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EXPERT COMMITTEE ON
BIOLOGICAL STANDARDIZATION

Report of the Subcommittee on
Fat-Soluble Vitamins

London, 26-29 April 1949

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WORLD HEALTH ORGANIZATION
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EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Subcommittee on Fat-Soluble Vitamins

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EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Report of the Subcommittee on Fat-Soluble Vitamins¹

The Subcommittee on Fat-Soluble Vitamins met in London, from 26 to 29 April 1949, on the premises of the Medical Research Council, 26 Old Queen Street.

The Assistant Director-General of WHO outlined the administrative procedure which led to the establishment of this subcommittee, the function of which was to advise the Expert Committee on Biological Standardization.² He welcomed the participation of a representative from FAO.

Sir Edward Mellanby, who presided over the international conferences on the standardization of vitamins of 1931 and 1934,^{3, 4} was elected to the chair.

I. Vitamin A

Preamble

It has always been recognized that β -carotene was not ideal as a standard of reference for determining vitamin A, but no satisfactory preparation of vitamin A was available at the last conference (1934) when β -carotene was adopted as a standard preparation.

Various esters of vitamin A have been prepared during the last few years. Of these esters the acetate appears to be the most suitable for adoption as a standard in place of β -carotene. It is desirable, nevertheless, to retain the β -carotene standard preparation as the standard of pro-vitamin A.

¹ The Executive Board, at its fourth session, adopted the following resolution:
The Executive Board

(1) NOTES the report of the Expert Committee on Biological Standardization on its third session and the report of its Subcommittee on Fat-Soluble Vitamins, and
(2) AUTHORIZES their publication. *Off. Rec. World Hlth Org.* 22, 3

² *Off. Rec. World Hlth Org.* 13, 307; 14, 23

³ League of Nations Health Organization (1931) *Report of the Conference on Vitamin Standards (London, June 17th to 20th, 1931)*, Geneva (C.H. 1055 (1))

⁴ League of Nations Health Organization (1934) Second Conference on Vitamin Standardization, *Quart. Bull. Hlth Org. L.o.N.* 3, 428

1. *International standard*

The subcommittee recommends that the international standard for vitamin A shall be crystalline vitamin A acetate having the characteristics specified below.

2. *Definition of unit*

It is recommended that the international unit shall be the activity of 0.344 $\mu\text{g.}$ of the standard preparation of crystalline vitamin A acetate, which is equivalent to 0.3 $\mu\text{g.}$ of vitamin A alcohol.

3. *Mode of issue*

It is recommended that the international standard preparation shall be issued as a solution in a suitable vegetable oil (see part I, 5) of such strength that 0.1 mg. of the solution contains 0.344 $\mu\text{g.}$ (= 1 International Unit) of vitamin A acetate.

4. *Properties of the standard preparation for vitamin A*

Pure crystalline all-*trans* vitamin A acetate ($\text{C}_{22}\text{H}_{32}\text{O}_2$) has the following characteristics :

Melting-point 57.8° to 59.0° C. (corr.)

$E_{1\text{ cm.}}^{1\%}$ 325 $\text{m}\mu$ = 1,525 (*iso*-propanol) corresponding to a molecular extinction coefficient of 50,000.

5. *Solvent for vitamin A standard*

An oil suitable as solvent for vitamin A acetate is one containing not less than 0.1% of tocopherol and not more than 32 parts per million total peroxide oxygen.^{5, 6} It is recommended that a sample of this oil shall be available on request.

6. *Factor for converting the results of spectrophotometric determinations into international units*

The subcommittee has recommended that the activity of 0.344 $\mu\text{g.}$ of vitamin A acetate shall be adopted as the international unit. This amount is equivalent to 0.3 $\mu\text{g.}$ of vitamin A alcohol.

The results of the large-scale biological tests carried out on rats for the Medical Research Council of Great Britain and for the United States

⁵ Greenbank, G. R. & Holm, G. E. (1934) *Industr. Engng Chem.* **26**, 243

⁶ Lea, C. H. (1938) *Spec. Rep. Food Invest. Bd, Lond.* No. 46; see also Lea, C. H. (1946) *J. Soc. chem. Ind., Lond.* **65**, 286.

Pharmacopoeia Commission show that this definition of the unit maintains continuity with the unit defined as 0.6 μg . of the international standard preparation of β -carotene which has been in use since 1934.

The value for $E_{1\text{ cm}}^{1\%}$ $325\text{ m}\mu = 1,750$ for vitamin A alcohol.

Since 0.3 μg . of vitamin A alcohol is equivalent to the value adopted for the international unit and 1,750 is the value for the absorption coefficient, it follows that the conversion factor must be 1,900

$$\left(\frac{10^6}{0.3 \times 1,750} = 1,900 \right)$$

and, since 0.344 μg . of vitamin A acetate is adopted as the value for the international unit, and since the value for $E_{1\text{ cm}}^{1\%}$ $325\text{ m}\mu = 1,525$, it follows equally that the conversion factor must be 1,900

$$\left(\frac{10^6}{0.344 \times 1,525} = 1,900 \right)$$

7. *Applicability of the conversion factor*

The conversion factor of 1,600 recommended in 1934 was intended to be applied to spectrophotometric data which could not at that time be conveniently corrected for irrelevant absorption.

The new conversion factor of 1,900 cannot be applied indiscriminately because few of the materials commonly tested are free from irrelevant absorption in the ultraviolet region of the spectrum, including the significant region 325 – 328 $\text{m}\mu$. It is therefore necessary:

- (1) to specify the conditions under which the factor is applicable, and
- (2) to indicate how, in principle, irrelevant absorption may be allowed for.

The conditions for (1) are:

- (a) that the absorption maximum shall be within the range 325 – 328 $\text{m}\mu$
- (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 310 – 350 $\text{m}\mu$ expressed as decimal fractions of the maximum should not differ between sample and standard by more than 0.02; the absorption curves for vitamin A alcohol and acetate, expressed in the above manner, are given by Morton & Stubbs.⁷

⁷ Morton, R. A. & Stubbs, A. L. (1948) *Biochem. J.* **42**, 195

To make the adjustment needed under (2), absorption curves failing to meet the above requirements may be corrected to allow for irrelevant absorption, provided that the maximum is not displaced in wave-length, by a geometric procedure.⁸

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 usually yield spectrophotometrically normal curves;^{9,10} whale-liver oils usually give a spectrophotometrically normal fraction after chromatography;¹¹ and many fish-liver oils yield a fraction exhibiting a normal curve after selective solvent extraction.¹² Selective photochemical destruction of vitamin A has also been used with success.^{13,14}

With some very low-potency materials, e.g. blood sera and some food-stuffs, it may be expedient to make use of the less specific colour test. Whenever colorimetric tests (e.g. antimony trichloride, glycerol dichlorohydrin) are used, the procedure should be calibrated with the international standard preparation. It must be borne in mind, however, that unless the colours, as between standard and sample, agree in tint, estimates of potency obtained in this way may be erroneous.

II. Provitamin A

1. *International standard*

It is recommended that the existing sample of pure β -carotene having the properties described below shall be retained as the international standard for provitamin A.

2. *Definition of unit*

It is recommended that the unit of provitamin A shall be the activity of 0.6 μ g. of the international standard preparation.

3. *Mode of issue*

It is recommended that the international standard preparation shall be issued in the form of a solution in a suitable vegetable oil (see part II, 5) of such a strength that 1 International Unit is contained in 5 mg.

⁸ Morton, R. A. & Stubbs, A. L. (1946) *Analyst*, **71**, 348

⁹ Society of Public Analysts (1933) *Analyst*, **58**, 203

¹⁰ Association of Official Agricultural Chemists (1945) *Methods of analysis*, 6th ed., Washington, D.C., p. 504

¹¹ Gridgeman, N. T., Gibson, G. P. & Savage, J. P. (1948) *Analyst*, **73**, 662

¹² Chevallier, A. & Dubouloz, P. (1936) *Bull. Soc. Chim. biol.* **18**, 703

¹³ Chevallier, A. & Manuel, S. (1941) *Bull. Soc. Chim. biol.* **23**, 1203

¹⁴ Neal, R. H., Haurand, C. H. & Luckman, F. H. (1941) *Industr. Engng Chem. Anal. Ed.* **13**, 150

4. *Properties of the standard for provitamin A*

The standard preparation of all-*trans* β -carotene ($C_{40}H_{56}$) has the following characteristics :

Melting-point $180^{\circ}C$. (corr.)

$E_{1\text{ cm.}}^{1\%}$ 465 $m\mu$ = 2,290 (benzene) corresponding to a molecular extinction coefficient of 122,700

$E_{1\text{ cm.}}^{1\%}$ 455 $m\mu$ = 2,440 (cyclohexane) corresponding to a molecular extinction coefficient of 130,800.

5. *Solvent for provitamin A standard*

An oil suitable to be used as solvent for the provitamin A preparation is one containing not more than 32 parts per million total peroxide oxygen^{15, 16} and to which 0.01% hydroquinone has been added.

6. *Use of the provitamin A standard*

Since two standards are available, it will now be necessary to express the vitamin A and provitamin A activity of foods or other substances in which one form only is present in terms of the respective units.

It should be emphasized that when the provitamin A standard preparation is used in biological assays, the results will be a combination of two effects :

- (a) the provitamin A content of the material tested ;
- (b) the availability to the animal of that content.

On the other hand, if the standard preparation is used for comparison by chemical or physical methods, the provitamin A content alone will be measured. The result will be strictly valid only if no form of provitamin A other than β -carotene is present.

III. Vitamin D

Preamble

The international standard for vitamin D, consisting of a solution of irradiated ergosterol, was adopted by the Permanent Commission on Biological Standardization of the Health Organization of the League of Nations in 1931. Being a solution mainly of vitamin D_2 , it has not proved to be sufficiently representative of the D vitamins.

¹⁵ Greenbank, G. R. & Holm, G. E. (1934) *Industr. Engng Chem.* **26**, 243

¹⁶ Lea, C. H. (1938) *Spec. Rep. Food Invest. Bd, Lond.* No. 46 ; see also Lea, C. H. (1946) *J. Soc. chem. Ind., Lond.* **65**, 286.

The suggestion made at the Second International Conference on Vitamin Standardization in 1934, that the standard of irradiated ergosterol should eventually be replaced by pure crystalline vitamin D₂ (calciferol), is therefore now unacceptable. It was even then recognized that the vitamin D₂ standard was not a suitable standard for determining the vitamin D activity for poultry. Crystalline vitamin D₃, which does not possess this defect, was not then available but has now been prepared in sufficient quantity to be adopted as an international standard. It should be noted, however, that the vitamin D activity for poultry, in international units, of materials that are not known to contain vitamin D₃ alone, can be obtained only by assay on poultry.

1. *International standard*

The subcommittee recommends that the preparation of crystalline vitamin D₃ as described below, at present held at the National Institute for Medical Research, London, shall be adopted as the international standard of vitamin D.

This new standard shall replace the existing solution of irradiated ergosterol. The latter shall be retained as a reference preparation only, and not as an international standard.¹⁷ Samples of the 1931 standard preparation shall be obtainable on request.

2. *Definition of unit*

The international unit of vitamin D recommended for adoption is the vitamin D activity of 0.025 µg. of the international standard preparation of crystalline vitamin D₃.

3. *Mode of issue*

It is recommended that the 14 g. of crystalline vitamin D₃ at present held in bulk in a nitrogen atmosphere at -5° C., at the National Institute for Medical Research, London, shall be divided into a number of ampoules such that each contains about sufficient for a half-yearly issue. For each issue the contents of an ampoule shall be dissolved in a suitable vegetable oil (see part III, 5) to make a solution of such a strength that 1 mg. of it contains 0.025 µg. (= 1 International Unit).

¹⁷ The argument which influenced the subcommittee to retain the original standard not as a definite international standard but as a reference preparation was:

The standard of crystalline vitamin D₃ has not yet been observed for a sufficiently long period for its stability to be beyond doubt. The original standard has proved over a course of years to be stable. It may be useful in case any difficulties arise in the use of the vitamin D₃ preparation.

4. Properties of the standard vitamin D preparation

The recrystallized vitamin D₃ (C₂₇H₄₃OH), freshly withdrawn from the sealed ampoule, had the following characteristics :

Melting-point 87° to 89° C. (corr.)

$$\left[\alpha \right]_{\text{D}}^{20^\circ} = + 110^\circ \text{ (ethanol)}$$

$E_{1\text{ cm.}}^{1\%}$ 265 m μ = 490 (ethanol) corresponding to a molecular extinction coefficient of 18,800.

It should be noted that determinations of the physical constants of this substance should be made as quickly as possible after withdrawal of the sample.

5. Solvent for vitamin D standard

An oil suitable to be used as solvent for the vitamin D preparation is one containing not more than 32 parts per million total peroxide oxygen^{18, 19} and to which 0.01% hydroquinone has been added.

IV. Estimation of Vitamin Content in Foodstuffs

The subcommittee considers that the value and usefulness of the international standards for vitamins might be increased if suitable methods of estimating the different vitamins in foodstuffs were to be proposed by WHO and FAO and recommended for general use.

¹⁸ Greenbank, G. R. & Holm, G. E. (1934) *Industr. Engng Chem.* **26**, 243

¹⁹ Lea, C. H. (1938) *Spec. Rep. Food Invest. Bd, Lond.* No. 46 ; see also Lea, C. H. (1946) *J. Soc. chem. Ind., Lond.* **65**, 286.

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