Vitamin Standards

REPORT OF THE PERMANENT COMMISSION ON BIOLOGICAL STANDARDISATION*

THE International Conference on vitamin standards was presided over by Professor E. Mellanby, and was attended by representatives of Denmark, France, Holland, Germany, Great Britain, Norway, Sweden, and the United States of America. The British participant was Professor J. C. Drummond.

It was the general opinion that, in the present state of our knowledge, only vitamins *A*, *D*, *B*, (also known as vitamin B_1), and *C* could be profitably discussed in connection with standardisation.

I. THE FAT-SOLUBLE VITAMIN *A*

(*a*) INTERNATIONAL STANDARD.—*The Conference recommends that carotene be accepted as an international provisional standard of reference for vitamin* A, *and that a selected sample of cod-liver oil be held in view as a possible secondary standard.*

(*b*) MODE OF PREPARATION.—It was decided that the information available does not yet justify the selection of one isomer of carotene as a standard. The similar biological activity of the two isomers that have recently been described is further justification for adopting as the provisional international standard a mixture prepared in an approved manner.

It was decided to employ a preparation of carotene made from carrots by Willstätter's method and purified by recrystallisation by the method described in the memorandum issued by the Department of Biological Standards, National Institute for Medical Research, London (Appendix, p. 76), until the melting-point determined is above 179° C. It was suggested that preparations should be made in various countries and despatched immediately and with all necessary precautions against decomposition to the National Institute for Medical Research, London, where they will be mixed to form a uniform preparation by the most suitable method. The details of this final purification are to be left to the discretion of the authorities of the National Institute. It was suggested that original preparations of, say, 4 to 5 grms., might be made in the following institutions:

Department of Physiological Chemistry, University of Amsterdam.
"Laboratoire de physiologie de la nutrition, École des Hautes Études," Paris.
"Tierphysiologisches Institut," Leipzig.
National Institute for Medical Research, Hampstead, London.
Department of Agricultural Chemistry, University of Wisconsin, U.S.A.
Biochemical Department, University of Stockholm, and "Institut für Organische Chemie der Universität," Zürich.
School of Hygiene and Public Health, Johns Hopkins University, Baltimore, U.S.A.

(*c*) PLACE OF PREPARATION.—It was decided that the National Institute for Medical Research, London, acting for this purpose as the *central laboratory on behalf of the Health Organisation of the League of Nations*, should be asked to undertake the final preparation of the sample of carotene to be used as the international standard for vitamin *A*.

(*d*) MODE OF DISTRIBUTION.—It was considered desirable that, as far as possible, the standard preparation should be distributed to workers through the appropriate official institution in each country, preferably that now responsible for the distribution of similar biological standards. The material should be sent out in tubes of 10 mgrms., as described in the Appendix, p. 76.

(*e*) DEFINITION OF UNIT.—The unit of vitamin *A* recommended for adoption is the vitamin *A* activity of 1γ (0·001 mg.) of the international standard.

Note.—Daily doses of about 3γ to 5γ of the international standard, when administered to young rats suitably prepared on a vitamin *A*-deficient diet, have been found adequate to restore growth and to cure xerophthalmia.

(*f*) PERMANENCE OF THE STANDARD.—The Conference recommends that this international standard and unit be accepted provisionally for two years.

(*g*) SUBJECTS RECOMMENDED FOR FUTURE INVESTIGATIONS.—It is highly desirable that, during the provisional period, further investigations of the standard should be made regarding

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the stability of the carotene preparation, both when sealed in the original tubes and after it has been removed and dissolved for biological testing. In the latter connection, emphasis is laid on the importance of minimising contact with air or oxygen of the solutions used for animal feeding. It is recommended that they be always stored in an inert gas and at low temperature.

It will be of great value if investigators submit to the League of Nations Health Organisation observations on the stability of the preparation, its behaviour in various solvents and under different conditions of storage, and any other information bearing on its use as a biological standard. The use of suitable "antioxidants" ("*antioxygènes*" in French) should, in particular, be studied.

(*h*) BIOLOGICAL METHODS FOR ESTIMATION OF VITAMIN *A*.—It was decided not to recommend any one particular method of conducting the biological assay, but to invite members of the Conference to submit to the League of Nations Health Organisation their observations on the value of the methods they have been using.

It is recommended that further attention be given to the methods based on the curative action of carotene for xerophthalmia and other lesions characteristic of vitamin *A* deficiency, as well as to those based on increase in weight.

(*i*) SELECTED SAMPLE OF COD-LIVER OIL FOR USE AS A POSSIBLE SECONDARY STANDARD.—It is recommended that a supply of an approved sample of cod-liver oil be obtained with the object of making a series of comparative tests to determine its suitability as an alternative standard. The Conference is informed that the United States Department of Agriculture is making arrangements for such a standard substance to be available in the United States during the coming year.

It was decided to ask the United States Department of Agriculture to obtain sufficient supplies of the oil for distribution in order to enable investigators of other countries to assay this oil in terms of the international unit of standard carotene. It is hoped thus to obtain evidence regarding the stability of vitamin *A* in cod-liver oil as affected by conditions and time of storage.

II. THE ANTIRACHITIC VITAMIN *D*

(*a*) INTERNATIONAL STANDARD TO BE ADOPTED AND ARRANGEMENTS FOR CONTROL.—*The Conference recommends that the standard solution of irradiated ergosterol at present issued from the National Institute of Medical Research, London, be adopted as international vitamin D standard for the next two years.*

If within this period it should become necessary, owing to threatened exhaustion of the present supply, to replace this solution by a fresh standard, the equivalence shall be determined by experts of different countries who have had the opportunity of comparing the proposed new standard with the one at present issued. It is suggested that the following Institutions, among others, be invited to co-operate in those tests:

"Allgemeines Chemisches Laboratorium," Göttingen.
"Tierphysiologisches Institut," Leipzig.
Food and Drugs Administration Laboratory, Department of Agriculture, Washington, D.C.
Biochemical Department, University of Stockholm.
Department of Agricultural Chemistry, University of Wisconsin.
School of Hygiene, Johns Hopkins University, Baltimore.
"Laboratoire de physiologie de la nutrition, École des Hautes Études," Paris.
Pharmaceutical Society, London.

(*b*) METHOD OF PREPARATION.—1. It is recommended that, in the preparation of the solutions of irradiated ergosterol, used as standards of reference for vitamin *D* (or as sub-standards), irradiation with ultra-violet light shall be done in ethereal solution in the absence of any appreciable traces of oxygen, and the solution should be kept meanwhile in rapid motion. The conditions of exposure should be such as to transform between 30 per cent. and 80 per cent. of the ergosterol. The solution of the product and further dilutions shall be made in a stable unsaturated natural vegetable oil, which has given a negative test for vitamin *D*.

2. The standard solution of irradiated ergosterol at present issued from the National Institute for Medical Research, London (Standard Solution III), was, however, prepared as follows, in January, 1929:

> A 0·1 per cent. solution of the ergosterol in absolute alcohol was exposed for half-an-hour in a silica cell, 1 cm. thick, to the unfiltered radiation from a K.B.B. (Kelvin, Bottomley and Baird) mercury vapour lamp, taking 2·5 amperes and 125 volts at atmospheric pressure, at 15 cm. distance from cell to lamp. The resulting solution was mixed with a little olive oil, and then evaporated at 45° C. at a low pressure to remove the alcohol. The concentrated oily solution thus obtained was diluted with pure olive oil to give a concentration corresponding with 1 mgrm. of the original ergosterol in 10 c.c. of olive oil at 18° C. The olive oil used was tested for stability and gave a negative test for vitamin *D*.

The additional larger quantity of standard (prepared January, 1931), which is available for distribution later if required, was prepared with observance of the general conditions indicated under 1.

(*c*) MODE OF DISTRIBUTION.—The National Institute for Medical Research, London, *acting for this purpose as the central laboratory on behalf of the League of Nations Health Organisation*, shall distribute to each country wishing to use the standard a sufficient quantity of the solution to enable the standard to be effectively applied according to the conditions in the particular country. Such quantity shall be supplied only to a central institution nominated for the purpose by the country concerned, which will be responsible for the distribution either of the portion of international standard solution received or, wherever possible, of an equivalent substandard prepared by comparison therewith.

(*d*) STABILITY OF THE INTERNATIONAL STANDARD.—The stability of the standard solution at present issued from the National Institute for Medical Research, London, has proved satisfactory on the results of tests over a period of two years, when preserved at or below 0° C., with exclusion of air.

(*e*) DEFINITION OF UNIT.—The unit of vitamin *D* recommended for adoption is defined as the vitamin *D* activity of 1 mgrm. of the international standard solution of irradiated ergosterol.

Note.—The international standard solution has been prepared to have such potency that approximately 1 mgrm. thereof given daily to a rachitic rat for eight successive days will produce a wide line of calcium deposits in the metaphysis of the proximal ends of the tibiae and of the distal ends of the radii.

(*f*) PERMANENCE OF THE STANDARD.—The international standard at present recommended shall be regarded as provisional for the next two years, in the hope that a more stable crystalline substance may in the meantime become available.

(*g*) SUBJECTS RECOMMENDED FOR FURTHER INVESTIGATION.—1. The influence of various oils as solvents upon the stability of solutions of irradiated ergosterol.

2. The crystalline antirachitic substances recently isolated from irradiated ergosterol. It was decided that Professor Windaus and Dr. Bourdillon shall be asked to investigate the constancy of the physical properties of the crystalline products recently isolated by them, respectively, in order to determine whether these may be regarded as pure substances. If so, the potency of these products should be accurately compared with that of the (present) international standard at intervals of three months during the next two years, in order to compare the stability in each case. If the results are satisfactory, it is hoped that one of these crystalline substances may eventually replace the solution of irradiated ergosterol as international vitamin *D* standard.

3. The toxicity of the present standard and of the crystalline products (see also Appendix, page 75).

(*h*) BIOLOGICAL METHODS FOR ESTIMATION OF VITAMIN *D*.—In using the international standard solution for the determination of the antirachitic potency of unknown preparations, it is recommended that not fewer than twenty rats (preferably more) be used for a determination, half of these to receive the standard and the remaining litter-mates the unknown substance. Provided this precaution is observed, it is considered permissible to use various biological methods of estimation, either prophylactic or therapeutic. For instance, the "line" test, *X*-ray examination, and determination of the bone ash, are all considered reliable methods.

III. THE ANTINEURITIC VITAMIN *B*

(*a*) INTERNATIONAL STANDARD.—*The Conference recommends the adoption, as international standard, of the adsorption product of the antineuritic vitamin* B *prepared in the Medical Laboratory, Batavia (Java), by the method of Seidell, as described by Jansen and Donath.*

(*b*) TERMINOLOGY.—The international standard preparation should be known as the "standard adsorption product of the antineuritic vitamin *B*."

(*c*) METHOD OF PREPARATION.—The international standard is prepared by extracting rice polishings with water, sufficient sulphuric acid being added to make the *p*H 4·5. Salicylic acid, to a concentration of 0·2 per cent., and toluene are then added to prevent bacterial decomposition. The process of extraction is continued for two days, after which the solution is filtered. For each 100 kilogrammes of the original rice polishings, 3 kilos. of fuller's earth (specially selected for its adsorptive powers) are added to the solution, which is then stirred for twenty-four hours. Subsequently, the solution is filtered off and the fuller's earth, after being washed with water and alcohol, is dried; 3 kilos. of the fuller's earth adsorbate represent the antineuritic vitamin *B* from 100 kilos. of rice polishings.

(*d*) PLACE OF PREPARATION.—It is recommended that the Medical Laboratory, Batavia, Java, should be asked, through Professor Jansen, of Amsterdam, to prepare a batch of 25 kilos. of the standard preparation. This should provide an adequate supply for many years.

(*e*) PLACE OF DISTRIBUTION.—It is suggested that this batch of standard adsorption product of antineuritic vitamin *B* should be kept at the National Institute for Medical Research, London, *acting for this purpose as central laboratory on behalf of the Health Organisation of the League of Nations*.

One hundred grms. would be an amount suitable for distribution to individual laboratories. No special precautions are necessary in keeping this preparation, except that it should be stored in a dry place. In the presence of moisture, bacterial decomposition readily takes place.

(*f*) DEFINITION OF UNIT.—The unit recommended for adoption is the antineuritic activity of 10 mgrms. of the international standard adsorption product.

Note.—A daily dose of 10 to 20 mgrms. of this preparation is required to maintain normal growth in a young rat on a diet deficient in the antineuritic vitamin *B*, but complete in all other respects, including the antidermatitis vitamin (B_2); the curative "day dose" for a pigeon (300 grms. weight) suffering from polyneuritis on a diet of polished rice is about 20 to 30 mgrms. (method of Kinnersley and Peters).

(*g*) PERMANENCE OF THE INTERNATIONAL STANDARD RECOMMENDED.—This standard adsorption product should serve as a provisional international standard for five years, or until advances in the knowledge of this vitamin make a revision desirable.

(*h*) RECOMMENDATIONS FOR FURTHER INVESTIGATIONS.—1. The standard adsorption product should be investigated for its content of other vitamins *B*.

2. Although there is no evidence that loss of potency is liable to occur in the standard adsorption product, the Conference suggests that the following laboratories be asked to undertake a further investigation of its stability:

Department of Physiological Chemistry, University of Amsterdam.
National Institute of Health, United States Public Health Service, Washington, D.C.
Institute of Hygiene, University of Copenhagen.
School of Biochemistry, University of Oxford.
"Tierphysiologisches Institut," Leipzig.
Biochemical Institute, University of Stockholm.
"Laboratoire de physiologie au Centre de recherches sur l'alimentation (Institut des recherches agronomiques)," Paris.
Lister Institute for Preventive Medicine, London.

(*i*) BIOLOGICAL METHODS FOR ESTIMATION OF THE ANTINEURITIC VITAMIN *B*.—The Conference expresses no opinion on the relative merits of current biological methods for estimation of the antineuritic vitamin *B* (as recorded in the report on this vitamin, presented to this Conference and in the literature generally). It considers that good evidence is provided by that report that the different methods described, either prophylactic or curative in type, and employing either the rat or the pigeon as experimental animal, may yield equally valid results.

IV. THE ANTISCORBUTIC VITAMIN *C*

(*a*) INTERNATIONAL STANDARD.—*The Conference recommends the adoption as international standard of the fresh juice of the lemon,* Citrus limonum.

(*b*) DEFINITION OF UNIT.—The unit of the antiscorbutic vitamin *C* recommended for adoption is the vitamin *C* activity of 0·1 c.c. of fresh juice of the lemon, *Citrus limonum.*

Note.—This is about 1/10th of the daily dose necessary to prevent development of macroscopic scorbutic lesions in a young guinea-pig maintained on a scurvy-producing diet.

(*c*) METHOD OF USE.—The fresh lemon juice used as standard may be decitrated as follows: to the expressed juice, after filtration through muslin, an excess of calcium carbonate is added until effervescence stops. After standing for one hour, the mixture is filtered through a Buchner funnel. The decitrated juice should have a reaction of *p*H about 6 and should be administered to the experimental animal within two hours after filtration.

(*d*) PERMANENCE OF STANDARD.—This international standard shall be regarded as provisional for the next two years.

NOTE ON THE TOXICITY OF IRRADIATED ERGOSTEROL

In view of the toxic effects which have been reported after administration of certain specimens of irradiated ergosterol, this Conference suggests the advisability of testing all preparations of irradiated ergosterol, destined for medicinal use, for toxicity as well as for antirachitic potency.

APPENDIX

MEMORANDUM ON CAROTENE SUPPLIED FOR TESTING ITS SUITABILITY AS A POSSIBLE STANDARD FOR VITAMIN *A*

PREPARATION.—The material provided has been prepared as follows:—Commercial carotene (B.D.H.) was dissolved in benzene and filtered, and the clear solution was poured into a large volume of warm absolute alcohol. The crystallisation was allowed to proceed at 37° C., and the crystals were filtered off at the same temperature. All these operations were carried out in an atmosphere of carbon dioxide. The crystalline material was dried *in vacuo*. Melting point 179° to 180° C. (taken in electrically heated "Berl block").

For distribution into tubes the material was dissolved in benzene at 37° C. to make a 2 per cent. solution; 0·5 c.c. of this solution was run into each of the brown glass tubes in which the material is distributed, the whole process of solution and filling out being again carried out in an atmosphere of carbon dioxide. The filled tubes were transferred to a desiccator containing paraffin shavings and calcium chloride. The desiccator was evacuated and left attached to the pump until the benzene had completely evaporated, and the carotene had been deposited, mainly as a crystalline residue, at the bottom of each tube. When drying was complete, the desiccator and contained tubes were again filled with carbon dioxide, evacuated, and refilled with carbon dioxide. The tubes, before filling, had been drawn out into narrow constrictions to facilitate sealing, which was thus rapidly effected with minimal contamination of the carbon dioxide by air.

Suggestions for Use.—It is assumed that less than the whole contents of one tube (10 mgrms.) will be required for a test. The tube having been opened, the necessary quantity of carotene can be removed with the aid of a fine glass rod or narrow platinum spatula, and immediately dissolved in the chosen solvent. The partly used tube should be enclosed in a test tube of suitable size, which should then be drawn out in preparation for sealing. The test tube and contained specimen tube can then be refilled with carbon dioxide in the vacuum desiccator, as above described, removed, quickly sealed, and preserved in a cold, dark place until again required. The prepared solution, if it is to be used for several tests, should be preserved from light and oxygen by similar or equivalent precautions.

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