CLXXXVIII. THE QUANTITATIVE DETERMINATION OF VITAMIN D BY MEANS OF ITS GROWTH-PROMOTING PROPERTY.

BY KATHARINE HOPE COWARD, KATHLEEN MARY KEY
AND BARBARA GWYNNETH EMILY MORGAN.

*From the Pharmacological Laboratories of the Pharmaceutical
Society of Great Britain.*

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A CERTAIN amount of distrust has sometimes been felt for those methods of estimation of vitamin D in which the basal diet of the rats either had an abnormally high calcium, low phosphorus content (*e.g.* the McCollum diet 3143 and the Steenbock diet 2965, in both of which the ratio Ca:P is about 4:1), or was devoid of various essentials for normal nutrition (*e.g.* the Pappenheimer diet on which rats do not grow). Dr J. H. Burn suggested that it should be possible, with an adequate basal diet, to use the growth-promoting property of vitamin D [Steenbock, Nelson and Black, 1924] as a means of estimating that vitamin with a degree of accuracy equal to that obtained in the estimation of vitamin A by means of its growth-promoting property [Coward, Key, Dyer and Morgan, 1930; 1931; Coward, 1932]. We therefore decided to use the same basal diet as that used in our vitamin A determinations but, instead of giving a supplement of vitamin D throughout the preparatory and test periods and graded doses of vitamin A during the test period, we gave a supplement of carotene (for vitamin A) throughout the preparatory and test periods and graded doses of vitamin D during the test period. We found that the rats ceased to grow on the diet rich in carotene but free from vitamin D in varying lengths of time, and resumed growth on being given vitamin D. The response was graded to the dose of vitamin D given. We thus had a method of estimation of vitamin D in which the basal diet of the animals supplied all needs except that for vitamin D. It is thus free from the objections sometimes made to the diets in general use for vitamin D determinations. While these objections still seem to be theoretically sound, they do not appear to be serious from the practical considerations of vitamin D estimations, for we have obtained the same results in estimating the vitamin D content of substances by using the line test and the growth test.

METHOD.

The rats used in these experiments were pure-bred albinos of about 30 g. weight, from our own colony. The members of each litter were kept together

in one cage, bedded on sawdust and given tap water and the basal diet *ad lib.* The basal diet consisted of:

Caseinogen, light white (B.D.H.)	...	...	15 %
Dextrinised rice starch	...	...	73 %
Dried yeast	...	...	8 %
Salt mixture (Steenbock's 40)	...	...	4 %

Each rat was given a dose of carotene in olive oil three times a week throughout the whole experiment. During the preparatory period (*i.e.* the time taken for the rats to exhaust their reserves of vitamin D) they were weighed twice a week. As each one became steady in weight it was placed in a separate cage for the test period of 5 weeks. Weighings were then made once a week.

Two experiments have been carried out. In the first the carotene was given as 0.02 mg. (and later as 0.04 mg.) daily of the light petroleum-soluble fraction of dried carrots. It was dissolved in olive oil so that 0.02 g. of the solution (the amount of one drop delivered from our dropping tubes for doses of cod-liver oil) contained the required dose. In the second, crystalline carotene (0.04 mg. in 0.02 g. olive oil) prepared by ourselves, or later supplied by the British Drug Houses, Ltd., was used. This was not easily soluble in olive oil but the insoluble part made a suspension satisfactory enough for the purpose of this experiment.

RESULTS.

(a) *Behaviour of the rats on the basal diet alone.*

Some of our rats grew very little after being given this diet. They did not, however, decline in weight and it was found to be quite safe to keep them on the basal diet for at least a month before giving them doses of vitamin D. Other rats increased 20 to 30 g. or even as much as 60 to 70 g. before becoming steady in weight and several had to be discarded at about the weight of 110 g. as too heavy for use in the test. It was evident that the reserves of vitamin D in the young rat varied in different individuals and in different litters. Moreover, none of the rats grew as rapidly as those in the preparatory periods of a vitamin A test, nor did they decline rapidly and die when their reserves were exhausted. Certain individuals which were given no dose of vitamin D remained practically level in weight for many weeks, and died without any great loss in weight. Occasionally a rat would resume growth without any apparent cause. In fact the behaviour of these rats recalled vividly the behaviour of rats in those early vitamin A tests which were made before vitamin D was recognised as a growth-promoting substance and added to the basal diet used in vitamin A tests. In those rats, almost certainly cessation of growth had been due to a lack of vitamin D rather than to a lack of vitamin A and growth had been resumed only when the test material contained vitamin D.

(b) *Response to doses of vitamin D.*

Occasionally the response to the daily dose of vitamin D was unnoticeable during the first week, but generally it was immediate and continued smoothly throughout the test period. This was prolonged for 8 weeks in the early part of the work, but later was cut down to 5 weeks. Doses of 0.01, 0.05, 0.1, 0.2, 0.25, 0.4, 0.75 and 0.8 unit of vitamin D daily were tested, each dose being contained in 0.02 g. olive oil. These have given responses graded logarithmically to the dose. That is to say, the mean increases in weight of the groups of rats plotted against the logarithms of the doses of vitamin D given fall practically on a straight line. The calculation of the straight line most accurately fitting these observations gives the equation of the curve of response between mean increase and dose [Fisher, 1930].

The curves are:

$$y = 92.82 + 30.02 \log x \text{ (bucks),}$$

$$y = 63.14 + 22.74 \log x \text{ (does),}$$

where y = the mean increase in weight in g. of the groups of rats,

and x = the dose of vitamin D in units.

In this experiment carotene in a daily dose of 0.04 mg. in 0.02 g. olive oil was supplied during the whole of the preparatory and test periods.

Fig. 1. Curves of response to doses of vitamin D of rats which had ceased to grow on a diet containing all factors known to be essential to growth (including a complete salt mixture) but lacking in vitamin D. The curves were determined by calculating the best straight line through the points relating mean increases in weight to the logarithms of doses of vitamin D given:

- × = Mean increases in weight of groups of bucks in response to doses of the international standard preparation of vitamin D.
- = Mean increases in weight of groups of does in response to doses of the international standard preparation of vitamin D.
- ⊗ and ⊙ = Mean increases of groups of bucks and does respectively in response to doses of a commercial sample of irradiated ergosterol already assayed by the line test.

Tests of the curves.

A commercial sample of irradiated ergosterol in maize oil was estimated in this laboratory (and also in two others) by means of the line test, to contain 2,000,000 units of vitamin D per g. Before the estimation was completed, however, we had begun to test a dose of 0.0005 mg. of the solution by means of the growth test. When we realised the exceedingly high potency of the preparation, we began to test a lower dose, 0.00001 mg. of the solution. The results are summarised in Table I B and the mean increases in weight have

Table I.

Dose of vitamin D units	No. of ♂ rats sur- viving 5 weeks	No. of ♂ rats dead in 5 weeks	Mean increase in wt. in 5 weeks g.	$\sigma = \sqrt{\frac{\sum d^2}{n-1}}$	No. of ♀ rats sur- viving 5 weeks	No. of ♀ rats dead in 5 weeks	Mean increase in wt. in 5 weeks g.	$\sigma = \sqrt{\frac{\sum d^2}{n-1}}$	Average increase* g.
(a) 0.04 mg. carotene as source of vitamin A:									
A { 0.01	0	0	—	—	2	1	11.0	1.41	11.0
0.05	5	2	42.4	14.37	5	0	39.8	3.12	41.1
0.1	5	2	70.8	7.01	6	0	37.5	12.98	54.15
0.2	5	0	83.6	14.37	5	1	52.0	5.20	67.8
0.25	1	1	75.0	—	2	0	54.5	4.95	64.75
0.4	5	0	78.4	25.97	6	0	63.1	15.69	70.75
0.75	1	1	88.0	—	1	0	51.0	—	69.5
0.8	4	0	84.75	9.22	5	2	54.8	9.42	69.77
10.0	2	0	84.0	36.77	1	0	57.0	—	70.5
B { 0.00001 mg. I.E. (=0.02 unit)	1	0	38.0	—	6	0	20.2	3.19	29.1
0.0005 mg. I.E. (=1.0 unit)	5	0	104.2	12.21	6	0	53.3	5.97	78.75
(b) 0.04 mg. light petroleum-soluble fraction of dried carrot as source of vitamin A:									
C { No dose	4	3	17.25	8.35	3	5	0	8.54	8.625
0.01	6	1	3.1	9.87	2	0	5.5	4.95	4.3
0.02	5	1	9.8	7.70	7	0	5.7	3.38	7.75
0.05	6	0	10.7	4.86	4	1	14.5	7.23	12.6
0.1	7	1	15.0	12.73	7	2	24.6	12.23	19.8
0.5	7	1	41.4	21.42	2	1	40.5	0.71	40.95
1.0	6	1	36.0	23.70	3	0	36.7	11.85	36.35

* This is the simple average of the values obtained from the bucks and does in each group. It seemed unsound to calculate the weighted means in the tests where large doses of vitamin D were given and the bucks made much greater increases in weight than the does.

Table II.

Dose of solution of I.E. mg.	No. of ♂ rats on test	Mean increase in wt. in 5 weeks g.	Dose of standard which would have brought about this increase, units	Dose of standard equiv. to 1 g. units	No. of ♀ rats on test	Mean increase in wt. in 5 weeks g.	Dose of standard which would have brought about this increase, units	Dose of standard equiv. to 1 g. units
0.00001	1	38	0.0149	1,490,000	6	20.2	0.0129	1,290,000
0.0005	5	104	2.3572	4,714,400	6	53.3	0.3692	738,400

The weighted mean of the values found from the bucks and does respectively on the two doses is 2,068,500 units per g., which is near to the estimation made by the line test.

been plotted on the curves of response of the bucks and does in Fig. 1, assuming, on the strength of the result obtained by the line test, that the lower dose contained 0.02 unit and the higher dose contained 1.0 unit.

The estimation of the potency of the solution would, however, be made direct from the curves of response by substituting the found values of y , and

taking the weighted mean of the calculated values for x . Thus the potency of this sample was found to be 2,068,500 units per g. by the growth method, which confirms the figure of 2,000,000 units found by the line test.

THE DEGREE OF ACCURACY OBTAINABLE BY THIS METHOD.

The standard deviation (σ) of the test.

The standard deviation ($\sigma = \sqrt{\frac{\sum d^2}{n-1}}$) of each result has been calculated (Table I). The mean (weighted) variances for the bucks and does respectively have also been calculated (Table III).

Table III.

	Average variance = $\sigma^2 = \frac{\sum d^2}{N-m}$	
	$N = \text{no. of observations}$ $m = \text{no. of tests}$	
	♂	♀
Tests in which vitamin A was supplied as the light petroleum-soluble fraction of dried carrots	223.06	74.93
Tests in which vitamin A was supplied as crystalline carotene (including two not quoted in this paper)	282.7	77.08
Weighted mean of whole series	249.5	76.31

Thus the standard deviation of the mean increase in weight during 5 weeks of bucks which have become steady in weight on a diet deficient only in vitamin D, and have then been given graded doses of that factor, is $\sqrt{249.5} = 15.80$. The standard deviation of does treated similarly is $\sqrt{76.31} = 8.74$. The value for the bucks is very similar to that found in the vitamin A (growth-promoting) test, which was 14.72. The value for the does is distinctly lower than the one found in the vitamin A test (11.02).

The standard error (ϵ) of the mean.

The standard error of the mean has been calculated from the formula, $\epsilon = \frac{\sigma}{\sqrt{n}}$ where σ = the standard deviation, n = the number of animals used in the test. When $n = 10$ (a reasonable number to use for a vitamin test) $\epsilon = 5.00$ for the bucks and 2.74 for the does. The degree of accuracy obtainable by the use of 10 animals has been calculated and stated in Table IV. The doses of vitamin D required to bring about mean increases in weight of 60 g. and 40 g. in groups of bucks and does respectively (seen from the curves in Fig. 1 to be convenient) have been calculated. The doses of vitamin D required to bring about mean increases of $60 \pm \epsilon$ and $60 \pm 2\epsilon$ in groups of 10 bucks and of $40 \pm \epsilon$ and $40 \pm 2\epsilon$ in groups of 10 does have been determined. The ranges of these doses have then been calculated as percentages of the original dose and the degree of accuracy of the test obtained. Thus, with 10 bucks, a result within 47 % above, and 32 % below the true value would be obtained 2 out of 3 times, and within 116 % above and 54 % below,

21 times out of 22. Similarly with 10 does a result within 32 % above and 24 % below the true value, would be obtained 2 out of 3 times and within 75 % above and 43 % below, 21 out of 22 times (see Table IV).

Table IV.

When the number of rats is 10, $\epsilon = 5.00$ for bucks and $\epsilon = 2.76$ for does.

Mean increase		Equiv. dose of vitamin D		Range of mean increase when $n=10$		Range of equiv. dose of vitamin D		Percentage range of equiv. doses	
♂ g.	♀ g.	♂ unit	♀ unit	♂ g.	♀ g.	♂ unit	♀ unit	♂	♀
(a) $\pm \epsilon$									
60	40	0.0806	0.0960	65	42.76	0.1184	0.1270	146.9	132.3
				55	37.24	0.0550	0.0726	68.3	75.6
(b) $\pm 2\epsilon$									
				70	45.52	0.1737	0.1679	215.5	174.9
				50	34.48	0.0375	0.0549	46.5	57.2

The logarithmic variance of the test.

The standard deviation of the logarithm of the result is obtained by dividing σ by the slope of the line connecting the response with the log (to the base 10) of the dose. The curves relating the response to the log of the dose are

$$y = 92.82 + 30.02 \log x \text{ (bucks),}$$

$$y = 63.14 + 22.74 \log x \text{ (does).}$$

Then the standard deviation (λ) of the logarithm of the result is

$$\frac{15.80}{30.02} = 0.526 \text{ for bucks} = \lambda_1,$$

$$\frac{8.76}{22.74} = 0.385 \text{ for does} = \lambda_2.$$

The logarithmic variance of the test is then

$$0.276676 \text{ for bucks } (\lambda_1^2),$$

$$0.148225 \text{ for does } (\lambda_2^2).$$

The error of the test is $\frac{\lambda}{\sqrt{n}}$. As λ_1^2 is about twice λ_2^2 , about twice as many bucks as does are required in this test for a given degree of accuracy.

Significant differences between the mean increases in weight of groups of rats may be calculated as in the vitamin A tests.

The relative degrees of accuracy obtainable by the growth method for vitamin D and for vitamin A are indicated in a comparison of the respective values of λ (Table V).

Thus the bucks give the more accurate result in a vitamin A test, while the does give a slightly more accurate result in a vitamin D test.

Table V. *The relative degrees of accuracy obtainable by the growth method for vitamins A and D.*

	Bucks		Does	
	Vitamin A	Vitamin D	Vitamin A	Vitamin D
λ	0.293	0.526	0.402	0.385
λ^2	0.086	0.277	0.162	0.148

Comparison of this method with the line test for the routine testing of samples for vitamin D.

The growth method and the line test appear to give the same results in the estimation of vitamin D. The line test appears to us the more useful for routine testing, as the preparatory period is limited to 3–3½ weeks and the test period to 10 days. In the growth method the preparatory period may be 1–3 months, the test period is 5 weeks, and the point at which a rat is ready for a test is less certain, *i.e.* one has to wait for 2–3 weeks in order to be sure that a rat has ceased to grow (*cf.* 10 days for a vitamin A test). The growth method is decidedly more tedious than the line test.

DISCUSSION.

Two points of biological interest emerge from this work. The first is noticeable in the behaviour of the animals on the basal diet, and also in their response to doses of vitamin D. Young rats on the vitamin D-free diet grow more slowly than rats on a similar vitamin A-free diet. Also, the rats on the vitamin D-free diet do not rapidly lose weight and die when their reserves of D are presumably exhausted (as evidenced by the cessation of growth). They often maintain weight for many weeks before they die. Rats on a vitamin A-deficient diet, on the other hand, seldom remain steady in weight for more than 10 days when their reserves of vitamin A are exhausted (as evidenced by their cessation of growth). They lose weight rapidly and die. Does this mean that the animal's need for vitamin D is less than its need for vitamin A? The response to doses of vitamin D is also considerably less than to doses of vitamin A, which is indicated by the slopes of the curves connecting response to dose of vitamin given. For bucks the slopes are given by the figures 50.3 for vitamin A and 30.03 for vitamin D, and for the does the slopes are given by the figures 27.4 for vitamin A and 22.74 for vitamin D. The second point of interest emerges from a comparison of the results from which the curves of response have been drawn with the results obtained in an earlier attempt to make such curves. The results of this previous experiment are collected in Table I C. It may be seen that the response to doses of vitamin D is consistently lower in the group of animals which received vitamin A as 0.04 mg. of the light petroleum-soluble fraction of dried carrots than in the group of animals which received vitamin A as 0.04 mg. crystalline carotene. If insufficient vitamin A were limiting the response of the animals

in Table I C one would expect that the lower doses of vitamin D in the two groups would produce equal responses, *i.e.* the shortage of vitamin D would limit the response equally in the two groups, and that the higher doses of vitamin D would only gain higher responses in the group having the larger amounts of vitamin A. But the influence of the shortage of vitamin A appears to be felt in the lower doses of D also. Does this indicate some inter-relationship between the effects of vitamins A and D?

SUMMARY.

A method for the estimation of vitamin D by means of its growth-promoting power has been worked out on similar lines to that proposed for vitamin A.

The responses of animals have been found to be graded to the dose of vitamin D given, that of the bucks being greater than that of the does. Equations (logarithmic) have been calculated to express the responses of the bucks and does respectively.

The degree of accuracy of this method of estimation of vitamin D is, by the use of bucks, only about one-third of that obtainable in a vitamin A test. By the use of does, the degree of accuracy of the vitamin D test is slightly greater than that of a vitamin A test.

The degree of accuracy obtainable by the line test in this laboratory has not yet been worked out. Tests of the same substances by the line test and by the growth method have given identical results. The growth method is, therefore, thought to measure the same factor as the line test. It is not however, as quick a method as the line test, and it is highly improbable that its degree of accuracy is any greater than that of the line test. It is therefore not recommended for routine testing.

Incidentally, the results reported here seem to indicate that the rat's need for vitamin A is greater than its need for vitamin D.

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