LXXXIV. INFLUENCE OF DIET AND SUNLIGHT UPON THE AMOUNT OF VITAMIN A AND VITAMIN D IN THE MILK AFFORDED BY A COW.

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1. INTRODUCTION.

THE present work forms a continuation of that carried out in this Institute by Luce [1924, 1, 2] who investigated the growth-promoting and anti-rachitic value of milk from a cow kept under varying conditions as regards diet and illumination. She showed that the growth-promoting power of the milk, as tested upon young rats, was greater when the cow received fresh green fodder than when taking a winter feed of cereals and roots, but was unaffected by the degree of exposure of the cow to sunlight. According to Luce's experiments the antirachitic value of the milk was also dependent mainly on the diet of the cow and to a slight extent only, upon the degree of insolation. Her results were not, however, very conclusive upon this point.

It seemed possible that in some of Luce's experiments the periods of time allowed for one set of conditions may have been too short for the maximum effect to have been exerted upon the cow. In Period III, for example [Luce, 1924, 1, p. 723], when the cow received fresh green food for two months from May-July, 1923, while living in a dark stall, the "growth-promoting power" of the milk rose continuously. The first sample (milk sample XI), taken after one month, had only half the value of that taken after two months, at the end of the period (milk sample XII). The anti-rachitic potency, on the other hand, showed no improvement and remained at the minimal value, equal to that of the previous Period II, during which the cow received a dry winter feed in the dark stall. Subsequently in Period IV after one month on pasture in the month of August 1923, the growth-promoting power of the milk had increased only slightly, and the anti-rachitic value very greatly, and both had attained their respective maximal values. It was not clear, therefore, whether the former might not have risen to a higher level; or even to the maximal value in Period III, if the grass feeding in the dark stall had been continued for a longer period than two months. And similarly it was left uncertain whether some additional anti-rachitic property might not also have developed if the time had been extended.

One of our principal aims, therefore, was to study the vitamin value of the milk during a more prolonged period of feeding with fresh green fodder in a dark stall and, by comparing with the milk yielded during a subsequent

period upon summer pasture, to investigate more completely the effect of the incidence of sunlight upon the cow.

Furthermore, the technique used by Luce for measuring the growth-promoting power of the milk can no longer be considered satisfactory in the light of the subsequent researches published by Steenbock and his co-workers. The conclusions arrived at may, therefore, need modification in view of the uncertainty as to what extent the method she used titrated vitamin A, or vitamin A jointly with the anti-rachitic vitamin D.

For the titration of the growth-promoting value Luce used the method of Zilva and Miura [1921], determining the daily dose of milk required to restore normal growth (increase in body-weight to the extent of 10–12 g. per week) in rats which had ceased to grow upon an artificial diet devoid of fat-soluble vitamins. The investigations of Steenbock and Nelson [1923] made it clear that not only vitamin A, but also the anti-rachitic vitamin D is concerned in maintaining growth and that cessation of growth upon the experimental diet may be caused by exhaustion of the animal's original reserve of vitamin A or of vitamin D or of both. Similarly, restoration of growth after addition of supplementary foods may be due to the presence in those foods of either fat-soluble vitamin or of both.

2. METHODS EMPLOYED.

Vitamin A. Steenbock, Nelson and Black [1924] justly maintain that when testing a food stuff for presence of vitamin A it is essential that the animal should be provided with an adequate supply of vitamin D. They suggest that this should be done by daily irradiation of the test animals with ultra-violet light. We have found that daily irradiation of 10 minutes' duration or longer, when continued over long periods, has a deleterious effect upon the rats' eyes, causing conjunctivitis and marked opacity in the cornea. This effect, which is independent of the ophthalmia which may develop as the result of vitamin A deficiency, reacts in time upon the animal's general health and capacity to grow. It is not practicable to shield the eyes of large numbers of rats during daily irradiation and we have found it more convenient to provide vitamin D in the form of irradiated cotton-seed oil included in the basal diet. The oil was irradiated for 30 minutes, at a distance of 33 cm. from the burner of a quartz mercury vapour arc lamp, in a film approximately 0.17 cm. deep.

The diet, known as F diet, was made fresh once a week and had the following composition:

Inactivated caseinogen	100 g.	Lemon juice	25 g.
Wheaten starch	250	Salt mixture ¹	25
Cotton-seed oil	75	Water	300
Marmite	25		

¹ No. 185 of McCollum, Simmonds and Pitz [1917], has the following composition, 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		

This diet has the same composition as that used by Luce, except that caseinogen is substituted for the egg-white used in her diet as a source of protein. The dried Chinese egg-white of commerce is an extremely convenient form of vitamin-free protein but Boas [1924, 1, 2] has recently shown that prolonged feeding with this material gives rise to a pathological condition in young rats independently of the presence or absence of fat-soluble vitamins. Caseinogen, therefore, inactivated by heating at 120° for 72 hours was used throughout the present work.

In the method we employed, young rats, bred under standardised conditions [see Smith and Chick, 1926], about 4–5 weeks old, and weighing 40–50 g., receive the above basal diet for a period of 3–4 weeks, or less if they cease to gain in weight. Irradiated cotton-seed oil is then included in the food in place of the ordinary oil. Growth is almost invariably restored for a further period of 3–4 weeks but eventually stops and there may be a sudden fall in weight. The ration of foodstuff whose content of vitamin A is to be tested must then be added without delay, for cessation of growth under these conditions indicates very complete exhaustion of this vitamin and the animal may suddenly succumb unless it is quickly provided. The daily dose of milk is given for a period of 3–4 weeks and the increase in weight noted. The vitamin A content of the different samples of milk is compared by determining the minimal dose necessary to restore normal growth—increase of 10 g. weekly in body-weight.

While, on the whole, this method may be relied upon to yield concordant results, it has some drawbacks. Firstly, the test is very tedious and from first to last takes not much less than three months to carry out. In the second place, the animals are often in poor condition at the end of the preliminary period, they frequently show signs of xerophthalmia and of respiratory trouble which may have progressed to a chronic pneumonia. If the milk dose contains a sufficiency of vitamin A the eye condition will quickly improve, but some degree of pneumonia may persist and although the animal may show a fair degree of good health, the rate of growth may be depressed. Lastly, the addition of irradiated oil to the diet almost invariably causes temporary diarrhoea or looseness of stools. This may persist for a week or two, but ultimately passes off. It appears to be due to the laxative action of some compound formed in the oil by the action of ultra-violet radiation. The same effect is obtained when rats on diets deprived of fat-soluble vitamins are irradiated directly and in some cases also when cod-liver oil is administered.

In a few instances the growth-promoting power of the same milk was titrated by the above method and also by the simpler method of Zilva and Miura. The results obtained with milk samples XXVII and XXVIII (see below, pp. 637–8) show clearly that by the former method vitamin A alone is estimated and that when there is no irradiation of rat or of diet the growth obtained may be due to the joint effect of both vitamins A and D supplied with the milk.

Vitamin D or anti-rachitic vitamin. The anti-rachitic power of the various

samples of milk was tested by a method similar to that used by Luce. Young rats of 40–50 g. weight were fed for 4 weeks upon McCollum's low phosphorus rachitic diet No. 3143, [McCollum, Simmonds, Shipley and Park, 1921: whole wheat, 33 %; maize, 33 %; gelatin, 15 %; wheat gluten, 15 %; sodium chloride, 1 %; calcium carbonate, 3 %] the milk being given as a supplement to the diet in various daily doses. In some cases the tests were made with butter prepared from the milk, which has the advantage that an inappreciable amount of phosphorus is introduced with the test dose. Luce [1924, 1, p. 729] called attention to this source of error when using milk. It is, however, clear that the varying anti-rachitic effect of different milk samples (see Table VI) cannot be thus accounted for, and in any case doses of milk up to 5 cc. daily have an insignificant effect upon the actual amounts or relative proportion of the calcium and phosphorus given in this diet (see below, pp. 642–3).

At the end of the experiment chemical analysis was made of the leg bones (femora, tibiae and fibulae) and histological examination made of the junctions of the 6th and 7th ribs on the right side. The methods used are described in full in the preceding paper by Chick, Korenchevsky and Roscoe [1926]. The degree of protection from rickets was judged by comparison with the bones of control animals receiving no addition to the diet and of others receiving a supplement of cod-liver oil. We gratefully acknowledge the help of Professor Korenchevsky, who kindly examined the histological specimens for diagnosis of rickets.

The proportion of calcium in the bones is reckoned on the fat-free bone, which is necessary in view of the varying content of fatty material. This variation is not so great in the bones of rachitic animals fed on McCollum's 3143 diet as in the bones of animals showing various degrees of osteoporosis [see Chick, Korenchevsky and Roscoe, 1926]. The proportion of calcified to uncalcified bone, or the value of the A/R ratio is probably the best chemical indication of rickets and gives the most trustworthy criterion of the degree of calcification of the bones.

Reserves of vitamins A and D in the test animals. The degree to which the young rats used for experiment are endowed with stores of fat-soluble vitamins exercises a very marked effect upon their behaviour, when used subsequently for the study of these vitamins. In this Institute, after long trial [Smith and Chick, 1926; Chick, 1926] we have succeeded in breeding young rats which are fairly constant in their vitamin reserves. Moreover, as regards vitamin A, the extended preliminary period on deficient diet, which is a feature of the newer method of estimating this vitamin, produces finally an experimental animal with its vitamin A reserves reduced to a minimum. It is nevertheless a good plan, in all comparative work, to use animals from the same litter. This was not always practicable in the present work, for the different milk samples were taken from the same cow at times separated by long intervals. The use of butter for vitamin D tests was an obvious advantage, for it can be kept for several months in the frozen condition.

Milk samples. The cow used was the same as was employed in the previous investigation of Luce and has been kept throughout at the Serum Farm of the Lister Institute at Elstree, Herts. She has remained in good health, has calved once, in January, 1923, and has yielded milk continuously from July, 1922–October, 1925. Our acknowledgments are due to Dr MacConkey and his staff for again kindly undertaking the care of the cow under strictly controlled conditions and for collecting the milk samples.

Each milk sample represented the morning's milk for one week. The milk was collected in bottles and pasteurised in a water-bath at 75° for 20 minutes, during which time carbon dioxide was bubbled through continuously. The bottles were sealed immediately, sent to Chelsea and thereafter kept in a refrigerator room. In order to avoid opening and re-pasteurising, the contents of the various bottles comprising each milk sample were not mixed before making the tests but a fair average of the whole week's yield was secured in the following way. The test was begun, using milk doses taken from one of the bottles filled on the first day of sampling; when this bottle was exhausted doses were taken from one of the bottles filled on the subsequent day, and so on.

During the period occupied by the present work (April, 1924–October, 1925) the milk yield has varied from 7–16 pints a day, the average being 9½ pints. The composition of the milk has remained fairly constant as regards content of fat and of calcium and phosphorus, the average values being 5·4 % fat, 0·138 % Ca and 0·105 % P respectively (see Table I).

Table I. *Details of the samples of milk used in the experiments.*

Period	Sample No.	Cow	Diet	Duration of period	Days	Incidence of sunshine. Av. No. of hours daily	Analysis of milk % (averages)			Remarks
							Fat	Ca	P	
1 (=Period V. Luce, 1924, 2)	XXII (=Sample XVII. Luce, 1924, 2)	In ploughed enclosure	Dry fodder; daily feed :— White maize meal, 3½ lbs. Gluten meal, 3 lbs. Oats and barley (crushed), 3 lbs. Mangolds, 50 lbs. Oat straw, <i>ad lib.</i>	Apr. 17– Aug. 6, 1924	111	6·3	5·3	0·138	0·119	Butter made in July, 1924
2	XXIII	In green field	Fresh grass and clover, <i>ad lib.</i>	Aug. 7– Nov. 1, 1924	86	3·9	5·5	0·142	0·097	—
3	XXIV	In dark stall	Dry fodder (as for XXII)	Nov. 2, 1924– Apr. 23, 1925	172	—	5·6	0·138	0·106	—
4	XXV	In dark stall	Fresh grass and clover	Apr. 29– June 22, 1925	54	—	5·0	—	—	—
	XXVII	„	„	Apr. 29– July 25, 1925	87	—	5·3	0·131	0·099	Butter made and tested
5	XXVIII	In green field	Fresh grass and clover, <i>ad lib.</i>	Aug. 1– Oct. 18, 1925	78	4·1	5·7	0·142	0·103	„
Mean							5·4	0·138	0·105	

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When butter was required for investigation, the cream was separated from the entire milk yield for one week. A "starter" was added and the cream sent to the Dairy Institute of the Reading University, where the butter was made. We desire to thank Captain Golding and Miss Dorothy Dearden in this place for their kind co-operation in this matter. The butter was stored in the frozen condition at -8° to -10° until the tests were made.

We have studied a complete cycle of changes very similar to those studied by Luce and details of these are set out in Table I. The milk samples for analysis were taken at the end of the respective periods. Most attention was given to period 4, in which the cow received fresh green grass and clover in the dark stall, and to period 5, the subsequent period on pasture.

3. GROWTH-PROMOTING VALUE OF THE MILK (VITAMIN A AND VITAMIN D).

In Tables II and III are given figures obtained for the growth-promoting value of milks XXIV to XXVIII, as tested by the old and the new methods respectively, that is to say, without and with irradiation of animals or diet. For purposes of reference the complete details of one titration (milk XXVII) are given in Protocol I and the results of the whole series are collected in Table IV.

Table II. *Growth-promoting value of milks XXIV, XXVII and XXVIII.*
Test made without irradiation.

Milk XXIV (Litters 212,166). Winter feed in dark stall							
Dose cc.	No. of animals	Av. body weight at end of preliminary period g.	Average weekly growth increments when receiving milk doses. g.				
			1st week	2nd week	3rd week	4th week	Mean
0	3	76	3	-1	1	-4.5	-0.5
5	1	54	7	7	9	2	6
8	2	58	9.5	8	8	4	7
12	2	89	12	12.5	9	7.5	8
20	1	75	14	18	7	9	12
Milk XXVII (Litters 218, 220, 271, 272, 277). Fresh green food in dark stall							
0	8	60	6	3.5	5	1	4
2	2	52	4.5	9.5	7	7	7
3	4	52	9	7	7.5	9	8
5	4	66	11	11.5	12.5	11	11.5
8	2	47	11	15.5	16	12	14
12	2	51	18	15.5	15.5	14	16
Milk XXVIII (Litters 271, 272, 277). Pasture							
0	6	61	7	3	5.5	0	4
1	3	60	9	6	6	5	6.5
2	3	59	9	11	9	6	9
3	3	57	10	13	11	9	11
5	3	51	15	14	13	11	13

We have adopted Dr Luce's arbitrary standard for a "growth unit," viz. an increase in body-weight of 10-12 g. per week [see Luce, 1924, 1, p. 723]. Column 4, Table IV, contains the doses of milk which proved insufficient and sufficient, respectively, to restore this degree of growth when the test was

Table III. *Vitamin A value of milks. Excess of vitamin D provided by irradiation.*

*Milk XXIV (Litter 181). Winter feed in dark stall							*Milk XXV (Litters 176, 181). Fresh green food in dark stall, 54 days						
Dose cc.	No. of animals	Body- weight at end of pre- liminary period g.	Average weekly growth increments (g.) when receiving milk doses				No. of animals	Body- weight at end of pre- liminary period g.	Average weekly growth increments (g.) when receiving milk doses				Mean
			1st week	2nd week	3rd week	Mean			1st week	2nd week	3rd week	Mean	
1	—	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	2	75	9	9.5	12	10	—
5	1	84	6	9	1	5	3	89	13	16	13	14	—
8	1	69	14	15	—1	9	3	72	11	14	12	12	—
12	1	78	12	9	11	11	4	83	14	18	12	15	—
20	1	87	17	11	11	13	—	—	—	—	—	—	—
Milk XXVII (Litters *212, 218, 220, †313, 318). Fresh green food in dark stall, 87 days							†Milk XXVIII (Litters 313, 318). Pasture						
1	4	98	4	6.5	4	5	1	90	11	2	8	7	—
2	5	91	9	14	8	10	2	118	12	13	10	12	—
3	5	86	10	11	10	10	3	88	11	12	9	11	—
5	5	81	13	16	10	13	1	91	14	9	10	11	—
8	1	59	15	16	8	13	—	—	—	—	—	—	—

* Animals irradiated directly.

† Fat in diet irradiated.

Table IV. *Growth-promoting value of milk samples XXIV to XXVIII.*

Period	Milk	Treatment of cow		Dose in cc. required to restore growth to the extent of 10 g. increase per week				Growth units per 100 cc. (Method I)	Vitamin A units per 100 cc. (Method II)
				Method I. Without irradiation		Method II. With irradiation			
				—	+	—	+		
3	XXIV	Winter feed.	Dark stall (172 days)	12	20	8	12	5-8	8-12
4	XXV	Fresh green grass.	Dark stall (54 days)	—	—	—	3	—	33
4	XXVII	Fresh green grass.	Dark stall (87 days)	3	5	1	2	20-33	50-100
5	XXVIII	Pasture (78 days)		2	3	1	2	33-50	50-100

made by the old method without irradiation (Method 1) and the figures obtained show good agreement with those published by Luce for similar samples of milk. In column 5 are given the doses of the same milks required when Method II was employed, and excess of vitamin D was provided by irradiation of the animal or its food. In columns 6 and 7 these results are expressed as the number of growth units per 100 cc. The results are very instructive.

Of sample XXIV, taken when the cow was on winter feed in a dark stall, 12-20 cc. were required to provide one growth unit when using the simple

growth test. When the rats were irradiated, however, the requirement was reduced to 8–12 cc., showing that in the former test growth was being limited by lack of vitamin D.

The same is true of milks XXV and XXVII, taken while the cow remained in the dark stall but after the winter feed had been replaced by fresh green grass. After 3 months on this diet (milk XXVII) the vitamin A value of the milk corresponded to 50–100 growth units per cc., but this high value was not accompanied by a corresponding increase in vitamin D. For when irradiation was omitted, as in Method I, only 20–33 growth units were demonstrated, showing that growth was again limited by deficiency of vitamin D.

In the “pasture-fed” milk XXVIII, on the other hand, while the vitamin A value remained equal to that of milk XXVII, there was a definite increase in the content of vitamin D. This is shown by the fact that the discrepancy between the growth-promoting value as determined by the two methods, viz. 33–50 and 50–100 growth units per cc. respectively, is less than in the case of any other sample (see also Fig. 1, sample XXVIII, doses 2 and 3 cc.).

It is clear, therefore, that, judged by this growth method, the incidence of sunlight upon the cow is needed in order to produce an appreciable amount of anti-rachitic vitamin D in the milk. These results are confirmed by the results of the second series of tests (see below, p. 642), in which the vitamin D value was determined more directly by observing the effect on histology and calcification of bone, when the test animals received McCollum's low phosphorus, rachitic diet.

The vitamin A content of the milk, on the other hand, as Luce concluded, depends entirely on the cow's diet. In periods 3 and 4, during which the cow remained in the dark stall, 54 days after changing the food from cereals and roots to fresh green grass and clover the vitamin A value of the milk (milk XXV) had increased about threefold, and a month later about five- to tenfold (milk XXVII). This was the maximum value for vitamin A attained. In period 5, when the cow was moved from the dark stall to the pasture field, without alteration in diet, no further increase in the vitamin A value of the milk occurred.

In Table V are collected some analyses of the bones of rats which served for the simple growth tests of milks XXIV, XXVII and XXVIII. The bones of the control animals receiving the basal F diet alone showed a condition of osteoporosis on histological examination and the high proportion of fat characteristic of this condition [see Chick, Korenchevsky and Roscoe, 1926]. The addition of milk to the diet caused a marked reduction in this fat content, but the proportion of calcium in the fat-extracted bones was increased very slightly, if at all. With milk XXIV (winter fodder in a dark stall) as high a dose as 20 cc. daily caused no increase in the percentage of calcium. With doses of milk XXVII (fresh green food in a dark stall) from 3 to 12 cc. and of milk XXVIII (pasture) from 3 to 5 cc., there was a definite small increase, depending on the size of the milk dose, in the percentage of calcium and in the value of the A/R ratio (ratio of the ash to the total organic matter of the fat-extracted bone). The differences are small however, and it is questionable whether, from these experiments, the conclusion is permitted that the process of calcification in the test animals has been influenced by the milk supplement to an extent that is significant.

Fig. 1. Growth-promoting value of milks XXIV to XXVIII, given in doses varying from 2 to 8 cc. The curves represent the average degree of restoration of growth (measured in g. increase in body-weight per week) in young rats during the period in which the milk doses were given.

In Method 1 the milk doses were given after body-weight had become stationary on diet F, deprived of fat-soluble vitamins; the growth observed is due to the joint effect of vitamins A and D contained in the milk.

In Method 2 the milk doses were given when body weight had become stationary on diet F, vitamin D being supplied by irradiation: the growth observed is a measure of vitamin A only.

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Table V. *Analysis of the bones of rats receiving F diet (devoid of fat-soluble vitamins but adequate in other respects) with milk supplements.*

Milk doses given on cessation of growth according to Method 1, without irradiation.

The figures represent average values.

Doses of milk cc.	Milk XXIV (Litters 212, 166) Winter feed in dark stall					Milk XXVII (Litters 212, 218, 220, 271, 272, 277) Fresh green food in dark stall				Milk XXVIII (Litters 271, 272, 277) Pasture in summer			
	No. of animals	% Ca on fat-extr.		% fat on wet wt.	Ratio A/R	No. of animals	% Ca on fat-extr.		% fat on wet wt.	No. of animals	% Ca on fat-extr.		% fat on wet wt.
		dry wt.	Ratio A/R				dry wt.	Ratio A/R			dry wt.	Ratio A/R	
0	1	20.7	1.26	8.3	—	8	19.8	1.21	10.6	6	19.5	1.19	10.4
1	—	—	—	—	—	—	—	—	—	3	19.9	1.23	8.5
2	—	—	—	—	—	2	19.6	1.19	9.8	3	19.8	1.23	6.5
3	—	—	—	—	—	4	20.7	1.28	7.2	3	20.1	1.35	8.1
5	1	20.2	1.27	6.5	—	4	21.5	1.44	5.7	3	20.5	1.32	4.8
8	2	21.2	1.38	7.6	—	2	22.3	1.52	4.0	—	—	—	—
12	2	21.6	1.51	5.6	—	2	22.2	1.50	6.4	—	—	—	—
20	1	20.4	1.32	4.8	—	—	—	—	—	—	—	—	—

In Table IV *c* of the previous paper in this *Journal* (p. 629) are set out the complete analyses of the bones of the whole series of animals used for the tests of milk XXVIII (pasture). The bones of the control rats, on basal F diet alone, show only a small departure from the normal in their composition and very little difference is to be seen when these figures are compared with those corresponding to rats which had received daily doses of this milk. There is a definite small increase in the A/R ratio and a very small change in the calcium content of the fat-extracted bones when the daily dose of the milk was equal to 5 cc. By other tests milk XXVIII was shown to possess definite anti-rachitic properties (see pp. 641-3). The conclusion, therefore, is inevitable that anti-rachitic qualities in supplementary foodstuffs are not demonstrated by changes in bone-composition if the test animals are fed upon a basal diet deprived only of fat-soluble vitamins.

4. ANTI-RACHITIC (VITAMIN D) VALUE OF THE MILK.

Milk samples XXII, XXIV, XXV, XXVIII, were tested for anti-rachitic potency by the more direct method, viz. observing the effect when fed as supplement to test animals upon a low phosphorus rickets-producing diet. The results are summarised in Table VI, while the complete details regarding the test made with milk XXVIII will be found in Table IV of the preceding paper by Chick, Korenchevsky and Roscoe [1926].

The effect of the doses of the different milks was observed (1) on the proportion of calcium in the fat-extracted bones of the test rats and (2) on the development of histological rickets. In both cases the results led to the same conclusions. Milks XXIV and XXV were about equal in anti-rachitic value as judged by either criterion, a dose of 12-20 cc. daily being required to correct the rickets-producing tendency of the diet. Substitution of fresh green grass for the dry feed had little effect on the anti-rachitic value of the milk as long as the cow remained in a dark stall. With "pasture-fed" milk XXVIII, on the other hand, a dose of 5 cc. reduced the severe rickets shown by the control animals to a condition diagnosed as "very slight," so that milk XXVIII may be considered to have an anti-rachitic value equal to 2-3 times that possessed by milks XXIV and XXV. Sample XXII, taken when the cow received dry winter food but was kept in the open, appeared to be definitely superior to

samples XXIV and XXV, though here the comparison is difficult because the young rats used for testing milk XXII were evidently better endowed with vitamin D reserves than those used for the other milk samples. The average calcium content in the bones of the control rats at the end of the test was 14.6 %, reckoned on the fat-extracted bones; in case of the control rats used in the tests of milks XXIV, XXV and XXVIII, this figure varied from 11.8 % to 12.1 % (Table VI).

Table VI. *Anti-rachitic value of milk samples.*

Examination of the bones of young rats after 28 days on McCollum's rickets-producing diet No. 3143. Rats placed on experimental diet at 40-50 g. weight.								
Milk XXII (Litter 155) Cow on winter feed: in the open in summer					Milk XXIV (Litter 233) Winter feed in dark stall			
Dose cc.	No. of animals	% Ca on fat-extracted dry weight	% increase in Ca	Histology	No. of animals	% Ca on fat-extracted dry weight	% increase in Ca	Histology
0	2	14.6	—	Severe R. and osteoporosis	1	11.8	—	Severe R.
5	2	17.5	19	Slight R. and osteoporosis	—	—	—	—
8	—	—	—	—	1	16.15	36	Moderate R.
10	2	18.6	27	Nearly normal, sl. osteoporosis	—	—	—	—
12	—	—	—	—	2	17.05	44	Slight R.
20	—	—	—	—	1	22.0	86	Nearly normal

Milk XXV (Litters 231, 232, 233) Fresh green food in dark stall					Milk XXVIII (Litter 302) Pasture in summer			
Dose cc.	No. of animals	% Ca on fat-extracted dry weight	% increase in Ca	Histology	No. of animals	% Ca on fat-extracted dry weight	% increase in Ca	Histology
0	5	12.1	—	Severe R.	2	12.0	—	Severe R.
2	—	—	—	—	1	13.6	13	Severe R.
3	1	11.4	—	Severe R.	2	15.4	28	Moderate-sl. R.
5	3	15.3	26	Sl.-moderate R.	2	16.75	39	Very slight R.
8	4	16.2	33	Sl.-moderate R.	—	—	—	—
10	—	—	—	—	—	—	—	—
12	4	17.4	44	Slight R, nearly normal	—	—	—	—
20	1	18.3	51	Nearly normal	—	—	—	—

It was pointed out above that, when employing rachitic diets deficient in phosphorus for testing the anti-rachitic value of milk samples, a complication is introduced by the administration of the phosphorus contained in the daily dose of milk. With doses under 5 cc. this effect would be insignificant, but in case of milks XXIV and XXV, where doses as large as 12-20 cc. were given daily, it is probable that the anti-rachitic effect observed is partly due to the inclusion of this extra phosphorus in the diet and that the anti-rachitic value of these milks is over-estimated.

McCollum's 3143 diet contains 0.19 % P and 0.76 % Ca reckoned on the fresh weight of the diet. It is not unusual for a young rat of good appetite to consume 15 g.-20 g. daily; if in addition 20 cc. milk were administered the total consumption of phosphorus would be nearly doubled. The disproportion between calcium and phosphorus in this diet, viz. 4 Ca : 1 P, an important factor in preventing normal bone calcification, is also altered (see Table VII).

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Table VII. *Amount of Ca and P ingested daily by rats on McCollum's 3143 rickets-producing diet, without and with supplementary doses of milk.*

Food	Ca g.	P g.	Total Ca g.	Total P g.	Ratio Ca/P
15 g. McCollum's diet	0.114	0.028	0.114	0.028	4 : 1
+ 5 cc. milk in addition	0.007	0.005	0.121	0.033	3.7 : 1
+ 20 cc. „	0.028	0.021	0.142	0.049	2.9 : 1

In order to obviate this source of error a further comparison was made between the butters made from milk samples XXVII and XXVIII, taken in periods 4 and 5 respectively. This was administered in varying doses to rats on McCollum's diet in place of milk. The results of one comparative experiment, using the same litter of rats for both samples of butter, is summarised in Table VIII and set out in full in Table IV of the previous paper (this *Journal*, p. 629). The results of a second experiment are given in full in Protocol II.

Table VIII. *Anti-rachitic value of butter made from milk samples XXVII and XXVIII respectively.*

Examination of the bones of young rats (Litter 289) after 28 days on McCollum's No. 3143 rachitic diet. Rats placed on experimental diet at 40-50 g. wt.

Dose, daily g.	No. of animals	% Ca in fat-extracted bones	% increase in Ca	Histology
0.0	2	12.55	—	Severe R.
Butter XXVII				
(Cow 87 days on green fodder in dark)				
0.1	1	16.19	30	Moderate-severe R.
0.25	2	14.95	19	Moderate R.
0.6	1	13.90	11	Moderate-severe R.
Butter XXVIII				
(Cow 78 days at pasture)				
0.1	1	14.51	16	Moderate-severe R.
0.25	2	15.44	23	Moderate R.
0.6	1	18.11	49	Osteoporosis. Very slight R.
Cod-liver oil, 3 drops (0.054 g.)	1	19.11	53	Practically normal

Butter XXVIII, made from the pasture-fed milk collected in period 5, is definitely superior in anti-rachitic value to butter XXVII, made from the milk of period 4, when the cow received fresh green food in the dark stall. If we compare the daily doses of the respective butters, required to prevent the development of histological rickets to about the same degree, we find 0.25 g. of butter XXVIII equivalent to about 0.6 g. of butter XXVII in one experiment (Table VIII) and 0.3 to 0.5 g. of butter XXVIII equivalent to about 0.5 to 0.8 g. of butter XXVII in the second (Protocol II).

The removal of the cow into the open without change of diet caused roughly a twofold increase in the anti-rachitic value of the butter made from the milk. In the experiment in which the milks were directly compared, the increase was two- to threefold (see Table VI).

A similar result emerges from a comparison of milks XXII and XXIV. During the periods 1 and 3, when these samples of milk were taken, the diet

of cereals and roots remained unaltered. But milk XXII, taken in period 1, when the cow was kept in the open in summer, has a definitely higher anti-rachitic value than milk XXIV, taken at the end of period 3, when the cow had been for several months in the dark stall.

Taken together the above results suggest that the insolation of the cow is the factor determining the anti-rachitic properties of the milk and that diet, whether fresh green grass and clover or the winter feed of cereals and roots, is immaterial. The same conclusion is drawn above (p. 641) when discussing the anti-rachitic value of milk samples XXIV and XXV.

A comparison of milk sample XXVIII with sample XXII, however, shows that this is not the whole truth, and that the fresh green food taken by the cow during the pasture period 5 has some contributory effect in raising the anti-rachitic power of the milk to the maximum value attained during this period. Both these samples of milk were taken when the cow was exposed to sunshine out of doors, but sample XXVIII, taken when the animal was on pasture, is definitely superior to either sample XXII or sample XVII of Luce's experiments [see Luce, 1924, 2, Table III). The latter were both taken in period 1 when the cow was kept out of doors but received a winter feed. The average daily incidence of sunshine during period 1 was 6.5 hours and during period 5 was only 4.1 hours. The fact that the anti-rachitic value was greater during period 5 can, therefore, only be attributed to the fresh green food which formed the cow's diet.

Fresh green leaves grown in summer under conditions of maximum natural illumination have been shown to possess a small but definite content of vitamin D [Chick and Roscoe, 1926; Boas, 1926] and the vitamin D content of fresh grass appears to be too small to exert any significant influence upon the anti-rachitic value of the milk, as long as the cow is deprived of sunshine. But when the cow is out at pasture the fresh green food together with the exposure to sunlight apparently combine to raise the vitamin D content of the milk to a higher level than can be effected by direct sunshine alone.

An interesting confirmation of the results obtained in this section was yielded by a re-examination of the material collected by Luce. In the course of her work she made a careful study of the milk produced when the cow was receiving a winter feed of cereals and roots, but was kept out of doors in a ploughed enclosure. This occurred in Period V of her cycle, a period which lasted, with one break, from October, 1923, to July, 1924, and overlapped with period 1 of the present research (see Table I). Milk sample XVII was taken in July, 1924, after the cow had been out of doors for nearly three months, during which time the daily incidence of sunshine had averaged 6.3 hours. The anti-rachitic potency of this milk was, however, found inferior to that of sample XIV, which was taken in October, 1923, after the cow had been on pasture for nearly three months and exposed to an average of 7.1 hours sunshine daily, an amount not very different from that occurring at the end of

Period V. In order to prevent development of rickets in rats receiving McCollum's No. 3143 rachitic diet, 8-10 cc. of milk XVII were required [Luce, 1924, 2, Table III], and 5 cc. of milk XIV [Luce, 1924, 1, Table III]. Luce, therefore, attributed the extra anti-rachitic value of the milk XIV to the fresh green food taken by the cow during the pasture period, a conclusion confirmed by us after comparison of our milk sample XXVIII with sample XXII (p. 644). It seems possible, however, that the anti-rachitic potency of sample XVII may have been somewhat under-estimated. If the increased calcification of the bones of the test rats be taken as the criterion of the anti-rachitic value of the milk doses rather than the prevention of structural deformity, Luce's tables show that sample XVII compares very favourably with sample XIV. In the tests of sample XVII the control rats, which received the rachitic diet alone, had 10.9 % calcium in their bones (reckoned on the dry weight), while those receiving in addition a daily dose of 5 cc. of milk XVII had this amount raised to 14.1 %. In the tests of milk XIV, the control rats had bones which were better calcified, the calcium content being equal to 13.4 % Ca (reckoned on the dry weight). Nevertheless, the animals which received 5 cc. daily of this milk sample showed an average of only 16.7 %, *i.e.* no greater proportional improvement than was manifested by the rats which had an equal dose of milk XVII.

Samples of butter were made from milks XVII and XIV at the time of collection. They were not investigated by Dr Luce, but were kindly placed by her at our disposal and tested in March, 1926. It was hoped that some additional evidence might be obtained by testing the relative anti-rachitic potency of these two samples of butter, even after so long a time of storage. The results obtained are summarised in Table IX, and show that butter XVII was in no way inferior to butter XIV, and, if anything, appears to possess a somewhat greater anti-rachitic value. This is so whether comparison is made between the degree of histological rickets or the degree of bone calcification displayed by the animals receiving equal daily doses of the two samples. It must be remembered, however, that when the tests were made butter XIV had been stored in the frozen state for 29 months and butter XVII for only 20 months, that is for 9 months less. Both samples appear to be of less anti-rachitic potency than the "pasture fed" butter sample XVIII (see Table VIII and Protocol II), which was tested after being kept for less than 6 months. It was to be expected, however, that some diminution in vitamin content should have taken place in samples XIV and XVII after their long period of storage and that this should have been greater in the case of XIV.

These results, taken in conjunction with those obtained by Luce with the respective samples of milk from which the butters were prepared, confirm the conclusion drawn from our own experiments and may be summarised as follows. The milk taken from a cow receiving a winter feed of cereals and roots, while exposed to a maximum degree of natural sunshine, was found to possess an anti-rachitic value only slightly, if at all, inferior to that of the milk produced

when the cow was on pasture under similar conditions of illumination, thus indicating that the degree of insolation of the cow is the important factor in determining the anti-rachitic potency of the milk.

Table IX. *Anti-rachitic value of butter made from milk samples XIV and XVII** [Luce, 1924, 1, p. 730, and 2, p. 1286 respectively].

Milk XIV was taken after the cow had been at pasture for 3 months (Aug. 1–Oct. 22, 1923).

Milk XVII was taken after the cow had been exposed to sunshine for 2½ months (April 17–July 2, 1924) but receiving a winter feed of cereals and roots.

The butter was preserved in the frozen condition until March, 1926.

Examination of bones (Litter 386 of young rats), after 28 days on McCollum's No. 3143 rachitic diet, rats placed on experimental diet at 40–50 g. weight.

Dose, daily g.	No. of animals	% Ca in bones		% increase in Ca content (ii)	A/R ratio	Histology
		(i) on dry weight	(ii) on fat-extracted weight			
0.0	2	8.3	8.7	—	0.308	Very severe R.
Butter XIV						
0.3	1	8.9	9.3	7	0.336	Very severe R.
0.5	2	10.7	11.4	31	0.446	Very severe—severe R.
0.8	1	11.5	13.35	53.5	0.564	Moderate R.
Butter XVII						
0.5	2	11.7	12.8	47	0.527	Moderate R.
0.8	2	12.1	13.8	58.5	0.597	Moderate-slight R.

* Sample taken during Period I, 5 weeks earlier than sample XXII.

DISCUSSION.

From the results of her experiments Luce [1924, 1 and 2] concluded that the cow's diet was the main factor determining the growth-promoting value of the milk and that the degree of insolation enjoyed by the cow played a minor part in determining the increased anti-rachitic potency of the milk when the animal was pasture fed. Since the discoveries of Steenbock and his co-workers it has been possible to discriminate between the influence of vitamin A and vitamin D upon growth and in the present work more trustworthy methods have been employed than were previously available.

The results indicate that the vitamin A value of the milk is entirely dependent upon the diet of the cow, and further that under certain circumstances, the diet, if of fresh green grass, may exert some small influence upon the vitamin D value of the milk. However, with regard to the latter, the degree of exposure of the cow to sunshine is the important determining factor.

There is no necessity to stress the need for nursing mothers to lead an open air life as far as possible or the desirability of exposing milch cows to direct sunlight out of doors if the milk is destined for the nutrition of infants and young children. In many European cities it is the custom to keep the cows, from which the town milk supply is obtained, confined in stalls situated in or near the city. The diet of these cows may vary at different times of the year and during spring and summer may include a proportion of fresh cut grass with beneficial effect upon the vitamin A content and growth-promoting value of the milk [see Reyher, 1924]. But the cows themselves remain indoors

and are never exposed to the direct rays of the sun. The anti-rachitic value of the milk may, therefore, be assumed to remain at a very low level.

In the course of the investigations on rickets carried out in the spring of 1923, at the University Kinderklinik in Vienna [Chick, Dalyell, Hume and others, 1923, pp. 73, 148] a practical test was made of the anti-rachitic value of milk derived from a cow living in a poorly lighted stall and fed exclusively on fresh green food—cabbage, fresh cut meadow grass and mixed graminaceous and leguminous green stuff. The milk was reserved for certain infants admitted to the Klinik with a diagnosis of active rickets. At the end of 1–2 months in which this milk alone was consumed, no healing of the rachitic lesions could be demonstrated in X-ray photographs of the epiphyses. When, however, specific measures of treatment were employed, such as administration of cod-liver oil or exposure of the infant to direct sunlight out of doors, healing rapidly occurred.

As far as we are aware, no trial has been made of the clinical value of “pasture-fed” milk in the healing of rickets. In most districts of Europe such milk is only available in summer, at a season of the year when cases of active rickets are rarely to be found.

SUMMARY AND CONCLUSIONS.

1. Experiments have been made, in continuation of those of Luce, in which a cow has been kept under varied conditions of diet and light and the milk obtained tested for its content of (growth-promoting, anti-xerophthalmic) vitamin A and of (growth-promoting, anti-rachitic) vitamin D.

2. The vitamin A content of the milk was found to be dependent on the diet of the cow, being at a maximum when the cow was fed upon fresh green food and least when the cow received a winter feed of cereals and roots.

3. The degree of exposure of the cow to sunlight had no influence upon the vitamin A value of the milk. When the cow received fresh green fodder in a dark stall the vitamin A content of the milk reached its maximum and no further increase was noted when the cow subsequently went out to pasture.

4. The anti-rachitic value of the milk (vitamin D content), on the other hand, was found to depend principally upon the degree of insolation of the cow. This result was obtained from experiments in which the rickets-preventing power of the milk (1) was estimated from the growth-promoting power of the milk and (2) was measured by its capacity to prevent rickets in rats receiving a low phosphorus rickets-producing diet.

5. At the same time there was some indication that the diet of the cow, if consisting of fresh green food, was a contributory factor. Our experiments confirmed those of Luce in showing that “pasture-fed” milk possessed the maximum anti-rachitic value while that yielded by the cow when receiving a diet of cereals and roots appeared on the whole inferior in this respect, in spite of exposure to maximum illumination out of doors in summer.

6. The milk contained the maximum amount of both fat-soluble vitamins, A and D, when the cow was out at pasture in summer.

7. Attention is called to the disadvantage of confining milch cows in stalls, when the milk is required for the nutrition of infants and young children.

8. Butter made from milk possessing anti-rachitic potency was found to preserve this property after storage in the frozen condition for more than two years.

Protocol I. *Vitamin A content of milk XXVII (cow three months on fresh green food in a dark stall).*

Restoration of growth in young rats whose growth had ceased upon a diet devoid of fat-soluble vitamins. Vitamin D supplied during the milk test and during the latter part of the pre-experimental period by direct irradiation (Litters 212, 218, 220) or by inclusion of irradiated cotton-seed oil in the diet (Litters 313, 318).

Rat No.	Litter	Milk dose cc. daily	Initial weight of rat g.	Time of preliminary period on deficient diet days	Weight of rat at end g.	Weekly increments of growth when receiving milk doses			Average weekly increment g.
						g.			
						1st week	2nd week	3rd week	
389	218	1	42	56	86	6	7	0	4
389	212	„	34	52	72	0	11	4	5
397	220	„	41	53	90	8	6	0	5
477	318	„	43	70	143	3	2	11	5
Average:			40	58	98	4	6.5	4	5
380	218	2	43	46	78	15	17	12	15
383	„	„	34	54	72	10	11	6	9
394	212	„	36	48	83	5	9	4	6
472	313	„	40	59	115	9	20	13	14
484	318	„	47	55	107	5	12	5	7
Average:			40	52	91	9	14	8	10
381	218	3	38	43	63	13	18	17	16
386	212	„	42	49	75	8	12	7	9
401	220	„	41	53	72	6	8	4	6
473	313	„	43	54	99	11	10	12	11
479	318	„	44	60	116	12	14	12	13
485	„	„	40	51	94	9	4	6	6
Average:			41	52	86	10	12	10	10
378	218	5	36	56	70	15	21	15	17
395	212	„	36	58	64	13	14	6	11
398	220	„	46	69	71	22	15	10	16
471	313	„	40	56	101	6	19	14	13
487	318	„	41	54	101	11	11	6	9
Average:			40	59	81	13	16	10	13
402	220	8	40	39	59	15	16	8	13

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Protocol II. *Anti-rachitic value (vitamin D content) of butter made from milk samples XXVII and XXVIII.*

Examination of the bones of young rats after 28 days on McCollum's No. 3143 low P rickets-producing diet.

No. of rat	Litter	Dose g.	Wet weight, %				Ca %		A/R	Histology of rib-junctions
			H ₂ O	Fat	Ash	Org. res.	On dry weight	On extracted weight		
492 ♂	360	0	64.0	2.7	9.0	24.3	9.2	10.0	.36	Very severe R. and osteoporosis
495 ♀	„	0	62.2	2.8	9.6	25.4	9.6	10.4	.38	Very severe R.
500 ♀	354	0	64.8	2.0	8.9	24.2	9.4	10.0	.37	„
									10.13	.37
Butter XXVII (87 days on fresh green fodder in dark).										
493 ♀	360	0.3	62.4	1.9	12.1	23.6	11.9	12.6	.51	Very severe R. and osteoporosis
498 ♀	354	„	61.9	2.9	10.8	24.4	10.5	11.4	.44	„ „
									12.0	.47
491 ♂	360	0.5	62.7	3.55	10.2	23.5	10.2	11.2	.44	Severe R.
501 ♀	354	„	59.2	2.85	14.2	23.8	12.85	13.8	.60	Severe R. and osteoporosis
									12.5	.52
489 ♂	360	0.8	59.3	5.6	12.8	22.35	11.6	13.5	.57	Moderate R.
502 ♀	354	„	58.3	4.1	15.4	22.25	13.7	15.1	.69	„
									14.3	.63
Butter XXVIII (cow 78 days at pasture).										
494 ♀	360	0.3	62.3	2.8	11.55	23.4	11.3	12.2	.49	Severe R.
499 ♀	354	„	61.9	2.4	12.35	23.3	12.0	12.8	.53	Moderate R.
									12.5	.51
488 ♂	360	0.5	55.95	5.0	17.2	21.8	14.5	16.3	.79	Nearly normal
497 ♂	354	„	58.8	4.1	14.8	22.3	13.3	14.9	.67	Moderate R.
									15.6	.73
490 ♂	360	0.8	58.5	5.5	13.1	22.9	11.7	13.5	.59	Slight R.
503 ♀	354	„	55.4	4.0	18.0	22.6	15.0	16.4	.80	„
									14.95	.695
496 ♂	354	3 drops (0.054 g.)	53.6	4.1	19.9	22.35	15.9	17.5	.89	Normal

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