XXII. THE ANTI-RACHITIC VALUE OF FRESH SPINACH.

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INTRODUCTION.

FRESH green leaves form the entire diet of many higher animals and may therefore be supposed to contain the various factors necessary for complete nutrition. Experimental proof is forthcoming for the presence of many of these, including vitamins A and C. As regards the presence of the anti-rachitic vitamin D, however, the evidence is conflicting. The majority of workers who have investigated the point have found that green leaves, whilst rich in the fat-soluble vitamin A, are devoid of anti-rachitic properties, even when administered in large doses. This constituted one of the most convincing pieces of evidence in favour of the view advanced by McCollum and his co-workers [1922] that vitamin A and the anti-rachitic substance found associated with certain fats are not one substance, but two separate entities.

The previous experimental work concerned with the detection and evaluation of anti-rachitic properties has, however, been almost exclusively carried out with the aid of diets deficient in phosphorus, but rickets may develop in children reared on cow's milk, which affords an abundant supply of calcium and phosphorus. The puppy behaves similarly, as has been demonstrated by Mellanby and others. But in order to induce rickets with certainty in the young rat—the animal which has been largely adopted in the experimental study of rickets, because it is in many respects the more convenient—it has been found necessary to use diets deficient in calcium or phosphorus as well as in other factors. The famous rickets-producing diets of McCollum and his co-workers [1921], of Sherman and Pappenheimer [1921], and of Osborne, Mendel and Park [1923] are instances of diets short, but not deprived, of fat-soluble vitamins and at the same time very low in phosphorus.

To deprive the growing animal of phosphorus, however, is to complicate the issue by setting up a severe competition for this element between the bones on the one hand and the various soft tissues on the other. The situation is somewhat otherwise in the case of calcium. The skeleton of the rat contains calcium and phosphorus in the ratio, approximately, of 2 to 1, and whereas all but about 7 % of the total calcium contained in the body of rats weighing from 70 to 100 g. is in the bones [Boas and Chick, 1924], the soft tissues

account for nearly 30 % of the total phosphorus. This striking quantitative difference suggests that to deprive the growing animal of phosphorus might cause a more severe competition for this element between the bones and soft tissues than would occur even in case of a shortage of calcium, for which a comparatively small demand is made by the soft tissues.

Using diets deficient in phosphorus, as well as in fat-soluble vitamins, anti-rachitic influences have been demonstrated in sunlight [Hess, Unger and Pappenheimer, 1921] and ultra-violet radiation [Powers, Park, Shipley, McCollum and Simmonds, 1922], in cod-liver oil and some other fats [McCollum, Simmonds, Becker and Shipley, 1922] and in egg-yolk [Hess, 1923]. Many other foodstuffs, on the other hand, have been denied anti-rachitic value, and among them fresh green vegetables. McClendon and Shuck [1923] were unable to detect appreciable anti-rachitic properties in dried spinach leaves, even when consumed in amounts equivalent to 75 % of fresh leaves in the total diet, whereas amounts equal to 0.1 % showed a definite retardation of the onset of xerophthalmia. Goldblatt and Zilva [1923] obtained a similar negative result with a daily dose of 3.0 g. fresh spinach given as supplement to McCollum's No. 3143 diet [McCollum, Simmonds, Shipley and Park, 1921], a diet deficient in phosphorus as well as in fat-soluble vitamins. At the same time the minimal daily dose of spinach found necessary to stimulate growth, which had ceased upon a diet deficient only in fat-soluble vitamins, was found to be between 0.1 and 0.25 g. Zucker and Barnett [1923] could detect no anti-rachitic value in extracts of spinach leaves, in the preparation of which alcohol, acetone and ether were employed, but their experiments were also made with diets very low in phosphorus.

Hess and Weinstock [1924, 1925] found no anti-rachitic properties in fresh lettuce leaves. But the leaves, whether grown in the light or etiolated by growth in the dark, acquired anti-rachitic potency if irradiated with the mercury vapour lamp for $\frac{1}{2}$ –1 hour, which potency was not lost after three days' storage in the dark. The doses given were however very large, 10 g. per diem for young rats of 30–40 g. weight, and the growth was very slight.

On the other hand, Hart, Steenbock, Hoppert and Humphrey [1922] and Gaessler and McCandish [1923], working with milch cows, found that the addition of fresh or dried alfalfa to the diet had a beneficial influence upon the calcium balance of the cow, provided the alfalfa had not been stored too long. Recently Shipley, Kinney and McCollum [1924], working with rats, have also found definite anti-rachitic value in dried alfalfa, when administered in the form of extracts made with ether, alcohol and acetone. They used amounts in the diet equivalent to 25 %, reckoned as fresh tissue; they failed however, to detect any evidence of anti-rachitic action in case of cabbage, spinach and other vegetables. McClendon [1922] states that rickets can be prevented on a low phosphorus diet by means of spinach if 40 % is included.

Luce [1924] found that milk from a cow on pasture in summer had a high anti-rachitic value compared with that of milk given by the same cow when

receiving dry fodder in a dark stall in winter and came to the conclusion that the increase of vitamin D in the milk was due partly to the fresh green food and partly to the insolation enjoyed by the cow. The criterion adopted was the power of the milk to prevent rickets in young rats upon McCollum's No. 3143, low phosphorus, rickets-producing diet; and in this case, as in other researches employing diets of this character, the criticism is justified that the anti-rachitic effect, at least in part, may be due to the extra phosphorus received with the rations of milk. Since the protective daily dose of the "pasture-fed" milk lay between 2 and 5 cc., whilst from 15 to 20 cc. daily of the winter milk was required, the salt content of both milks being approximately the same, it is reasonable to assume a considerable superiority in the former of anti-rachitic properties.

In view of these somewhat conflicting results and of the importance of the subject, it seemed worth while to make further attempts to ascertain whether any anti-rachitic value could be demonstrated in fresh green leaves of other plants than alfalfa and in so doing to abandon altogether the use of diets deficient in phosphorus. The fresh leaves of the "prickly seeded" variety of spinach (*Spinacea oleracea*) were chosen because this variety of spinach can be grown in this country at most times of the year and is readily eaten by young rats; under favourable conditions it is possible to administer as much as 2 to 3 g. daily.

METHODS.

Young rats, about three weeks old and 25 to 35 g. in weight, were placed upon diets devoid of fat-soluble vitamins, but adequate as regards salts of calcium and phosphorus. The behaviour of rats receiving various daily rations of fresh spinach leaves was compared with that of control animals receiving the basal diet without addition, during a period of about six weeks. The increase in body weight during the period was taken as an indication of the amount of vitamin A contained in the spinach (possibly of the vitamin A combined with the vitamin D), whilst the degree of calcification of the bones at the end of the time provided a measure of the calcium-depositing power of the ration (vitamin D content). In most of the experiments comparison was also made with one or more animals from the same litter, which animals received cod-liver oil, or irradiation with ultra-violet light, or a ration of irradiated spinach.

Basal diet. The experimental diet, devoid of fat-soluble vitamins, which is usually employed in this Institute has the following composition:

Inactivated caseinogen 100 g., wheaten starch 250 g., cotton-seed oil 75 g., marmite 25 g., lemon juice 25 cc., salt mixture¹ 25 g. This diet which is known as "F" diet contains 0.4 % Ca and 0.9 % P reckoned on the dry weight and

¹ The salt mixture was the following:

NaCl	...	...	43 g.	CaH ₄ (PO ₄) ₂ · H ₂ O	...	136 g.
MgSO ₄ · 7H ₂ O	...	...	137	Ca lactate (5H ₂ O)	...	327
NaH ₂ PO ₄ · H ₂ O	...	...	87	Ferric citrate	...	30
K ₂ HPO ₄	...	...	240			

was used for the tests in Exps. 1, 2, 5. These amounts of calcium and phosphorus were, however, thought to be rather in excess of those occurring in more natural food and a second diet was devised, containing smaller proportions of Ca and P, viz.:—"F.R. diet" used in Exps. 3 and 4. In arranging this diet, the food eaten by the stock animals in this Institute was taken as a guide. This food consists mainly of bread, brown and white, cow's milk and fresh carrot and cabbage, with occasional additions of marmite and raw lean meat. It was estimated that a normal young rat of 70 g. weight would consume daily about 2 g. cabbage, 10 cc. milk, 15 g. bread. Such a diet would contain about 0.23 % Ca and 0.32 % P, reckoned on the dry weight of the diet.

	g.	Dry wt., g.	Ca, g.*	P, g.*
Cabbage	2	0.2	0.0023	0.0022
Milk	10	1.0	0.011	0.0083
Brown bread	15	9.0	0.009	0.022
Total	27	10.2	0.023	0.032

* Figures taken from Schall-Heisler [1917].

The following diet was prepared to reproduce the above diet of cabbage, bread and milk as regards the inorganic constituents: inactivated caseinogen 100 g., wheaten starch 250 g., hardened cotton seed oil 75 g., salt mixture (MgCO_3 14 parts, NaHCO_3 53 parts, NaCl 13 parts) 9 g., ferric citrate 0.4 g., calcium carbonate 1.5 g., K_2HPO_4 0.8 g., distilled water 300 cc. Direct estimations made on different batches of the diet, for which we are indebted to Dr M. Boas, gave the following mean values:

	Water	...	...	45 %	} on the fresh weight
	Ca	...	...	0.15 %	
	P	...	...	0.20 %	
and	Ca	...	...	0.27 %	} on the dry weight.
	P	...	...	0.37 %	

About one-half of the total calcium is derived from the calcium carbonate and about one-half from the caseinogen. Of the phosphorus also about one-half is accounted for by the caseinogen and the other half by the potassium phosphate and other constituents. So that, as in the natural diet which was taken as a model, a large proportion of the salts both of calcium and of phosphorus are presented in the form in which they exist in milk.

The rats were considerably younger than those usually employed in such tests, this being arranged to secure that the vitamin reserves in the test animals should be small. There is usually a check in growth and a tendency to diarrhoea when young rats, immediately after weaning, are placed upon artificial diets devoid of fat-soluble vitamins. To tide them over the change it is a good plan to provide bedding of purified cotton wool and to give a ration of dried cows' milk of low vitamin content for a day or two. In the earlier experiments control rats or those receiving the same additional rations were placed together, 2 or 3 in the same cage, but in the end it was found

more satisfactory to keep each animal separate after the first few days of the experiment. The looseness of stools was found to be more persistent on the F.R. than on the F diet, owing to the lower proportion of calcium salts in the former. In Exp. 4 the rats receiving cod-liver oil and the irradiated control animals were so severely affected that many had to be killed before the end of the experiment and thereafter only the F diet was used. Even so, irradiated animals and those receiving irradiated rations of spinach underwent a period of mild diarrhoea at the beginning of the observation; this, however, did not last more than 1 to 2 weeks.

All the animals except those receiving irradiation were kept in a poorly illuminated laboratory, screened from direct daylight. At the end of the experiment the rats were killed, the junctions of the 6th and 7th ribs on the right side were preserved for histological examination and the leg bones (femora, tibiae and fibulae) dissected out for chemical analysis. The water content was estimated by the loss of weight on drying for 24 hours at 105° to 110° and the fat by the loss of weight on boiling in alcohol for about 16 hours followed by extraction with ether in a Soxhlet apparatus for 24 hours. If the bones when fresh are split with a sharp scalpel, it is safe to reduce the time of the ether extraction to about 8 hours.

In Exps. 1, 2, 3 calcium was determined in the dried or fat-extracted bones by the method of Aron and Sebauer [1910]; in the later experiments (4 and 5) the fat-extracted bones were ashed in porcelain crucibles and the calcium calculated as 36 % of the weight of the ash. This figure was arrived at as the result of a series of analyses of similar bones in which the calcium was estimated directly in the ash by the method described by Boas [1924] based on that of McCrudden¹ [1909, 1911]. The mean figure found for 12 determinations was 35.94 % of the ash, the extremes being 34.85 and 37.18 %.

The histological diagnosis was kindly undertaken by Prof. V. Korenchevsky, who has added a note to this paper, explaining the criteria adopted.

EXPERIMENTS.

Exps. 1 and 2 (Tables I and II). Table I contains the details of Exp. 1 made with a litter of 12 young rats divided into four groups of three. One group received the basal diet only, whilst three similar groups consumed respectively daily rations of 0.1, 0.5 and 1.0 g. fresh raw spinach leaves in addition. The observation lasted 50 days. The rats which received the 0.1 g. spinach ration had a mean calcium content of the bones of 16.4 %, as compared with 14.1 % in control animals, both figures being reckoned on the dry weight of the bones (femur, tibia and fibula). The daily rations of 0.5

¹ In a series of estimations, in which the Ca content was determined separately in the two leg-bones of the same animal by the two methods respectively, the figure obtained by McCrudden's method was found to be on an average 3 % lower than that given by the method of Aron and Sebauer. The calcium determined by the latter method was found to be equal to 37.14 % of the weight of the ash.

and 1.0 g. spinach raised the Ca content to 16.5 and 16.8 % respectively. At the same time the growth of these rats and their general condition were much in advance of those of the control animals. The concentration of inorganic phosphorus in the blood was within normal limits in all cases.

Table I. Exp. 1. *Influence of fresh spinach upon calcium deposition in bones of young rats maintained on F diet deficient in fat-soluble vitamins (Ca = 0.4 %, P = 0.9 %, reckoned on dry weight of diet).*

Litter of 12 young rats, 21 days old at beginning of experiment.
Duration of experiment = 50 days. March-May 1924.

Spinach daily ration g.	Rat No.	Sex	Body weight			Increase % (males only)	Leg bones, femur, tibia and fibula		Average intake of basal diet g. daily	Blood P mg. per 100 cc.	
			Initial g.	Max. or final g.	Increase g.		H ₂ O %	Ca % (reckoned on dry wt.)		Inorganic	Total acid-soluble
0.0	1	♂	34	65	31	74	48.9	13.6	8.0	9.8	40
	2	♂	35	55	20		42.7	13.0			
	3	♀	38	69	31		45.4	15.7			
						Mean	45.7	14.1			
0.1	4	♀	35	55	20	122	51.2	16.0	9.0	8.4	36
	5	♂	37	76	39		47.0	17.35			
	6	♂	35	84	49		50.1	15.7			
						Mean	49.4	16.4			
0.5	7	♀	33	52	18	167	51.5	15.6	9-10 } 10-14 }	8.4	36
	8	♀	33	71	38		47.0	16.4			
	9	♂	36	96	60		49.1	17.4			
						Mean	49.2	16.5			
1.0	10	♀	34	74	40	200	47.2	16.9	10-11 } 10-17 }	8.3	42
	11	♂	38	129	91		49.6	18.0			
	12	♂	36	93	57		49.0	15.6			
						Mean	48.6	16.8			

Table II. Exp. 2. *Influence of a small daily ration of fresh spinach in inducing calcium deposition in bones of young rats maintained on F diet.*

Rats 19 days old at beginning of experiment. Duration of experiment = 43 days. April-July 1924.

Spinach daily ration g.	Rat No.	Sex	Body weight			Mean % increase	Leg bones. Ca % reckoned on dry wt.	Histology of rib-junctions
			Initial g.	Max. or final g.	Increase g.			
0.0	22	♂	29	56	27	109	11.9	Osteoporosis and slight rickets
	23	♀	29	65	36		14.9	Infraction and osteoporosis
	24	♂	30	54	24		15.9	Osteoporosis
	25	♀	27	53	26	88	15.3	Osteoporosis
						Mean	14.5	
0.1	26	♂	29	75	46	167	15.5	Osteoporosis and very slight rickets
	27	♀	31	85	54		16.5	Osteoporosis
	28	♀	26	46	20		15.6	Osteoporosis and slight rickets
						Mean	15.9	

The details of a second experiment in which a litter of seven young rats was employed are set forth in Table II. This experiment lasted 43 days. At the end of this period three rats, which consumed a daily ration of 0.1 g.

Table III. Exp. 3. Diet F.R. (Ca = 0.3 %; P = 0.4 %; reckoned on the dry weight.) Early spring spinach. Feb.-April 1925.

Litter	Rat no.	Sex	Dose, daily	Duration of experiment, days	Body weight		Analysis of bones, calculated on fresh weight, %				% Ca			Histology of rib-junctions	Remarks
					Initial	% increase	H ₂ O	Fat	Ash*	Residue	A/R	on dry wt.	on extr. dry wt.		
140	127	♂	0, Control	50	27	81	Not determined				0.836	16.2	17.3	Osteoporosis	Growth poor
131	131	♀	0, Control	45	24	87	56.8	0.73	19.36	23.15	0.836	16.6	16.85	Osteoporosis	Growth poor. Died on 45th day
129	129	♂	Spinach, 0.1 g.	51	29	203	56.0	5.18	17.58	21.19	0.830	14.8	16.8	Slight	Growth subnormal
132	132	♀	" 0.5 g.	51	22	245	55.7	3.55	18.90	21.85	0.965	15.8	17.2	"	"
130	130	♂	" 2.6 g. (average)	51	27	289	56.4	3.65	18.01	21.91	0.922	15.3	16.7	Very slight	Growth normal. P.M. 4 ribs fractured, fibula fractured
128	128	♂	Cod-liver oil, 1 drop	51	27	263	48.6	3.73	26.92	20.73	1.298	19.4	20.9	Nearly normal	Growth subnormal, but fair
146	133	♂	0, Control	39	35	103	51.6	8.42	18.17	21.82	0.833	13.9	16.8	Very slight	Growth poor
139	139	♂	Spinach, 2.2 g. (average)	37	30	167	56.1	3.89	16.63	23.39	0.711	14.0	15.4	Slight	Growth nearly normal. P.M. 1 rib fractured
135	135	♂	Cod-liver oil, 1 drop	39	32	231	48.3	5.68	24.78	21.21	1.168	17.8	20.0	Normal	Normal growth
<i>Animals irradiated:</i>															
134	134	♀	0	39	32	150	46.0	6.81	26.78	20.37	1.315	18.4	21.0	Slight	Growth fair
138	138	♀	Spinach, 0.5 g.	36	31	123	47.7	5.89	24.60	21.82	1.127	17.4	19.6	? Very slight	Growth good at first. Died on 26th day, after an accident; animal lost appetite

* Calculated = (calcium content reckoned on fresh weight) × 2.60.

fresh spinach, were found to have an average calcium content of 15.9 % in their bones, whereas the four control animals, which received the basal diet without supplement, had an average calcium content of only 14.5 %. The average growth in weight was also better in the former group, the weight of the male animals being increased by 170 %, whereas in case of the control rats the increase was 110 %. Histological examination of the rib-junctions showed a condition of osteoporosis in all the animals and a slight degree of rickets in certain animals of each group.

If the proportion of calcium in the dry bones at the end of the experiment, compared with that of the control animals, be taken as a measure of the calcium-depositing power of the spinach ration, it must be concluded that so small a dose of fresh spinach as 0.1 g. daily has a definite anti-rachitic effect. But it is a curious fact that, whereas this small dose raised the calcium content in Exp. 1 by 10 to 15 %, an increase in the spinach ration to ten times this amount, to 1.0 g. daily, made no further difference. The figure obtained for animals receiving the larger ration, 16.8 % Ca, was still far below the normal for animals of this age, viz. 20.5 % [see Korenchevsky, 1922]. The body weight was, however, increased in proportion to the spinach ration.

In the subsequent experiments the analyses of the bones were made more complete by extracting the fatty material before making the calcium determinations.

In general it is true to say that the bones of the control animals contained much more fat than those of animals receiving spinach (see Table VI), exceptions occurring only in those control animals which became sick and died before the end of the experiment or were killed after a period of semi-starvation and loss of body weight (*e.g.* rat 131, Table III). The difference in bone calcium between control animals and those receiving spinach therefore appears considerably less if the calculation is made upon the weight of the fat-extracted bones instead of upon the dry weight, see Tables VI and VII. It is probable, therefore, that the apparent improvement in calcification due to the spinach ration in Exps. 1 and 2 would have disappeared had the analyses been made on the fat-extracted bones. In any case this supposition affords the most likely explanation of the fact that increase of the ration failed to cause any corresponding increase in the calcium content of the bones.

Exp. 3. Early spring spinach grown February–April, 1925. This experiment included 28 rats from 5 different litters and the doses of spinach varied from 0.1 g. to 2.6 g. daily. Complete details concerning 2 of the 5 litters are set out in Table III and a summary of the whole experiment is given in Table IV. The growth of the animals receiving spinach was much stimulated, the ophthalmia was less or was entirely prevented, and the general condition was much improved in comparison with that of the control animals. But the calcium content of the bones, whether reckoned on the dry, or the extracted dry weight, showed no increase over that of the control animals even in the case of the animals receiving the larger rations. The total variation is from

15.1 % to 15.7 % Ca, reckoned on the dry weight and from 16.6 % to 17.5 % Ca, reckoned on the fat-extracted weight. These values, on the other hand, are definitely lower than those obtained for the animals receiving cod-liver oil or ultra-violet irradiation; in this case the figures range from 17.4 % to 18.4 % Ca, reckoned on the dry weight and from 19.6 % to 21.9 % Ca, reckoned on the fat-extracted dry weight (see Table IV).

Table IV.

Exp. 3. Diet F.R. Early spring spinach. February–April 1925.
Duration of experiment 37–52 days.

Addition to basal diet, daily	No. of animals	Average increase in body wt. %	Average calcium content of bones		Ratio A/R
			% on dry wt.	% on extracted dry wt.	
0, Control animals	8	86	15.1	17.1	0.85
Spinach 0.1 g.	6	187	15.4	17.2	0.90
0.5 g.	4	212	15.7	17.5	0.94
(averages) 1.4–2.6 g.	5	248	15.1	16.6	0.82
Cod-liver oil, 1 drop=0.02 g.	2	247	18.6	20.4	1.23
<i>Irradiated animals:</i>					
0, Control animals	1	150	18.4	21.0	1.32
Spinach 0.5 g.	1	123	17.4	19.6	1.13

The detailed analysis of the fresh bones given in Table III shows a striking constancy among all groups of animals in the organic residue of the bone after the fat had been extracted. The larger variations in bone composition seem to be confined to the water, fat and mineral constituents. These three components consistently amount to 77 % to 80 % of the total weight, leaving 23 % to 20 % for the organic residue. It seems probable that the value of the ratio *mineral ash/organic residue* might after all be the most trustworthy chemical guide to the efficiency of the process of calcification. In the tables A/R is used to signify this ratio.

In Exp. 3 this ratio showed no consistent increase corresponding to the inclusion of spinach in the diet, though the effect of irradiating the animal or of giving cod-liver oil is well marked. The histological investigation of the rib-junctions of the rats confirmed the results of the chemical analysis. The bones of the control rats showed a condition of osteoporosis. Those of the animals receiving spinach had in addition an increased zone of proliferating cartilage and more osteoid tissue, indicating a slight degree of rickets and a less degree of osteoporosis. In litter 140, for example, rat 130, receiving an average of 2.6 g. spinach daily, had a fractured fibula and four fractured ribs. The post-mortem examination and the histology of the rib-junctions showed a slight degree of rickets of which there was no trace in the control animals of the same family. Rat 130 had grown at the normal rate during the 51 days of the experiment, whilst the growth of the control animals was at less than one-half the normal rate.

The spinach used in this experiment was grown in the winter and early spring. The later experiments were therefore made with leaves grown in summer, to see whether the increased amount of ultra-violet light falling on the leaves might improve their anti-rachitic value.

Exps. 4 and 5. Summer-grown spinach, May–October, 1925. Tests were made with spinach grown from May to October and for comparison with each batch raised in open ground ("open" spinach), a crop was raised simultaneously in the same situation, but covered with a well-ventilated frame glazed with Crookes' "A" glass ("frame" spinach). This glass, which is clear but possesses a slight yellow tint, has the property of absorbing ultra-violet radiations of wave-length shorter than 3780 Å while transmitting 88 % of the visible light. To provide a standard for comparison with the two types of spinach, the "open" spinach fed to certain animals in Exp. 5 was irradiated immediately before consumption for 30 minutes at a distance of 36 cm. from a quartz mercury vapour arc lamp.

Exp. 4 was made with spinach grown from May to July, the period of the year when the sun's illumination lasts longest and is most intense. In 1925, this period included some intervals of very bright weather. The animals received the F.R. diet and the experiment was somewhat complicated by the occurrence of persistent looseness of stools among many of the animals, especially those which were irradiated. Table V gives a summary of observations on 36 rats from 5 litters, the various doses of spinach being distributed as evenly as possible among the animals of the different families.

Table V.

Exp. 4. Diet F.R. Summer spinach. May–July 1925.

Duration of experiment 40–44 days.

Addition to basal diet, daily	No. of animals	Average increase in body wt. %	Average calcium content of bones		Ratio A/R
			% on dry wt.	% on extracted dry wt.	
0, Control animals	9	73	13.6	16.9	0.88
"Frame" spinach 0.1 g.	2	178	14.9	17.4	0.94
0.5 g.	3	199	16.1	17.5	0.95
(averages) 1.0–2.5 g.	5	171	15.0	17.0	0.90
"Open" spinach 0.1 g.	3	154	14.8	17.3	0.93
0.5 g.	2	210	15.7	17.7	0.99
(averages) 0.9–2.0 g.	4	182	15.7	17.3	0.93
Cod-liver oil, 1 drop = 0.02 g.	5	172	17.0	18.7	1.08
<i>Irradiated animals:</i>					
0, Control animal	1	47	15.9	17.8	0.97
"Frame" spinach 0.1 g.	1	155	17.6	19.6	1.27
"Open" spinach 0.1 g.	1	93	16.5	18.2	1.03

In this experiment the spinach rations do appear to exert a small but definite influence in increasing the calcification of the bones, and the "open" spinach seems to be more effective than the "frame," whichever criterion is adopted for judging the effect. The calcium content of the bones is raised,

Table VI.

Exp. 5. Diet F. (Ca=0.4 %; P=0.9 %, reckoned on the dry weight.) Late summer spinach. August-October 1925.

Litter	Rat no.	Sex	Dose, daily	Duration of experiment, days	Body weight g.	Initial	Increase %	Analysis of bones, calculated on fresh weight, %				Ratio A/R	Ca %		Histology of rib-junctions	Ophthalmia	Remarks
								H ₂ O	Fat	Ash	Residue		on dry wt.	on extr. dry wt.			
260	188	♂	0, Control	43	31	45		47.3	13.7	19.0	20.0	0.95	12.9	17.5	Osteoporosis	Severe	Growth very poor
196	189	♀	0, "Open"	44	31	58		41.7	13.3	23.8	21.2	1.12	14.7	19.0	"	Slight	Growth poor
191	191	♀	0, "Open"	43	33	94		53.0	7.6	19.6	19.8	0.99	15.0	17.9	Osteoporosis and very slight rickets	Very slight	Growth poor, P.M. 5 fractured ribs
192	192	♀	"	43	33	106		48.6	5.9	23.9	21.7	1.10	16.8	19.0	Slight osteoporosis	None	Growth subnormal
190	190	♀	"	43	31	116		50.5	3.8	21.7	24.0	0.90	15.8	17.1	Slight osteomalacia	Very slight	"
189	189	♀	"	43	34	150		52.7	4.6	20.9	21.8	0.96	15.9	17.7	"	None	"
193	193	♀	"Frame" spinach 0.5 g.	43	34	121		48.2	6.7	23.4	21.7	1.07	16.2	18.6	Nearly normal	Doubtful	"
195	195	♀	"Irradiated" 0.5 g.	43	35	89		46.0	8.0	24.3	21.7	1.12	16.2	19.0	"	Slight	"
194	194	♀	"Irradiated" "open" spinach, 0.5 g.	43	31	190		45.7	2.3	29.4	22.6	1.30	19.5	20.3	Normal	None	Growth slightly subnormal
264	197	♂	0, Control	44	35	54		43.3	16.9	19.8	20.1	0.98	12.6	17.8	Osteoporosis and ? slight osteomalacia	Doubtful	Growth very poor
204	204	♀	0, "	43	38	37		44.9	15.8	19.2	20.2	0.95	12.5	17.5	Osteoporosis and ? slight osteomalacia; infraction	"	Growth very poor, P.M. 2 fractured ribs
200	200	♂	"Open" spinach, 0.5 g.	43	40	55		46.5	14.0	19.1	20.4	0.94	12.9	17.4	Osteoporosis	"	Growth poor
198	198	♀	"	43	37	100		46.8	11.5	20.5	21.1	0.97	13.9	17.7	Osteoporosis and very slight osteomalacia	"	Growth subnormal
202	202	♂	"Frame" spinach, 0.65 g. (av.)	43	37	81		48.2	9.7	20.7	21.4	0.97	14.4	17.7	Slight osteoporosis	Slight	Growth poor
199	199	♂	Irradiated "open" spinach, 0.5 g.	43	39	138		43.7	9.5	25.5	21.3	1.20	16.3	19.6	Slight osteoporosis, nearly normal	Doubtful	Growth subnormal
205	205	♀	Irradiated "open" spinach, 1.9 g. (av.)	43	36	200		44.4	5.5	28.4	21.7	1.31	18.3	20.4	"	None	Growth subnormal, improving later
201	201	♂	Cod-liver oil, 3 drops	44	36	200		49.5	7.5	27.7	21.3	1.30	17.7	20.4	Normal	Doubtful	"
203	203	♀	"	43	33	164		45.7	5.1	27.6	21.6	1.28	18.3	20.2	"	None	"
212	212	♀	"	44	35	83		43.4	11.5	24.0	21.2	1.13	15.2	19.1	Osteoporosis	Very slight	Growth poor
208	208	♀	0, Control	44	37	59		43.3	11.1	24.5	21.1	1.16	15.6	19.4	"	Doubtful	Growth very poor
213	213	♂	"Open" spinach, 0.5 g.	44	36	72		46.3	8.6	23.6	21.5	1.10	15.8	18.9	Less osteoporosis; increased osteoid in shaft	None	Growth poor
209	209	♂	"	44	39	103		45.1	8.6	24.5	21.8	1.08	16.0	19.0	"	"	Growth subnormal
211	211	♂	"Frame" spinach, 0.5 g.	44	37	76		46.2	10.5	23.2	21.1	1.05	14.9	18.5	Osteoporosis	Doubtful	Growth poor
210	210	♂	"	44	35	94		45.5	7.2	24.1	22.3	1.08	16.0	18.4	Osteoporosis; increased osteoid in shaft	"	"
207	207	♀	Irradiated "open" spinach, 0.5 g.	44	34	156		44.2	4.7	29.5	21.6	1.36	19.0	20.8	Nearer normal than control	None	Growth subnormal

* Calculated = 36% of ash.

whether calculation is made on the dry, or on the extracted dry weight, and the value of the ratio A/R is also definitely increased. In all three cases however the figures are lower than those of corresponding animals which had received ultra-violet irradiation (Table V).

Exp. 5 (Tables VI and VII) was made with spinach grown in late summer, from August to October. The results recall those of Exps. 1 and 2. The addition of "open" or "frame" spinach, whilst stimulating growth and preventing xerophthalmia, caused no improvement in the calcification of the bones. The calcium content, reckoned on the dry weight, was raised relatively to that of the control animals, but the figures calculated on the fat-extracted weight showed no increase and there is none in the A/R ratio.

Table VII.

Exp. 5. Diet F. Late summer spinach. August–October 1925.
Duration of experiment 43–44 days.

Addition to basal diet, daily	No. of animals	Average increase in body wt. %	Average calcium content of bones		Ratio A/R
			% on dry wt.	% on extracted dry wt.	
0, Control animals	6	56	13.9	18.4	1.05
"Frame" spinach 0.5 g.	3	95	15.8	18.7	1.08
(averages) 0.65–1.2 g.	2	88	15.2	18.0	1.02
"Open" spinach 0.5 g.	4	82	15.1	18.3	1.03
(averages) 1.0–2.6 g.	4	117	15.4	17.9	0.96
Irradiated "open" spinach 0.5 g.	3	161	18.3	20.2	1.29
(average) 1.9 g.	1	156	18.3	20.4	1.31
Cod-liver oil, 3 drops = 0.06 g.	2	182	18.0	20.3	1.29

The histological investigation of the ribs of the animals receiving spinach showed on the whole a less degree of osteoporosis than was present in the control animals, and in addition a tendency to slight osteomalacia as evidenced by an increased amount of osteoid tissue in the shaft of the bone. The bone cartilage junction showed no increased tendency to disorganisation as in the rats receiving spinach in Exp. 3. The animals in Exp. 5 were a little heavier at the beginning of the experiment, 31 to 40 g. in weight instead of 20 to 30 g., and the F diet was used with its higher proportion of Ca and P, instead of the F.R. diet. This may account for the different type of the departure from the normal.

The artificially irradiated spinach had a very marked effect upon the calcification of the bones. The average calcium content of the fat-extracted bones was increased from 18.4 % Ca to 20 % Ca and the A/R ratio was increased from 1.06 to 1.31 (Table VII). The histology of the rib-junctions was more nearly normal than in the case of the control animals (see Table VI).

DISCUSSION.

The above experiments confirm the observations made by Hess and Weinstock [1924, 1925] showing that young green wheat or fresh lettuce leaves had no power to prevent rickets in rats, but acquired anti-rachitic properties when irradiated with ultra-violet light from an artificial source. These tests were made with diets deprived of fat-soluble vitamins and at the same time very low in phosphorus, whereas the diets employed in the present work were devoid only of fat-soluble vitamins. It was thought that anti-rachitic properties might be revealed under the latter conditions which were masked under the more severe tests made with diets deficient in phosphorus.

This has proved not to be the case with the exception of Exp. 4, in which spinach grown under the most favourable conditions of natural illumination appeared to possess a slight calcium-depositing effect on the bones of the rats which consumed it.

It is difficult to understand why direct sunlight falling on the green leaves should not confer definite anti-rachitic properties, seeing that irradiation with ultra-violet light from artificial sources does so in so marked a degree. In other cases the biological action of the two types of radiation has been found to be exactly comparable. Rickets in children or in animals is prevented or cured equally by exposure to either. The reaction of animals sensitised with eosin or haematoporphyrin is also similar when exposed to either type of light. The difference found in the case of green leaves might be due

(1) to a difference in the *degree of intensity*, the sun's radiation even under the most favourable circumstances being much feebler in ultra-violet rays of certain wave-lengths, or

(2) to a rapid removal or decomposition of any anti-rachitic substances that might be produced in the growing leaf.

In the present case the spinach leaves were usually cut two or three times a week after sunset, placed in airtight tins over night and then kept in the dark at a low temperature until fed to the animals. This procedure was adopted in order to prevent any exposure of the "frame" spinach to unfiltered sunlight. It was therefore conceivable that any anti-rachitic powers possessed originally might have been lost during the period of storage.

Hess and Weinstock, however, found that irradiated cut lettuce leaves and green wheat seedlings retained their anti-rachitic potency after being stored for 3 days and 2 weeks respectively. It does not therefore seem likely that anti-rachitic substances are formed in the leaf tissue and then rapidly destroyed, although the conditions of a growing leaf attached to the plant might be different in this respect from those of a cut leaf or shoot. It would be interesting to investigate the leaves of summer-grown spinach fed to animals immediately after being gathered in the middle of the day.

It is conceivable that the high vitamin A content of green leaves might prevent the detection of vitamin D, if present in small amount. The rats

receiving spinach grew much larger than the control animals and were better animals in every way. The histology of the rib-junctions showed a greater tendency to rickets, but the bones were equally well, and in Exp. 4 better, calcified, notwithstanding the greater strain imposed by the greater growth. This fact is also brought out by the work of Boas [1926] in which direct measurements of calcium and phosphorus retention were made in animals upon diets deprived of fat-soluble vitamins with and without additions of the fresh leaves of winter spinach. It is possible that a marked disproportion between the respective amounts of vitamins A and D provided in the additional ration might limit the effectiveness of the latter if present in very small quantity. The recent work of Mellanby [1925], showing the effect of certain cereals in increasing the severity of rickets in dogs when added to a rickets-producing diet, may have an analogous explanation. The potency of the different cereals will be found to be in proportion to their content of vitamin A, wheat germ, oatmeal and whole wheat flour being more potent than rice or white wheat flour. At the same time definite anti-rachitic properties were demonstrated in the unsaponifiable fractions isolated from the fat contained in both oatmeal and wheat germ.

The failure to demonstrate any appreciable anti-rachitic potency in green leaves exposed to natural sunlight raises the question of the origin of the anti-rachitic vitamin in nature. It may well be that vitamin D is present but that the amount is small and can only be detected in cases in which large quantities of leaves are consumed, as in the experiments of Shipley, Kinney and McCollum [1924] with dried alfalfa. Herbivorous animals in the natural state are certainly protected from rickets, but they take a diet composed almost exclusively of fresh green food and are at the same time themselves exposed to the sun's direct radiation. The origin of the rich stores of anti-rachitic vitamin in the liver of the cod and other fishes is a more difficult problem. For these animals spend most of their life at a depth below the surface of the water at which they must be exposed only to a very small fraction of the total ultra-violet radiation of the sun. Their food has been traced ultimately to green algae which are intermittently exposed to direct sunlight, but these, if at all similar to the green leaves of land plants, may be assumed to be but poorly endowed with the anti-rachitic substance. It would be worth while to make a more complete study of some marine green diatoms, such as *Nitzschia*, which has been shown by Jameson, Drummond and Coward [1922] to be rich in vitamin A, but whose anti-rachitic value has not yet been investigated.

SUMMARY.

1. Fresh leaves of spinach are a rich source of vitamin A, a small daily ration (0.1 g. and upwards) encouraging growth and lessening or preventing xerophthalmia in young rats on diets devoid of fat-soluble vitamins.
2. Spinach grown in the open in winter, spring or autumn possesses no

anti-rachitic properties that can be demonstrated by the methods employed. Spinach grown in midsummer has a slight but appreciable anti-rachitic value.

3. Spinach leaves when irradiated with ultra-violet rays from a mercury vapour quartz lamp become powerfully anti-rachitic.

4. There is no obvious explanation for this discrepancy between the effect of direct sunlight and of artificial ultra-violet radiation. The difference may be one of degree or there may be a rapid removal or destruction of newly formed anti-rachitic substance in the leaves while these are attached to the living plant. It is also possible that the failure to demonstrate anti-rachitic properties in naturally illuminated spinach may be due partly to their disproportionate content of vitamin A.

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Note on the criteria adopted for the diagnosis of rickets, based upon histological examination. By V. KORENCHEVSKY.

THE principal pathological change found in the bones of the rats described above was one of osteoporosis. In addition to this, the rib-junctions of some rats showed a slight or very slight degree of rickets. This consisted of an increased amount of osteoid tissue in relation to the thickness of the trabeculae or of the shaft walls. The proliferating cartilage was also increased in some cases to a slight or moderate degree and there was also a partial deficiency in the calcification of the zone of provisional calcification.

In some rats of Exp. 5 there was an increased amount of osteoid tissue in the shaft of the bones without any increase in the proliferating cartilage or deficiency of calcium in the zone of provisional calcification. These cases were diagnosed as osteomalacia.

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