CXXI. VITAMINS A AND D OF SPINACH.

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RECENT work on the differentiation of vitamins A and D has emphasised the need of a vitamin D-free source of vitamin A. This has been chiefly sought for in fresh spinach leaves, which have been shown by many investigators to contain considerable amounts of vitamin A. However, in spite of much work on the problem, the possible presence of vitamin D in spinach leaves still appears to be a matter of debate.

The first investigators to use spinach for the supply of fat-soluble vitamins appear to have been Steenbock and Gross [1920], who found that the dried leaves when fed to rats at the level of 5 % of the diet as the sole source of vitamin A, produced continued but subnormal growth over periods of 6 or 7 months. Osborne and Mendel [1920], employing an ether extract of dried spinach leaves, obtained adequate growth and cure of xerophthalmia with doses of 66 mg., equivalent to about 10 to 20 g. of fresh leaves. McClendon and Sedgwick [1921] secured satisfactory growth in rats with dried spinach administered in amounts equivalent to about 5 or 6 g. of the fresh leaves, and also found it of benefit for children. In these investigations vitamin A was not distinguished from vitamin D.

After the differentiation of the fat-soluble vitamins attention was turned to the possible antirachitic properties of spinach. McClendon and Shuck [1923], using dried spinach in amounts equivalent to about 8 or 9 g. of the fresh leaves, observed no antirachitic action. Zucker and Barnett [1923], who employed an acetone-ether-alcohol extract of the dried leaves, found its growth-promoting potency comparable with that of cod-liver oil, but no significant amount of vitamin D was detected. The amount fed, however, was not stated. Goldblatt and Zilva [1923], feeding the fresh leaves, obtained some growth with less than 0.25 g., but no increase in calcification in rats on a rachitogenic diet with doses up to 3 g., as much as it was found practicable to administer, and concluded that "there is a great disparity between the growth-promoting and the antirachitic potency of spinach."

The next step came when it was discovered that the antirachitic vitamin could be formed by the action of ultra-violet light, either that naturally present in sunlight, or artificially produced. Hess and Weinstock [1924] found

no appreciable amount of vitamin D in spinach or other green leaves, but were able to produce it by irradiation. Following up this idea, Chick and Roscoe [1926] showed that fresh spinach might contain slight amounts of vitamin D if collected in the summer, while Boas [1926] concluded that winter spinach contains an amount of vitamin D which is negligible compared with its content of vitamin A. Later, an attempt was made by Roscoe [1927] to increase the content of vitamin D by further exposure of the leaves to the ultra-violet rays in sunlight, but the results obtained were not conclusive. Thinking that this might be due to rats not eating sufficient greenstuff, Roscoe also tested "sunned" spinach on rabbits. It could not be shown conclusively that exposure to sunlight increased the content of vitamin D, but Roscoe succeeded in demonstrating that fresh spinach contains more of this vitamin than had previously been suspected.

While the substitution of the rabbit for the rat as an experimental animal in testing for vitamin D enables larger proportions of spinach to be included in the diet, our opinion was that the use of this modified technique involves new difficulties. First, the digestive and assimilative processes in a herbivorous animal are different from those in an omnivorous animal, the gastro-intestinal p_H varying considerably and being closely connected with the rôle of the vitamin [Redman, Willimott and Wokes, 1927]. Secondly, from the recent work of Mellanby and Killick [1926], it seems probable that the optimum conditions for calcium absorption in the rat differ from those in the rabbit. Thirdly, the administration of large doses of fresh spinach leaves necessarily involves the ingestion of considerable quantities of calcium and phosphorus, with consequent alteration of the calcium/phosphorus ratio. (Fresh spinach contains 0.09 % CaO and 0.13 % P_2O_5 according to Sherman [1911].) For these reasons we decided to employ a practically mineral-free extract of dried spinach leaves which could be administered to rats in large doses without upsetting the calcium/phosphorus ratio.

EXPERIMENTAL.

Preparation of the spinach extract.

7167 g. of fresh English spring spinach (picked at the beginning of May, 1926) were sorted, spread out on sheets of paper and dried at 18° for 2 days. The resulting product was a light green material weighing 726 g. This was first broken up by rubbing between the hands, the midribs and stalks were then removed, and the residue was ground in a mortar and sieved through a no. 10 sieve. The product was a light green powder weighing 640 g. This material was shaken with dry ether at room temperature for 3-hour periods, three successive lots of about 3 litres each being used. The first extract was dark green leaving a residue coloured light yellow-green. The marc was pressed on a Büchner funnel by hand so as to remove as much of the solvent as possible. This pressed marc on treatment with a second lot of ether gave a

much less darkly coloured extract, while the third extraction yielded a product only slightly coloured. The residue was then extracted with two successive lots of dry acetone, but little colour was obtained. The whole of the ether and acetone extracts were filtered, combined, and the solvent removed as rapidly as possible *in vacuo*. The resulting product was a dark green pasty mass, weighing 32.5 g. and representing 0.45 % of the fresh leaves. This was put aside in a desiccator protected from light, over sulphuric acid, and after a few days small red crystals were observed, probably of carotene and xanthophyll. The extract was stored in a desiccator filled with nitrogen and protected from light while in use.

Tests applied to the extract showed it to be free from oxidising enzymes (oxygenase and peroxidase). The ash was about 4.5 %. Since the fresh leaves contain about 2 % of ash [Plimmer, 1921], and the extract was concentrated over 200 times, only about 1 % of the mineral matter in the fresh leaves had passed into the extract. On taking up in light petroleum a few grams of the extract, decolorising with norite, filtering and evaporating, a colourless oily residue was obtained which gave transient blue colours with both arsenic trichloride and antimony trichloride [Willimott and Wokes, 1927]. Takahashi [Takahashi *et al.* 1925] using a vitamin A concentrate prepared both from cod-liver oil and from spinach, obtained transient blue colours with concentrated sulphuric acid, but we have not been able to trace any record of these colours, supposedly due to the vitamin, having been previously obtained with the former two reagents on a spinach extract.

Feeding experiments using high fat diets.

In a previous communication [Redman, Willimott and Wokes, 1927] it was pointed out that in tests for vitamin D two types of diet have been principally used by different investigators, viz. a high fat diet containing 15 to 17 % of fat, and a low fat diet containing less than 3 %. The high fat diet resembles the natural diet of the infant the more closely both in fat content and in its calcium/phosphorus ratio. It has been largely used by English workers, who have adopted bone changes as their chief criteria of antirachitic potency, but so far as we are aware, no results have yet been published of the faecal p_H of rats on high fat diets free from vitamin D. In view of the findings of Zucker and Barnett [1923] and the more recent work of Jephcott and Bacharach [1926] that the faecal p_H in the rat can be used to test for vitamin D, we thought it of interest to feed to rats a high fat diet in which the spinach extract previously described served as sole source of vitamin A, and vitamin D if any. The faecal p_H was observed as well as the bone changes more commonly employed as criteria for vitamin D.

Standard albino rats of Wistar Institute ancestry were placed on the basal diet usual in this laboratory containing 15 % fat, vitamin B being supplied by an extract of yeast and vitamin C by lemon juice. The spinach extract was administered at first by hand in advance of the basal ration but as the animals

consumed it readily without objection, the required dose was afterwards uniformly mixed in with the basal diet. By following the food intake, it was possible to give an amount of the extract such that each animal received on the average about 25 mg. Much larger amounts are consumed by rats without difficulty.

Table I. *Spinach extract used with a high fat diet as sole source of vitamins A and D.*

Group	Sex	No. of rats	Supply of vitamins	Daily dose (mg.)	Average weekly weights (g.)						Rickets		Xerophthalmia						
											Microscopic	A.R. ratio							
Experimental 1	♂	3	Spinach extract	25	55	72	79	91	102	109	Very slight	1.15	Absent						
	♀	3	Spinach extract	25	126	130	133	141	48	60	74	82	87	95	98	Very slight	1.21	Absent	
Positive control	♂	3	Cod-liver oil	50	100	101	106	56	72	94	119	141	164	178	Absent	1.25	Absent		
	♀	3	Cod-liver oil	50	194	210	221	48	62	82	96	107	118	129	138	142	143	Absent	1.29
Negative control	♂	2	Vitamin D as direct	—	55	59	72	85	88	87	Absent	—	Present in two animals						
	♀	3	irradiation of the animals	—	84	86	75	53	55	68	80	81	83	83	76	74	Absent	—	Present in two animals
Average faecal p_H																			
Experimental 1	♂ & ♀	6	—	—	6.8	7.0	7.1	7.4	7.4	7.4	—	—	—						
					7.5	7.5	7.5												
Negative control	♂ & ♀	5	—	—	6.8	7.0	6.9	6.8	6.7	6.8	—	—	—						
					6.7	6.8													

On account of the different rates of growth in the two sexes, separate records were made of the average weights of males and females, in each of the three groups. The groups were chosen so as to be well balanced in regard to age, weight, sex and litter origin, each containing originally three males and three females, but in the negative control group one male died just after the beginning of the experiment from some unknown cause, and was not included in the average data. Of the female negative controls, one died in the third week and a second in the eighth week, and their weights are included in the average data. The surviving female, and the two males, were chloroformed and autopsied at the end of 8 weeks, when vitamin A deficiency was pronounced.

The experimental results are summarised in Table I. It will be seen that 25 mg. of the spinach extract, equivalent to about 6 g. of the fresh leaves, secured freedom from xerophthalmia and steady though subnormal growth in all the six experimental animals over a period of 10 weeks. It is noteworthy that Chick and Roscoe [1926], using a similar high fat diet, and fresh spring spinach, did not succeed in entirely preventing xerophthalmia with doses reaching in one case as high as 2.6 g., although the growth obtained was rather better.

In regard to rickets, the bone changes in our experiments were not conclusive. Autopsies on the animals revealed very slight rickets in several but not in all cases, which is in agreement with the findings of Chick and Roscoe. The average A.R. ratio was only very slightly lower than normal. On the other hand, the faecal p_H became alkaline and, while fluctuating somewhat, remained on the average faintly alkaline throughout the experiment. From this it would appear that an alkaline faecal reaction is specific to absence of vitamin D, whether rickets develops or not, but the matter requires further investigation. At the end of the experiment, vitamin D was supplied to the remaining animals in two ways, either as cod-liver oil or by direct irradiation of the animals, the diet not being altered, and in each case the faecal reaction changed within a few days to definitely acid (p_H below 6.8).

Feeding experiments using low fat diets.

The low fat, vitamin D-free diet has been used mainly by American investigators, who find that it produces within a few weeks a definite rachitic condition recognisable either by bone changes or by an alkaline faecal p_H . Since our previous experiments with the high fat diet had shown that a 25 mg. dose of the spinach extract was apparently too low to supply sufficient vitamin D, we decided to administer a much larger dose of 100 mg., equivalent to over 20 g. of fresh leaves, or more than twice as much as had previously been given by other workers testing for vitamin D in spinach.

Four standard albino rats were put on Zucker's modified low fat diet, consisting of patent flour 85, powdered egg-albumin 10, calcium lactate 2.8, sodium chloride 2, ferric citrate 0.2 parts by weight, with 2 g. of fresh spinach leaves per animal per day. The faecal p_H was ascertained daily, both colorimetrically and electrometrically, employing the methods previously described [Redman, Willimott and Wokes, 1927]. The results are shown in Fig. 1, in which the curve is built up from the average p_H of the total faeces collected from the group of animals. The individual variations between different animals, and between samples obtained from the same animal at different times on the same day, were investigated by means of the capillary electrode, and it was found that the greatest variation was 0.3 p_H , but on the average the results agreed to within 0.2 or less.

After 9 days on this diet, the faecal reaction had become definitely alkaline (average p_H 7.7). When it had remained alkaline for several days, each animal was given separately a daily dose of about 100 mg. of the spinach extract by hand, in advance of the basal ration, the supply of fresh spinach leaves then being discontinued. This was readily consumed without exception, and the food intake rose slightly from about 9 g. per head per day before dosing to about 11 g. after dosing. Xerophthalmia was absent throughout the experiment. A slight drop in alkalinity resulted on administration of the 100 mg. doses of extract, but at the end of another 12 days the faeces were

still alkaline (average p_H 7.3, Fig. 1). On reducing the dose of extract to 50 mg., the faecal p_H became slightly more alkaline (average p_H 7.5). After the reaction had remained at this level for another 10 days, cod-liver oil was administered in doses of 0.1 g., and the change of average faecal p_H to below 6.8 within a week showed that the animals were reacting normally to the administration of vitamin D. The animals were immediately chloroformed and autopsied. Definite rickets was observed, and the A.R. ratio was found to range between 0.7 and 0.8. It would appear, therefore, that the spinach extract, when fed at a level supplying adequate amounts of vitamin A, does not contain significant amounts of vitamin D.

Fig. 1. Effect of feeding spinach extract to rats on Zucker's low fat rachitogenic diet.

Table II. *Summary of results obtained by various workers for content of vitamins A and D in spinach leaves.*

Reference	Type of diet	Form in which spinach was fed	Approx. equivalent in fresh leaves (g.)	Growth	Xerophthalmia	Rickets
Steenbock and Gross [1920]	—	Dried leaves	5-6	Steady but subnormal	Absent	—
Osborne and Mendel [1920]	High fat	Ether extract	15-20	Adequate	Cured	—
McClendon and Shuck [1923]	Low fat	Dried leaves	8-10	Adequate	Cured	No antirachitic property
Chick and Roscoe [1926]	High fat	Fresh leaves	0.5-2.6	Adequate	Very slight	Slight (? traces of vitamin D present)
Roscoe [1927]	Low fat	Fresh leaves	1.5	—	—	Severe, but small amount of vitamin D present
Willimott and Wokes [1927]	High fat	Ether-acetone extract	5	Steady but subnormal	Absent	Very slight
	Low fat	Ether-acetone extract	10	Adequate	Absent	Definite
	Low fat	Ether-acetone extract	20	Adequate	Absent	Some vitamin D present

NOTE. In calculating the approximate equivalent in fresh leaves of the doses of dried leaves, the fresh leaves have been assumed to contain about 92 % of moisture [Plimmer, 1921].

DISCUSSION.

In many previous investigations on spinach as a possible source of vitamins A and D, sufficient attention does not appear to have been paid to various factors which might affect the content of these two vitamins. Amongst these factors are exposure of the spinach to ultra-violet light, either artificially produced or naturally present in sunlight, to the action of oxidising enzymes known, from the work of Ciamician and Ravenna [1919], to be present in spinach leaves, or to heat and oxidation during the drying processes. Moreover, the variations in vitamin content of the fresh leaves, according to the amount of sunlight they have received, and the difficulty of feeding them regularly to rats in large doses, have thrown serious obstacles in the way of obtaining satisfactory quantitative data. In Table II are summarised results obtained by different workers, which show considerable variations in their estimate of the content of fat-soluble vitamins in spinach leaves. These differences are probably accounted for by the factors indicated above and possibly also by factors still unknown.

In our opinion, these varying factors can best be controlled by the use of an acetone-ether extract of the dried leaves which, when carefully prepared under given conditions, is a potent and stable source of vitamin A practically free from calcium and other mineral matter and from oxidising enzymes. The employment of such an extract has allowed the feeding of much larger doses of spinach than hitherto, and it has been found that the extract when fed at a level supplying ample quantities of vitamin A, does not contain more than traces of vitamin D. However, the fact that vitamin D may be present in these small amounts, which can be greatly increased by exposure to the action of the same agent (ultra-violet light) which under certain conditions appears to be responsible for the destruction of vitamin A, raises the interesting question of a possible balance between these two vitamins, a problem for further investigation.

SUMMARY.

1. An acetone-ether extract of dried spring spinach has been prepared more than 200 times as concentrated as the fresh leaves, and practically free from mineral matter and oxidising enzymes.
2. The extract was a potent and stable source of vitamin A in that 25 mg. daily doses secured complete freedom from xerophthalmia and induced satisfactory though slightly subnormal growth in rats.
3. The extract, when fed at this level, does not appear to contain significant amounts of vitamin D.

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