XX. SOURCES OF ERROR IN THE TECHNIQUE EMPLOYED FOR THE BIOLOGICAL ASSAY OF FAT-SOLUBLE VITAMINS.

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THE dual character of the fat-soluble vitamins, which was discovered by McCollum and his co-workers [1922], has thrown doubt upon the validity of much previous work involving their titration. This discovery has been confirmed by many subsequent workers and it has now been accepted generally that fat-soluble A, the vitamin concerned with prevention of ophthalmia and respiratory disorders in rats, must be studied separately from the anti-rachitic substance, or vitamin D, which occurs in association with vitamin A in many naturally occurring fats and oils.

Steenbock and Nelson [1923] were the first to point out that to designate vitamin A as the "growth factor" in any special sense was inappropriate. They brought forward evidence which they have since greatly amplified to show that the anti-rachitic vitamin is also necessary for the maintenance of growth in young animals. In short, the view of these workers is that both vitamins A and D are to be regarded merely as two members of a whole group of essential factors in diet, any one of which may limit growth, notwithstanding the presence of all the others.

This constitutes a serious criticism of much of the previous work upon fat-soluble A, notably that concerned with the evaluation of this vitamin in various foodstuffs. It is probable that much of our present knowledge of the distribution of vitamin A in nature will need revision. Hitherto the criterion has been the growth induced after administration of the substance in question to young rats upon a diet deprived of *both* fat-soluble vitamins, as, for example, in the method of Zilva and Miura [1921].

Steenbock and his co-workers maintain that vitamin A can only be estimated appropriately under circumstances in which the experimental animal is provided with an adequate supply of the anti-rachitic substance, as, for example, by irradiation with ultra-violet light. They have, therefore, recommended a new method for determination of vitamin A [Steenbock, Nelson and Black, 1924]. The young rats, placed upon a diet deprived of fat-soluble vitamins, are at the same time irradiated daily with the rays from a mercury vapour quartz lamp. The degree to which growth is maintained after addition of various foodstuffs is then a measure of the vitamin A content of these¹.

¹ Note added Feb. 8th. Drummond, Coward and Handy [1925] have recently described a similar method in which the anti-rachitic vitamin is supplied to the diet in the form of irradiated crystals of pure cholesterol.

Not only do these new facts and theories require a re-examination of much previous work in this field, but they also serve to explain much previous confusion.

There was a whole series of facts which made the dual character of the fat-soluble vitamins less easy of acceptance. Their parallel distribution in nature and the apparent possibility of replacing vitamin A as well as vitamin D by ultra-violet light [Hume, 1922; Goldblatt and Soames, 1922] seemed to argue, if not unity, at least some close relationship. Cod-liver oil, for example, the richest known source of vitamin A, was found to be also the richest source of vitamin D. Goldblatt and Zilva [1923], while investigating the destruction by heat and oxidation of the two fat-soluble vitamins in cod-liver oil, attempted to titrate both factors separately in the original oil. Using the method of Zilva and Miura [1921] they found the minimum daily dose required to restore growth in rats upon a diet devoid of fat-soluble vitamins to be 2.2 mg., and that needed to protect similar rats from rickets while receiving McCollum's No. 3143 rickets-producing low-phosphorus diet, to be 3.3 mg. The discrepancy between these two figures is not great when account is taken of the large experimental error inseparable from work of this kind. It is, however, possible in the light of the theories advanced by Steenbock and his colleagues that, in this estimate of vitamin A, the restored growth of the rats may have been limited by the animals' supply of the anti-rachitic vitamin D, *i.e.* that received together with the vitamin A in the dose of cod-liver oil, as well as what could be mobilised from any reserves that the animal may have possessed. If during the test the experimental animals had been provided with excess of anti-rachitic substance from another source, by means of irradiation or otherwise, it is possible that growth might have been restored with a smaller dose of oil. If, on the other hand, the experimental animals when first placed upon the deficient diet possessed reserve stores of vitamin A in excess of those of vitamin D (which would appear to be a usual condition), the failure to grow after 2-3 weeks might have been due to exhaustion of vitamin D reserves. The size of the cod-liver oil ration required to restore growth would then be a measure not of the vitamin A, but of the vitamin D contained in the oil. The result (minimal effective dose 2.2 mg.) published by Goldblatt and Zilva then shows a satisfactory concordance with that obtained by them using the more direct method of measuring vitamin D, *viz.* 3.3 mg., the dose required to prevent occurrence of rickets in young rats upon a standard rickets-producing diet, and the coincidence that vitamins A and D are present in equal concentration disappears. In any case the results cannot now be regarded as proof that cod-liver oil contains both vitamins in approximately equal concentration.

These new theories regarding the interrelation of the two fat-soluble vitamins have also helped to explain difficulties and irregularities which were suspected to lie in errors of technique but which were difficult to trace. One such irregularity forms the subject of the present paper.

In this Institute, in the course of work extending over several years, we have found a great deal of unexplained irregularity in the behaviour of our stock rats when placed upon diets deprived of fat-soluble vitamins. Although all the animals are bred in the Institute under apparently constant, standardised conditions of management and diet, a large variation has been noticed in the time elapsing after the young rats are placed upon the experimental diet before they cease to grow. This is shown in Table V, where are collected data, obtained from winter 1923-4 to the autumn of 1925, concerning the growth in weight, and calcification of bones, of young rats placed on diets devoid of fat-soluble vitamins at 25-35 days old and of weight 40-55 g. In the 6th column of Table V are given the average weights after 21 days upon the experimental diet for male and female rats, respectively, of the different litters. In the 7th column are given the maximum weights attained. Only litters in which 6 rats and over survived are included in the table. Fig. 1 (p. 128) shows graphically the variation in percentage increase in weight after 21 days upon the experimental diet.

In the course of attempts to discover the cause of the irregularity, a detailed investigation has been made of every stage in our technique for estimating fat-soluble vitamins and the results are set out in the following pages. In general, the irregularity appears to have some connection with the time of year in which the experiments are carried out, the young rats on the deficient diet showing a greater impulse toward growth, and better calcification of their bones, in summer and autumn than in winter and spring. The animals under experiment are kept in rooms with glass windows facing north, and except in early morning in summer, no direct sunshine reaches the interior. Still, the amount of illumination varies greatly at different times of the year and it was thought that this might be significant. However, the results of special observations made to investigate this point showed that it was unimportant.

Attention was next turned to the experimental diet, to see whether there might not be irregularities introduced in the details of the constituents or of their preparation. The results here were also negative.

Finally, the possibility was explored that there might be unrecognised variations in the breeding stock and in the state of nutrition of the young animals themselves at different periods of the year. It is in this connection that we have found the theories put forward by Steenbock and his co-workers to be helpful in guiding us to what we now believe to be the real cause, viz. variations in the reserves of the two fat-soluble accessory factors, and in particular of the anti-rachitic vitamin, with which the young are equipped at the beginning of their independent existence. Numerous observations have led to this conclusion and are set out below in detail. They are collected from the experiments of various workers in this Institute. Special thanks are due to Miss Hume, Prof. Korenchevsky and Dr Zilva for placing the results of their work at my disposal for publication in this paper.

Influence of the degree of illumination of the animals under experiment.

A litter of 12 young rats, born in the month of June, was divided directly after weaning into two groups of 6 rats each. In each group 2 of the 6 animals received cod-liver oil and acted as controls. All received a diet deprived of fat-soluble vitamins, but the two groups were subjected to different degrees of illumination. Both groups of rats were indoors behind glass windows, the better illuminated animals occupying a well lighted laboratory with a north aspect, whilst the poorly illuminated ones were placed in a darkened corner screened from all direct sunlight. The experiment was made in bright weather, during July and August 1924, and lasted 7 weeks. Rough comparative measurements have been made of the illumination of the two situations by means of a simple photometer (Eder-Hecht Dauer-Graukeil-photometer [see Hecht, 1918]), and it was found that the illumination of the darker position was not more than about 1/200th of that of the brighter one.

The results showed little difference between the animals of the two groups either in the degree of growth achieved or the degree of calcification of the bones. The calcium content of the dissected leg bones was determined by the method of Aron and Sebauer [1910] and the amount calculated on the dry weight of the bone. Phosphorus was determined in the pooled blood of the animals in each group, using Briggs' [1922] modification of the method of Bell and Doisy with the precautions described by Martland and Robison [1924]. The differences between animals of the two groups were insignificant and within the error of experiment. The advantage, if any, was in favour of the better illuminated animals (see Table I).

In a second experiment (see Table II), also carried out during July and August 1924, the diet was modified to contain white wheat flour in place of wheaten starch. Here again there was no significant difference between the animals in the two groups after a period of 50 days upon the experimental diet.

Investigations of the basal diet.

Attention was next directed to the possibility of variation in the constituents of our basal diet, more especially as regards the fat and caseinogen employed. The diet usually employed in this Institute, known as "F" diet, contained

Inactivated caseinogen	...	...	...	100 g.
Wheat starch	...	...	...	250 g.
Marmite	...	...	...	25 g.
Hardened cotton seed oil (or sesame oil)	...	...	...	75 g.
Lemon juice	...	...	...	25 cc.
Salt mixture	...	...	...	25 g.
Water	...	...	...	250 cc.

In some cases egg-white was used to replace 90 % of the caseinogen. This was tried in order to minimise error, when it was feared that the heating

Table I. *Influence of degree of illumination.*

Litter of 12 young rats of weight 25-31 g., aged 21 days. Maintained on diet F-R deprived of fat-soluble vitamins and containing 0.27 % Ca and 0.37 % P, reckoned on dry weight of the diet.

Duration of experiment=52 days. July-August, 1924.

(a) In well-lighted laboratory.									
Rat No.	Sex	Body weight			Time days	Analysis of bones mg. per 100 cc.			Histology of rib-junctions
		Initial g.	Max. or final g.	Increase %		Ca on dry wt. %	Inorg. soluble	Total acid-soluble	
65	♀	30	71	137 134	52	54	15.5	—	Ribs No. of fractures 0 Infractions present
66	♀	25	59	—	52	53	16.6	6.6	Osteoporosis
67	♂	30	86	187	52	51	16.0	0	Osteoporosis and very slight rickets
68	♂	29	68	134	52	50	15.0	0	Osteoporosis and ? very slight rickets
					Mean	52	15.8		
69	♂	31	154	397	52	47	19.7	6.4	Practically normal } cod-liver oil added Practically normal } to the diet
70	♂	26	121	365	52	46	19.7	46.3	
					Mean	46.5	19.7		
(b) In dark corner, screened from direct daylight.									
71	♀	31	85	174	52	52	17.0	0	Osteoporosis
72	♀	28	60	—	34	—	—	38.1	Norw. No. 72 found dead and partly eaten on the 34th day; thereafter growth of 73 and 74 much improved
73	♂	31	93	200	52	53	17.2	6.3	
74	♂	30	62	107	52	55	14.8	8	
					Mean	53	16.3		
75	♂	28	127	353	52	59	18.8	6.4	Nearly normal but more osteoporosis in comparison with 69 and 70
76	♂	29	127	338	52	46	15.8	43.4	
					Mean	47	17.3		Cod-liver oil added to the diet

* Animals killed fasting, fed only on previous day; analyses made on the pooled blood of the group.

of the caseinogen might not have been effective in destroying all traces of fat-soluble vitamins.

Table II. *Influence of Degree of Illumination.*

Litter of 10 young rats of weight 27-33 g., aged 21 days. Maintained on diet deficient in fat-soluble vitamins (F diet with starch replaced by white flour).

Duration of experiment=50 days. July-August, 1924.

(a) In well-lighted laboratory.

Rat No.	Sex	Body weight			Mean	Analysis of bones Ca on dry wt. %	Blood P* mg. per 100 cc.	
		Initial g.	Max. or final g.	Increase %			Inor- ganic	Total acid soluble
77	♀	27	96	255	256	19.19	7.10	42.3
78	♀	30	97	223		19.24		
79	♀	29	115	296		19.33		
80	♀	27	95	252		18.60		
					Mean	19.09		
81	♂	31	125	303	315	18.53	6.80	43.9
82	♂	32	137	328		18.95		
					Mean	18.74		
Mean value Group (a)						19.0		

(b) In dark corner, screened from direct daylight.

83	♀	30	111	270	265	20.01	6.80	43.9
84	♀	28	98	250		18.75		
85	♀	27	102	278		18.54		
86	♀	28	102	264		18.21		
					Mean	18.88		
87	♂	33	123	282	291	17.15	6.80	43.9
88	♂	29	116	300		17.78		
					Mean	17.46		
Mean value Group (b)						18.4		

* Determined in the pooled blood of the whole group.

The salt mixture was No. 185 of McCollum and his co-workers [1917] and contained in 1000 parts:

NaCl	43.5	CaH ₄ (PO ₄) ₂ H ₂ O	136
MgSO ₄ 7H ₂ O	137	Ca lactate (5H ₂ O)	327
NaH ₂ PO ₄ H ₂ O	87	Ferric citrate (3H ₂ O)	30
K ₂ HPO ₄	240		

Fat. A large consignment of hardened cotton seed oil had served the needs of the Institute for some years, but when it was exhausted no more could be obtained and only hardened sesame oil was available. Special experiments therefore were made to determine whether the latter was equally devoid of fat-soluble vitamins and to compare it with the cotton seed oil used previously. The results given in Table III show that no consistent difference exists between the two oils judged either by the degree of growth or by the calcification of the bones. The mean calcium content in the leg bones (femur, tibia and fibula) reckoned on the dry weight was 14.5 % for animals receiving hardened cotton seed oil and 14.3 % for those receiving hardened sesame oil.

Table III. *The effect upon growth and calcification of bones of substituting hardened sesame oil for hardened cotton seed oil in the experimental diet.*

F diet in which 10 % of the protein is provided in the form of caseinogen and 90 % in the form of egg-white.

Duration of experiment = 40 days.

Litter	Rat No.	Sex	Body weight			Duration of experiment days	Analysis of long bones (leg)		Character of fat employed
			Initial g.	Max. or final g.	Increase %		H ₂ O %	Ca on dry wt. %	
I	2	♂	50	89	78	40	50.3	13.0	Cotton seed oil
	3	♂	50	60	20	"	45.8	11.8	"
	7	♀	49	85	73	"	51.7	15.1	"
	1	♀	50	89	78	"	47.4	14.2	Sesame oil
	4	♀	50	83	66	"	48.0	14.5	"
	5	♀	49	77	57	"	48.4	13.2	"
II	6	♀	50	74	48	"	45.2	13.8	"
	8	♂	57	91	60	38	48.6	14.3	Cotton seed oil
	9	♀	50	72	44	"	48.4	14.7	Sesame oil
	11	♀	50	81	62	"	45.6	16.3	"
III	14	♀	51	103	102	38	47.4	16.3	Sesame oil
	12	♀	48	76	58	"	48.0	15.0	"
IV	19	♀	49	93	90	38	52.2	15.0	Cotton seed oil
	18	♀	50	73	46	"	51.8	13.8	"
	20	♀	49	82	67	"	52.0	14.8	"
	16	♂	50	90	80	"	49.5	14.5	Sesame oil
	15	♀	50	93	86	"	51.2	15.0	"
	17	♀	49	58	18	"	46.3	13.8	"
Normal for rats of the same age							44.0	20.5	

Caseinogen. The caseinogen used for experimental diets in this Institute is commercial caseinogen, prepared by precipitation with acetic acid. It is inactivated by being heated in an electric oven at 120–130° for 36 hours. The dry caseinogen is spread evenly on shallow trays and once every hour during the day-time is thoroughly raked over to expose fresh surfaces and to facilitate the oxidation of any traces of fat-soluble vitamins originally contained. It was conceivable that whilst vitamin A might be completely destroyed by such treatment, the anti-rachitic vitamin, which is known to be more resistant to heat and oxidation [McCollum, Simmonds, Becker and Shipley, 1922; Goldblatt and Zilva, 1923], might survive. Accordingly, diets made with caseinogen prepared in the above manner were compared with those containing caseinogen of the same batch, but heated at 120–130° for an additional period of 36 hours. During this total heating of 72 hours the caseinogen was raked over every hour during the three day-time intervals.

The results of three separate experiments made with three separate litters of young rats are given in Table IV. In each case one-half of the litter received the experimental diet prepared with the twice heated caseinogen. The time of observation varied from 8–12 weeks. In Exps. 1 and 3, comparing the males with males of the same litter and females with females, there does appear to be a slight inferiority in the bone calcification of the animals receiving the more drastically heated caseinogen. In Exp. 2, however, there

is no significant difference between the two groups in this respect. As regards growth in weight, the variation between the two groups may also be regarded as coming within the error of experiment. The blood phosphorus (inorganic and total acid-soluble) was determined in case of the animals of Exps. 2 and 3 and all the results were found to lie within normal variations. The values were slightly lower in the groups of animals receiving the more strongly heated caseinogen, but it is doubtful whether the differences are significant. A mild degree of xerophthalmia was apparent in most animals in both groups, but there was no apparent difference in the time of onset or severity of the symptoms.

Table IV. *Growth and calcification of bones in young rats on F diet deprived of fat-soluble vitamins. Influence of degree of heating undergone by the caseinogen incorporated in the diet.*

	Rat No.	Sex	Body weight			Mean	Duration of experiment, days	Time of experiment	Analysis of bones		Blood phosphorus mg. per 100 cc.	
			Initial g.	Max. or final g.	Increase %				H ₂ O %	Ca on dry wt. %	Inorganic	Total acid-soluble
<i>Exp. 1. Litter 33:</i>												
(a) Caseinogen heated 36 hours	13	♂	48	118	146	118	55	April-June, 1924	44.5	18.0		
	14*	♀	47	88	91				48	17.0		
	17	♀	49	86	76	88	54		47	18.0		
	18	♀	49	93	90				46	19.1		
							Mean		46	18.0		
(b) Caseinogen heated 72 hours	15	♂	45	89	94	95	55		45	16.1		
	16	♀	48	94	96			45.5	16.3			
	19	♀	49	96	96	97	54	46.5	17.6			
	20	♀	47	93	98			46	18.1			
	21†	♀	46	91	98		50.5	16.6				
						Mean	47	17.0				
<i>Exp. 2. Litter 36:</i>												
(a) Caseinogen heated 36 hours	29	♂	45	175	289	218	76	May-July, 1924	41.5	19.0	5.9§	39.4§
	30	♀	47	116	147				45	16.5		
	31	♀	42	110	162	154			42	13.8		
	32	♀	49	117	139				44	17.3		
	33	♀	47	123	162	75	42		18.3			
						Mean	43	17.3				
(b) Caseinogen heated 72 hours	37	♂	50	147	194	171	76		40.0	17.9	5.6§	37.9§
	38	♀	47	117	149		74	44.0	18.0			
	34	♀	45	131	191	155	76	41	18.9			
	35	♀	42	88	110			43	16.0			
	36	♀	48	131	173		40	(lost)				
						Mean	42	17.5				
<i>Exp. 3. Litter 38:</i>												
(a) Caseinogen heated 36 hours	49	♀	48	127	165	160	84	May-July, 1924	37	22.8	5.5	43.3
	50†	♀	50	109	118				43	(lost)	4.2	38.3
	51	♀	48	142	196				36	20.5	4.7	36.8
							Mean	38	21.7	4.8	39.5	
(b) Caseinogen heated 72 hours	52	♀	48	110	129	145	84		38	19.4	4.6	38.1
	53	♀	46	122	165			36	20.4	3.9	37.0	
	54	♀	49	118	141			39	18.5	4.6	37.7	
							Mean	37	19.4	4.4	37.6	

* Had three fractured ribs.

† Had two fractured ribs.

‡ Had four fractured ribs.

§ Determined in pooled blood of group.

Table V.

No. of litter	No. of surviving rats in litter	Date of birth	Age when placed on experimental diet, days (extremes)	Body wt. g. (extremes)	Average body wt. after 21 days on experimental diet, g.		Average maximum wt. attained, g.		% Ca in leg bones, reckoned on dry wt.		Time on experimental diet, days
					♂	♀	♂	♀	♂	♀	
X	7	16. x. 22	31/34	50/53	69	64	85	105	—	—	—
A	6	30. xi. 22	25	50/53	75	66	99	90	—	—	—
1	7	10. xii. 23	25/26	49/50	69	66	—	—	13.0	14.1	40/41
3	8	11. xii. 23	27	48/51	75	65	—	—	16.3	15.0	38
4	6	14. xii. 23	24	49/50	70	57	—	—	14.8	14.1	38
8	9	4. i. 24	28	50	77	—	93	—	—	—	—
11	9	5. i. 24	27	50	69	—	75	—	—	—	—
17	7	18. i. 24	27	48	68	—	88	—	—	—	—
19	6	25. i. 24	31/33	50	81	65	147	114	—	—	—
33	9	23. iii. 24	28	45/49	67	62	98 +	92 +	16.8	17.9	54
36	11	1. iv. 24	28/30	42/50	78	67	139	117	17.8	16.9	76
38	13	2. iv. 24	33/36	46/50	77	72	129	119	—	20.3	84
39	13	4. iv. 24	33	50/51	87	79	138	108	19.0	18.8	87
40	10	5. iv. 24	30	50	86	70	130	114	17.3	18.4	89
69	8	21. viii. 24	30	49	98	84	152	118	18.7	20.0	86
73	11	25. viii. 24	28/29	49/52	110	96	139	128	18.3	19.9	86
75	12	2. ix. 24	29	50	87	—	138	—	19.8	—	114
74	13	9. ix. 24	29/30	45/57	87	74	154	113	17.8	—	94
									18.7	—	75
76	6	20. ix. 24	28	48	—	79	—	101	18.1	—	96
77	11	3. x. 24	29	44/47	86	75	118 +	88 +	16.0	17.9	62
80	10	5. x. 24	30/31	49/50	99	99	160 +	127 +	17.8	—	58
86	9	8. x. 24	27/28	49/51	108	88	146	98	17.3	18.2	64
105	9	19. xii. 24	29/32	45/51	88	75	130 +	110 +	—	—	—
114	8	31. xii. 24	23/27	47/51	90	69	141 +	92 +	—	—	—
141	11	13. ii. 25	28/31	48	66	69	88	82	16.7	17.8	73
142	10	14. ii. 25	32/34	47/51	66	66	91	93	16.3	18.7	82
144	7	13. ii. 25	29/31	47/51	90	78	—	—	—	—	—
147	10	18. ii. 25	28	50	—	69	—	—	—	—	—
148	11	20. ii. 25	31/32	46/47	77	73	—	—	—	—	—
177	9	4. iv. 25	24	40/43	58	56	—	—	—	—	—
178	8	7. iv. 25	23	41/45	57	59	—	—	—	—	—
218	9	24. v. 25	26/27	41/43	54	55	—	—	—	—	—
220	7	26. v. 25	28/29	40/46	57	47	—	—	—	—	—
227	11	29. v. 25	31	40/45	60	52	—	67	—	16.7	84
228	9	30. v. 25	26	40/41	51	49	—	—	—	—	—
271	8	20. viii. 25	25/27	40/48	61	51	82	68	14.5	13.7	53/59
272	11	21. viii. 25	26/31	40/46	58	62	88	86	16.6	17.4	50/54
277	8	25. viii. 25	27/29	41/48	69	59	73	81	15.6	17.8	44/50
313	8	19. x. 25	31/33	40/44	61	48	—	—	—	—	—
318	11	20. x. 25	31/34	41/47	66	61	—	—	—	—	—
324	9	22. x. 25	29/30	43/46	63	—	—	—	—	—	—
316	6	22. x. 25	29	45	—	60	—	—	—	—	—
319	9	26. x. 25	24	43/44	71	—	—	—	—	—	—
322	9	28. x. 25	23/30	43/46	61	59	—	—	—	—	—
323	10	30. x. 25	25	43	67	—	—	—	—	—	—
325	9	30. x. 25	28/29	44/45	62	—	—	—	—	—	—

Reserves of fat-soluble vitamins in the experimental animals.

The reserves of fat-soluble vitamins possessed by the young rats at the beginning of the experimental period are known to affect greatly their behaviour when placed later upon diets deficient in these vitamins, and the diet of our breeding stock was, therefore, carefully scrutinised. The accompanying paper [Smith and Chick, 1926] (published in conjunction with Miss Henderson Smith, who is in control of the breeding of rats designed for nutritional work in this Institute), deals with the question of diet and of the

technique which has been evolved for maintaining a supply of young rats which shall be reasonably constant as regards their reserves of fat-soluble vitamins. The diet of the breeding stock, which contains a fair proportion of whole cereals and of fresh green food, is moderately rich in vitamin A, but comparatively poor in vitamin D, except in summer, when the ration of fresh cow's milk contains appreciable amounts of the latter vitamin [see Luce, 1924, 1, 2; Boas and Chick, 1924]. Luce [1924, 1] found the vitamin A content of summer "pasture fed" cow's milk to be about 2-3 times that of milk from the same cow when fed on dry fodder in a dark stall in winter. The relative increase in anti-rachitic value (vitamin D) was somewhat greater viz. 4-5 times. When the whole diet of the breeding stock is taken into account, the change in total vitamin A due to the seasonal variation in the vitamin A content of the milk ration will probably be small in comparison with the change in the total vitamin D.

Fig. 1. Influence of the month of birth upon the average percentage increase in body weight shown by young rats (40-50 g. original weight) after 21 days upon diets deprived of fat-soluble vitamins.

It was conceivable, therefore, that variations in the nutritional value of the fresh milk supplied to the lactating mothers and to the young up to the time of experiment might explain the irregularity which was being investigated. Accordingly, arrangements were made to provide the mothers after the birth of the young with dried milk prepared from winter milk, enough being prepared in March 1925 to last over the summer and autumn. The lower portion of Table V from litter 177 downwards and the right-hand section of Fig. 1 summarise our experience since the alteration was made. The average weight after 21 days on the experimental diets devoid of fat-soluble vitamins, as well as the average maximum weight attained, remain roughly constant for

animals born during the summer and autumn of 1925, although the young animals are seen to possess lower vitamin reserves than previously, judged by the amount of growth.

Table VI. *Variations shown by young rats after 4 weeks on McCollum's low P rickets-producing diet, No. 3143.*

All rats bred in the Institute on stock diet. Egg-yolk included in stock diet from Sept. 10 to Dec. 10, 1924.

Full cream dried milk substituted for fresh milk from Dec. 2, 1924 to March 6, 1925.

Litter No.	Date of birth	No. of rats in litter	No. of rat	Sex	Age when placed on experimental diet, days	Body weight			Calcium content of long bones, % calculated on dry wt. of bone	Degree of rickets judged by histology of rib-junctions	Notes
						Initial g.	Final g.	Increase %			
78	2. x. 24	11	30	♂	32	50	78	56	13.1	Moderate	Mother received egg-yolk on one or two occasions during period of lactation (21 days)
			37	♀	38	49	82	67	15.1	"	
87	10. x. 24	5	41	♂	25	51	90	76	16.0	Slight	" "
111	19. xii. 24	8	70	♂	33	48	92	92	14.3	Moderate to severe	
			68	♀	34	50	86	72	12.3	Severe	" "
113	29. xii. 24	6	82	♂	28	52	75	44	12.9	Moderate to severe	
155	2. iii. 25	11	337	♂	29	48	76	58	12.2	Severe	" "
			338	♀	31	44	81	84	13.8	"	
166	11. iii. 25	11	342	♂	36	47	61	37	17.0	Slight	Mother received dried March milk during lactation period
165	14. iii. 25	11	354	♀	33	43	68	58	15.4	Moderate	" "
232	4. vi. 25	8	410	♂	26	48	89	85	9.8	Severe	" "
			414	♀	26	46	75	63	12.4	"	" "
233	11. vi. 25	9	420	♀	30	46	62	35	11.8	"	" "
289	14. ix. 25	11	454	♂	33	45	63	40	10.7	"	" "
			458	♀	33	44	63	43	11.9	"	" "
301	18. ix. 25	10	228	♂	29	44	67	52	10.2	"	" "
			230	♀	29	46	59	28	10.2	"	" "
302	30. ix. 25	7	462	♂	26	44	62	41	9.9	"	" "
			465	♀	26	45	54	20	10.5	"	" "

It is our belief that this is due chiefly to a diminution of the vitamin D in the mothers' diet, for the substitution of dried winter milk for the fresh milk previously supplied in summer and autumn had reduced the intake of this vitamin, while affecting less seriously the total intake of vitamin A. It so happened that the young born during the previous winter had received an unusually large endowment of vitamin D, due to an unwise addition of egg-yolk to the diet of the breeding stock during the autumn (Sept.-Dec. 1924). Egg-yolk is a rich source of vitamin A and also very rich in anti-rachitic properties [Hess, 1923; Casparis, Shipley and Kramer, 1923; Hess and Weinstock, 1924], and this addition endowed the young with such large reserves of vitamins A and D that they proved useless for the assay of either vitamin. When placed at 40-50 g. weight upon the diet deprived of fat-soluble vitamins, they often grew normally for several weeks (*e.g.* Table V, litters 74, 80, 105) and developed well-calcified bones. Upon McCollum's No. 3143 low phosphorus, rickets-producing diet an abnormal amount of growth was made

in many cases, and at the end of 4 weeks the macroscopic signs of rickets were slight or altogether absent. The degree of rickets diagnosed histologically was often slight or moderate in place of the severe type usually produced, whilst the calcium content of the bones varied from 12–17 % in place of the 9–13 % usually found [see also Luce, 1924, 1, 2]. In Table VI are collected the results obtained on McCollum's diet with litters born from Oct. 1924 to Sept. 1925. It will be seen that after March 1925, when the mothers received only dried winter milk, a satisfactory degree of constancy is attained in the behaviour of the young.

SUMMARY.

1. An important source of error in the biological method of estimating the fat-soluble vitamins A and D in various foodstuffs consists in variation in the reserves of these two vitamins which may be present in the young rats used for experiment. These reserves depend on the diet upon which the young rats are reared.

2. With the diets ordinarily used in breeding rats for such work, the reserves of vitamin D are usually low in comparison with those of vitamin A. Great irregularity is caused if materials rich in vitamin D are at times included in the diet. Substances to be avoided are egg-yolk and cod-liver oil. Summer, "pasture fed," cow's milk should be used sparingly.

3. The degree of illumination of the test rats while under experiment (within the limits attained by ordinary daylight passing through glass windows at different seasons of the year) is of slight importance.

4. If the hypothesis of Steenbock and his co-workers be accepted that the anti-rachitic vitamin D is essential for growth equally with vitamin A, and that lack of one in a diet may limit growth in presence of adequate amounts of the other and of the remaining essential factors—it follows that much previous work concerned with the distribution and estimation of vitamin A needs revision.

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