LX. THE DETERMINATION OF VITAMIN D¹.
**I. THE RELATIONSHIP BETWEEN GRADED DOSES OF A
STANDARD SOLUTION OF VITAMIN D, ADMINISTERED
TO YOUNG RATS ON A RACHITOGENIC DIET, AND THE
ASH CONTENT OF THEIR BONES.**

BY ELEANOR MARGARET HUME, MARGARET PICKERSGILL
AND MARY MONTGOMERY GAFFIKIN.

From The Division of Nutrition, Lister Institute.

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INTRODUCTORY.

THE methods for the biological estimation of vitamin D, which are commonly used, are all based on a measurement of the calcification of the bones of rats, on a diet very low in phosphorus and high in calcium. The measurement of calcification is made either by X-ray photography of the epiphysis or growing end of a selected bone, or by staining the calcium deposited in it ("line" test), or by gravimetric analysis of the ash in certain selected whole bones. The first two methods measure essentially the same thing and the antirachitic treatment may be administered prophylactically or curatively; both these methods involve a certain subjective element in judging with the eye, by matching, the degree of calcification attained. The third method measures calcification in the whole bone, including the growing end; the test is usually prophylactic and there is no subjective element of error, but there might be an equal degree of error due to manipulation in the course of the analysis. The absence of any subjective element renders the third method attractive, though it is very much longer and more tedious than the other two.

For the application of any of these three methods some standard of comparison is desirable, in the form of a previously prepared scale, which shall relate a series of doses of a known standard preparation of vitamin D (cod-liver oil or irradiated ergosterol) to the degree of calcification produced and measured by any of the three methods mentioned. The preparation of such a scale, for use with the X-ray method, is described by Bourdillon *et al.* [1931] and by Everse and van Niekerk [1931], and for use with the "line" test by Bills *et al.* [1931] and by Dyer [1931]. No attempt has heretofore been made

¹ The three papers grouped under this heading describe the results of work undertaken at the instance of the Accessory Food Factors Committee of the Lister Institute and Medical Research Council. The second paper includes, in addition, some account of a comparative study initiated by Dr Zilva who kindly placed his results at the disposal of the authors.

to construct a scale which shall correlate different values of percentage ash in the bone with different graded doses of vitamin D. Indeed Adams and McCollum [1928] seem to have reached the conclusion that it would not be possible to construct such a scale, for they conclude (p. 521) that "data obtained in the present investigation, however, indicate that there is scarcely a standard behavior of animals as evidenced by bone composition."

The construction of such a scale, correlating the percentage ash in the bones with graded doses of vitamin D, would have the added interest, if it could be done, that estimation of the percentage ash is a method which reveals the condition of calcification in the whole bone, while estimation by X-ray or "line" test reveals only what the eye can detect; from the former set of data, therefore, biological facts of fundamental interest may conceivably be deduced, while from comparable data obtained by means of the X-ray or "line" test, this is hardly likely to be possible. This point has, however, no bearing on the relative merits of the various methods as means for the evaluation of vitamin D.

The present paper describes an attempt to prepare such a scale and contains some other data which have a bearing on the general accuracy and application of the method, but it is not proposed to deal exhaustively with this aspect of the subject. Certain sources of error are inherent in the biological method, in whatever way the measurement of the degree of calcification may be made; these are, particularly, the variation of the stock of rats in sensitivity to rickets, the variation in the response of individual rats, even when litter-mates, to identical treatment, and variation in the rickets-producing potency of the basal diet or of certain elements in it. These sources of error have been discussed very fully by Bourdillon *et al.* [1931] who reached the conclusion that the variation in the responses of litter-mates to the same dose of vitamin D was the chief of all the sources of error. It might be expected that the responses of animals in a prophylactic experiment, in which the state of calcification of the whole bone is measured, would be less subject to individual variations, than would the responses of animals, already abnormal, in a curative experiment, where only calcification at the epiphysis is measured. Inspection of the results included in this paper shows, however, that the range of individual variation is also very high when the prophylactic method is used, and that it is not at all infrequent for a rat receiving a certain dose of vitamin D to show a lower calcification than another rat, its litter-mate, receiving half the dose.

The question, therefore, at once arises as to the number of rats which it is necessary to employ in testing each single dose, in order to give average results in which a series of doses will fall smoothly into line. Section I provides a small amount of data, which, although the experiments were not planned to answer that particular question, are relevant.

The writers have only been able to find in the literature one considerable series of ash estimations on the bones of rats, in which the values for the two hind legs were worked out separately [Jones and Robson, 1931]. With a view to throwing light upon the degree of error which is to be ascribed to the

manipulation of the worker, a large part of the results in this series has been worked out in that way; these results are dealt with in Section II.

Finally Section III deals with the actual construction of the curve correlating the ash content of the bones with the given doses of a standard solution of irradiated ergosterol.

The same series of rats described in Section III was also X-rayed at the end of the experiment, through the kindness of Dr R. B. Bourdillon and Miss H. M. Bruce, and the evaluation of this identical experimental series by the X-ray method is dealt with by them in the ensuing paper (p. 506).

TECHNIQUE.

The rats used were of the Lister Institute piebald strain, aged 21–28 days, weighing 35–40 g. in the first experiment in Section I, and 40–45 g. in the other experiments.

Each rat was housed separately in a metal cage, with a fine wire grid as flooring. Cotton wool was given as bedding. Equal distribution of litters and, as far as possible of sexes, between the different experimental groups was strictly observed. The diet used was McCollum 3143 [McCollum *et al.*, 1921]. The rats received the experimental diet for a period of 28–33 days, the number of days being strictly the same for all rats in any one comparative series; they also received the given dose of vitamin D daily throughout the experiment, except on Sundays, the doses on Saturday and Monday being adjusted to make up the omission.

The standard used was one or other of the standard solutions of 0.01 % irradiated ergosterol, dissolved in inactive olive oil, prepared at the National Institute of Medical Research, Hampstead. In the two experiments in Section I, standards II and III were used; for the construction of the scale (Section III) only standard III was used, this being part of the solution now in use as the international standard. Both the original solutions and any dilutions were kept in the cold room at about 0° throughout, except when brought out for use. Dilutions were made by weight with olive oil as the diluent; dosage was so arranged that every animal in a given comparison received the same amount of olive oil. The oil was dropped into the animal's mouth from a capillary pipette, the drops given being the number calculated to give the required dose.

At the end of the experiment the rats were killed with coal gas, and after being X-rayed, the hind legs were removed and the bones (femur, tibia and fibula) were cleaned, broken across, dried, extracted with alcohol and ether, and ashed to constant weight. The results are expressed as the percentage ash in the dried extracted bone, but, for the average values on which the scale is based, they are also calculated as the percentage ash in the dry bone and as the A/R ratio, the latter being the value of the ash divided by the value of the organic part of the dried extracted bone (*i.e.* the difference between dried extracted bone and ash).

SECTION I. (By E. M. Hume and M. Pickersgill.)

The original object of the two experiments described in this section was to compare the antirachitic values of two different specimens, Nos. II and III, of standard solution of irradiated ergosterol. From this particular point of view the results of the experiments are not relevant to the subject of this paper and it may be taken from evidence published by Bourdillon, Bruce and Webster in the third paper in this series (p. 522) that the two samples, II and III, were of like antirachitic value. The results do, however, serve as a preliminary observation, determining roughly the number of animals which it is desirable to use for any single dose in a comparison, in order to obtain satisfactory average values correlating dose and degree of calcification. They also yield information as to the size of dose which it would be desirable to use in such prophylactic experiments.

Exp. 1. Daily doses of 0.05, 0.1 and 0.2 international units of vitamin D, being the equivalent of 0.005, 0.01 and 0.02 γ (1 unit = 0.1 γ) of irradiated ergosterol, of each of the solutions II and III, were chosen for administration to groups of rats. There was also a group of negative controls receiving olive oil only and a group of positive controls receiving 2.0 units (solution III). Each of these 8 groups contained only 4 animals; 4 litters of 8 animals each were used so that each group contained one animal from each of the 4 litters.

Table I. *Percentage ash in the bones of rats, on a rachitogenic diet (McCollum 3143) and receiving various doses of two solutions of irradiated ergosterol (solutions II and III) of approximately equal strength, prophylactically for 30–35 days.*

Results calculated as percentage ash in dried fat-extracted bone.									
Daily dose of irradiated ergosterol expressed in units of vitamin D									
Litter	Sex	0	0.05		0.1		0.2		2.0
			Solution II	Solution III	Solution II	Solution III	Solution II	Solution III	
1539	♂	25.5	—	—	46.0	35.5	38.5	39.3	45.5
	+	—	40.1	40.0	—	—	—	—	—
1542	♂	24.9	33.5	31.8	—	—	46.7	40.0	44.5
	+	—	—	—	38.6	35.9	—	—	—
1537	♂	27.8	30.1	28.7	32.7	31.5	—	—	—
	+	—	—	—	—	—	32.5	37.4	42.3
1557	♀	Lost	31.5	39.8	40.2	34.8	41.0	44.1	52.5
Average		26.1	33.8	35.1	39.3	34.4	39.7	40.3	46.2
Combined av., solutions II and III		26.1	34.4		36.8		40.0		46.2

The results, expressed as percentage ash in the dried extracted bone (see Table I), are typical of what might be expected; there is great variation between individual values for the percentage ash in rats receiving the same dose, and great irregularity within the same litter, so that higher doses quite frequently give lower values than lower doses. On averaging the values from the 4 animals in each group, the results showing the degree of calcification corresponding with the different doses begin to fall into series, although the value for the dose

of 0.1 unit (solution II) is almost exactly the same as the value for twice that dose, and the value for the dose of 0.1 unit (solution III) is less than the value for half that dose. If, however, solutions II and III are regarded as being of the same antirachitic strength (as there is every reason to believe that they were), then results derived from 8 animals on each dose are available for calculating the average value for the percentage ash corresponding with each of the doses of 0.05, 0.1 and 0.2 units of vitamin D. When the results are averaged in this way, a much smoother series is obtained in which the values are at least nowhere reversed.

Exp. 1 therefore suggests that the number of animals in an experimental group should be more than 4 and probably at least 8.

Exp. 2. In the second experiment, the original object was again to compare the antirachitic values of the two standard solutions II and III, by comparing the results obtained from groups of 10 animals on a single dose. At the same time it was desired to determine the variation in the average result, which might be given by two groups of 10 animals receiving the same dose of the same solution. One group of 10 animals therefore received a certain dose of solution II, while two groups of 10 animals, comparable with the first in litter and sex, received the same dose of solution III. In effect, therefore, taking the two solutions II and III as being of equal antirachitic potency, the experiment should show the variation in the average result which is obtained when three comparable groups of 10 comparable animals are comparably treated.

The dose selected was one of 0.1 international unit of vitamin D daily, a dose which in the previous experiment had resulted in a suitably intermediate degree of calcification.

Reference to Table II shows that a generally higher level of calcification was attained in this experiment than in the previous one with the same dose,

Table II. *Percentage ash of the bones of rats on a rachitogenic diet (McCollum 3143) and receiving a daily dose of 0.01 γ (= 0.1 unit) of irradiated ergosterol, as two solutions II and III, of approximately equal strength, prophylactically for 30–35 days. Treatment of groups A and B, receiving solution III, was identical.*

No. of litter	Sex	Percentage ash in the dried, fat-extracted bone		
		Solution II	Solution III group A	Solution III group B
1679	♂	40.0	43.1	42.2
	♀	40.2	43.8	42.7
1682	♂	51.6	41.7	39.8
	♀	42.4	36.8	42.1
	♂	38.1	41.8	36.7
1680	♂	44.5	52.4	47.6
	♀	—	—	41.7
	♂	45.1	45.8	—
	♀	49.6	50.6	51.1
1691	♂	41.3	36.7	41.3
	♀	37.7	38.8	38.0
Average		43.05	43.15	42.32

but the percentage ash in the dry extracted bone in this series is still much below the maximum attainable on the rachitogenic diet, when optimal anti-rachitic treatment is given. Though the individual values vary very widely, particularly within one litter (No. 1682), yet the average values (43.05, 43.15 and 42.32 %, in the three similar series) are very close together. It may therefore be concluded that where groups of as many as 10 comparable animals are used for each dose of material tested, an average result will be obtained upon which reliance may be placed and that with a graded series of doses, average results will be obtained which will arrange themselves in a fairly smooth series. A similar conclusion was reached by Sherman and Stiebling [1929]. They used groups of 10 or more rats and determined the content of calcium in the femur and concluded that reliance could be placed on the average result yielded by such groups, but the diet and conditions of their experiment were so different from the ones just described that argument from such data to the present case would not be valid.

The difference between the value obtained for the percentage ash content of the bones corresponding with a dose of 0.1 unit of vitamin D daily, in Exp. 1 (Table I, ash in dried fat-extracted bone, average of 8 rats, 36.8 %) and in Exp. 2 (Table II, ash in dried fat-extracted bone, average of 30 rats, 42.8 %) has already been pointed out, and this difference offers another example of the variation in results which is liable to be obtained in this kind of work when carried out at different times. Whether such a variation in the response of the experimental animals at different times is due to variation in the animals' reserves of vitamin D, or to variation in the rickets-producing power of any of the constituents of the basal diet or to some other unrecognised cause, is at present not known, but it is one of the greatest causes of difficulty in the biological estimation of vitamin D.

SECTION II. (By E. M. Hume and M. M. Gaffikin.)

Any evidence as to the measure of accuracy which may be expected in the actual manipulation of preparing and ashing the bones, in the method under discussion, is desirable. Estimations of ash content in the bones of the two hind legs of each rat were, therefore, carried out separately, in a large part of the series. The values for about 90 animals are available and are set out in Table III.

While the present work was in progress, a similar set of 50 estimations, made separately on the two hind legs of the same rat, was published by Jones and Robson [1931]. Their results show a variation very comparable with that obtained in the present series. The average difference between estimations of percentage ash in the dried extracted bone, carried out separately on pairs of legs, is in their case 0.56 % and in the present series 0.50 %.

Relatively to the error due to the variation in the individual responses of the rats, this degree of error is small. For instance, if for the 20 pairs of rats receiving doses of 0.1 and 0.2 unit of vitamin D (see Table IV A) the average

Table III. *Comparison between the percentage ash in the dried, fat-extracted bones (femur and tibia) of the right and left legs of rats, estimated separately.*

No. of rat	Percentage ash in dry, extracted bone		No. of rat	Percentage ash in dry, extracted bone		No. of rat	Percentage ash in dry, extracted bone		No. of rat	Percentage ash in dry, extracted bone	
	L. Tibia and femur	R. Tibia and femur		L. Tibia and femur	R. Tibia and femur		L. Tibia and femur	R. Tibia and femur		L. Tibia and femur	R. Tibia and femur
834	39.4	39.7	856	49.9	45.7	879	41.2	42.1	902	43.9	44.8
835	38.4	38.8	857	41.6	41.6	880	41.3	40.3	903	44.0	43.0
836	41.9	40.4	858	41.1	41.7	881	39.4	38.6	904	43.9	44.0
837	40.3	40.2	859	44.7	43.9	882	47.0	48.0	905	47.8	47.8
838	47.6	47.8	860	45.4	45.2	883	47.6	47.3	906	37.8	38.2
839	43.6	43.2	861	48.2	48.8	884	50.8	51.7	907	49.4	49.0
840	47.3	47.6	862	39.6	40.5	885	49.5	49.2	908	48.7	49.1
841	42.5	42.8	863	48.3	47.5	886	48.6	48.7	909	44.7	44.2
842	47.2	46.4	864	42.5	43.5	887	48.0	48.3	910	40.2	39.6
843	47.7	48.2	865	39.9	40.1	888	44.8	45.3	911	43.9	43.8
844	44.8	44.1	866	42.8	44.0	889	42.8	42.1	912	44.4	44.0
845	43.1	43.5	867	47.8	47.9	890	45.2	44.5	913	46.6	46.2
846	37.9	37.9	868	47.3	47.6	892	41.4	41.7	914	42.9	43.6
847	41.2	40.5	869	46.3	47.2	893	46.9	47.2	925	45.3	45.8
848	43.8	44.5	870	41.1	40.4	894	41.5	41.3	926	32.6	32.5
849	42.5	42.6	872	48.2	48.3	895	43.5	43.0	927	43.0	42.9
850	48.7	48.8	873	47.9	48.1	896	46.9	47.9	928	43.1	43.7
851	47.8	46.9	874	46.9	47.7	897	35.1	34.8	929	27.0	27.9
852	48.6	48.9	875	50.6	49.7	898	50.0	49.6	930	34.7	34.3
853	43.4	43.5	876	52.2	51.9	899	47.5	48.7	931	39.3	38.5
854	39.6	40.1	877	52.3	52.5	900	38.9	39.3	932	38.9	39.2
855	49.3	50.2	878	53.7	53.4	901	46.9	46.6	933	44.8	44.7

difference from the mean for the differences between pairs be calculated, it is found to be as large as 1.5 % of ash value, while the similar value for the doses of 0.1 and 0.05 unit is 2.6 % of ash value.

The question of errors is further considered by Bourdillon and Bruce in the ensuing paper (p. 506).

SECTION III. (By E. M. Hume and M. M. Gaffikin.)

The preparation of the scale.

In preparing a scale which should best show the relation of the calcification of the bones of rats on a rachitogenic diet to a series of graded doses of vitamin D, the selection of the doses is of paramount importance.

Dutcher *et al.* [1925] constructed charts, reproduced in Fig. 1*a*, showing the progressive changes taking place with age in the calcification of the bones of young rats, maintained for periods varying from 0 to 8 weeks on a rachitogenic diet of the low phosphorus type (Steenbock 1965), both uncorrected and corrected with an optimal supply of vitamin D. They compared these with a similar chart, showing the corresponding values for young rats after similar periods of time on a normal diet. The values for the ash content of the bones thus obtained for rats on the corrected rachitogenic diet may be taken as the maximum which could be attained, the insufficiency of the phosphorus in the diet acting as a limiting factor which made the attainment of normal calcification impossible. Although the basal diet used by Dutcher *et al.* was not the same as that used by the writers, it was of the same type, and the young rats were similar in size and age, so that the values of Dutcher *et al.* make a very useful approximate

standard of reference. It is true that their curves are constructed using only a small number of animals, and the curve for rats on the uncorrected rachitogenic diet could certainly have no universal application, yet the values for rats on the normal diet and for rats on the corrected rachitogenic diet would be less likely to be greatly influenced by variations in the sensitivity of the rats to rickets.

Table IV A. *Percentage ash in the dried fat-extracted bones of young rats on McCollum's rachitogenic diet and receiving various doses of irradiated ergosterol, prophylactically for 24-33 days.*

No. of litter	Sex	Dose of irradiated ergosterol in international units of vitamin D (1 unit = 0.1 γ of irradiated ergosterol)										
		0	0.01	0.02	0.03	0.05	0.08	0.1	0.2	0.32	0.64	1.28
1826	♂	41.1	—	40.7	41.6	—	41.1	—	45.2	—	—	—
	♀	—	38.3	—	—	42.2	—	41.3	—	44.1	—	—
1827	♂	—	—	—	—	38.7	—	39.3	—	41.4	—	—
	♀	36.2	34.3	42.2	38.9	—	39.7	—	44.1	—	—	—
1914	♂	39.5	38.6	—	41.2	40.3	—	42.7	43.4	—	—	46.8
	♀	—	—	—	—	—	—	—	—	47.5	47.7	—
1917	♂	—	—	—	—	—	—	44.5	42.6	—	—	—
	♀	43.3	37.9	—	40.9	44.2	—	—	—	48.8	48.0	47.4
1915	♂	39.9	41.6	—	41.4	—	—	—	—	44.3	—	—
	♀	—	—	—	—	43.5	—	45.3	—	—	48.8	49.8
1943	♂	—	—	—	—	43.0	—	—	—	46.8	48.5	47.9
	♀	40.1	47.9	—	40.0	—	—	43.4	47.5	—	—	—
1954	♂	40.8	45.3	—	48.0	—	—	52.1	52.4	—	48.3	50.2
	♀	—	—	—	—	47.3	—	—	—	53.6	—	—
1963	♂	41.7	40.8	—	39.0	47.5	—	47.5	49.4	48.7	—	—
	♀	—	—	—	—	—	—	—	—	—	51.3	48.2
1965	♂	—	—	—	42.5	—	—	—	43.3	—	45.1	47.1
	♀	44.9	43.8	—	—	41.6	—	41.4	—	47.4	—	—
1991	♂	38.0	39.9	—	43.9	44.2	—	43.3	—	—	—	—
	♀	—	—	—	—	—	—	—	44.5	46.4	49.2	48.9
1828	♂	32.6	31.8	—	33.5	—	40.7	38.3	40.3	43.1	—	—
	♀	—	—	38.1	—	39.7	—	—	—	—	—	—
1834	♂	34.6	38.1	36.8	36.7	39.3	39.6	—	—	—	—	—
	♀	—	—	—	—	—	—	38.7	40.4	41.2	—	—
1854	♂	—	—	—	—	36.7	—	—	—	—	—	—
	♀	32.5	33.7	36.0	36.5	—	39.5	45.0	46.0	45.8	—	—
1869	♂	—	33.8	31.9	31.4	—	—	—	—	—	—	—
	♀	33.7	—	—	—	—	—	—	—	—	—	—
1867	♂	—	—	—	—	31.4	36.4	34.6	41.4	43.3	—	—
	♀	28.4	33.1	28.5	27.0	27.1	30.3	34.9	31.7	37.3	—	—
1875	♂	—	—	36.4	—	38.2	—	44.4	—	41.6	—	—
	♀	31.0	37.3	—	38.8	—	39.6	—	47.4	—	—	—
1988	♂	35.0	39.1	—	—	44.4	—	43.5	44.0	47.8	—	—
	♀	—	—	—	48.1	—	—	—	—	—	49.8	46.8
2057	♂	—	—	—	—	—	—	—	—	43.4	45.6	43.0
	♀	27.5	32.6	—	34.5	38.9	—	39.1	44.8	—	—	—
2060	♂	31.3	30.1	—	36.1	39.7	—	43.2	45.4	46.3	—	—
	♀	—	—	—	—	—	—	—	—	—	48.8	48.2
2095	♂	24.9	29.1	—	32.6	35.1	—	—	41.9	44.1	—	—
	♀	—	—	—	—	—	—	40.1	—	—	47.2	47.6
Av. of 20 litters		35.9	37.4	—	38.6	40.2	—	42.2	44.1	45.2	—	—
Av. of first 10 litters; 0 dose, ash over 35 %		40.6	40.8	—	41.7	43.3	—	44.1	45.8	46.9	—	—
Av. of last 10 litters; 0 dose, ash under 35 %		31.2	33.9	—	35.5	37.1	—	40.2	42.3	43.4	—	—
Av. of 8 litters including 0.02 and 0.08 unit doses		33.8	35.1	36.3	35.6	36.7	38.4	39.6	42.1	42.2	—	—
Av. of 12 litters including 0.64 and 1.28 unit doses		37.3	38.9	—	40.7	42.5	—	43.9	45.4	47.1	48.2	47.7

Table IV B. *The same series of averages, calculated as the A/R ratio.*

		Dose of irradiated ergosterol in international units of vitamin D (1 unit = 0.1 γ of irradiated ergosterol)										
		0	0.01	0.02	0.03	0.05	0.08	0.1	0.2	0.32	0.64	1.28
Av. of 20 litters	...	0.58	0.61	—	0.64	0.69	—	0.74	0.80	0.83	—	—
Av. of first 10 litters; 0 dose, ash over 35 %		0.69	0.70	—	0.72	0.77	—	0.80	—	0.89	—	—
Av. of last 10 litters; 0 dose, ash under 35 %		0.46	0.52	—	0.56	0.60	—	0.68	0.74	0.77	—	—
Av. of 8 litters including 0.02 and 0.08 unit doses		0.52	0.55	0.58	0.56	0.59	0.63	0.66	0.74	0.73	—	—
Av. of 12 litters including 0.64 and 1.28 unit doses		0.61	0.65	—	0.70	0.75	—	0.79	0.84	0.90	0.93	0.91

Table IV C. *The same series of averages, calculated as percentage ash in the dry non-extracted bone.*

Av. of 20 litters	...	31.7	32.6	—	34.0	35.1	—	37.0	37.8	38.4	—	—
Av. of first 10 litters; 0 dose, ash over 35 %		34.9	35.8	—	36.9	37.9	—	39.0	39.7	40.6	—	—
Av. of last 10 litters; 0 dose, ash under 35 %		28.4	29.4	—	31.1	32.3	—	34.9	35.9	36.1	—	—
Av. of 8 litters including 0.02 and 0.08 unit doses		29.4	29.9	32.6	31.3	32.2	33.8	34.6	35.9	36.3	—	—
Av. of 12 litters including 0.64 and 1.28 unit doses		33.1	34.4	—	35.8	37.1	—	38.5	39.1	39.7	40.8	39.3

Fig. 1 a. (Reproduced by kind permission from the paper of Dutcher, Creighton and Rothrock [1925].) "Bone ash expressed in per cent. of dry extracted femurs." Values obtained by killing groups of about 4 rats at weekly intervals: curve A, rats on normal diet; curve B, rats on Steenbock's (2965) rachitogenic diet, irradiated; curve C, rats on Steenbock's (2965) rachitogenic diet, without addition of vitamin D in any form.

Consideration of Dutcher's values and of those set out in Table I suggests that a series of doses ranging from one rather larger than 0.2 unit down to no dose at all should cover the range of calcification possible of attainment on the rachitogenic diet.

For the present purpose the number of doses between which comparison

could fairly be made was limited by the number of rats which could regularly be obtained in one litter, since it was desired to make the groups for each dose absolutely uniform in their litter constitution. The maximum number of young rats which could regularly be obtained in one litter was found to be 9, and it was therefore decided to select 9 doses, one animal from each litter receiving each dose.

The sexes were distributed as regularly as possible amongst the groups. Reference to Table IV A shows that, in the full series of 20 litters examined, the animals on each of those doses for which 20 animals were used include either 11 males and 9 females, or 10 of each sex.

As it appeared from the values obtained in Exp. 1 that the response to increase of dose was much greater with smaller doses than with larger doses, the smaller doses were spaced more closely than the larger ones. The doses chosen were therefore 0, 0.01, 0.02, 0.03, 0.05, 0.08, 0.1, 0.2 and 0.32 international units of vitamin D.

When 8 litters, *i.e.* 8 animals on each of the 9 doses had been used, the average results were plotted, and it was found that the smaller doses appeared to be somewhat too close together and that one or two larger doses might give useful information. Doses of 0.02 and 0.08 units were therefore omitted in the later experiments and doses of 0.64 and 1.28 units were substituted. In all 20 litters were used, the 7 doses of 0, 0.01, 0.03, 0.05, 0.1, 0.2 and 0.32 units being represented in all these litters, so that for each of these doses the average value for the percentage ash derived from the bones of 20 animals is available for the construction of the curve. For the doses of 0.02 and 0.08 units, the average of only 8 animals is available, and for the doses of 0.64 and 1.28 units, the average from 12 estimations.

The values thus obtained, calculated as percentage ash in the dried, fat-extracted bone, are set out in Table IV A. The corresponding average values, expressed as percentage ash in the dry bone and as A/R ratio, have also been calculated and are included in Tables IV B and C.

In Fig. 1 the curves are plotted showing the average calcification values for the 7 doses for which 20 animals were used, the values for calcification being calculated as percentage ash in the dried, fat-extracted bone (curve a), as percentage ash in the dry bone (curve a_1) and as the A/R ratio (curve a_2). In the further analysis of the results only the values expressed as percentage ash in the dried, fat-extracted bone are used, but by reference to Table IV B and C values calculated in either of the other two ways can be substituted. Comparison of the 3 curves in Fig. 1 shows that those derived from the values for the dried fat-extracted bone and from the A/R ratio are, as would be expected, practically identical in form. The curve plotted with the values derived from the dry bone is somewhat different. Inspection of the amount of fatty material extracted from the bone by ether and alcohol shows that this amount is larger for animals receiving higher doses of vitamin D than for those receiving lower doses. For the doses of 0.01 unit, the percentage fat extracted from the dry bone

lies between 12 and 13 but for doses of 0.2 and 0.32 units it rises to 14.4 and 15.7 respectively, thus tending to depress the value for the percentage ash in the dry non-extracted bone for these higher doses.

Fig. 1. Average values for the calcification of the bones of groups of 20 rats on McCollum's (3143) rachitogenic diet and receiving graded doses (0–0.32 international units) of the international standard solution of vitamin D (1 unit = 0.1 γ irradiated ergosterol). Calcification values calculated as: curve *a*, percentage ash in the dried extracted bone (Table IV A, first line of averages); curve *a*₁, percentage ash in dried bone (Table IV C, first line of averages); curve *a*₂, A/R ratio (Table IV B, first line of averages).

Fig. 2. Average values for the percentage ash in the dried extracted bones of groups of 12 rats on McCollum's (3143) rachitogenic diet and receiving graded doses (0–1.28 international units) of the international standard solution of vitamin D (1 unit = 0.1 γ irradiated ergosterol) (Table IV A, fifth line of averages).

In Fig. 2 the curve is plotted for the series of values derived from 12 animals for each dose, and this includes the two higher doses of 0.64 and 1.28 units (see Table IV A, last line of averages). The percentage ash for these 2 doses is 48.2 and 47.7 % respectively, while that for the dose of 0.32 unit, in the same series, is 47.1 %, showing that the maximum calcification attainable has already been reached in this series with the dose of 0.64 unit.

The form of the curve thus plotted in Fig. 2 suggests a logarithmic relationship between the two variables.

The question at once arises, can this curve be accepted as a standard, relating the responses of all young rats of a given age and weight, on the given diet, to a given series of doses of vitamin D? And if not, how are similar curves, constructed in other laboratories, with rats bred under other conditions, likely to differ from it? The greatest difference will certainly be due to the varying resistance which different stocks of rats, or the same stock of rats at different times, will offer to the decalcifying action of the rachitogenic diet. The great variation which exists in sensitivity to rickets in an apparently uniform population of young rats is shown by the wide variation in the levels of calcification attained by the rats receiving no dose in the present series (see Table IV A); this level ranges from 24.9 to 44.9 % ash content of the bones.

Fig. 3. Average values for percentage ash in the dried extracted bones of groups of young rats on McCollum's (3143) rachitogenic diet and receiving graded doses (0-0.32 international units) of the international standard solution of vitamin D (1 unit = 0.1 μ irradiated ergosterol): curve *a*, average values from 20 litters, *i.e.* 20 animals for each dose (same curve as Fig. 1, curve *a*) (Table IV A, first line of averages); curve *b*, average values from 10 litters, *i.e.* 10 animals for each dose, being that half of the series used for curve *a* in which the animals receiving no dose showed a percentage ash over 35 % (Table IV A, second line of averages); curve *c*, average values from 10 litters, *i.e.* 10 animals for each dose, being that half of the series used for curve *a* in which the animals receiving no dose showed a percentage ash under 35 % (Table IV A, third line of averages).

The present series of 20 litters can, by selection, be divided into two series, of which one, comprising the first 10 litters in Table IV A, shows a percentage ash of over 35 % for the rats receiving no dose of vitamin D, while the other series, comprising the last 10 litters in Table IV A, shows a percentage ash of under 35 % for the rats receiving no dose. Inspection of the serial numbers of these 20 litters shows that the more and less rachitic litters tend to occur in batches, but their occurrence at any given time has not as yet been correlated with any known set of environmental conditions.

In Fig. 3 the average values obtained with these two series of 10 litters are plotted against the doses, as two separate curves, together with the curve obtained with the whole 20 litters, which is reproduced from Fig. 1 for the sake of comparison. The 3 curves thus produced do not run parallel with one

another at different levels of calcification. For the higher doses they appear to do so, but for the lower doses the calcification of the more rachitic series of rats is relatively much lower than that of the less rachitic series.

Curves published by Dyer [1931], correlating doses of vitamin D with degrees of healing, as judged by the "line" test, show a similar divergence when a division is made into a more and a less rachitic series, although measurement in that case is made of deposition of calcium at the epiphysis of the bone, and in the present instance of prevention of decalcification in the whole bone.

Interpretation of the scale.

If the average values derived from estimations on 12 animals for the percentage ash in the dried fat-extracted bone of rats receiving doses of 0.03 to 1.28 units of vitamin D (Table IV_A, fifth line of averages; see also Fig. 2) are plotted against the logarithms of the doses (Fig. 4, curve (e)), the points are

Fig. 4. The ash percentages, plotted against the doses in Figs. 2 and 3, are replotted against the logarithms of the doses. Curves (a), (b) and (c) correspond with curves a, b and c in Fig. 3. Curve (d) is plotted from the series of average values for 8 animals on each dose (4 only for the dose of 2 units), taken from Table I. Curve (e) corresponds with the curve in Fig. 2.

found to lie approximately on a straight line, except in the case of the dose of 1.28 units, which falls completely out of series. This behaviour of the points may be interpreted as indicating that a logarithmic relation exists between degree of calcification and dose of vitamin D. In other words, when the doses of vitamin D are increased in geometrical series, the corresponding values for degree of calcification, as measured by ash content of the dried fat-extracted bones, advance in arithmetical series. This relation holds good until a point is reached with doses of vitamin D as large as 1.28 units, where calcification is limited by the insufficiency of phosphorus available in the diet. The relation also fails with the lowest and no dose, so that the graph, though a straight line for the major part of its course, is really S-shaped.

If the curves plotted in Fig. 3, representing the series derived from values for 20 animals on each dose, and its two subdivisions into a more and a less rachitic series, are similarly replotted, using the logarithms of the doses from 0.03 to 0.32 unit of vitamin D, the three sets of points lie approximately along three converging straight lines (Fig. 4, curves (a), (b), (c)), indicating that the constant increment in bone calcification, corresponding with the same multiple of dose, is less for the less rachitic series and greater for the more rachitic one.

If a similar curve (Fig. 4, curve (d)) is plotted for the small series of values for 8 animals each, on 4 different doses (0.05, 0.1, 0.2 and 2.0 units of vitamin D), described in Section I, Exp. 1 (see Table I), the points corresponding with the logarithms of the 3 lowest doses lie on yet another straight line, but the value for the logarithm of the high dose of 2 units, like that of 1.28 units in Fig. 4, curve (e), lies completely out of series.

The five series of points described above lie only approximately on five converging straight lines. The positions of these lines have been calculated according to the formula given by Fisher [1925, § 26]; when they are drawn and produced in the direction towards which they are converging, they tend to meet about a point (*A*) located by a percentage ash value of about 54, corresponding with the logarithm of a dose of about 5 units of vitamin D. One of the curves (Fig. 4, curve (b)), that for the least rachitic series, tends to cut the others rather than converge with them to a point, a state of affairs which appears to depend on the apparent aberrance of one ash value, that obtained for the dose of 0.05 unit; if this one value is omitted from the series in the calculation, the resulting line for curve (e) tends to converge with fair accuracy to the common point.

It is conceivable that this point of convergence represents the value for normal calcification for the bones of rats of the age used in this work (7–8 weeks at the end of experiment), which would be attained with increasing dose of vitamin D, if deficiency of phosphorus in the diet did not act as a limiting factor. Reference to the curve of Dutcher *et al.* shows that in their series the value for the ash in the dried extracted bones of rats of corresponding age on normal diet, was about 56 %, a value which tends to support the supposition. Further research is, however, required to establish that the point, thus obtained by extrapolation of the curves, really has this significance, and further investigation of the suggestion is contemplated. In any case, even if the point did represent the value for normal calcification, its position and indeed that of the curves themselves, could only be regarded as fixed for rats of a given age, since the percentage ash in the normal bone rises steadily, reaching a height of about 62 % at about 12 weeks old [Dutcher *et al.*, 1925].

The significance of the variation in susceptibility to rickets of the different litters of young rats is also discussed by Bourdillon and Bruce in the ensuing paper (p. 506). The superficially obvious interpretation is that the rats showing the lowest calcification are those which at the beginning of the experimental period have the lowest bodily reserve of vitamin D. We have no knowledge of

the way in which such reserves may be drawn upon, but it is hard to imagine any regular way in which they might be put out, which would yet preserve the logarithmic relation observed between dose and calcification, in different series of rats, showing different degrees of sensitivity to rickets.

It should not, however, be forgotten that the action of the rachitogenic diet is a decalcificatory one, and if we may take the value of 42 % obtained by Dutcher *et al.* for the ash in the dried extracted bone, as roughly representative of the normal value for the bones of young rats of the weight and age (40–45 g. and 3–4 weeks) at the beginning of the experiment of those used in the present series, we must regard all rats showing, at the end of the experiment, a percentage ash of less than 42, as animals in which an active process of decalcification of the bone has been going on. This makes it very hard to envisage what may be actually happening in the bone, though it is easy to conceive that the more rachitic the bone was becoming, the more economical might be the use made of any small amounts of vitamin D available.

There is no doubt also that differences in the amount of phosphorus available to the rat from any source, by altering the unbalanced Ca/P ratio (4/1) in the rachitogenic diet, would raise or lower the level of calcification on a given dose of vitamin D. Key and Morgan [1932] have shown that by lowering the ratio of calcium to phosphorus from 4/1 to 2/1 healing of rickets in the rat is induced equal to that brought about by a daily dose of 0.7 unit of vitamin D. There is, however, no evidence to show whether, when the ratio is altered in this way, the curve relating calcification to dose of vitamin D would still show the characteristics shown by the curves in this series. An examination of these possibilities is contemplated.

The fact, that when the effect is plotted against the logarithm of the doses the resultant graph, over the major part of its course, is a straight line, was noted also by Bourdillon *et al.* [1931] in constructing an X-ray scale for use with curative experiments, but since they were dealing with empirical numbers allotted to appearances in the bone, it could not be deduced that their curve possessed the fundamental significance, which emerges clearly from the present study. As they point out, the possibility could not be quite excluded that the logarithmic nature of the scale was due to an effect on the eye, in accordance with the Weber-Fechner law.

The existence of the logarithmic relation between dose and effect on calcification, coupled with the fact that it is preserved even when rats of different levels of sensitivity to rickets are used, so that the relation can be expressed by straight lines inclined at different angles and probably converging to a point, provides material which should be capable of application in the evaluation of unknown substances for vitamin D.

Application of the scale to the evaluation of unknown substances for vitamin D.

In applying the scale thus prepared to the estimation of the value of an unknown substance for vitamin D, the curves derived by plotting the percentage ash against either the dose (Fig. 3, curves *a*, *b*, *c*) or the logarithm of the dose (Fig. 4, curves (*a*), (*b*), (*c*)) may be used. The experimental procedure would be in either case the same; two groups of 8–10 young rats would receive suitable doses, 0.01 and 0.02 γ of irradiated ergosterol in a standard 0.01 % solution, the doses thus equalling 0.1 and 0.2 units of vitamin D, while two other groups of 8–10 rats, similar in litter constitution, would receive two doses of the unknown substance. The results for each dose would then be averaged. If the resulting values were such that the values of the percentage ash obtained for the doses of standard fell directly on one of the curves in Figs. 3 and 4, the values for the unknown substance could be read off against the dose or logarithm of the dose, on that particular curve, provided that the values for the unknown fell within the range of the curve. If they did not it would be necessary to repeat the experiment, selecting fresh doses of the unknown, until doses were arrived at which gave average values for the percentage ash, falling within the effective range of the curve.

It might, however, happen that the average values for the percentage ash given by the two doses of the standard did not fall on any of the curves already obtained; in that case it would be very much easier to use the curves in which the ash values are plotted against the logarithms of the doses, since these graphs are straight lines and knowledge of two points is sufficient to construct the whole graph.

The scale should not be used for doses of material having a value lower than the equivalent of 0.03 or higher than the equivalent of 0.32 or perhaps 0.64 unit of vitamin D.

If a sufficiently definite position could be ascribed to the point (*A* in Fig. 4) of convergence of the various straight lines correlating the ash values with the logarithms of the doses, it would be possible, with the knowledge of the position of this point and of that of the average ash value derived from 8–10 rats on a single dose of the standard, to construct the whole graph for rats of that given degree of sensitivity. It is improbable, however, that the position of this point can be more than approximately determined.

No attempt has so far been made to evaluate any unknown substance against the scale and instances cannot be found in the literature where groups of animals as large as 8–10 have received serially graded doses of irradiated ergosterol, yielding results which could be compared with those in the present paper. Schultz [1931], it is true, gives average results from ashing the bones of groups of 9 rats, receiving doses of 0, 1, 2, 3, 5, 10, 20 and 40 mg. of cod-liver oil. The average ash percentage in this series does not show any increase for doses above 3 mg., so that doses of 1, 2 and 3 mg. are the only ones

available for comparison. The percentage ash plotted against the logarithms of these doses gives points which are far from being on a straight line. The ash is, however, calculated as percentage ash in the fresh bone, and when thus calculated the values are subject to even greater irregularities than when calculated as percentage ash in the dry fat-extracted bone. Also his experimental groups, on the various doses, are not uniform in litter composition and unless such uniformity is very strictly preserved there would appear to be little hope of a smooth result, since variation between litters in their sensitivity to rickets is so great.

SUMMARY.

1. The construction is described of a scale correlating doses of a standard solution of irradiated ergosterol (international standard for vitamin D), administered prophylactically, with the percentage ash in the bones of young rats on a rachitogenic diet.

2. The groups of animals on the different doses had the same litter composition, and, when 10–20 animals were used for each dose, smooth curves were obtained.

3. When the values for the percentage ash thus found were plotted against the logarithms of the doses (with the exception of the lowest and the highest) a linear relationship between the two variables was revealed, showing that as the doses were increased in geometrical progression the calcification increments advanced in an arithmetical series.

4. The whole series of 20 litters of rats could be divided into a more and a less rachitic series of 10 litters each. When the values for the percentage ash for these two series were similarly plotted against the logarithms of the doses, the logarithmic relation was preserved, but the values for the percentage ash, though advancing in arithmetical series, did so by smaller increments in the case of the less rachitic series, so that the straight lines in the graph tended to converge.

5. The application of the scales thus plotted to the evaluation of unknown substances for vitamin D is discussed.

The work here described was begun in connection with the inquiry, initiated by the Medical Research Council, into the stability of standard solutions of vitamin D and was further pursued at the request of the Accessory Food Factors Committee of the Lister Institute and Medical Research Council, the assistance being financed by the Medical Research Council. The writers are, therefore, indebted to a large number of people for help and advice of various kinds, but particularly to Dr Burn who suggested the construction of the scale, to Sir Charles Martin, to Dr Chick and to Dr Coward.

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