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The Biological Assay of Vitamin D₃

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Two methods are generally used for the biological assay of vitamin D₃ on chicks, viz. the A.O.A.C. method which uses the bone ash of the tibia as a criterion of response, and the method of Olsson [1936], in which the tarso-metatarsal distance (t.m.t.) measured on an X-ray film is so used. Both methods use criteria which are measured in fundamental units (mass or length, respectively). This gives them an advantage over those methods used in rat assays where a visual estimate is made by comparison with an arbitrary set of standards. The Olsson radiographic method also involves less time, expense and labour, since the chicks are not killed.

The technique has been described elsewhere [Olsson, 1936; 1939; Baker & Wright, 1940] and the method has been adopted (1940) as standard by the British Standards Institute.

The present paper deals with some investigations on the nature of the test diet and its effect on the t.m.t. distance, and shows that on a suitable diet, gain in weight runs parallel with decrease in t.m.t. distance and affords a criterion for assay of vitamin D₃ of the same order of accuracy as the t.m.t. method. Experience with diet no. 1 of the British Standards Institute Specification 911/1940 indicated that differences in t.m.t. distances between

high and low doses of vitamin D were too small to be satisfactory, and resulted in a very low slope to the response curve. Baker & Wright [1940] quote cases where the difference between the t.m.t. distances for their extreme doses over an 8:1 range, is approximately 0.5 mm. Their experience is confirmed in this paper. Alternative diets were investigated with a view to increasing this difference. These diets comprised the A.O.A.C. diet used in the bone-ash test; a diet used by Olsson (private communication); and a diet used by Dr Wilkinson of Lever Bros. (private communication). Olsson's diet was a modification of his published diet [1936] in which bone meal is included.

METHODS

Chicks used. Sex-linked cockerels (Light Sussex and Rhode Island Red) were used in all experiments except in test 2, where pullets were used as well in equal number.

Housing. The chicks were housed in all-metal brooders, each of 6 units, arranged in three rows of two, each unit having a warm chamber, and an unheated run. The brooders were installed in a thermostatically controlled room with air circulation controlled by fans. Temperature in the heated chamber was 85–90° F. and in the room 80° F. during the first week, and it was lowered 5° F. each week for the next 4 weeks.

Food. Food was prepared in an electrically driven Hobart mixer. In all, six series of tests were made. Complete analyses of the diet were made in certain cases. Calcium and phosphorus determinations were done in all cases except series I. The composition and analysis of the diets are given in Tables 1 and 2. Variations between different batches of diet made to the same formula make it necessary to determine at least the Ca and P content of each batch, and to adjust these values to a constant figure, e.g. in diet A, suggested to us by Dr Wilkinson, values for CaO and P₂O₅ of 2.83 and 1.3–1.4% respectively were recommended, but the diet when made up for the test showed 1.09 and 2.24% respectively.

Table 1. *Composition of various diets fed to chicks*

Diets	B.S.I.	Diet A	A.O.A.C.	Olsson
Bran	16	15	—	—
Ground yellow corn	35	33.25	57	34.4
Middlings	23	38	28	28.4
Crude acid pptd. casein	—	—	12	—
Dried skim milk	8	—	—	9.4
Meat meal	10	—	—	—
Grass meal dried	5	—	—	5.5
Soya-bean meal	—	—	—	15.4
Bone meal	—	—	—	4.4
Fish meal	—	5	—	—
Ca ₃ (PO ₄) ₂	—	—	2	—
Iodized salt 0.02% KI	0.5	—	1	1.0
MnSO ₄ ·4H ₂ O	—	0.0094	0.02	—
CuSO ₄	—	0.0068	—	—
Charcoal	—	0.256	—	0.5
Ground limestone	1.5	4.0	—	—
NaCl	—	0.368	—	—
Fe ₂ O ₃	—	0.111	—	—
Yeast	—	—	2	—
Dried cattle food yeast	—	2.5	—	—
Olive or arachis oil	1.0	1.0	1	1.0
Vitamin A oil (1000 i.u./g.)	—	0.5	—	—

Table 2

(a) *Analyses of various diets*

Material	% B.S.I. A.O.A.C. Olsson		
	B.S.I.	A.O.A.C.	Olsson
Water	9.12	6.54	1.39
Fat	5.88	5.10	6.91
Protein (N × 6.25)	17.08	20.73	17.28
Starch	1.20	6.00	2.00
Sugar (reducing)	0.71	0.25	0.28
Ash	4.48	4.86	6.63
Ca	1.45	0.50	0.83
P	1.36	1.34	1.09
Fe	0.017	0.009	0.018
Mg	0.29	0.23	0.41

(b) *Ratio of calcium to phosphorus in various diets fed to chicks*

Diet	Test no. 2 3 4 5 6				
	2	3	4	5	6
B.S.I.: Ca	1.45	1.37	0.84	—	—
P	1.36	1.14	1.25	—	—
Ratio	1.063	1.193	0.669	—	—
A.O.A.C.: Ca	0.50	0.43	—	—	—
P	1.34	0.91	—	—	—
Ratio	0.358	0.450	—	—	—
Olsson: Ca	—	—	0.83	0.94	1.17
P	—	—	1.09	1.10	1.07
Ratio	—	—	0.764	0.856	1.093

respectively. The ratio was, therefore, adjusted by the addition of CaCO₃ so that the final quantities were 4.54% CaO and 2.24% P₂O₅. The results obtained with this diet differed from those obtained by Dr Wilkinson, possibly owing to the higher level of Ca and P, though the ratio is the same. There is evidence that doubling both Ca and P in Olsson's diet leads to smaller t.m.t. distances, but most of the chicks died within 6 weeks.

The Ca and P in the Olsson diet were maintained at 0.85–1.0 and 1.0–1.1% respectively, giving a ratio of Ca : P of approx. 1 : 1. In these separate experiments no adjustment was found necessary, the figures for Ca being 0.83, 0.94, 1.17, and for P 1.09, 1.10 and 1.07% respectively. The amount of food consumed by each group of birds was determined every week.

Vitamin D supply. The B.S.I. standard solution, i.e. crystalline vitamin D₃ dissolved in arachis oil to give a solution containing 1 unit/mg. of solution, was used, the units being calculated on the basis of 40 × 10⁶/g. crystalline product. The solution was stored in a refrigerator and diluted with arachis oil to correspond to 1% of the ration, as required.

Procedure. 15–20 birds were allocated to a group (6–8 in negative controls). Day-old chicks were placed immediately on the basal diet without any addition of vitamin for 1 week. They were weighed and marked and again weighed at weekly intervals during the experiments. The dose of vitamin D₃ was incorporated in the diet from the 8th day onwards, and the birds were X-rayed when 4, 5 or 6 weeks old.

X-Ray technique. The tarso-metatarsal joints were X-rayed on Kodak Crystallex film with exposure of 1½ sec. at 12 in., 30 mA., 39 kV. Prints were made from the negatives on ultra-contrasting bromide paper, and the t.m.t. distances were measured, at a magnification of approx. 5,

with a travelling microscope (vernier) reading to 0.05 mm. An accuracy greater than this is not considered feasible. Whereas in general the edge of the tarsus was well defined even when magnified 5 times, the edge of the metatarsus was sometimes difficult to locate, and the best solution was found to be that of Halnan [Baker & Wright, 1940] which consists in marking with a scalpel a print viewed without magnification. Recent experiments show that the new Ilford Kryptoscreen X-ray paper gives good results and avoids the labour of printing. The reproducibility of such measurements is shown in Table 3A, which gives the data obtained from two sets of photographs, each of 20 bones, measured on each of 5 separate days, by two observers. The photographs were shuffled each time by a third person.

Table 3A. Mean values of t.m.t. distances for sets of 20 photographs (mm.)

	Set 1		Set 2	
	Observer A	Observer B	Observer A	Observer B
Reading 1	3.73 (5)	3.69 (8)	0.84 (3)	0.89 (5)
2	3.71 (3)	3.68 (0)	0.86 (0)	0.82 (5)
3	3.74 (5)	3.63 (0)	0.89 (0)	0.82 (0)
4	3.73 (0)	3.70 (0)	0.86 (8)	0.80 (3)
5	3.73 (8)	3.69 (5)	0.84 (5)	0.85 (0)
Overall mean	3.73 (2)	3.68 (1)	0.86 (1)	0.83 (9)
Mean difference between extreme readings for each bone	0.15 (5)	0.19 (7)	0.10 (5)	0.15 (0)

In a series of measurements of this same photograph therefore there is liable to be a difference between extreme readings of:

0.2 mm. in distances of approx. 4 mm.,
i.e. $\pm 2.5\%$ from the mean,

and of 0.15 mm. in distances of approx. 1 mm.,
i.e. $\pm 7.5\%$ from the mean.

In one experiment two sets of prints were made and marked and measured by two separate observers. The results are shown graphically in Fig. 1 and are summarized in Table 3B.

Fig. 1. Uniformity of t.m.t. measurements.

Table 3C shows assay results calculated from independent measurements made on separate sets of prints by two observers. The results calculated by the gain-in-weight method are included for comparison.

Table 3B. Results of measurements by different observers on the same prints

Dose group	Observer 1		Observer 2	
	Mean t.m.t.	Standard deviation	Mean t.m.t.	Standard deviation
5 u./100 g. diet	3.891	0.8112	3.771	0.7206
10 u./100 g. diet	3.739	0.8360	3.683	0.7856
15 u./100 g. diet	3.336	1.2020	3.178	1.1340
20 u./100 g. diet	2.131	0.8823	2.144	0.5825
40 u./100 g. diet	1.359	0.3871	1.382	0.5074
Weighted mean and pooled standard deviation	2.897	0.8151	2.838	0.7790

Table 3C. Results of assays by different observers, t.m.t. method

Material: C.L.O. emulsion reputed to contain 50 u./g.

Assay period weeks	Observer 1		Observer 2		Gain in wt. method %
	Potency	Fiducial limits P 0.95 %	Potency	Fiducial limits P 0.95 %	
4	47	76-126	52	79-123	48
5	46	78-124	46	78-124	46
6	48	75-129	—	—	47

RESULTS

Six experiments were made, but one, no. 3, was discarded because the chicks failed to grow. This was attributed by the hatchery to a breakdown of the incubators. The remaining experiments were as follows:

Exp. 1. An attempted assay using B.S.I. diet no. 1. It is considered unsatisfactory owing to the low slope of the regression line.

Exp. 2. Comparison of B.S.I. and A.O.A.C. diets. The results on the B.S.I. diet confirmed those in Exp. 1; those on the A.O.A.C. diet showed a greater slope of the regression line and a parallelism between t.m.t. distance and the reciprocal of the gain in weight.

Exp. 3. Discarded.

Exp. 4. Comparison of B.S.I., A & Olsson diets. B.S.I. and A diets showed similar results, which confirmed Exps. 1 and 2, viz. they gave relatively uniform growth at all dose levels, but very low slopes to the regression line. Olsson's diet gave results similar to the A.O.A.C. but appeared to be less severe in its effect at low doses. There was a linear relation (a) between gain in weight and vitamin D₃ intake, and (b) between t.m.t. and vitamin D₃ intake.

Exp. 5. Was confined to the Olsson diet. In addition to an assay of two oils, the effect of varying the Ca and P in the diets was examined. Doubling both Ca and P in the diet led to reduced t.m.t. distances and to reduced gains in weight, and many birds died. Doubling the P alone (sodium phosphate) led to death of all birds within 4 weeks. Doubling the Ca alone led to reduced t.m.t. distances and increased gain in weight.

Table 4. *B.S.I. Standard, t.m.t. data*

Test no.	Diet	Period of test, weeks	Dose levels, units/100 g. diet	t.m.t. distances	Slope	Pooled variance	Coefficient of pooled variation, %
1	B.S.I.	5	3-23	(1.767); 1.063; 0.88; 1.021; 0.885; 0.854	-0.1887	0.0932	32.45
		6	3-23	(1.325); 0.653; 0.618; 0.634; 0.485; 0.591	-0.1137	0.0689	40.04
2	B.S.I.	4	3-23	(0.819); 0.720; 0.889; 0.875; 0.961; 0.946	—	—	—
		5	3-23	(1.265); 0.947; 0.714; 0.760; 0.720; 0.700	-0.2212	0.0500	29.10
4	B.S.I.	4	2.5-20	(1.320); 1.361; 1.363; 1.650; 0.942	-0.5897	0.1820	36.15
		5	2.5-20	(1.280); [1.194]; 1.516; 0.932; 0.266	-2.2412	0.3686	67.35
2	A.O.A.C.	4	3-23	(3.757); 3.608; 3.353; 2.681; 2.436; 1.291	-2.0515	0.6264	29.43
		5	3-23	(2.769); 2.601; 2.368; 1.928; 1.604; 0.953	-1.4910	0.9074	49.57
4	A	4	2.5-40	(1.310); 1.420; 1.259; 1.132; 1.106; 1.222	-0.1826	0.1128	27.35
		5	2.5-40	(1.008); [1.547]; 2.059; 1.394; 1.165; 1.181	-0.9496	0.2666	35.70
4	Olsson	4	2.5-40	(3.450); [3.253]; 3.508; 2.494; 1.375; 0.808	-2.6184	0.5310	35.90
		5	2.5-40	(3.338); [3.500]; 3.767; 2.747; 0.761; [0.403]	-3.6800	0.8039	36.96
5	Olsson	4	5-40	(4.378); [3.590]; 2.950; 1.100; [0.788]	-4.8635	0.5983	38.20
		5	5-40	(3.925); 3.648; 2.700; 0.833; [0.759]	-3.7068	0.6981	34.91
		6	5-40	(3.372); 3.836; 2.898; 0.817; [0.691]	-3.7999	1.5408	49.00
6	Olsson	4	5, 10, 15, 20, 40	(3.643); [3.700]; 3.542; 2.768; 1.856; 0.765	-3.9777	0.6824	36.78
		5	"	(4.000); [3.944]; 3.817; 3.091; 2.111; 0.682	-4.1662	0.6042	31.85
		6	"	(3.760); [3.559]; 3.686; 3.123; 1.869; 0.912	-3.4988	0.8195	37.58

Table 5. *B.S.I. Standard. Gain-in-weight data*

Test no.	Diet	Period of test, weeks	Dose levels, u./100 g. diet	Gain in weight	Slope	Pooled variance	Coefficient of pooled variation, %
2	A.O.A.C.	4	3-23	(100.2); [94.89]; 83.56; 116.11; 158.28; 192.94	138.02	1103.4	24.28
		5	3-23	(166.75); [150.76]; 141.56; 188.00; 270.59	215.11	3059.6	24.21
4	Olsson	4	2.5-40	(138.8); [121.4]; 124.3; 204.5; 257.2; [258.4]	178.75	2685.5	26.36
		5	2.5-40	(182.5); [147.8]; 150.3; 277.7; 351.2; [370.6]	255.79	4563.2	25.63
5	Olsson	4	5-40	(119.05); 143.65; 175.75; 273.90; 322.71	182.19	2193.1	20.8
		5	5-40	(142.82); [169.50]; 239.35; 413.35; [475.4]	418.17	4577.0	20.70
		6	5-40	(162.55); 192.61; 281.20; 524.65; [583.82]	433.61	7369.5	25.43
6	Olsson	4	5, 10, 15, 20, 40	(101.5); 125.1; 162.6; 190.2; 212.7; 251.2	119.74	1416.6	19.87
		5	"	(123.2); 165.0; 201.5; 256.9; 312.2; 397.0	212.57	3486.6	22.17
		6	"	(139.6); [185.0]; 234.0; 310.1; 400.6; 539.1	373.19	7494.8	23.43

Table 6. *Assays by t.m.t. method*

Test no.	Diet	Period of test, weeks	Material	Dose levels		Combined slope	Pooled variance	Potency	Fiducial limits $P=0.95$ %
2	A.O.A.C.	4	C.L.O.	Standard	Sample mg.				
				3-23	25 and 76.4	-1.9588	0.6072	112	64-151
5	Olsson	4	C.L.O.	10-20	100 and 200	-5.1106	0.5973	65	82-118
		5	"	10-20		-4.7128	0.8074	62	78-125
		6	"	10-20		-4.5866	0.8492	86	80-123
5	Olsson	4	Veterinary C.L.O. blended with seal oil	10-20	500 and 1000	-4.9687	0.6636	17	85-119
		5	"	10-20		-4.3095	0.7756	16	80-123
		6	"	10-20		-4.5724	0.8084	18	81-124
6	Olsson	4	C.L.O. emulsion	5, 10, 15, 20, 40	200, 300, 400	-3.7933	0.8081	47	76-126
		5	"	"	"	-3.8322	0.9620	46	75-124
		6	"	"	"	-3.4700	1.1161	48	75-129

Table 7. Assays. Gain in weight

Test no.	Diet	Period of test, weeks	Material	Dose levels		Combined slope	Pooled variance	Potency	Fiducial limits <i>P</i> = 0.95 %
				Standard	Sample mg.				
2	A.O.A.C.	4	C.L.O.	3-23	25 and 76.4	119.75	925.9	143	74-131
5	Olsson	4	C.L.O.	16-20	100 and 200	230.23	2163.2	71	77-124
		5	"	—	—	400.45	4232.3	80	85-120
		6	"	—	—	488.03	11710.4	74	77-126
5		4	Veterinary C.L.O. blended with seal oil	16-20	500 and 1000	214.08	2047.1	21	81-128
		5	"	—	—	377.05	4211.9	21	84-121
		6	"	—	—	498.90	9869.9	20	81-124
6	Olsson	4	C.L.O. emulsion	5, 10, 15, 20, 40	200, 300, 400	119.20	1293.6	47	75-131
		5	"	"	"	210.95	3587.3	46	79-125
		6	"	"	"	362.31	8114.7	47	79-123

Fig. 2. T.M.T. responses.

Fig. 3. Gain in weight responses.

Exp. 6. Was also confined to the Olsson diet in an assay. A parallel set of standards was run in which the vitamin was separately administered to each chick by micrometer syringe. This mode of administration showed slightly greater weight increases for the same vitamin intake, but paradoxically, with the higher doses, greater t.m.t. distances. This point is being further investigated. The observations of Fritz, Halpin, Hooper & Kramke [1942] on oxidative destruction of vitamin D in poultry foods makes it important to investigate these two methods of dosing for assay purposes.

The essential results of the experiments are given in Tables 4-7 and in Figs. 2 and 3. Tables 4 and 5 are concerned only with responses to the B.S.I. standard vitamin D₃ preparation; Tables 6 and 7 give certain assay results. Dose levels were (in addition to negative controls) 3, 5, 8.33, 13.68 and 23 units/100 g. diet or 2.5, 5, 10, 20 and 40 units/100 g. diet. In some cases, as will be obvious from the figures, extreme doses had to be omitted to satisfy criteria of linearity, and these values are enclosed in square brackets. Negative control values are shown in curved brackets. There is

general agreement that the linear response occurs with doses from 10 to 25 units/100 g. diet. Above 25 units no great change occurs, and below 10 there is little calcification. This is confirmed in test 6, which includes a dose level of 15 units/100 g. diet.

One of the advantages claimed for the B.S.I. diet is that growth is equalized and is independent of vitamin intake. Olsson's data show that over the active dose range 8–32 units/100 g. food (he shows no point between 16 and 32 units), there is a gain

Table 8. Assay by log t.m.t. method

Test no.	Diet	Period of test, weeks	Material	Combined slope	Pooled variance	Potency	Fiducial limits P=0.95 %
5	Olsson	4	C.L.O.	-1.0384	0.03615	61	78-123
		5	"	-1.0090	0.03196	57	78-122
5	Olsson	4	Veterinary C.L.O. blended with seal oil	-1.2171	0.04182	17	84-119
		5	"	-1.099	0.037973	16	82-120

DISCUSSION

It will be seen that the slope of the regression line for the B.S.I. diet is very low, and that there is a tendency to greater regularity when the test period is longer than 4 weeks. In test 2, using this diet, the t.m.t. after 4 weeks on the highest doses is greater than that on the lowest doses, but the difference is not statistically significant. After 5 weeks, though there is a reduction in t.m.t. with increasing dose, the difference between responses for extreme groups is not statistically significant.

In marking the end of the metatarsus for measurement, there are occasions when the end of the bone is so ill defined that a difference of 0.5 mm. may occur in marking the same photograph on different occasions. The greater the t.m.t. distance, the less the relative magnitude of such an error, and since such photographs occur mostly on low doses, it is highly desirable that the t.m.t. distances should be great on such doses so as to minimize this error.

On the A.O.A.C. diet the slope of the regression line is much increased, but after 5 weeks it is less so than after 4 weeks. The Olsson diet gives good slopes, which, as shown in the diagrams for tests 4–6, are consistent and not affected by the duration of the test between 4 and 6 weeks.

In these tests, the t.m.t. distance has been used rather than its logarithm, as recommended in the B.S.I. specification. Assays in this series calculated by both methods (t.m.t. and log t.m.t.) gave almost identical results for potency and fiducial limits (Tables 6 and 8). The object of using the log t.m.t. is presumably to satisfy the mathematical purist's desire to equalize the variance in the different groups. It may be mentioned here that this is not necessary when the gain in weight is used as the criterion of response (see below).

in weight of about 40–50 g. after 4 weeks. This is about one-third of the gain in our experiments, and Olsson introduces a correction for his t.m.t. values to compensate for the differences in weight of the chicks. Where, however, as in our results, the gain and t.m.t. are related, such a correction is not necessary. A probable reason for the difference between our results and Olsson's lies in the Ca and P content of the diet. Olsson gives 1.76% Ca and 1.04% P, against our figures of 1.0 and 1.1% respectively.

On the Olsson diet, as used in these experiments, there is a marked difference in growth (and a corresponding difference in food intake) with increasing vitamin D dose level; this makes it necessary to use intake instead of dose level in calculating the results. It will be seen from the figures that this increase is linearly proportional to the log of the intake, and this being so, there is no need to correct the t.m.t. distances for growth differences. On the contrary, the weight increase is in itself a satisfactory criterion of response, and has the advantage of giving an equalized variance between groups, and also a lower coefficient of pooled variation. In some assays it tends to give higher results, which however, are more consistent over different test periods than the t.m.t. results and are of a similar degree of reliability. The slope of the response curve increases when the test is prolonged and we favour a 5 weeks' test period when gain in weight is used as a criterion.

SUMMARY

In the light of the results described above, it is suggested that the method of using the growth response of chicks is a simple, convenient and accurate method for assays of vitamin D₃, when the rachitogenic diet is suitably chosen and controlled, with special regard to its Ca and P content.

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