XIX. THE QUANTITATIVE DETERMINATION OF VITAMIN D.

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(Received December 23rd, 1927.)

THE ordinary procedure for the quantitative determination of vitamins consists in giving the experimental animals during a preparatory period a diet which is complete in every respect, with the one exception that it lacks the vitamin with which the experiment is concerned. As soon as characteristic deficiency symptoms have definitely developed, the test period is initiated, during which the minimum curative dose is determined. As is already well known, we have in the case of vitamin C to determine the protective dose, for the condition of the teeth in scorbutic animals interferes with their development.

Certain difficulties are encountered in the quantitative determination of vitamin D which has of late years attracted much attention. The first requirement is that the animals (rats) shall develop rickets during the preparatory period. Very suggestive evidence of this condition are certain peculiarities in the animals' gait and movements, but these clinical signs do not warrant definite conclusions as to the degree which the disease has reached, and still less do they indicate the stages in a recovery which may be taking place. For the changes which occur in response to the administration of the missing vitamin are so gradual that the degree of the improvement is difficult to estimate.

Definite information can, of course, be obtained by a post-mortem examination. There are also the well-known analytical methods, such for example, as the determination of the water, ash and calcium, or of the ratio of the inorganic to the organic material in the long bones. These methods require considerable time and care; the bones have first to be most carefully freed from the soft tissues, and have then to be dried and analysed. Considerable simplification has been achieved by McCollum and his fellow-workers with their delicate and much-used line test. This renders possible a quantitative determination both of the severity of the rickets, and of the progress of recovery should this occur. Lastly, we may mention the reaction of the faeces [Zucker and Barnett, 1923; Jephcott and Bacharach, 1926] which

normally are acid, but which under a high calcium, low phosphorus rickets-producing diet become alkaline, turning acid again when an antirachitic diet is given. It is, however, not known whether changes in the p_H are proportional to the severity of the disease or not. In the most recent publication [Willimott and Wokes, 1927] the opinion is held that the alkaline reaction of the faeces is specifically indicative of absence of vitamin D, whether rickets do or do not develop.

At the end of the preparatory period, *i.e.* at the beginning of the test period, the quantitative diagnosis of rickets is thus made by chemical analysis or the line test, supported by the anatomical examination¹.

A fault common to all these methods is, however, that the same animals are not employed for the preparatory and the test periods. One is obliged to proceed in such a way that a certain number of animals are put on a rachitogenic diet and some of them are, after a suitable interval, killed and examined. If all are found to be rachitic, it is assumed that the survivors are so also. This procedure, as will shortly be pointed out, entails the possibility of a serious error which cannot be evaded even if some of the animals are kept as pathological controls.

The first condition for a uniform reaction is that the breeding stock as well as the lactating mothers and their progeny should have a diet which is both suitable and of a constant composition. The importance of this condition can be realised by a study of, among others, the exhaustive researches which have recently been published from the Lister Institute [Chick, 1926; Smith and Chick, 1926; Chick and Roscoe, 1926]. It was observed that the difference between winter and summer milk was sufficient to cause fluctuations in the reactions, the greater content of vitamin D in summer milk conferring greater resistance to a rachitogenic diet in the summer than in the winter. This difficulty was avoided by the use of dried winter milk in the summer. But, as Chick's tables [see 1926, p. 29] show, all the fluctuations were not abolished by this procedure. Of 10 young rats whose mothers had been given the dried winter milk, two were found to have long bones with a calcium content (dry weight) of 9.8 and 9.9 % respectively, whereas the rest showed higher figures, the percentage in the case of one animal being as high as 15.4. When such variations occur in tests carried out by experts, the only conclusion to be drawn is that these variations depend on fundamental, individual differences.

The existence of such differences has been confirmed by us by numerous tests. They may at any rate appear under a diet (Steenbock and Black, 1965) employed by us, either as familial (one litter differing from another) or as individual, half the number of animals in one litter, for example, developing well-defined rickets during the preparatory period, while others of the same

¹ The determination of the phosphorus content of the blood by micro-analysis is very useful when applied to large animals, but can hardly be used in connection with young rats, which have only a few cubic centimetres of blood.

litter remain nearly free from rickets, or show only early signs of it. These individual variations are most striking, notably when the litters are limited, as is our usual practice, to six or seven, so that all shall receive sufficient milk from the mother.

Similar variations recurred continually among a great number of litters which included a total of about 1000 young rats. Yet the extraneous conditions seemed to have been perfectly identical for all. Their parents had, throughout several generations, 5 to 6 years, lived under very uniform conditions—the same diet, the same quantity of light, the same opportunity for exercise, and so on. In spite of this, however, it was observed that, for example, of 30 animals on a rachitogenic diet, about 80 % showed well-developed rickets after 3 to 4 weeks, whereas the remainder did not suffer from rickets or only to a slight extent. Had 5 animals been killed and examined, they might well have been among the 80 %. One would, in such a case, have had in the test groups many almost normal animals, and incorrect conclusions might have been drawn with regard to the efficiency of the anti-rachitic substance tested. We have repeatedly let such resistant animals continue for an extra week on the rachitogenic diet without the development of any serious changes.

It may be of interest to remember that the human being is also subject to similar, very puzzling variations in susceptibility to rickets. On one and the same diet, and even on the milk of the same wet-nurse, it is not rare to find that one infant becomes rachitic and another not. Still more striking is the observation that twins are not the same with regard to rickets, although it would be impossible to have more uniform conditions than those under which they live. They have the same mother and father, and have lived in the same uterus for 9 months. Sex also plays a part. In Priestley's copious statistics, dealing with more than 75,000 cases of rickets, this disease was twice as common among boys as among girls [quoted by Pfaundler, 1927]. We have not made any observations as to sex incidence of the disease in rats.

We must, therefore, conclude that, in animals as well as in human beings, susceptibility to rickets depends on certain unknown factors which play a part in spite of uniform extraneous conditions, a uniform diet, etc., which do not eliminate these factors. The consequent mistakes can only be avoided by the exclusive use of animals with definitely demonstrable rickets, and by *employing the same animals for the preparatory and the test periods*, as is done with determinations of vitamins A and B. The radiographic observations made by many workers from time to time constitute a sure and logical way out of the difficulty. Many, including the present author, have been apt to regard this process as complicated and difficult. On closer study, it has proved to be easy and simple, and in our experience it provides a reliable, quantitative means of estimating both the degree of the disease and the changes provoked by the administration of an antirachitic substance. The following is a description of the technique we have employed.

The breeding stock employed has for several years lived during summer and winter at an approximately uniform temperature, about 21–22°, in a room facing north and provided with ordinary window glass. The dietary consisted originally of bread, yellow whole maize and whole milk *ad libitum*. Twice a week fresh vegetables (carrots, turnips and cabbages) and boiled meat were given. When we suspected that the young rats were getting too large a reserve supply of vitamins A and D from the milk of this dietary, we have, during the past year, mixed the milk with equal quantities of water, and we have been satisfied with this change. We have not encountered the difficulties, depending on the seasonal variations in the milk, described by the authors from the Lister Institute to whom we have referred above. Observations made on butter at different seasons of the year have shown us that, at any rate with regard to vitamin A, and probably also with regard to vitamin D, there is not such a great difference between summer milk and winter milk in Norway as there is in other countries. The explanation is probably to be found in the fact that roots and seeds are used less in the winter in Norway than elsewhere. The cows' winter fodder consists principally of hay, much of which comes from mountain levels favoured by active and prolonged insolation. Even as far south as Oslo, daylight during June and July lasts about 18 hours, the sun rising between 3 and 4 a.m., and setting between 9 and 10 p.m. Yet another factor acting in the same direction is the extensive use during the winter of the herring meal or cake, which contains vitamins A and D.

The young rats are taken from the mother when about 24 days old, and are put on a rachitogenic diet when they weigh 45–50 g. (the first weighing). After we had tested several dietaries, we have continued to use, for the time being, No. 2965 of Steenbock and Black [1925]. This consists of yellow corn 76 %, wheat gluten 20 %, calcium carbonate 3 % and sodium chloride 1 %, or McCollum's modification of the same diet (25 parts of maize replaced by 25 parts of whole wheat—information which we owe to the courtesy of E. M. Houghton). We have not been able to observe any definite difference in the action of these two dietaries. The animals develop well on both, and at the worst show naked eye signs only of commencing xerophthalmia.

After the animals have been about 25 days on the rachitogenic diet, they are weighed for the second time, and then a skiagram is taken of the left knee-joint which is chosen in every case for the sake of uniformity (a long series of comparative observations showed that the two knee-joints were always involved to the same extent). The rat is anaesthetised in a large tumbler into which a few cc. of ether are poured. The anaesthesia must be light to avoid the risk of being fatal. It is maintained, when necessary, by the use of a little funnel-shaped mask on to which some drops of ether are dripped. The rat is then immobilised, the left knee being gently extended. With practice, the taking and the developing of the photograph is very easy and does not require much time. A skilled laboratory assistant takes and

develops 20 films in 2 to 3 hours. Thus, a very great and welcome economy of time and labour is effected, as compared with the time and labour needed for the line test, not to mention the chemical analysis. When the small dental films measuring 3 by 4 cm. are used, the cost is very moderate. (We have used Eastman Duplicitised X-ray films.)

The test period.

The time needed for an antirachitic substance, such as cod-liver oil, to make its action demonstrable, depends on the dosage. Large doses act much more rapidly than smaller doses. We use 3 mg. of cod-liver oil a day as the dosage from which we start. After exactly 6 days, the rats are weighed for the third time. It is important that their growth should be satisfactory, for, as is well known, starvation helps to deposit calcium in the metaphysis and effects improvement in the rickets (cf. the modern treatment of rickets in human beings by a moderate underfeeding). We might also weigh the daily food intake, but this would entail giving each animal a separate cage and complicating the work to an unnecessary degree. Throughout the test period, the rats are, of course, still kept on the rachitogenic dietary, and if they continue to develop just as well as during the preparatory period, any improvement which occurs must be traced only to the antirachitic substance added to the dietary. If cod-liver oil is the subject of the test, growth is often promoted, and thus a further assurance is given that the positive results are correctly interpreted. Rats, which during the test period remain at the same weight or lose weight, are discarded.

Both during the preparatory and the test periods, the rats are kept in lots of four each in large cages which permit of exercise. The floor is covered with sawdust, and the rats have, therefore, free access to faeces.

Estimation of the results.

The degree of the rickets is indicated by one to four crosses. Thus, + + + + indicate a 4 to 5 mm.-wide, uncalcified, or almost uncalcified (see below) metaphysis which lies between the diaphysis and epiphysis of the tibia. A corresponding picture is also to be seen in the distal end of the femur but is less clear. A slightly narrower metaphysis, *e.g.* one of 3 to 4 mm. is indicated by + + +. Occasionally the transition from diaphysis to metaphysis is not quite sharply defined, or a narrow calcified strip, lying just over the proximal end of the diaphysis, is visible. In such cases the degree of the rickets is put at a lower figure, *e.g.* in class 3 instead of in class 4. Narrower metaphyses, with or without calcified strips, are indicated by + + and +. The metaphysis is often almost rectangular, but in the slighter degrees of rickets its shape is that of a blunt-cornered triangle, the base of which faces the fibula. The more advanced the disease, the more clearly do changes in the bones appear. In every group of four rats it is, therefore, necessary that at least two of them suffer from + + + + or + + + rickets. The different

stages in the disease are indicated in Figs. 1 to 4 (Plate I) which are obtained by first taking a diapositive of the film and then copying it. In this way the same distribution of light and shade is obtained as in the original negative.

The action of the substance tested is judged by comparing the pictures taken at the beginning and end of the test period. The sign $\div$ indicates that the disease has become worse, and the sign $\pm$ that no change has occurred, while recovery is indicated by one to four crosses. It is easy to decide whether the substance tested is, or is not, potent. The quantitative evaluation of a recovery involves, of course, a certain subjective element, but with practice it can be done with satisfactory regularity. Commencement of recovery is indicated, as a rule, by a somewhat ill-defined calcification shadow (a light strip on the film) in the proximal portion of the metaphysis, separated from the epiphysis by an uncalcified zone which appears black on the film. The commencing calcification often fails to extend throughout the whole of the horizontal width of the metaphysis, there being a gap in the middle. As recovery proceeds, the calcified strip becomes wider, a couple of zones of calcification may appear, and finally the whole of the metaphysis shows calcification, and the picture is practically normal.

The efficacy of the substance tested is indicated in units per g. on a principle analogous to that adopted by the American pharmacopoeia in its prescriptions for vitamin A. Thus, if a daily dose of 2 mg. yields a positive result, the substance contains 500 vitamin D units per g. etc.

Examples.

Lot 225. Cod-liver oil 346. Daily dose 4 mg.

Rat No.	Rickets	Healing	Change in wt' during the test period g.
1192	+++	++++	+14
1195	+++	+++	+10
1200	+++	++++	+7
1204	+++	+++	+9

Result. Very great antirachitic action, together with great gain of weight.

Conclusion. New test with a smaller dose, e.g. 2 mg.

Lot 296. Cod-liver oil 386. Daily dose 3 mg.

1860	+++	++	+5
1866	+++	+++	+4
1871	++	+++	+6
1880	+++	+++	+1

Conclusion. About 300 vitamin D units per g.

SUMMARY.

A quantitative method for determining vitamin D is described. Its chief features are the following.

(1) The same rats are used for the preparatory period as for the test period.

Fig. 1.
Rickets
+

Fig. 2.
Rickets
++

Fig. 3.
Rickets
+++

Fig. 4.
Rickets
++++

(2) *Preparatory period.* The rats are put on a Steenbock and Black diet 2965, or a similar diet. After about 25 days a skiagram of the left knee-joint is taken, and the rats are again weighed.

(3) *Test period.* The antirachitic substance to be tested is given for 6 days, after which another skiagram is taken and the rats are again weighed.

(4) The efficacy is indicated in units per g. on a principle analogous to that adopted by the United States Pharmacopoeia in its prescription for vitamin A.

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