LXVI. THE ASSAY OF THE ANTIRACHITIC VITAMIN D.

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IN the past the methods for assay of vitamin D in foodstuffs have consisted usually in determining the power of the foodstuffs to prevent or cure rickets induced in rats by a diet containing unfavourable amounts, or an unfavourable balance, of calcium salts and phosphates. Such diets have been devised and used by McCollum and his co-workers [1921], by Sherman and Pappenheimer [1921] and by Steenbock and Black [1925].

When young rats are nourished on these diets for two or three weeks without any supplement they are in effect starved of calcium or of phosphorus or of both, and almost invariably develop a degree of rickets which can be recognised by typical histological appearances, and they have an abnormally low proportion of calcium in the bones. The rickets can be prevented or cured by any powerful antirachitic agent. Such are irradiation of the animal or its food with ultra-violet light, or the administration of cod-liver oil or irradiated ergosterol [Rosenheim and Webster, 1927]. In animals fed on low-phosphorus rachitogenic diets, these measures seem to result in diverting the inadequate supply of phosphorus to the bony tissues in preference to the soft ones [Chick and Roscoe, 1926, 1]. The animal does not grow, but calcium phosphate is laid down in the bones, and these show no marked divergence from the normal in structure. The amount of any supplementary foodstuff which is found necessary to prevent rickets on such a diet is in inverse proportion to its antirachitic value.

The above method does not, however, satisfy the primary requirement which should be demanded of any biological method for vitamin assay, viz. that the basal diet of the experimental animal, while devoid of the vitamin in question, should contain optimal amounts of all other necessary constituents. All the diets mentioned above, in addition to being devoid of vitamin D, contain a defective salt mixture and an inadequate provision of vitamin A: in some there is also an inadequate supply of protein and of vitamin B.

It is obvious that the basal diet should contain a perfectly balanced salt mixture, and adequate protein supply, as well as optimal amounts of vitamins A and B. The problem of finding such a perfect basal diet ultimately resolves

itself into a search for some suitable source of vitamin A which shall be devoid of vitamin D. For these two fat-soluble vitamins are usually found together in nature and although vitamin D is much less abundant there are few known sources of vitamin A in which traces of vitamin D cannot also be demonstrated.

Steenbock, Nelson and Black [1924] found that "hog millet," when given to the extent of 84 % of the diet, provided adequate vitamin A and did not add materially to the antirachitic value of the diet. They also investigated dried alfalfa but found that the action of vitamin D could be demonstrated if more than 1 % was present in the ration. Miss E. M. Hume, in the course of work not yet published and kindly placed at our disposal, used wheat embryo as a source of vitamin A for young rats. She found that a daily dose of 2 g. of wheat embryo provided no significant amount of vitamin D, as judged by the percentage of ash in the fat-extracted bones at the end of the experiment. The leg bones of control animals, receiving no addition to the experimental diet deprived of fat-soluble vitamins, had an ash content of from 50.9 to 55.3 %, while in those receiving the wheat embryo, this value varied from 48.5 to 52.9 %.

CRITERIA ADOPTED.

(a) *Calcification of bones.* When young rats are fed upon diets which contain an adequate supply of calcium salts and phosphates, even when vitamin D is absent, it is not usual to obtain a degree of rickets that can be recognised histologically. Defective calcification of the bones is nevertheless shown by the occurrence of osteoporosis and by a lowered calcium content, which can be demonstrated by chemical analysis.

The effect on the bony tissues of diets containing optimal salt content, but deprived of fat soluble vitamins, has been studied by Chick, Korenchevsky and Roscoe [1926]. These authors came to the conclusion that the best measure of bone calcification was given by the value of the A/R ratio or the ratio of the amount of mineral ash to the amount of organic material contained in the fat-extracted bone. The value of this ratio in a normally nourished young rat was found to be about 1.5, and if the diet were deficient in vitamin D ("F" diet + fresh autumn-grown spinach, see their Table, p. 626) this value fell to about 0.9 to 1.0. When nourished on a low-phosphorus rachitogenic diet, the value of A/R can be as low as 0.5 to 0.3 [Chick and Roscoe, 1926, 2]. Histological rickets is prevented on such a diet by supplying vitamin D as cod-liver oil or by ultra-violet irradiation, but the calcification of the bones never approaches the normal level although A/R may be raised to a value of about 1.0.

(b) *Growth; increase in body weight.* Steenbock, Sell and Jones [1923] and Steenbock and Nelson [1923], were the first to insist that the antirachitic vitamin D was essential in a diet to secure normal increase in weight in young rats. Steenbock and his collaborators [1924] have shown further that increase in weight may be used as a criterion for vitamin D assay if the young animals

are provided with all other essential dietary factors, including vitamin A. These investigators have done service to this branch of nutritional study in maintaining that no single vitamin can properly be considered as an especial growth factor, seeing that under appropriate circumstances, growth can be limited or entirely prevented by deficiency of any one of a whole series of necessary constituents, including vitamins.

DIETS.

The work described in the present paper resolves itself into an attempt to discover a satisfactory basal experimental diet which shall contain adequate vitamin A unaccompanied by vitamin D. In consideration of the results obtained by Steenbock and his co-workers and by Hume, the three materials selected for trial were wheat embryo, hog millet and alfalfa meal. The following four diets were prepared.

1. Diet *F*, a diet complete in all other respects but devoid of both fat-soluble vitamins A and D.

Diets 2, 3 and 4 in which vitamin A was supplied respectively, as 20 % wheat embryo, 50 % hog millet, and 1 % alfalfa meal.

In Diets 2, 3 and 4 the amounts of starch and caseinogen were modified so that there should still be about 50 % carbohydrate and 20 % protein, reckoned on the dry weight of the diet.

Table I.

	Parts by weight			
	1. <i>F</i>	2	3	4
Wheat starch	50	43.7	14.2	49
Inactivated caseinogen	20	14.3	14.3	20
Cottonseed oil	15	12.4	13.3	15
Marmite	5	5.0	5.0	5
Salt mixture (McCollum No. 185)	5	5.0	5.0	5
Lemon juice	5	5.0	5.0	5
Wheat germ	—	20.0	—	—
Hog millet	—	—	50.0	—
Alfalfa meal	—	—	—	1
Distilled water	50	50.0	50.0	75

RESULTS.

These diets were tested upon young rats about four weeks old, of 40–50 g. body weight. There were nine groups of such rats, each containing four animals. Group 1 received Diet *F* and may be regarded as the “negative control” group. The animals in Group 5, which supplied a series of “positive controls,” also received Diet *F*, but in addition 20 mg. daily of cod-liver oil. Groups 2, 3 and 4 received, respectively, Diets 2, 3 and 4. The remaining four groups, viz. 1*R*, 2*R*, 3*R* and 4*R* received respectively, Diets *F*, 2, 3 and 4, but vitamin D was supplied in addition by means of irradiation of the hardened cottonseed oil contained in the diet. The oil was exposed for 30 minutes in a thin film at a distance of 35 cm. to the irradiation from a mercury vapour quartz lamp.

The 36 animals used for this test were derived from five different litters,

arranged as shown in Table II; they were maintained for 50 days on their respective diets. Table II shows the average increase in body weight and also the results of the chemical analysis of the leg bones (femora, tibiae and fibulae) at the end of this period. The proportion of calcium in the bones may be taken as equal to about 37 % of the total ash [see Chick, Korenchevsky and Roscoe, 1926].

The results show how disappointingly great is the variation between the individual rats in the various groups, both as regards increase in weight and the proportion of ash contained in the bones. This variation is sometimes greater than that occurring between the averages calculated for different groups.

Groups 2, 3 and 4 show an increased rate of growth compared with Group 1. It is difficult to decide to what extent this increase (41, 43, 33 g. in 50 days, in Groups 2, 3 and 4 respectively, compared with 25 g. in Group 1) is significant, having regard to the variation between individual rats in each group. The growth shown by the rats in Group 1 is an indication of the amount of the reserves of vitamin A and vitamin D possessed by the rats at the beginning of the experimental period and is limited by deficiency of both these vitamins. In Groups 2, 3 and 4 the extra provision of vitamin A in the diets may account for the small extra amount of growth. That this growth was limited by deficiency of vitamin D only and that diets containing 20 % wheat embryo and 50 % hog millet contain abundant vitamin A, while devoid of vitamin D, is indicated by the following facts. The rats in Groups 2 and 3 have no greater proportion of ash in their bones (% ash in fat-extracted bones, 46.7 and 48.1; $A/R = 0.88$ and 0.93 respectively) than the rats in the control Group 1 (% ash in fat-extracted bones 49.1; $A/R = 0.97$). When, however, abundant vitamin D is provided, as in Groups 2R and 3R, both growth and bone calcification become optimal.

The results also suggest that 1 % alfalfa meal provides slightly inadequate vitamin A, and contains traces of vitamin D. The bone calcification (51.7 % ash) of the rats in Group 4 is, on the average, slightly better than in the control Group 1 (49.1 %). On the other hand, the average increase in body weight during the experimental period (33 g.), while greater than that of rats in Group 1 (25.2 g.), is less than that of those in Groups 2 and 3 (41.5 g. and 42.7 g. respectively).

The rats in Group 5, which received 20 mg. daily of a cod-liver oil which has been in use for some time in this Institute, were intended to serve as "positive control" animals. The results obtained, however, suggest that this oil may have deteriorated as regards its content of vitamin A, and that the growth of the rats in this group may have been limited slightly in consequence. The average growth, 12 g. weekly, though not markedly subnormal, is certainly lower than in Groups 2R, 3R and 4R. That the vitamin D supplied in 20 mg. daily of this oil was, however, adequate is shown by the values of the ash content of the fat-extracted bones (60.2 %), and of the A/R ratio (1.54), which are both up to normal value.

Table II. *Growth in weight and bone calcification of young rats maintained for 50 days on diets (a) devoid of vitamins A and D, (b) containing vitamin A as supplied from various foodstuffs, and (c) with vitamin D in addition. The weights of the rats at the beginning of the experiment varied from 36 to 52 g.*

Analysis of bones (femur, tibia and fibula)																
Group	Diet		Rat no.	Sex	Litter	Increase in body weight g.	Average increase g.	Dry weight of fat-extracted bones		Average weight of extracted bones g	Weight of ash g.	Average weight of ash g.	% ash in extracted bones		Average % ash in extracted bones g.	A/R Average
	Vitamin A	Vitamin D						g.	g.				g.	g.		
1	0	0	875	F	1	13		0.390		0.1774		45.5		0.83		
			893	F	3	4		0.281		0.1351		48.1		0.93		
			901	F	4	24		0.359		0.1882		52.4		1.10		
			911	M	5	60	25.2	0.635	0.416	0.3194	0.205	50.3	49.1	1.11	0.97	
2	20 % wheat embryo	0	877	M	1	61		0.492		0.2271		45.1		0.82		
			885	F	2	27		0.405		0.1990		49.1		0.97		
			894	M	3	40		0.452		0.1959		43.3		0.77		
			902	F	4	38	41.5	0.405	0.438	0.2007	0.204	49.5	46.7	0.98	0.88	
3	50 % hog millet	0	878	M	1	70		0.586		0.2776		47.4		0.90		
			886	F	2	41		0.483		0.2313		47.9		0.92		
			895	M	3	24		0.426		0.1928		45.3		0.83		
			903	F	4	36	42.7	0.428	0.481	0.2222	0.231	51.9	48.1	1.08	0.93	
4	1 % alfalfa meal	0	879	M	1	37		0.500		0.2472		49.4		0.98		
			887	F	2	32		0.505		0.2628		52.0		1.09		
			896	M	3	42		0.532		0.2675		50.3		1.01		
			904	F	4	21	33.0	0.371	0.477	0.2048	0.245	55.2	51.7	1.23	1.08	
5	Cod-liver oil		884	F	1	85		0.794		0.4851		61.1		1.57		
			892	F	2	77		0.823		0.5006		60.8		1.55		
			909	F	4	94	85.3	0.805	0.807	0.4843	0.490	60.2	60.7	1.51	1.54	
			883	F	1	28		0.422		0.2351		55.7		1.26		
1 R	0	Irradiated cotton-seed oil	908	F	4	56		0.639		0.3921		61.4		1.59		
			913	M	5	99		0.867		0.5067		58.4		1.41		
			914	M	5	102	71.2	0.910	0.709	0.5380	0.418	59.1	58.7	1.44	1.42	
			880	F	1	78		0.745		0.4383		58.8		1.43		
2 R	20 % wheat embryo	Irradiated cotton-seed oil	888	F	2	110		0.951		0.5993		63.0		1.70		
			897	M	3	140		1.010		0.6048		59.9		1.49		
			905	M	4	128	114	0.949	0.914	0.5765	0.555	60.7	60.6	1.55	1.54	
			898	M	3	141		0.986		0.5930		60.1		1.51		
3 R	50 % hog millet	Irradiated cotton-seed oil	906	F	4	100		0.863		0.5315		61.6		1.60		
			912	F	5	103	115	0.961	0.937	0.5898	0.571	61.4	61.0	1.59	1.57	
			882	F	1	85		0.801		0.4949		61.8		1.62		
			890	M	2	130		1.023		0.6381		62.4		1.66		
4 R	1 % alfalfa meal	Irradiated cotton-seed oil	899	F	3	93		0.896		0.5579		62.3		1.65		
			907	M	4	124	108	1.028	0.937	0.6279	0.580	61.1	61.9	1.57	1.62	

SUMMARY.

1. Experiments are described with two diets suitable for the assay of vitamin D, in which vitamin A was provided by 20 % wheat embryo (Hume) or 50 % hog millet (Steenbock, Nelson and Black).
2. Steenbock and Black's observations that alfalfa meal contains too much vitamin D for the purpose are confirmed.
3. The conclusion of Steenbock and his colleagues [1923], that the growth curve of the animal on an otherwise adequate diet can be employed for the assay of vitamin D, is substantiated.

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