LXII. THE DETERMINATION OF VITAMIN D.

III. THE STABILITY OF PREPARATIONS OF VITAMIN D.

BY ROBERT BENEDICT BOURDILLON, HILDA MARGARET BRUCE
AND THOMAS ARTHUR WEBSTER.

From the National Institute for Medical Research, London, N.W. 3.

(Received February 5th, 1932.)

THE stability of preparations of vitamin D is of importance, both for the commercial products now on the market, and especially for the standard solutions which are widely used in estimations of the activity of other preparations. At present the International Standard adopted at the London Conference in 1931 consists of a solution of irradiated ergosterol in olive oil, and although this is likely to be replaced within two or three years by preparations of the crystalline solid vitamin recently isolated, sub-standards of solutions in oil will probably continue in use for many years.

Although it is well known that cod-liver oil retains its antirachitic activity for a long time, little work has been done on the stability of artificially prepared vitamin D. Steenbock and Black [1925] found that irradiated olive oil lost all activity in nine months when freely exposed to laboratory air, but retained its activity when kept in a stoppered bottle for the same period. We are, however, aware of no detailed work on the stability of irradiated ergosterol in oily solutions, and we therefore undertook the following tests.

Owing to the considerable stability of vitamin D at room temperature, and the inaccuracy of current methods of estimation, tests of stability are difficult and laborious. Hence the tests described in this paper are admittedly incomplete. They do, however, show that preparations cannot be relied upon to retain their activity for a year at room temperature, and they give a rough guide to the probable keeping qualities of standard and other preparations. The majority of these tests were done at the request of the Vitamin D Subcommittee of the Accessory Food Factors Committee of the Lister Institute and Medical Research Council, in preparation for the International Conference of 1931.

TYPES OF TEST EMPLOYED.

A direct test of the stability of vitamin D can only be made by studying the effect of one sample on large numbers of animals at long intervals of time. However, this procedure is slow and inaccurate, owing to the varying sensitivity of successive stocks of animals to this vitamin. We have therefore used, in addition, the following indirect methods.

(a) *Comparison of the strength of similar preparations made at widely different dates.* If it were possible to use an identical procedure in the preparation from ergosterol of two samples of vitamin D made at widely different dates, a comparison of the two samples could be relied upon to show any change that had occurred in the first sample during the interval between the preparation of the two. Unfortunately, it is impossible to secure constant sources of ultra-violet radiation, and it would therefore be unsafe to assume that successive preparations were initially of exactly equal activity (except in so far as this could be controlled by biological tests). Hence tests of this type can only be relied upon to show gross changes (unless made on the pure isolated vitamin).

(b) *Determinations of the temperature coefficient of decomposition of the vitamin.* Tests at elevated temperatures show decomposition of an extent and velocity that can easily be measured. By plotting a curve of results at higher temperatures, and extrapolating the results to lower temperatures, it is possible to form a rough estimate of stability at 0° . This method has been used for insulin by Krogh and Hemmingsen [1928] and for oxytocin by Gaddum [1930].

(c) A further series of tests was made to determine the effect of adding to a standard solution large quantities of such impurities as might be expected to occur in small quantities in preparations of the vitamin. If impurities of a destructive type were likely to be present in standard solutions, no general tests of stability would be of value, and each solution would have to be tested separately. Fortunately our results show no such necessity.

TECHNIQUE OF TESTS.

The estimations of antirachitic activity were carried out by the radiographic method described in detail in a report already published [Bourdillon *et al.*, 1931]. Albino rats (Wistar strain) of age 3 to 4 weeks were given a modification of Steenbock's rachitogenic diet for 4 weeks. After 2 weeks on this diet, the rats were anaesthetised with ethyl chloride, and radiographs were taken of their right knees, in order to determine the degree of rickets produced. During the second 2 weeks, daily doses of vitamin D dissolved in oil were given by capillary pipettes into the animals' mouths. The animals were then killed, and again radiographed. The radiographs of the rats taken after dosing were compared with a standard scale of healing divided into 12 stages, and the results averaged as described in the report referred to. In the tests at varying temperatures half the rats in each litter were dosed with the heated solution, and half with some of the same solution that had been stored at -4° .

ESTIMATION OF ERRORS IN THE BIOLOGICAL TESTS.

An estimate of the accuracy of these tests was made by examining the standard deviations for 1000 of the rats used during the period covered by this work, and showed that, for tests based in each case on 20 pairs of rats, the

error corresponding to twice the standard deviation, *i.e.* the error expected to be reached or exceeded only once in 22 tests, was *plus* 25 % or *minus* 21 %. The probable error (or error expected once in 2 tests) is 8 %. These values are in close agreement with those calculated for earlier tests in the report referred to above.

Most of the tests described in this paper were done with approximately 20 pairs of rats for each test. In the cases where fewer rats were used the number is specified in the text or placed in brackets directly after the figure giving the result.

NATURE OF PREPARATIONS SUBJECTED TO TEST.

The following preparations were used.

(a) *Standards I to V.* These all consisted of 0.01 % solutions in olive oil of the irradiated ergosterol. These were prepared by dissolving ergosterol to 0.1 % solution in alcohol (Standard III) or ether (Standards I, II, IV and V) and exposing the solution in a closed vessel filled with nitrogen to unfiltered radiation from a mercury vapour lamp until 40 to 70 % of the ergosterol had been transformed. Standards I, II and III were irradiated in silica cells with plane sides enclosing a layer 1 cm. thick. Standard V was irradiated while flowing rapidly through a water-cooled spiral of silica tubing of bore 4 mm. After irradiation the solutions were evaporated at a low pressure, at or below 40°, to a small bulk. Some olive oil was then added, and evaporation continued in order to remove as much solvent as possible. The concentrated solution was then diluted with commercial olive oil. The dates of preparation were as follows: Standard I, February 1927; Standard II, April 1928; Standard III, February 1929; Standard IV, November 1928; Standard V, January 1931. The tests were mostly done during 1931. Standard III was part of the solution issued during 1930 as the Medical Research Council Standard, and during 1931 as the International Standard. Standard V is the solution now under International trial for adoption when the stock of Standard III becomes exhausted. Except where otherwise stated (as in Table I) all solutions were kept in stoppered bottles at about - 4° from preparation until the date of test.

The solid preparations referred to as "impure" or purified crystalline vitamin D are described in a paper previously published [Askew *et al.*, 1932]. They are now believed to have contained 50 %, and over 90 %, of the pure vitamin respectively.

DIRECT TESTS OF ONE SOLUTION AT DIFFERENT PERIODS.

For this test samples of Standard IV were withdrawn from cold store every 2 or 3 weeks during a period of 21 months, and used as a standard during routine testing of vitamin D. If the general sensitivity of the rat stock had remained constant, the average degrees of healing of rickets produced during successive periods would have served as a measure of the stability of the

standard. However, in spite of careful control of the diet of the test rats, and that of their parents, the stock showed a large and irregular variation in sensitivity. The effects of this variation were partly, but only partly, obviated by selecting for this test only rats which had shown a full and satisfactory development of rickets (approximately 1.6 mm. of wholly uncalcified space at the head of the tibia) before dosing with vitamin began. Fig. 1 shows the results for successive groups of 20 rats thus selected, all dosed with one solution during 1929 and 1930. The abscissae show the dates. The ordinates show the average degree of healing per group of 20 rats, expressed on the right of the figure as scale numbers, and on the left as numbers proportional to the apparent activity of the solutions, taking that of the initial activity for the larger dose as 100. The upper curve shows the results for doses of approximately one International Unit per day and the lower one for doses of half a unit. The graph shows irregular variations large enough to indicate the impossibility of

Fig. 1. Direct test of one solution during 21 months. Each point on the curves represents the mean result for 20 rats.

checking the stability of vitamin D by this method, unless a very long period of time is available, or unless a stock of rats of more constant sensitivity can be obtained. It can only be said that the average degree of healing produced at the end of the period of 21 months is slightly higher than that at the beginning, so that no great deterioration of the standard can have occurred.

We do not know the "errors of sampling" in this test, since our previous estimate refers to comparisons between litter-mates, and does not include the interlitter deviations which also have influence in this case. It is, however, clear from the similarity of the two curves, and from other similar tests [Bourdillon *et al.*, 1931], that the major variations are due to changes in the general sensitivity of the stock. We have as yet found it impossible to avoid such changes, in spite of attempts to keep the diets constant.

INDIRECT TESTS.

(a) *Comparison of similar products prepared at long intervals of time.*

An indirect test of stability was made by comparing the activities of Standards I, II and III. Although the conditions of preparation of these three solutions were not identical, there is reason to think that the initial activities of the three were roughly equal (*i.e.* probably within $\pm 50\%$). The activities of these solutions were compared by Dr Coward and Miss Key at the Labora-

tory of the Pharmaceutical Society, and by Miss Hume and Miss Pickersgill at the Lister Institute, and one comparison was made in this laboratory. The results are given in Table I.

Table I.

Test No.	Date of test	Observer	Method of test	No. of pairs of rats used for test	Age of standard preparations in months			Temp. at which standards had been stored	Result
					I	II	III		
1	Nov. 1929	Hume and Pickersgill	% ash in bones (prophylactic test)	12	—	19	10	0°	III = II
2	Apr. 1930	Hume and Pickersgill	% ash in bones (prophylactic test)	10 rats on II 20 rats on III	—	24	15	0°	III = II
3	Oct. 1929	Coward and Key	Line test (curative)	17	32	—	9	Room temp.	III/I = 84/100
4	Oct. 1929	Coward and Key	% ash in bones (prophylactic test)	17	32	—	9	Room temp.	III = I
5	Apr. 1930	Coward and Key	Line test (curative)	24	—	24	15	Room temp.	III/II = 100/50
6	Apr. 1930	Coward and Key	% ash in bones (prophylactic)	21	—	24	15	Room temp.	III/II = 100/50
7	Feb. 1929	Webster	Radiography (curative test)	12	—	9	0	- 4°	III = II

Tests 1, 2 and 7 show that no appreciable change occurred in Standard III during 9 months at 0° (unless it was initially stronger than II). Tests 3 and 4 show that Standard I did not deteriorate appreciably in 23 months at room temperature (unless it was initially stronger than III). Tests 5 and 6, however, suggest that when kept at room temperature, Standard II lost at least half its activity in 24 months.

(b) *Tests of deterioration at different temperatures.*

Consideration of the effects of temperature was first suggested by the experiment of Dr Coward given in Table I, in which one sample of Standard II showed a slow but definite deterioration at room temperature. It was not

Table II. *Percentage fall in activity of vitamin D preparations after exposure to different temperatures.*

0.01 % solution of solid products in olive oil.									
Temp.	Duration of heating (days)	Irradiated ergosterol in stoppered bottles				Impure crystalline vitamin D in stoppered bottles		Purified crystalline vitamin D (calciferol) in evacuated ampoules	
		Standard III		Standard V					
		% fall	Velocity constant, <i>k</i>	% fall	<i>k</i>	% fall	<i>k</i>	% fall	<i>k</i>
Room temp. approx. 18	43	9	—	—	—	—	—	—	—
	83	0	—	—	—	—	—	—	—
	321	51	0.000965	—	—	—	—	—	—
40	66	33	0.002634	—	—	—	—	—	—
60	23	53	0.01430	—	—	—	—	—	—
	42	73	0.01354	—	—	—	—	—	—
80	10	56	0.03565	—	—	—	—	—	—
100	3	37	0.06690	—	—	—	—	—	—
	5	53	0.06558	25	0.025	27*	0.0273	43	0.0488
	10	75	0.06021	50	0.030†	51	0.031	59	0.0387

* 16 pairs.

† 11 pairs.

certain whether the change in this sample was due to true instability at 20°, or to slow oxidation. It was, however, evident that a study of deterioration at higher temperatures was likely to give a rough guide to the stability at lower temperatures, and the series of tests shown in Table II was undertaken.

A further series of tests was made on solvent-free crystalline preparations with the following results. At room temperature a sample of impure crystalline vitamin showed no evidence of deterioration after 44 days in dry air or after 56 days *in vacuo*. At 150° a sample of purified crystalline vitamin (calciferol), in evacuated ampoules, gave the following results:

Duration of heating: hours	1	2	3.4	6	8
% fall in activity ...	25	45	63	83	90
<i>k</i> ...	3.00	3.11	3.05	3.08	3.00
					Mean 3.05

These results are of interest in three respects.

(1) The decomposition of any one preparation at one temperature closely follows the unimolecular law, as is shown by the values of the velocity constant $k = 1/t (\log a - \log (a - x))$ where t = time in days, $a = 100$, and x = percentage of activity destroyed. These values are closely concordant at 150°, and fairly concordant for Standard III at 100°, and at 60°. The values for the other specimens at 100° are less concordant, but reasonably good, in view of the errors of biological testing. It follows that, if the reaction is bimolecular, the second reactant is present in considerable excess. In view of the low concentration of the vitamin such a possibility is not in itself improbable. However, the purest specimen of vitamin that we can obtain shows a rate of decomposition in a fused state *in vacuo* at 150° that does not differ greatly from the value expected from observations on a dilution of 1 in 10,000 in olive oil at lower temperatures, and this makes it very probable that the main reaction is truly unimolecular. Were it bimolecular the change of concentration should cause a very great change in velocity.

(2) The variation between different samples is appreciable, and exceeds the error to be expected from the biological tests. The causes of this variation are unknown, but variations in the different samples of olive oil, or in the relative proportions of the mixed irradiation products, may be responsible for the observed effect by causing subsidiary reactions. Further, the presence of varying volumes of air in the bottles below the stoppers might easily be of importance, since 0.07 cc. of air at 760 mm. and 0° would be sufficient to oxidise the whole of the vitamin in one 5 cc. bottle. To test this possibility a solution of purified vitamin was sealed in evacuated ampoules, after careful removal of dissolved gases by a rotary pump. However, this test showed a rate of decomposition closely similar to the other tests.

(3) The temperature coefficient of the velocity constants is of much interest. Fig. 2 shows the logarithm of the velocity constants for oily solutions plotted against the reciprocal of the absolute temperature. The mean result at each temperature is marked by a cross, the enclosed cross showing the result of test 5, Table I. The points deviate seriously from a straight line owing to

some unknown factor, possibly the side reactions already suggested as a possible cause of variations. The continuous line shows the most probable values for extrapolation, and suggests that at 0° 50 % of the vitamin would be destroyed in 3.6 years. The irregularity of the points allows of some latitude in extrapolation, but even by neglecting altogether one or other of the two lowest points, it is only possible to extend the half life period to 5½ years or to

Fig. 2. Velocity of decomposition at different temperatures.

shorten it to 2.6 years. Hence 3.6 years can be taken as the probable time for 50 % destruction. This period is short enough to indicate grave difficulty in maintaining a permanent standard of the vitamin, and it is perhaps fortunate that a crystalline product which is believed to be pure, and of a reproducible activity, is now available. However, the extrapolation may be seriously at fault, owing to the fact that olive oil freezes at about 10°. There is some reason to think that decomposition of the vitamin occurs, or is accelerated, at the surface of contact with the glass containing vessels. Thus, *in vacuo* at 100° very small samples of irradiated ergosterol lose their activity much more quickly than larger samples. If decomposition is due to such a surface effect, or to oxidation, the enormous rise in viscosity of olive oil between 20° and 0° would stop the reaction almost entirely, and it is therefore not impossible that the vitamin is stable in oily solution for many years at 0°.

The value of μ calculated from the (Arrhenius) equation

$$\ln k_1 - \ln k_2 = \mu/2 (1/T_2 - 1/T_1)$$

(where k_1 and k_2 are the velocity constants at absolute temperatures T_1 and T_2) varies rather widely, from 7750 to 17,340, at different parts of the curve. The mean value calculated from the graph is 11,800, which is of the usual order of magnitude. It may be noted that if $\log k$ is plotted directly against temperature a somewhat similar graph is obtained from which the mean temperature coefficient of the velocity constant per 10° is 1.74 between 20° and 100°.

(c) *Effects of impurities on stability.*

1. *Oxygen.* The effect of oxygen present during preparation of the vitamin is still uncertain. Tests show that oxidation products can accelerate decomposition *in vacuo* at 150°, and it is possible that the same is true for oily solutions at 18°. However, if such acceleration occurs, it is not excessive, since the Standards I and II mentioned above were prepared without complete exclusion of oxygen.

The effect of air on oily solutions of vitamin was tested as shown in Table III. These tests show that oxidation at room temperature is slow, but can be serious. In this connection it should be observed that in one of the cases of deterioration at room temperature already quoted (Table II) the bottle concerned was opened once only during the test, and in the other case (Table I, Standard II) the bottle was not opened at all until tested.

Table III. *Effect of air on Standard Solution III.*

Temperature	Duration of exposure days	No. of pairs of rats used	Conditions of exposure	Activity expressed as % of original
Room temp.	108	20	Exposed to air in a layer at the bottom of a beaker	75
„	305	20	Exposed to air in a layer at the bottom of a beaker	Less than 10
„	84	10	5 cc. in a bottle with stopper removed	107
-4°	50	11	5 cc. in 50 cc. stoppered bottle, containing air above solution	112

2. *Effect of addition of reagents which might possibly be present as contaminants.* These tests were made on 30 cc. samples of Standard V, kept at 40° for 29 days in full stoppered bottles. The results are only rough approximations, since the number of rats used, 3-5 pairs per test, was too small for accuracy. They suffice, however, to show whether gross deterioration did or did not occur.

Destruction of not less than 75 % of the original activity was caused by the addition (in each case to a sample of 30 cc.) of either (a) 0.2 cc. of a saturated solution of sulphur in benzene, or (b) 20 mg. of flowers of sulphur (arsenic-free) for 20 days, or (c) 0.4 cc. of concentrated sulphuric acid. On the other hand, no deterioration was detected after the addition of 0.6 cc. 25 % aqueous sulphuric acid; 1.9 cc. 40 % aqueous oleic acid; 0.2 cc. 0.880 ammonia solution; 0.7 cc. *N*/5 NaOH solution; 0.2 cc. 20 % aqueous KNO₃ solution; pieces of metallic silver, copper, lead, steel, and calcium; 0.1 cc. acetone or acetaldehyde; 0.1 cc. perhydrol (H₂O₂).

Similar tests, also at 40°, on samples of Standard V, diluted with two different brands of commercial "olive oil" bought in small bottles, showed no serious deterioration in the 29 days of the test, but a third sample showed an apparent loss of 37 % in activity, in a test based on 10 pairs of rats. This fall is within the margin of possible error for that number of rats, but is large

enough to be suspicious. In view of the commercial importance of oily solutions of the vitamin, tests with other oils than olive oil seem desirable.

The results of these experiments indicate that most of the inorganic or organic impurities likely to be present in small quantities in solutions of irradiated ergosterol cause no great reduction in stability, with the exception of sulphur. It appears important to avoid this impurity in such preparations, and the possible danger from the use of vulcanised rubber in tubing or stoppers should be noted.

SUMMARY.

Tests of the stability of vitamin D in solution in olive oil show that such solutions may deteriorate seriously if kept at room temperature for periods of a year or more. Deterioration at 0° has not been detected in the time available. Extrapolation from results at higher temperatures suggests that preparations at 0° may lose half their activity in about 3 years, but there is some reason to think that the solidification of the oil has a preservative effect, and that the stability at 0° may be greater than the experiments suggest.

The addition of various common substances likely to be present as impurities has no very great effect, with the exception of sulphur, which causes rapid deterioration.

We wish to express our thanks to Miss E. M. Hume and Dr K. H. Coward for permission to reproduce their results, and to Miss C. Fischmann and Mr F. Askew for assistance in this work.

REFERENCES.

- Askew, Bourdillon, Bruce, Callow, Philpot and Webster (1932). *Proc. Roy. Soc. Lond.* B **109**, 488.
Bourdillon, Bruce, Fischmann and Webster (1931). *Sp. Rep. Ser. Med. Res. Coun. Lond.*, No. 158.
(H.M. Stationery Office.)
Gaddum (1930). *Biochem. J.* **24**, 939.
Krogh and Hemmingsen (1928). *Biochem. J.* **22**, 1231.
Steenbock and Black (1925). *J. Biol. Chem.* **64**, 266.