary (1) to specify the conditions under which the factor is applicable and (2) to indicate how, in principle, irrelevant absorption can be allowed for. The conditions for (1) are:—(a) that the absorption maximum shall be within the range 325 to 328 mμ and (b) that the shape of the absorption curve shall agree closely with that of the international standard measured under the same conditions and compensated with a solution of the diluent oil. Intensities of absorption in the region of 310 and 350 mμ expressed as decimal fractions of the maximum should not differ between sample and standard by more than 0·02 (for standard curves see Ref. 13). To make the adjustment needed under (2) absorption curves failing to meet the above requirements may be corrected to allow for irrelevant absorption, provided the maximum is not displaced in wavelength, by a geometric procedure[12]. Absorption curves in which the maximum occurs outside the stipulated wave-length range indicate a need for purification of the material prior to spectrophotometric analysis. For example, cod-liver non-saponifiable fractions[20,21] usually yield spectrophotometrically normal curves; whale-liver oils usually give a spectrophotometrically normal fraction after chromatography[15] and many fish-liver oils yield a fraction exhibiting a normal curve after selective solvent extraction[22]. Selective photochemical destruction of vitamin A has also been used with success[23,24]."

Experience may show a need for modifications in matters of detail, but the principle of vitamin A determination seem to be firmly laid down.

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