REVIEW ARTICLE

THE INTERNATIONAL STANDARD FOR VITAMIN D

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THE International Standards for the determination of the different vitamins are part of the indispensable equipment of the laboratories in which this work is carried out. Though the standard materials themselves are so well known, the amount of labour which has been devoted to their creation and adoption and the infinite care which has been taken to ensure their suitability, accessibility and integrity are not so well known or appreciated. The adoption of a crystalline preparation of vitamin D_3 to serve as the International Standard for Vitamin D affords a suitable opportunity for outlining the nature of this work, the importance of which to scientific research, medical practice, the problems of nutrition, and the international exchange of ideas, results and materials is now well recognised.

In this country the Therapeutic Substances Act (1925) was passed to deal with the administrative control of those substances of therapeutic value, the potency of which could not be adequately tested by chemical and physical means. Such substances are of many different kinds and, since their potency could only be determined by biological tests carried out in strict comparison with a standard preparation, the necessity for these was fully recognised; and, since the same materials and medicaments are used in many lands, it was obvious that the standards for their measurement should have an International standing and application. International Standards for the assay of antitoxins, insulin, pituitary (posterior lobe) powder and digitalis had been established before the similar work for vitamins was begun. Very few firms, however, were equipped to carry out the necessary biological determinations on their products of therapeutic application. Accordingly, to meet the demand for such tests, the Pharmaceutical Society of Great Britain established its Pharmacological Laboratory in January, 1926. Within a very few months requests for the determination of vitamin D in cod-liver oils were made by firms who realised that the vitamin D content of these oils varied within wide limits and who knew also that it was possible to extract the vitamin D of the oil without detection of the loss, and the Pharmaceutical Society after consulting the Accessory Food Factors Committee of the Medical Research Council set up a Vitamin-testing Department within the Pharmacological Department. The writer of this review was appointed to take charge of it and in January, 1927, the available laboratories had been put into a condition suitable for the satisfactory performance of the tests. Actually, the task of devising a reasonable uniform diet for albino rats—on which they would grow, reproduce and lactate and, moreover, would yield a progeny having minimum reserves of vitamins A and D, so that they could be used at an early age for the comparative tests—proved

to be of paramount importance and has so remained throughout the years. The account of the work in this field is described elsewhere[1]. Suffice to say here that the diet found to be satisfactory by 1928 has, with only minor modifications such as the exigencies of the times demanded, proved to be satisfactory up to the present time.

Up to the time of setting up this laboratory, tests had been really only qualitative, and the relative vitamin potencies of foods indicated by the signs +, ++, +++, or ++++, according to the amounts, roughly, of the different foods required for bringing a certain amount of reaction which was known to be extremely variable and which could only be controlled by comparative tests in relation to Standard preparations; and it was not until 1931 that the extremely important step of establishing International Standards for the assay of vitamins A, B_1, C and D was taken by the Permanent Standards Commission of the Health Organisation of the League of Nations[2].

By 1926 certain criteria for measuring the response of an animal to vitamin D were already known and considered to be adaptable for use as the basis for quantitative assay; of these, the ash content of the bone (generally expressed as the percentage ash of the dry, fat-extracted bone) had been used in various laboratories for comparing different cod-liver oils; and the " line test " had been used for the same purpose and, particularly in Steenbock's laboratory, for following the antirachitic activation of foods, food-constituents and cholesterol by irradiation with ultra-violet rays. In his laboratory the amount of healing, indicated by the width of the line of calcification, was assessed in five grades, 0, +, ++, +++, ++++, the last indicating complete healing. It was decided to adopt the " line test " in our own laboratory as it was quicker and less laborious than the " ash content of the bone " method. While we were raising our colony of rats to the right condition for these tests, we used litters drawn partly from our own colony and partly from those of other laboratories. The inconvenience of this was offset by the value of the information it gave on how greatly rats obtained from different colonies and subjected to the same treatment may differ in their response to the same dose of vitamin D. Ample evidence was also obtained of the difference in response of different litters from the same colony. All this afforded proof—if any were needed—of the necessity to provide for the comparative tests some stable preparation of vitamin D which could be used as a standard of reference and tested simultaneously with every vitamin D determination.

About this time Rosenheim and Webster[3] were irradiating ergosterol with ultra-violet light and getting intensely active antirachitic material as shown by tests on rats very similar to the line test, the width of the line of healing in their experiments being measured by X-ray examination of the bones. They had determined that a certain degree of activity could be produced in a solution of ergosterol by irradiating it for a certain time, that further irradiation for some time did not increase or decrease the activity but that finally the activity did fall to zero. They were therefore able to undertake to make a solution of activated ergosterol, and by

using the same conditions of irradiation, strength of solution, etc., they could reasonably expect to make a similar solution of equal potency at any future time. Such a preparation would be very nearly ideal for a standard of reference, the only point left to be determined being the stability of the preparation. This could only be done in the course of time or by expedited experiments in which a solution was subjected to more stringent conditions of temperature, etc., than it was likely to encounter during its normal use. Such experiments were carried out and no loss of activity found[4]. In February, 1927, a particular sample of irradiated ergosterol in olive oil, containing the equivalent of 0·0001 mg. of the original ergosterol in 1·0 mg. of oil, was prepared by Mr. T. A. Webster of the Rosenheim and Webster team at the National Institute for Medical Research, London and adopted by the Pharmaceutical Society as its standard of reference. The unit of activity adopted was that contained in 1 mg. of this oily solution. This was the first standard of reference for any vitamin to be adopted in any country[5].

The first way in which the standard was used was generally to give to three rats of a litter daily doses of, say, 0·00001, 0·00002, and 0·00005 mg. of original sterol (i.e., 0·1, 0·2, 0·5 units) respectively, and to three other rats of the same litter doses of the oil under test in the same proportions. Comparisons were then made between the responses of rats on corresponding doses, and an estimate of potency made by the consideration of all the possible comparisons without the litter. Several litters were used in exactly the same way and an average of the several estimates calculated.

Later, in 1931, Dyer[6] worked out a method of making the estimation more quantitative. He selected a series of cut and stained bones of rats which had been used in " line tests " which showed graded stages in healing, the widths of the lines chosen being as nearly proportional to the figures 0 – 6 as could be judged by the naked eye, 0 representing no healing and 6, complete healing. Thus it became possible to assign a numerical value to the healing of each rat and then total and average the healing of any number of rats all of which had received the same dose. He then constructed a curve of response to graded doses of vitamin D. 15 litters of 7 rats each were prepared in the usual way. In each litter different rats received the following daily doses of vitamin D:— no dose, 0·0625, 0·125, 0·250, 0·5, 1·0, 2·0 units. The responses of the 15 rats receiving each dose were averaged and plotted against the dose given. The curve of response was used in future tests to compare the average response of a group of rats receiving one dose of, say, cod-liver oil with that of another group receiving one dose of the standard, by finding the abscissa of the curve corresponding with the healing of each group and calculating the potency from the respective abscissae. Further work, however, showed that the curve of response to vitamin D was logarithmic in shape, whether the ash content of the bone or the line of healing was the criterion used. The slope varied from time to time and, accordingly the method of testing 2 doses of Standard against 2 doses of test substance was adopted, the average slope of the 2 curves thus obtained in each

assay being used for the calculation of potency and of error of that assay.

Meanwhile, the need of a standard of reference for vitamin D was being recognised in other laboratories and in other countries. It was obviously desirable that workers in different laboratories should use the same standard and unit of activity. A second larger batch of irradiated ergosterol in olive oil had been prepared in 1928 at the National Institute for Medical Research under conditions similar to those of the first batch, part being kept at $-4°$ to 0°C. and part at room temperature. A further large batch was prepared early in 1929 and extensive biological comparisons made between it and the second and first batches. It proved to have the same activity as the first batch and also the same as the part of the second batch which had been kept at the very low temperature. The part of the second batch which had been kept at room temperature, however, was very much less active than the third batch and a direct comparison between the two parts of the second batch confirmed the loss of activity of the part kept at room temperature. The Medical Research Council[1] therefore made available a standard of reference of vitamin D and recommended its adoption. It also recommended that the unit of activity should be defined as the antirachitic potency of a quantity of this preparation corresponding to 0·0001 mg. of the ergosterol used in its production. Thus the unit, as far as biological tests could determine, had the same value as the one already used for some years in the Pharmaceutical Society's laboratory. It was of convenient size for laboratory use on test animals; that is to say, the doses required for tests were neither a large multiple nor a small fraction of the unit proposed. The solution for distribution was made up to contain 1,000 units/g. It was ready for issue the following September, and a memorandum giving details for the use of the standard was sent out with the first issue to each laboratory proposing to use it. Thereafter, fresh supplies were sent out every 6 months. Still another large preparation of irradiated ergosterol was made in 1931 and by biological tests it was shown to be indistinguishable in antirachitic activity from the previous one.

The need for standards of reference for other vitamins also had become urgent, not only in Great Britain but also in other countries. In 1931 a conference on Vitamin Standards[2] was held in London at the invitation of the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations and standards of reference were adopted for International use for vitamins A, B_1, C and D.

The 1931 Conference recommended that the Standard solution of irradiated ergosterol at that time issued from the National Institute for Medical Research, London, should be adopted as International Vitamin D Standard for the next two years. If, within that period, it should become necessary, owing to exhaustion of the supply, to replace that solution by a fresh standard, the equivalence should be determined by experts of different countries. It also recommended for adoption as international

unit of vitamin D, the activity of 1 mg. of the International Standard solution of irradiated ergosterol.

It was hoped at that time that a crystalline substance more stable than the solution of irradiated ergosterol was expected to be would soon become available. Bourdillon in this country, Windaus in Germany and Reerink and van Wijk in Holland had each prepared a crystallised substance which they thought was pure vitamin D. The three substances, however, differed in certain physical properties and each worker realised that his product was not pure. Callow purified Bourdillon's preparation by treating it with 3-5-dinitrobenzoyl chloride in pyridine and crystallising the product from acetone. Close examination revealed the fact that the product consisted of two kinds of crystals, one yellow and the other orange. Callow separated them into two groups by means of a pin and obtained 50 mg. of the yellow and 40 mg. of the orange form. He hydrolysed each and found one to be inactive biologically and the other active. He gave the name pyrocalciferol to the inactive form and retained the original name of the mixture, calciferol, for the active substance, which had about twice the antirachitic activity of the original. Meanwhile Windaus showed that his original product (which he now called D_1), really consisted of a molecular compound of lumisterol and a substance which he called D_2. This he sent to Hampstead for comparison with Bourdillon's calciferol and it proved to have the same potency as that substance and the same physical properties. Windaus and Bourdillon concluded that they had at last arrived at the same substance. It has since been called calciferol or vitamin D_2. It is now made on a small manufacturing scale and has been available for some years to replace the standard preparation of irradiated ergosterol, as recommended by the Second International Conference on Vitamin Standardisation. This has, however, never been necessary except on one occasion during the War when the dilution of the Standard was made with an oil which rapidly inactivated it. Reports from various workers that the standard did not appear to have its usual activity resulted in an immediate comparison between that issue and the remains of previous issues in several laboratories. It showed beyond question that that issue was not "up to Standard" and a solution of pure calciferol was made up with a carefully chosen oil and compared by several workers with a fresh dilution of the standard made with the fresh oil. The two solutions proved to be of equal potency and the activity of the original solution has never since been doubted.

The Second International Conference on Vitamin Standardisation[8] held by the Permanent Commission on Biological Standardisation of the League of Nations in London, 1934, recommended that the Standard Solution of irradiated ergosterol prepared at the National Institute for Medical Research, London, and issued as International Vitamin D Standard, should be retained. It also recommended that when the present International solution was exhausted, or if it should become unsatisfactory for any reason, it should be replaced by an equivalent solution of pure crystalline vitamin D in olive oil of such strength that 1 mg.

contained 0·025 μg. of crystalline vitamin D, a definition for which was added. The unit recommended for adoption by the 1931 Conference was to remain unaltered: namely, the vitamin D activity of 1 mg. of the International Standard solution of irradiated ergosterol which had been found equal to that of 0·025 μg. of crystalline vitamin D.

By this time it was becoming more and more evident that irradiated ergosterol had very little antirachitic activity for chicks; hence a determination of the vitamin D potency of a cod-liver oil by comparison with the standard by means of tests on chicks would give a very much larger value for the oil than a similar test carried out on rats. Therefore, the Conference stated that the assay of materials for vitamin D content should be carried out by comparative tests on rats, and their value in International units should be derived from the results of such tests. If other species were employed for these tests, the values could not be expressed in International units.

With regard to the method of determination, the 1931 Conference considered it permissible to use various biological methods of estimation, either prophylactic or therapeutic, e.g., the "line-test," X-ray examination or determination of the bone ash, provided that not less than (and preferably more than) 20 rats were used, one half receiving doses of the Standard and the other receiving doses of the substance under test. The Conference did not consider it desirable to draft detailed procedures for the determinations. Indeed, Dr. R. Gautier[9], now Assistant Director-General to the World Health Organisation and in 1945 Secretary to the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations, wrote at that time (with regard to all biological assays), "When the value of an international unit has thus been given an agreed definition, it has been recognised that the nature and details of the biological method used in making the comparative measurements in units should be left, so far as possible, to the free choice and judgment of the expert conducting the test. Each worker is likely to make the most accurate assays when using a method with which experience and opportunity have made him familiar. Freedom of choice in this matter also affords an incentive to researches aiming at the improvement of existing methods and the discovery of better ones, whereas the adoption of a single method by agreement and the standardisation of its details must tend to stereotype knowledge and hinder progress in this field. There are certain conditions, however, by which the choice of a method of biological measurement must be guided. It should, of course, be capable of yielding results of the greatest practicable precision; but the fact is apt to be overlooked that the most accurately reproducible results are of doubtful value, and may even be misleading, unless it is certain that the method is measuring the therapeutically important constituent of the product under test. In a case where the assay can be assumed to be dealing with a single and uniform active principle, the position is simple. Any biological

test will then be acceptable if it will give an accurate comparative measure of the quantity or proportion of that principle; and provided it so measures the right thing, and measures it accurately enough, the activity on which the measurement depends need have no more relation to the therapeutic action than would the property forming the basis of a chemical determination of the active principle, if such were applicable. The position becomes at once more complicated when several active principles are in question, which may be present in varying proportions, and concerning the relative therapeutic importance of which there is no sufficient ground for decision or differentiation. . . ."

The Second Conference on the Standardisation of Vitamins held in 1934 recommended that "the experience gained in the use of cod-liver oils standardised against the International Standard by means of tests on rats might be devoted to an attempt to elucidate the questions involved in the anomalous action on certain species of different sources of vitamin D." It was Waddell[10] who first threw light on this subject. Reverting to the antirachitic substance generated in cholesterol on irradiation, he found it to be at least as active as the vitamin D of cod-liver oil in the prevention of rickets in chicks, the respective doses being determined by tests on rats, and he concluded that the pro-vitamin D of cholesterol was not ergosterol. Work by Windaus, Lettre and Schenck, Grab, Brockmann, Brockmann and Busse led to the isolation of a substance from the products of the irradiation of 7-dehydrocholesterol and its identification with the vitamin D of tunny-liver oil and halibut-liver oil. This substance was named vitamin D_3 and it was reported to have the same antirachitic potency (40,000 I.U./mg.) for rats as calciferol. A collaborative test to compare the antirachitic potencies of calciferol (vitamin D_2) and vitamin D_3 was organised by the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute.[11]. Workers in 9 different laboratories in Great Britain made the comparison by means of tests on rats, and the relative potencies of the two preparations, as indicated by the different workers, were all so nearly equal to unity that statistical analysis of the result was considered unnecessary. Vitamin D_3 was declared to contain 40,000,000 International units of Vitamin D per gram. Moreover, a comparison of these two preparations on the healing of rickets in children carried out by Morris and Stephenson in Glasgow, on cases of osteomalacia and late rickets by Wilson in India and on a case of parathyroid tetany by Himsworth and Maizels in London failed to show any difference in antirachitic potency and, indeed, offered a certain amount of positive evidence that the two substances were equally potent for human beings. Various other workers had shown that the vitamin D of cod-liver oil (whatever might be its nature) and calciferol were equally potent for human beings. Thus it was possible to adopt vitamin D_3 as a standard of reference for determining the potency of oils intended for rats, chicks or human beings. The possibility of its adoption was to have been included in the Agenda of the Third International Conference

on Vitamin Standardisation planned for September, 1939, but not held at that time on account of the outbreak of war.

Meanwhile the British Standards Institution had adopted a particular sample of vitamin D_3 as a Provisional Standard Preparation for assaying the vitamin D potency of cod-liver oils intended for chick feeding. It was dissolved in pure vitamin D-free olive oil; 1 mg. of the solution contained 0·000025 mg. of the crystalline vitamin, and this amount was adopted as the B.S.I. unit of antirachitic activity. Since rats appear to use vitamin D_2 and vitamin D_3 equally well, 1 unit of the B.S.I. Standard was equivalent to 1 International unit as far as rats were concerned. It was obvious, however, that a cod-liver oil intended for chick feeding must be assayed by tests on chicks, for a cod-liver oil containing added calciferol would have a high value according to tests on rats, but would be less potent when given to chicks. The British Standards Institution drew up a plan[12] for determining the vitamin D content of cod-liver oils by using chicks as test animals and the ash content of the bone as criterion. An example worked out for potency and approximate and fiducial errors was added. The result of such a test, however, could only be expressed in B.S.I. units since the oil was compared with the B.S.I. Standard.

The work carried out on this B.S.I. Standard in many laboratories in Britain, Canada and the U.S.A. proved of the greatest value and paved the way towards the adoption of an International Standard of vitamin D_3. As soon as the World Health Organisation was formed, the International Standards for vitamin A and vitamin D came under review. The story of the vitamin A standard will be told later. Work for this vitamin D standard began in 1946. The Accessory Food Factors Committee of the Medical Research Council and Lister Institute organised, through its vitamin D sub-committee, a large collaborative experiment to investigate the possibility of adopting vitamin D_3 as the International Standard of reference for vitamin D. Several manufacturers were making crystalline vitamin D_3, and samples of this substance were generously contributed by them. The total weight contributed was about 29 g. The physical constants of each sample were determined. The samples were pooled, and the physical constants of the pooled sample were determined. The solution for issue was prepared at the National Institute for Medical Research, London, and was of the same strength as that issued by the B.S.I., viz., 0·000025 mg. in 1 mg. of oil. It was decided at a meeting of the Vitamin D sub-committee to compare the following preparations:—

1. The present International Standard for vitamin D (irradiated ergosterol in olive oil).
2. The new preparation of pooled samples of vitamin D_3.
3. The British Standards Institution Standard for vitamin D_3.
4. A preparation of the purest sample of calciferol obtainable.

The Committee invited laboratories in as many countries as possible to take part in the comparative tests. Eventually workers in Great

Britain, Denmark, Holland, Norway, Sweden, New Zealand, Canada and the U.S.A. did so. It was impossible to find experts able to carry out the comparisons in France, Spain, Portugal, India or South Africa. A most complete and exhaustive series of comparative tests was carried out and investigators in 33 laboratories sent in reports, some concerning tests with chicks, some with rats, some with both. Some even sent in duplicate tests with rats, one to compare results obtained with albino and piebald rats respectively.

The British participants and Dr. J. O. Irwin met to arrange the design of the experiment, and a scheme put forward by N. T. Gridgeman was adopted. It entailed randomisation of litters between doses (x, 2x, 4x) and randomisation of rats within each litter between the four samples. Thus all rats of any one litter had the same size of dose, but of different preparations. Altogether there were to be at least 10 litters on each dose, i.e., at least 30 litters in all, comprising at least 120 rats. Details of procedure (diet, criterion, duration of test, etc.) were left to individual workers. The work in the U.S.A. and Canada was organised by the U.S.P. Committee, who accepted the design planned by the British workers and issued certain instructions to their participants to accord with their own experience. In addition, they issued 2 bottles of cod-liver oil, 1 to be assayed on the assumption that it contained 115 U.S.P. units (International units)/g. and the other as 96 U.S.P. units (International units)/g. Actually these were one and the same oil, but it seemed a good opportunity for investigating the possible influence of "level" of testing on the result obtained.

In all, 54 assays were completed, 29 using rats and 25 using chicks. Dr. J. O. Irwin and his colleagues calculated the results of each test and its fiducial limits of accuracy and the ratios of the potencies (with their fiducial limits) of the various preparations. Their summary[13] is as follows:—

	Result	Fiducial Limits (P = ·95)
(a) New Standard/Old Standard	0·981	0·925 to 1·009
(b) B.S.I. Standard/Old Standard	0·916	0·902 to 0·983
(c) Calciferol/Old Standard	0·933	0·896 to 0·972
(d) U.S.P. Ref. C.L.O./Old Standard...	0·869	0·839 to 1·004
(e) New Standard/B.S.I. Standard	1·071	1·036 to 1·098
(f) U.S.P. Ref. C.L.O./B.S.I. Standard	0·949	0·843 to 0·958

Irwin says, "Thus the B.S.I. Standard and the calciferol are less potent than the Old Standard and the New Standard may be slightly so. The new standard is somewhat more potent than the B.S.I. Standard, while the calciferol and B.S.I. Standard do not differ significantly. The U.S.P. Reference Oil is less potent than the B.S.I. Standard. This could not be concluded from the rat assays only, and so may be due to the presence of a little vitamin D_2 in the oil. The results are remarkably uniform. For (a), (b), (c), the fiducial range is less than 10 per cent., for (e) it is about 6 per cent., for (d) and (f) it is rather greater, but still of a satisfactory order, for a biological test."

A Sub-Committee on Fat-soluble Vitamins of the Expert Committee on Biological Standardisation of the World Health Organisation held a meeting in London in 1949[13], and recommended that the preparation of crystalline vitamin D_3, as described below, at present held at the National Institute for Medical Research, London, should be adopted as the International Standard for vitamin D. This new standard should replace the existing solution of irradiated ergosterol and the latter should be retained as a reference preparation only and not as an International Standard. The Committee also recommended that the International Unit of vitamin D should be the vitamin D activity of 0·025 μg. of the International Standard preparation of crystalline vitamin D_3. The Standard should be issued as a solution containing 1000 I.U. per gram. The properties of the recrystallised vitamin D_3 freshly withdrawn from a sealed ampoule were determined as:—

m.pt. 87° to 89°C. (corr.)

$[\alpha]_D^{20°C.} = +110°$ (ethanol)

$E_{1\ cm.}^{1\ per\ cent.}$ 265 mμ = 490 (ethanol) corresponding to a molecular extinction coefficient of 18,800.

The recommendations of the sub-committee were adopted by the General Assembly of the World Health Organisation held in Rome in June, 1949.

Step by step with the growing use of international standards of reference for assaying the biological potency of therapeutic substances has been the adaptation of existing methods and development of further methods and refinements of methods for estimating the accuracy of assays. The first edition of Fisher's "Statistical Methods for Research Workers" was published in 1925. It has now reached its tenth edition; several other writers have published somewhat similar books and a few mathematicians, such as J. O. Irwin, E. C. Fieller, D. J. Finney and C. I. Bliss, have applied themselves to original investigations in this subject. Biologists have followed the trail these pioneers have blazed and now they know just how much as well as how little faith they may place in their results. The one is quite as important as the other.

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