CCLXXX. TECHNIQUE OF THE LINE TEST ASSAY FOR VITAMIN D.

BY ALFRED LOUIS BACHARACH, EDITH ALLCHORNE
AND HARRY EPHRAIM GLYNN.

From Glaxo Laboratories Ltd., Greenford, Middlesex.

(Received 10 September 1936.)

COWARD & KEY [1934] have reported the successful use of a modified technique in the now well-known "line" test for the assay of vitamin D. In this test animals of suitable age and pre-experimental history are given a rachitogenic diet for about 3 weeks. They are then divided into groups, the sexes and litter-mates being distributed as uniformly as is necessary to give trustworthy results, and the animals in each group receive a measured dose of the substance under test or of a standardized preparation of the vitamin. Coward & Key, and other workers, had previously divided these doses into equal portions, one for each day of the test period, which is usually 10 days. In the paper cited, the authors report that, in a direct comparison between litter-mates, they obtained identical results, of equal accuracy, whether they gave to the animals a certain amount of the vitamin either as a tenth of the total dose daily throughout the 10 days' test period or as one dose at the beginning of that period.

Obviously such a modification of technique presents advantages, mainly in economy of time, and it seemed to us necessary to discover whether it could be adopted in the somewhat different circumstances of our own laboratory. For reasons published elsewhere [Bacharach, 1936] we find it necessary to use a much more severely rachitogenic diet than the 2965 of Steenbock & Black [1925], which was used by Coward & Key. Another, but a minor, difference is that we examine the femora instead of the ulnae and radii.

In the first instance two litters of animals were used, and approximately half the animals in each litter were dosed with a solution of previously assayed irradiated ergosterol diluted so that 1 drop contained 0.5 International Unit (I.U.) of vitamin D. Each of these animals was given 1 drop daily for 10 days. The other half of the animals each received on the first day of the test a single drop of a solution containing the same irradiated ergosterol at ten times the concentration of the first solution. The degrees of healing found in the stained bones are shown in Table I.

Table I.

Dose	Individual healing	Average healing
Single	0, 0, $\frac{1}{2}$, $\frac{1}{2}$, $\frac{1}{2}$, $\frac{1}{2}$, $\frac{1}{2}$, 1	0.4
Divided	1, 1, 1, 1, 1, 1, $1\frac{1}{2}$	1.1
Negative control	0, 0	0

It was thought possible that the single dose method might, through incomplete consumption of a single drop by the animals, have involved a purely mechanical error of unduly high effect. A further test was therefore made, using a dilution of the International Standard Préparation (VD 10) such that 1 drop contained approximately 0.25 I.U. of vitamin D. This was fed at two levels, both by the single and the divided dose methods; the single dose was given as 10 or 20 drops on the first day of test, the divided dose as 1 drop or 2 drops on each day (Table II).

Table II.

Dose	Individual healing	Average healing
0.25 unit Single	0, 0, 0, 0, 0	0
Divided	0, 0, 2, 1	0.75
0.5 unit Single	0, 0, 0, ②, $\frac{1}{2}$, 1	0.25
Divided	0, ②, $\frac{1}{2}$, 1, 1	0.6
Negative control	0, 0	0

② indicates only one or two spots of calcified tissue.

Here one litter was used for the 0.25 unit and the other for the 0.5 unit dose, so that the degrees of healing on the two doses were not necessarily comparable. Again, however, we seemed to have evidence that the divided dose method gives greater healing.

The final experiment involved the use of 8 litters, including 64 animals. An intermediate course was adopted between those taken in the preliminary experiments, as it was considered undesirable to administer as much as 20, or even 10, drops (each 22.5 mg.) of oil solution on one day to an animal.

Accordingly one test solution contained 0.005 γ of calciferol per drop; 3 drops were given daily, so that the daily dose was 0.6 I.U., and the total experimental dose 6 units. This solution also contained sufficient β -carotene¹ to give every 3 drops a vitamin A equivalence of 5 I.U.

The second solution contained 0.0375 γ of calciferol per drop, of which 4 were given on the first day of the test period. Thus the total dose was also 0.15 γ of calciferol, or 6 I.U. Carotene¹ equivalent to 50 I.U. was administered separately during the first 2 days of the test period (Table III).

Table III.

Dose	Number of animals	Range of healing	Average healing
Single	28	② to 2	0.91
Divided	27	1 to 2	1.41
Negative control	9	0 to $\frac{1}{2}$	0.11

An examination was also made of the differences between males and females (Table IV).

Table IV. *Average healings.*

	Males	Females	Average of males and females
Single dose	1.07 (15)	0.73 (13)	0.90
Divided	1.47 (17)	1.30 (10)	1.39

Numbers in brackets represent number of animals.

The averages shown in Tables III and IV are all calculated from the appropriate bone-scores, irrespective of the number of animals in any given litter that may be receiving a particular dose. Table V has been drawn up by calculating the means of the litter means for each dose, taking the sexes separately or together.

Table V.

	Average of both sexes	Average of healing by litters		
		Males	Females	Males and females
Single	0.93	1.20	0.76	0.98
Divided dose	1.41	1.41	1.33	1.37

¹ The feeding of carotene in this experiment had no bearing on the problem under examination and was practised for reasons not germane to it.

DISCUSSION.

It seems clear that, even under the best conditions, the technique recommended by Coward & Key cannot be applied to animals of our particular stock and upbringing. Whether some limitation to the maximum possible daily absorption or utilization of vitamin D, or to both, is imposed by the intensely rachitogenic nature of our diet 401, or whether some unsuspected difference operates, we are unable to decide.

There are indications that males respond somewhat better to cure by a given dose, that is, are less rachitic than females. This is contrary to the view of Morgan [1932] but it is not inconsistent with the slight superiority in bone-ash content of females over that of males of approximately the same age [Bacharach, 1936]; the males have less distance to go to achieve normal calcification. Slight differences in result can be obtained by various methods of calculating the average degrees of healing, but these differences seem to become unimportant when as many as eight different litters are used and over 25 animals receive any one dose, even though the sex distribution be not perfectly symmetrical. Provided that a grand average is finally taken, the results are little affected. In the present instance the single and the divided dose healings showed figures of

0.91 to 1.41,
0.90 to 1.39,
0.93 to 1.41,
0.98 to 1.37,

according to the method of averaging.

We have no evidence to show whether, with our animals and under our experimental conditions, the two methods of administering vitamin D supplements, if used independently, would give the same accuracy of result. But it seems clear to us that, with those animals under those conditions, it is not justifiable to compare one source of vitamin D with another unless both are administered in distributed doses. It is possible that it might be satisfactory to give both sources by the single dose method, but of this we have no direct proof.

SUMMARY.

The single dose method of feeding vitamin D supplements in the line test, as advocated by Coward & Key, was found not to be applicable to a different stock of rats receiving a more severely rachitogenic diet and gave significantly less good healing than the divided dose method.

Males appear to be slightly less rachitic than females under similar conditions of slowly healing rickets.

We take this opportunity of acknowledging the kindness of Dr K. H. Coward, who read the manuscript of this paper before it was submitted for publication and made certain suggestions for modification, all of which have been adopted.

REFERENCES.

- Bacharach (1936). *Z. Vitaminforsch.* (in the Press).
Coward & Key (1934). *Biochem. J.* **28**, 870.
Morgan (1932). *Biochem. J.* **26**, 1147.
Steenbock & Black (1925). *J. biol. Chem.* **64**, 263.