65. THE DETERMINATION OF VITAMIN D IN FOOD SUBSTANCES CONTAINING PHOSPHORUS

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THE diet of the rats used in most determinations of vitamin D has a high-Ca, low-P content, the ratio Ca:P being 4:1 or as much as 8:1. These figures are always based on total P in spite of the fact that some of it may be in organic combination and not available [Bruce & Callow, 1934, 1]. It is now a matter of common knowledge that the rickets so produced can be largely, if not completely, healed by the addition of phosphates to bring the Ca:P ratio more nearly equal to 1. The absolute concentrations of Ca and P in the rachitogenic diets are rarely reported, and Shohl [1937] has in fact shown that in each of 8 different Ca:P ratios there were zones where rickets was produced when the absolute amounts of Ca and P were low, and other zones where no rickets was produced when the amounts were increased. The healing produced by changing the Ca and P content is apparently similar to that produced by giving vitamin D in addition to the rachitogenic diet. Hence the healing brought about by a dose of a food substance whose vitamin D content is being determined may be due partly to the vitamin D and partly to the P contained in the substance. This might be considered acceptable as a measure of the healing effect of this food substance if all cases of rickets were due to the high-Ca, low-P content of the diet consumed; but as rickets is also produced by a low-Ca, high-P diet, the giving of a food substance containing more P and even a certain amount of vitamin D also might do more harm than good. The determination of vitamin D in a food substance should therefore be a measure of this factor and of nothing else. Key & Morgan [1932] made an attempt to simplify determinations of vitamin D in food substances which contained enough P to alter the Ca:P ratio of the rachitogenic diet used by them. By replacing part of the calcium carbonate of the diet by the appropriate amounts of calcium phosphate they made diets with Ca:P ratios of 4:1, 4:2, 4:3 and 4:4. The effects of giving these diets to rats rendered rachitic by feeding on a diet with a Ca:P ratio of 4:1 were then compared with the effects of feeding graded doses of vitamin D, viz. 0.25, 0.5, 1.0 and 2.0 units daily, by simultaneous comparisons. From two experiments on rats with "slight" and "severe" rickets respectively, they concluded that changing the Ca:P ratio from 4:1 to 2:1 was equivalent to giving a dose of 0.7 unit of vitamin D daily. They concluded, however, that it was preferable to adjust the Ca:P ratio of the diets of the rats receiving the standard to that of the rats receiving the test substance rather than to make an arithmetical allowance in calculating the result, particularly as they had so little information as to the effect of other changes in the Ca:P ratio.

Bruce & Callow [1934, 2] pointed out that if the relation between healing and dose of vitamin D given was logarithmic when a diet with a particular Ca:P ratio was used and the effect of giving both extra P and vitamin D was an additive one, then the curve of response to graded doses of vitamin D plus a constant amount of P would not be logarithmic. By experiment they found that the

effect of altering the Ca : P ratio and at the same time giving vitamin D was indeed multiplicative and not additive. The curves of response relating healing to the logarithm of graded doses of vitamin D given, obtained with diets of different Ca : P ratios, were all straight lines and parallel to each other within the limits of experimental error. It seemed then desirable to test this point for ourselves with an arrangement of the experiment more like that of a determination of vitamin D in a foodstuff which might contain enough Ca and P in the dose tested to upset the Ca : P ratio of the basal diet. In two series of experiments we found that the response to a particular dose of vitamin D was not constant but that it bore a logarithmic relation to the dose of potassium phosphate given at the same time. Since this paper was written, O'Brien & Morgareidge [1939] have reported similar findings.

EXPERIMENTAL

We consider it essential in vitamin D determinations to divide each litter of rats equally between the groups given the one (or more) dose of standard and the one (or more) dose of test substance. The number of groups used and hence the number of doses tested at any one time are thereby limited. Therefore we carried out a number of separate experiments in each of which we compared the amounts of healing produced by:

(a) a certain dose of vitamin D (5 units in one series of experiments and 10 units in the other);

(b) a certain decrease in the ratio Ca : P made by adding potassium phosphate to the diet;

(c) the same dose of vitamin D plus the same decrease in the Ca : P ratio.

The comparisons were made by means of the "line test". For calculating changes in the Ca : P ratio, it was assumed that all the P in the rachitogenic diet was available.

In a preliminary experiment it was found that the rats would not take potassium phosphate as a separate dose even when mixed with dextrin and a little water. In all experiments reported in Table 1 therefore the amount that it

Table 1. *The healing brought about in rachitic rats by giving (a) 5 or 10 units of vitamin D, (b) graded doses of potassium phosphate and (c) 5 or 10 units of vitamin D plus graded doses of potassium phosphate, as judged by the "line test"*

No of rats	Amounts of K_2HPO_4 added to 70 g. diet, mg.	Ca : P ratio of diet	Average healing from			Difference between (c) and (a) healing
			(a) 10 units vit. D	(b) Additional P	(c) 10 units vit. D + added P	
45	60	3.84:1	1.46	0.00	1.55	0.09
42	115	3.69:1	1.51	0.00	2.34	0.83
45	230	3.43:1	2.08	0.08	3.29	1.21
45	460	3.0:1	2.28	0.12	4.29	2.01
45	920	2.4:1	1.28	0.15	4.08	2.80
45	1380	2.0:1	1.38	0.88	4.87	3.49
			(a) 5 units vit. D	(b) Added P	(c) 5 units vit. D + added P	
45	460	3.0:1	0.94	0.13	2.85	1.91
42	920	2.4:1	0.74	0.53	2.86	2.12
45	1380	2.0:1	0.73	0.82	4.20	3.47

was desired to give each rat in 10 days was mixed with 70 g. of food so that there would be the same change in the Ca : P ratio of all rats on any particular dose, though there would not necessarily be the same absolute amounts eaten.

RESULTS

The results of the experiments are summarized in Table 1. In every experiment the healing brought about by giving both vitamin D and additional P was greater than the sum of the healings brought about by the two treatments separately. Thus there was abundant evidence that the effect of giving both vitamin D and extra P was much more than additive. In particular it may be noted that in the series in which a dose of 10 units of vitamin D was always given, the lower doses of phosphate alone were not enough to bring about measurable healing but with the constant amount of vitamin D the healing was measurable and graded to the dose of phosphate given. Curves were obtained from these experiments by plotting average healing against:

(a) Graded doses of phosphate only, omitting lower doses which gave negligible healing (i) in comparison with 10 units vitamin D plus phosphate,

$$y = 4.145x - 12.136,$$

(ii) in comparison with 5 units vitamin D plus phosphate

$$y = 1.647x - 4.351.$$

(b) Graded doses of phosphate + 5 units vitamin D,

$$y = 2.524x - 4.076.$$

(c) Graded doses of phosphate + 10 units vitamin D,

$$y = 2.324x - 2.398.$$

The average slope of the two curves (a) (i) and (ii) is 2.896 which is very nearly equal to the slopes of the curves (b) 2.524 and (c) 2.324. Thus we have three curves of response to graded doses of phosphate given of equal slopes and at heights dependent upon the dose of vitamin D given at the same time.

For practical purposes this is as far as the calculation need be taken. It demonstrates very clearly that the vitamin D potency of a substance containing enough Ca and P in the dose tested to alter the Ca : P ratio of the rachitogenic diet cannot be determined directly by comparison with the standard. Moreover, it would be well-nigh impossible to construct a curve or table of sufficient accuracy to use for correcting the healing apparently due to vitamin D but partly due to changes in Ca : P ratio. Even if such a table were once constructed, the labour of determining the Ca : P ratio of the particular batch of diet used in any experiment and the amounts of Ca and P in the dose of test substance given, and especially of the amounts of available P in the two products, would be almost prohibitive. Even the labour of making these determinations on the test substance only, with a view to giving the appropriate amounts of salts of Ca and P to the control rats on the standard, would be heavy. The safest plan seems to be to extract the ether-soluble portion after saponification of the substance under examination and make a determination of the vitamin D content of the extract by direct comparison with the standard in the ordinary way.

A point brought out in the last column of Table 1 is worth considering. The figures in this column were obtained by subtracting the healing brought about by the dose of vitamin D only from the healing brought about by the dose of vitamin D plus the dose of phosphate. When the differences of the first series of experiments are plotted against the doses of phosphate given a curve of response is formed

whose equation is $y = 2.398 \log x - 4.242$. The slope of the curve is similar to the slopes of the curves of response to doses of vitamin D plus phosphate, which was to be expected, but the points lie more nearly on the line calculated than do the points of either of these other curves. This also would be expected since the variation in response between litters has been virtually eliminated by the subtraction of the healing produced by vitamin D only in each separate experiment. In fact for those workers who still doubt that there is a variation in response to vitamin D between different litters in addition to the variation in response between members of one litter, this finding should prove strong enough to remove their doubt. It is tempting to make calculations of the significance of the distances between the curves of response to 5 units plus phosphate and to 10 units plus phosphate respectively but that procedure cannot be sound because there was no "litter-mate" control between the different experiments.

SUMMARY

A reinvestigation has been made of the effect of giving vitamin D plus phosphate to rats fed on a rachitogenic diet of high-Ca, low-P content. In six separate experiments the average healings produced by (a) 10 units of vitamin D, (b) a dose of potassium phosphate and (c) 10 units of vitamin D plus a dose of phosphate were compared. The dose of phosphate was the same in groups (b) and (c) of each experiment but ranged from 60 to 1380 mg. per rat approximately in the different experiments. A shorter series of three experiments was carried out with doses of (a) 5 units of vitamin D, (b) a dose of phosphate and (c) 5 units of vitamin D plus a dose of phosphate. In this series the doses of phosphate were 460, 920 and 1380 mg. respectively per rat.

The lower doses of phosphate alone were practically without effect on calcification but all doses affected the calcification brought about by either 5 or 10 units of vitamin D. The curves of response to (a) 10 units of vitamin D plus graded doses of phosphate, (b) 5 units of vitamin D plus graded doses of phosphate and (c) graded doses of phosphate alone (omitting those which were very nearly zero) were all logarithmic and of similar slope, though at different heights, the curve (a) being the highest and (c) the lowest.

Therefore, as shown by Bruce & Callow [1934, 1, 2] by a slightly different method, it has been determined that giving both vitamin D and phosphate to rats receiving a rachitogenic diet which has a high-Ca, low-P content has a multiplicative effect on healing, not an additive one.

The only practical method, therefore, of determining the vitamin D content of the food substance which contains enough P in the dose tested to alter the Ca:P ratio of the diet given is to extract the ether-soluble portion after saponification and determine the vitamin D content of the extract.

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