XXIV. INFLUENCE OF A MILK DIET ON THE SKELETON.

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RICKETS is developed principally at the age when milk forms the staple diet. Most authors (Aron [1908], Schabad [1910], Dibbelt [1910], Korenchevsky [1922, 3]) draw attention to the fact that a child fed on a milk diet is liable to suffer from calcium starvation, which is an important factor in the development of rickets. Pfeiffer [1885] found that the milk of mothers of rickety children might be less rich in phosphorus. According to the investigations of McCollum and co-workers [1921, 1922] this deficiency, in conjunction with the deficiency of a fat-soluble factor, may be one of the causes of rickets. Daniels and Loughlin's experiments [1920], if confirmed by experiments on a greater number of animals, might explain the cause of the decreased content of salts in the milk: these two authors found that pasteurisation and prolonged heating caused the loss of calcium salts from milk. Rats fed on such milk did not grow, and even perished from exhaustion. The addition of calcium salts (especially tricalcium phosphate or calcium glycero-phosphate) was followed by a resumption of normal growth. This result could not be obtained by adding vitamin A or B to the milk.

The work of Mellanby [1919, 1920, 1921] and Korenchevsky [1921, 1922, 1, 2, 3; 1923, 1, 2] showed the importance of a deficiency only of a fat-soluble factor in the production of rickets, and Korenchevsky's investigations showed the importance of the simultaneous deficiency of the fat-soluble factor *plus* calcium. In the present work, "fat-soluble factor" is applied to a factor or factors connected chiefly with animal fats, and conducing to the normal metabolism of calcium in the organism, to growth, nutrition, and appetite in animals. We consider "fat-soluble factor" a suitable term in view of the fact that the existence of a fourth, anti-rachitic, vitamin, apart from vitamin A [McCollum and co-workers, 1921, 1922], has not yet been fully proved. The existence, in milk, of a fat-soluble factor possessing anti-rachitic properties raises the question of its possible deficiency in some milk, and therefore, of the possibility of a milk diet producing rickets in consequence of this. The investigations of Hughes, Fitch and Cave [1921], Drummond, Coward and Watson [1921], Dutcher [1921], Kennedy and Dutcher [1922] showed that the content of vitamin A in cow's milk or in butter made from it may fluctuate very greatly, principally in dependence on the vitamin content in the food of the cow.

In making butter, part of the vitamin A contained in the milk is inactivated [Drummond, Coward and Watson, 1921]. In some samples of butter these authors found a surprisingly small content of vitamin A. On the other hand, Hopkins [1912, 1920] found that the addition of 2 cc. of fresh milk to the food deprived of vitamins was enough to maintain the normal growth and health of rats, that is to say, some kinds of milk are really very rich in vitamins. The factor acting destructively on vitamin A is oxidation, especially on heating, but not heating alone [Hopkins, 1920; Drummond and Coward, 1920; Zilva, 1920; Mellanby, 1921; McCollum and co-workers, 1922].

In the present investigation we shall not quote all the literature on the influence of fresh, heated or desiccated milk on the growth, nutrition and reproduction of animals. Such literature has been collected and properly compared in the works of Mattill and Conclin [1920], and Daniels and Loughlin [1920]. In the summary of their investigation Mattill and Conclin give a clear idea of the present position regarding this question. In their experiments, young rats after weaning were placed on various rations, consisting primarily of cow's milk, fresh and desiccated. On fresh milk rats made good initial growth, but beginning between the 50th and 100th day of life, especially in the females, there was a decided retardation. There was no reproduction. The addition of yeast filtrate temporarily increased the rate of growth, and one female became pregnant, but the litter was eaten. Addition of wheat embryo also increased the rate of growth, with no further effect thus far. When milk was supplemented with iron citrate, growth was much more satisfactory, but reproduction was not successful. The growth failure was in part the result of the dilute form of the food, for on dry milk rats made much better growth, and their average food consumption in milk solids was considerably greater than that of those fed on fresh milk. The females, however, remained somewhat below normal after 75 days, and again there was no reproduction. Neither substitution of dry skim milk to which butter fat was added equivalent to that in dry whole milk, nor the addition of 10 % of butter fat to dry whole milk, was successful.

On a ration containing 55 % of dry whole milk, 40 % of starch, and 5 % of butter fat, both male and female rats made practically normal growth, and the females bore young, but did not rear them. A similar ration has been used by other investigators, who secured not only satisfactory growth, but also reproduction, while others again have, like ourselves, not had success with whole dry milk alone. Dilution of dry milk by 25 % lactose resulted in poor growth and no reproduction. The testes were, with one exception, of normal weight, and contained motile spermatozoa. The ovaries, on the other hand, were much under normal weight, even 50 % and more. In Mattill and Conclin's opinion, possibly milk is lacking both quantitatively and qualitatively in substances necessary for successful adolescent growth and reproduction, especially in the female, and it may contain substances that are inhibitory to the growth of an animal in the third or mature growth cycle. A ration containing dry milk and 1 % of yeast is the only one on which normal growth and partially successful reproduction were obtained, and the growth of rats on this ration, and on one containing 5 % of yeast, is thus far practically normal. Whether yeast supplies something unique remains to be seen.

As far as we know, there have been only three investigations of the influence of a milk diet on the skeleton. Bolle [see Bartenstein, 1905] found that milk, either fresh or boiled for five minutes, on which guinea-pigs were fed almost exclusively, produced no skeletal changes in the latter. Milk boiled for 10 or 20 minutes produced osteoporosis, fragility of the bones and a partial separation of ephiphyses in the skeleton of guinea-pigs. The skeletons were not examined either microscopically or chemically.

Keller [see Bartenstein, 1905] obtained negative results on repeating Bolle's experiments on mice and dogs.

Bartenstein [1905], on the contrary, obtained, on the whole, the same results as Bolle. In his experiments, the daily ration of the guinea-pigs consisted of milk *ad libitum*, with the addition of cream (up to 8–12 % fat), 2 g. of hay and 1 g. of wheat bran. Unfortunately, for the purpose of histological examination the bones were completely decalcified in H_2SO_4, and therefore the question of the presence of rickets could not be solved. The author considers the resultant histological picture analogous to the osteotabes infantum of Ziegler [1901].

Thus, up to the present time there have been no investigations, carried on in accordance with modern technical requirements, for the purpose of determining the influence of a milk diet on the skeleton. The object of the present investigation was to ascertain the influence on the skeleton of rats of diets consisting of various doses of fresh, heated and oxidised milk. Oxidation was attempted for the purpose of diminishing, or destroying, its antirachitic properties in order to produce rickets in the rats by such a diet. In all there were three series of experiments. The object of the first series was to study the effects of large doses of milk; that of the second series—the effects of medium doses, and of the third series—of small doses. The histological and chemical methods of investigating the skeleton were the same as those used in previous investigations [Korenchevsky, 1921, 1922].

First series of experiments. Large doses of milk.

Seven experiments were conducted on 51 rats, milk being given *ad libitum*. 3 % of cane sugar was added to the milk, in order to increase its calorie content. The milk was supplied by a large London firm, and was a mixture of milk from different cows. The experiment lasted from December to 10th March, *i.e.* "winter" milk was used.

The milk supplied was divided into three parts: one was left fresh (FM), the second was heated in a bath of boiling water in a flask for seven hours (HM); the third (OM) was aerated by a brisk stream of air and at the same time heated in a bath of boiling water for seven hours, for the purpose of oxidising the fat-soluble factor. Before being passed through the milk, the air was heated in a coil, likewise immersed in boiling water. Owing to this the temperature of the milk during oxidation averaged 98°·8 C. Milk thus oxidised was used in experiments Nos. 36–42 of this series.

The calcium content of the milk when boiled for ten minutes, heated in a bath of boiling water, with or without aeration, was hardly altered, as shown by our analysis of nine samples of milk. The average figures were as follows:

(1) Fresh milk	0·122 % Ca
(2) Boiled for ten minutes	0·123 % Ca
(3) Heated for seven hours in a bath of boiling water	0·121 % Ca
(4) Oxidised and heated for seven hours	0·113 % Ca

That is to say, only the oxidised milk showed a slight decrease (about 7 %) below the normal in its calcium content. The milk itself became thicker on aeration: this was not due to the evaporation of water, as a reflux condenser was placed over the flask during oxidation for the purpose of preventing evaporation. In order to make sure that the resulting skeletal changes on a milk diet were due solely to the changes in the amount of the fat-soluble factor, and not to that of vitamins B and C, each rat received daily, over and above milk with 3 % sugar, 3 g. of BC I paste, composed of the following:

Paste BC I:	Starch ...	57 g.
	Yeast ...	11·4 g.
	Orange juice	13·6 cc.
	Oxidised milk	18 cc.

For control purposes, during the last third or half of the period of milk diet, some of the rats were given, in addition to paste BC I, a daily additional 0·03 g. cod-liver oil (paste ABC), or, in some experiments, the control rats were given "normal paste" containing about 1·7 % cod-liver oil[1].

Milk was given to the rats *ad libitum*; their average consumption was as follows:

Table I.

	Beginning of experiment cc.	End of experiment cc.
Fresh milk	37	50
Heated milk	35	70
Oxidised milk	35	58
„ „ + ABC paste	35	56

That is to say, on the whole, the rats consumed a slightly larger quantity of heated, and even oxidised milk than of fresh milk. The very high average

[1] The composition of N paste was the same as that used by Korenchevsky [1921, 1922].

figure of heated milk consumed may be explained by the fact that this average was obtained from three rats, the rest of the rats of this litter also consuming more than the average of fresh or oxidised milk shown in Table I (65 cc. and 63 cc. respectively). The average amount of "normal" paste consumed by the rats was about 20 g.

In the various experiments of the first series the rats were divided into the following groups (see Table II):

Table II. *Diet of rats.*

No. of experiment	FM + 3 g. BC I	HM + 3 g. BC I	OM + 3 g. BC I	OM + 3 g. ABC	"Normal" paste
36	1	—	3	1	1
37	2	—	3	2	2
38	1	—	2	—	1
39	2	—	4	—	1
40	1	1	2	—	1
41	1	2	2	1	1
58	7	—	—	—	1
Total	15	3	16	4	8

In every experiment the rats were taken from the same litter. The rats in experiment 58 were born of parents fed on FM diet, and were themselves fed on that diet after weaning.

Besides these experiments, this series includes one more experiment, No. 42, conducted on five rats, described later.

The average figures of the age of the rats, of the duration of the milk diet, as also the average chemical composition of the skeleton of the rats in experiments 36 to 41, are shown in Table III. As the results were practically identical, no tables of separate experiments are given.

Table III. *Chemical composition of the skeleton of rats fed on milk ad libitum.*

No. of experiment	Age in days		Feeding in days	FM + BC I in bones			HM + BC I in bones			OM + BC I in bones			OM + N paste in bones			OM + ABC in bones		
	Initial	Final		H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %
Exp. 40 (5 rats)	23	66	43	42·0	12·1	20·8	38·7	13·2	21·6	37·7	13·6	21·9	37·9	13·4	21·5	—	—	—
,, 38 (4 rats)	17	105	88	39·7	13·2	21·9	—	—	—	37·3	13·6	21·7	37·9	14·2	22·7	—	—	—
,, 41 (7 rats)	24	109	85	36·7	13·9	22·0	36·5	14·1	22·0	37·3	13·9	22·1	39·7	12·9	21·3	39·4	12·6	20·7
,, 36 (6 rats)	21	118	97	37·6	13·9	22·3	—	—	—	37·6	14·1	22·7	39·4	13·0	21·5	36·7	14·5	22·5
Average of exps. 38, 41, 36	21	111	90	38·0	13·7	22·1	36·5	14·1	22·0	37·3	13·9	22·2	39·0	13·4	21·8	38·0	13·5	21·6
Exp. 37 (9 rats)	36	130	94	38·7	14·0	22·9	—	—	—	36·6	14·4	22·7	38·2	14·0	22·6	37·9	14·2	22·8
,, 39 (7 rats)	40	140	100	37·7	14·1	22·6	—	—	—	38·3	13·6	22·2	40·3	12·8	21·5	—	—	—
Average of exps. 37, 39	38	135	97	38·2	14·1	22·7	—	—	—	37·5	14·0	22·5	39·2	13·4	22·1	37·9	14·2	22·8

These data show that in the different groups of rats consuming milk *ad libitum* no essential difference in the chemical composition of the skeleton was observed, even when, for instance, the oxidised milk diet was begun at a very early age, immediately after weaning (17th day after birth), and was continued for about three months.

Histological examination likewise showed no difference between the structure of the skeleton of rats fed on FM, HM or OM. Sometimes the microscopical picture of the skeleton of rats on a milk diet differed from that of rats on a normal diet only in being slightly more osteoporotic, this condition being more perceptible in rats fed on OM. The osteoporosis was due to the lowered activity of osteoblasts (the number of osteoclasts being normal).

The weight curves of rats of various categories in experiments 36–41 *were identical, and in no way different from the weight curves of rats on a normal diet.* In view of this, they are not shown here. There was likewise no difference in the external appearance and nutrition of the various groups of rats.

Thus the large ration of OM consumed by the rats retained sufficient of the fat-soluble factor for the growth of the rats, and the composition and structure of their skeleton were practically normal even when air was passed through the milk at a temperature of 98°·8 C. during seven hours.

In experiment 58, the second generation of rats on FM diet showed a marked difference from the normal as regards external appearance: rats killed at the age of 57 days were some 60 % below the normal weight; in many places the fur was so thin that some rats were nearly hairless. Moreover, they all suffered from emaciation and anaemia. Chemically, the skeleton was normal in structure. Histologically, the bones showed a moderate increase of the proliferating cartilage with an insufficient deposition of calcium into the zone of provisional calcification in the case of some rats. There was considerable osteoporosis, but the amount of osteoid was not increased.

The object of experiment 42 was to ascertain the influence on the skeleton of rats of an addition to the milk diet either of vitamin B alone (in the form of a paste with yeast), or of vitamin C alone (paste with orange juice). The milk given to the rats was either fresh or oxidised.

Neither the chemical nor the histological examination of the skeleton showed any effects of the addition of vitamins B and C to the milk given to the rats *ad libitum*. The composition of the skeleton of the rats in experiment 42 was compared with that of the control rats from the same litter, fed on a "normal" diet. In view of the negative results of the experiment, we give no detailed data.

Second series of experiments. Influence of medium doses of milk (15 to 30 cc. per day).

Thirty-nine rats, belonging to six litters, were used in this series of experiments. The milk was given to the rats in the same form as in the first series of experiments, *i.e.* FM, HM and OM; except that, for the purpose of intensifying the process of the oxidation of the fat-soluble factor, 1 cc. of a 20 % solution of hydrogen peroxide was added per 100 cc. milk. On investigation it was found that after heating the milk for seven hours and passing air through it, any reaction of hydrogen peroxide had disappeared.

The experiments were conducted from February to the first half of May, *i.e.* during the first third or half of the experimental period "winter" milk was used, and the rest of the time "spring" milk.

In this series of experiments the young rats were weaned at the age of 19 to 20 days, after which, for a period of from 13 to 34 days (Period I), they were fed exclusively on OM. From the age of 33–35 days the rats were transferred to a diet consisting of a moderate ration of milk (15 to 30 cc.) (Period II). The number of calories taken in by each rat was equalised by the starch given to the rats in pastes BC II and BC III. In these pastes the rats received approximately the same amount of vitamins B and C in the daily dose. The composition of the paste was as follows:

	Paste BC II	Paste BC III
Starch	61·5 g.	66 g.
Yeast	3·5 g.	5 g.
Orange juice	5·0 cc.	6 cc.
Water	30·0 cc.	23 cc.

To the daily ration of 15 cc. milk was added 16 g. of paste BC II; to 30 cc. milk 11 g. of paste BC III.

For purposes of comparison some rats received, even during Period II, large doses of milk with 3 g. of paste BC I, or 3 g. of paste ABC (see p. 190).

During Period II the rats were fed for 86 to 103 days, after which they were killed at ages varying from 122 to 151 days.

Thus, during Period II, the rats in the experiments of the second series, as compared with those of the first series, received a considerably smaller amount of protein, fat, salts, and fat-soluble factor.

In the various experiments of the second series the rats were divided into the following groups (see Table IV):

Table IV. *Diet of rats during Period II.*

No. of exp.	50 cc. FM +3 g. BC I	15 cc. FM +16 g. BC II	50 cc. OM +3 g. BC II	50 cc. OM +3 g. ABC	15 cc. OM +16 g. BC II	30 cc. OM +11 g. BC III	30 cc. OM +11 g. BC III + 0·03 g. cod-liver oil
52	—	2	—	1	4	—	—
53	1	2	—	1	3	—	—
54	—	—	—	—	2	2	2
56	1	2	1	—	2	—	—
57	1	2	1	—	2	—	—
Total	3	8	2	2	13	2	2

Besides this, in experiment 55 there were seven rats the distribution of which will be mentioned later, in describing that experiment.

In experiments 52, 53, 54, 56 and 57, during Period I, *i.e.* that of an exclusive diet of OM, the first few weeks after weaning the rats consumed, on an average, about 23 cc. of milk per diem, and later about 40 cc. Those rats which received milk *ad libitum* during the whole experimental period, consumed about 50 cc. during the last third of the time, there being no difference in the consumption of FM and OM. The ration of 15 to 30 cc. of milk and pastes BC II and BC III was completely consumed by the rats, with the exception of one rat, fed on OM.

There was very little difference in the appearance of the rats of each group. The curves of the average weight of the rats of each group in these experiments are shown in Fig. 1 (males) and Fig. 2 (females). The weight curves of rats on OM are somewhat below those of rats on FM, this being more marked in the case of rats on a ration of 15 cc. milk. A similar, merely slight, retardation of the weight curves was observed in the case of rats fed on 15 cc. milk, as compared with those of rats receiving and consuming about 50 cc. milk. The age, duration of feeding on special diets, and the chemical composition of the skeleton of rats in the experiments described are given in Table V. This table shows the following:

Table V. *Chemical composition of the skeleton of rats fed on 15–50 cc. of milk.*

No. of exp.	Age in days: When put on OM only	Age in days: When put on milk and paste	Age in days: Final	Duration of feeding in days: On OM only	Duration of feeding in days: On milk and paste	50 cc. FM+ABC in bones: H_2O %	Fresh Ca %	Dry Ca %	50 cc. FM+BC I in bones: H_2O %	Fresh Ca %	Dry Ca %	50 cc. OM+ABC in bones: H_2O %	Fresh Ca %	Dry Ca %	50 cc. OM+BC I in bones: H_2O %	Fresh Ca %	Dry Ca %	15 cc. FM+BC II in bones: H_2O %	Fresh Ca %	Dry Ca %	15 cc. OM+BC II in bones: H_2O %	Fresh Ca %	Dry Ca %
Exp. 52 (7 rats)	19	53	146	34	93	—	—	—	—	—	—	35·2	14·7	22·6	—	—	—	35·1	13·7	21·1	36·0	13·3	20·8
Exp. 53 (7 rats)	19	54	151	35	97	31·4	16·3	23·7	—	—	—	32·5	14·6	21·7	—	—	—	32·7	14·7	21·9	35·3	14·5	21·3
Exp. 54 (2 rats)	20	45	144	25	99	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	39·9	12·1	20·1
Average of exps. 52 and 53	19	54	149	35	95	31·4	16·3	23·7	—	—	—	33·8	14·7	22·2	—	—	—	33·9	14·2	21·5	37·1	13·3	20·7
Exp. 56 (6 rats)	20	33	133	13	100	—	—	—	32·6	15·9	23·7	—	—	—	32·1	14·9	22·0	35·4	13·7	21·2	34·4	14·0	21·5
Exp. 57 (6 rats)	20	33	136	13	103	—	—	—	30·5	15·5	22·4	—	—	—	34·1	13·8	20·9	34·1	14·1	21·3	38·9	12·7	20·6
Average of exps. 56 and 57	20	33	135	13	102	—	—	—	31·6	15·7	23·0	—	—	—	33·1	14·3	21·4	34·7	13·9	21·3	36·6	13·4	21·1

On an OM diet, a slight diminution of calcium in the skeleton was observed: *e.g.* on 50 cc. OM the skeleton, as compared with that of rats on FM, was more deficient in dry bone (about 7 %) and in fresh bone (9 %); on a diet of 15 cc. milk it was not in every experiment that even this slight difference was observed. In any case, the difference seen in the composition of the skeleton was so slight that it might have been due to physiological fluctuations. Histologically, there was also no considerable difference between the structure of the skeleton of rats in different groups. Only a slight degree of osteoporosis might be observed in the rats especially fed on OM.

Experiment 55 was conducted in such a manner as to approximate the conditions to those of bringing up children by hand, that is to say, the rats were given diluted milk *ad libitum*. The requisite number of calories was provided by a 17 % solution of cane sugar, with which the milk was diluted. The milk was heated in a bath of boiling water for seven hours. The seven rats used in this experiment were divided into four groups, and at the age of 36 days were placed on the following diets:

Table VI.

Denomination of milk	No. of rats	Dilution of milk: Percentage of 17 % solution of cane sugar contained in milk	Consumption of milk in cc.	
			Beginning of experiment	End of experiment
HM	2	—	27	46
DM_1	2	60	22	50
DM_2	2	40	23	50
DM_3	1	50	24	28

Besides milk, each rat received 3 g. of paste BC I. After 86 days of feeding on the above-mentioned diets, the rats were killed. The rats fed on DM_1 and DM_2 were slightly (about 8 %) lighter in weight than those on HM, but, on the whole, were not different from them in any other respect. The rat on DM_3 weighed 37 % less than the rats on HM, and was thin. As may be seen from Table VI, its appetite was worse than that of any of the others.

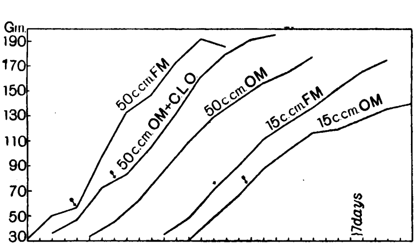

Fig. 1. Weight curves of average rats (males) from experiments 52, 53, 54, 56 and 57. Up to the point shown approximately by the arrows rats were kept on OM only. After that the amount and kind of milk are shown above each curve. C.L.O. = cod-liver oil.

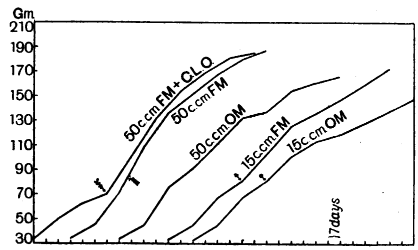

Fig. 2. Weight curves of average rats (females) from experiments 52, 53, 54, 56 and 57. Up to the point shown approximately by the arrows rats were kept on OM only. After that the amount and kind of milk are shown above each curve. C.L.O. = cod-liver oil.

In all the rats fed on diluted milk, as compared with the rats fed on HM, the composition of the skeleton was worse: the average water content of the bones was 8 % greater, the calcium content in fresh bone was about 15 % less, and about 12 % less in dry bone. Histologically, the rats fed on diluted milk, as compared with those fed on HM, showed more marked osteoporosis, and in some cases there was a slight enlargement of cartilage and an irregular line of osseo-chondral junctions. A slightly increased amount of osteoid was observed only in the case of one rat fed on DM_3.

Thus, in this series of experiments also, no considerable skeletal changes could be obtained. It is a striking fact that the seven-hours' aeration of milk at 98°·8 C. did not diminish the amount of the fat-soluble factor in the milk to such an extent that the growth of the rats and the calcification of their skeleton was affected considerably, even by the ingestion of only 15 cc. of milk per diem. On the other hand, larger quantities of milk (up to 30 cc.), diluted with 17 % sugar solution, caused greater skeletal changes, that is to say, apparently an exclusively *liquid* diet has an unfavourable effect on animals if continued for a long time.

THIRD SERIES OF EXPERIMENTS. INFLUENCE OF SMALL DOSES OF MILK (5 CC.) ON THE SKELETON OF RATS FED ON A DIET DEFICIENT IN THE FAT-SOLUBLE FACTOR.

In this series there were 40 rats, belonging to five litters. In each experiment the rats, after being put on a diet deficient in the fat-soluble factor (– A diet)[1] for 33–39 days, were divided into the following groups:

Group I remained on –A diet;
,, II transferred to normal diet (N)[1], containing butter and cod-liver oil as a source of fat-soluble factor;
,, III fed on –A diet + 5 cc. FM;
,, IV fed on –A diet + 5 cc. HM; and
,, V fed on –A diet + 5 cc. OM.

The distribution of the rats into various groups in each experiment, their weight, age, duration of various dieting, the chemical composition of their skeleton and the histological diagnosis, are shown in Tables VII–XI.

The amount of N paste consumed by the rats fluctuated between 16 and 21 g.; the amount of –A paste consumed per diem varied in the different experiments: in experiments 43 and 44 from 10 to 14 g. were eaten; in experiment 45 from 9 to 11 g., but in the case of rats fed on FM and of one (490) fed on HM, the paste was consumed in larger quantities—14 to 15 g.; in experiment 47 the average consumption reached 10 g.; in rats fed on FM it was 11 g. Thus with some rats the ingestion of fresh milk alone increased their appetite.

Table VII.

Experiment 43. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on – A diet without milk 38 days (period I), with milk 20 days (period II). Final age: 96 days.

			Weight in g.			In bones			
No. of rat	Sex	Diet in period II	When put on – A diet	At the beginning of period II	Final	H_2O %	Fresh Ca %	Dry Ca %	Histological results
474	♂	N + FM	43	58	132	38·9	13·0	21·2	*Normal
477	♀	– A + FM	51	70	97	47·8	8·7	16·6	Osteoporosis
480	♂	– A + FM	46	74	95	50·0	7·5	15·1	Slight rickets and osteoporosis
			Average of 477 and 480:—			48·9	8·1	15·9	
475	♀	– A + HM	56	73	98	46·2	8·4	15·5	Slight osteomalacia
476	♂	– A + OM	55	75	92	48·0	7·7	14·7	Slight osteomalacia
479	♂	– A + OM	48	64	87	47·5	7·3	14·4	Slight rickets
			Average of 476 and 479:—			47·7	7·5	14·6	
478	♂	– A alone	53	84	87	51·3	7·5	15·4	Slight rickets. Osteoporosis. Died from cachexia 3 days before the end of the experiment

* Analyses lost; these figures of the chemical composition are taken from rat 501 which was of a corresponding age. (See also very similar figures from rats aged 80–120 days [Korenchevsky, 1922, 3, p. 65].)

[1] For the composition of the diets N and – A, see previous communications [Korenchevsky, 1921, 1922].

Table VIII.

Experiment 48. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on – A diet without milk 33 days (period I), with milk 26 days (period II). Final age: 104 days.

			Weight in g.			In bones			
No. of rat	Sex	Diet in period II	When put on – A diet	At the beginning of period II	Final	H_2O %	Fresh Ca %	Dry Ca %	Histological results
510	♀	– A + FM	62	72	110	43·5	9·8	17·3	Slight rickets
513	♂	– A + FM	62	78	132	49·4	8·3	16·5	Slight rickets, osteoporosis
			Average of 510 and 513:—			46·5	9·1	16·9	
507	♀	– A + HM	69	60	89	48·1	8·5	16·5	Slight rickets, osteoporosis
511	♀	– A + HM	85	96	128	42·9	10·4	18·3	Slight rickets
			Average of 507 and 511:—			45·5	9·5	17·4	
508	♀	– A + OM	63	72	87	48·1	8·1	15·6	Slight rickets, osteoporosis
512	♂	– A + OM	88	120	144	45·5	9·5	17·4	Slight rickets
			Average of 508 and 512:—			46·8	8·8	16·5	
509	♂	– A alone	85	112	128	45·7	8·4	15·5	Moderate rickets

Table IX.

Experiment 47. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on – A diet without milk 39 days (period I) with milk 48 days (period II). Final age: 109 days.

501	♀	N + FM	47	59	158	38·9	13·0	21·2	Normal
502	♂	– A + FM	49	70	113	51·0	8·6	17·5	Very slight rickets
506	♂	– A + FM	56	74	137	49·2	8·5	16·7	Slight rickets
			Average of 502 and 506:—			50·1	8·6	17·1	
504	♂	– A + HM	57	70	118	50·4	6·9	13·8	Slight rickets, osteoporosis
500	♂	– A + OM	54	70	117	53·3	6·6	14·0	Moderate rickets
503	♀	– A + OM	41	63	65	56·7	6·7	15·6	Slight rickets. Osteoporosis. Hyperaemia of bone marrow. Died from cachexia
			Average of 500 and 503:—			55·0	6·7	14·8	
505	♀	– A alone	43	62	81	49·5	7·6	15·0	Moderate rickets

Table X.

Experiment 45. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on – A diet without milk 35 days (period I), with milk 63 days (period II). Final age: 139 days.

493	♀	N + FM	35	55	180	38·1	13·3	21·5	Normal
494	♂	N + FM	36	60	248	44·3	11·7	21·0	Normal
			Average of 493 and 494:—			41·2	12·5	21·2	
485	♀	– A + FM	43	79	106	41·2	11·2	19·0	Slight rickets
488	♂	– A + FM	32	68	163	45·2	8·9	16·2	Slight rickets
			Average of 485 and 488:—			43·2	10·0	17·6	
486	♀	– A + HM	34	58	103	49·0	7·8	15·3	Slight rickets
490	♂	– A + HM	34	75	162	46·7	8·3	15·7	Slight rickets
			Average of 486 and 490:—			47·8	8·1	15·5	
484	♀	– A + OM	35	65	94	44·5	8·0	14·3	Slight rickets
491	♂	– A + OM	33	74	125	44·0	7·3	13·1	Slight rickets, osteoporosis
492	♀	– A + OM	37	62	99	50·4	7·5	15·0	Moderate rickets
			Average of 484, 491 and 492:—			46·3	7·6	14·2	
487	♂	– A alone	35	71	82	50·9	6·4	13·1	Severe rickets
495	♂	– A alone	47	86	117	47·6	7·3	14·0	Moderate rickets, osteoporosis
496	?	– A alone	56	84	122	49·9	6·9	13·9	Severe rickets
			Average of 487, 495 and 496:—			49·5	6·9	13·6	

Table XI.

Experiment 44. The influence of 5 cc. of milk on the chemical composition of the skeleton of rats kept on – A diet without milk 38 days (period I), with milk 66 days (period II). Final age: 144 days.

No. of rat	Sex	Diet in period II	Weight in g. When put on – A diet	Weight in g. At the beginning of period II	Weight in g. Final	In bones H_2O %	In bones Fresh Ca %	In bones Dry Ca %	Histological results
445	♀	N + FM	57	67	143	40·3	12·2	20·5	Normal
451	♀	– A + FM	49	62	105	43·5	11·2	19·8	Nearly normal
447	♂	– A + HM	67	104	134	44·6	10·1	18·3	Slight rickets
449	♀	– A + HM	58	71	103	41·4	11·0	18·7	Nearly normal
			Average of 447 and 449:—			43·0	10·5	18·5	
448	♂	– A + OM	60	78	151	40·8	10·3	17·6	Slight rickets
450	♂	– A + OM	54	84	133	49·5	8·9	17·7	Slight rickets
			Average of 448 and 450:—			45·2	9·6	17·7	
446	♂	– A alone	66	87	99	47·2	8·8	16·6	Slight rickets. Moderate osteoporosis

The weight curves of rats are given in Figs. 3–6, and show that the addition of 5 cc. milk to –A diet improved the growth of the rats. Moreover, the greatest effect was produced by FM, to a lesser degree by HM, the least effect being produced by OM; that is to say, the mere heating of the milk for seven hours at 100° C. in most cases caused a slight disintegration of the growth-promoting properties of milk, but the greatest effect was produced by oxidation combined with heating. At autopsy, the following peculiarities were observed in the various groups of rats: on N diet the rats were fat and normal; on – A diet they were thin or emaciated, with opaque teeth, frequently with depressed thorax and numerous fractures of the ribs. In some rats the ribs and the bones of their hind legs were curved.

On –A diet + 5 cc. OM the macroscopical changes hardly differed from those on – A diet alone, the only difference being that the rats were somewhat better nourished.

Table XII. *Summary table showing in percentage the influence of small doses (5 cc.) of milk on the chemical composition of the skeleton of rats kept on –A diet.*

Above (+) or below (–) – A standard. In bones

No. of exp.	N + FM H_2O	N + FM Fresh Ca	N + FM Dry Ca	– A + FM H_2O	– A + FM Fresh Ca	– A + FM Dry Ca	– A + HM H_2O	– A + HM Fresh Ca	– A + HM Dry Ca	– A + OM H_2O	– A + OM Fresh Ca	– A + OM Dry Ca
I. Milk given during a period of 20–26 days (average 23 days).												
Exp. 43 (7 rats)	– 24·2	+ 73·4	+ 37·5	– 4·7	+ 8·0	+ 2·7	– 10·1	+ 12·0	+ 0·8	– 7·0	– 4·0	– 5·6
„ 48 (7 rats)	—	—	—	+ 1·6	+ 7·6	+ 9·0	– 0·5	+ 12·8	+ 12·1	+ 23·6	+ 4·4	+ 6·5
Average of exps. 43 and 48	– 24·2	+ 73·4	+ 37·5	– 1·6	+ 7·8	+ 5·9	– 5·3	+ 12·4	+ 6·5	+ 8·3	+ 0·2	+ 0·45
II. Milk given during a period of 49–66 days (average 60 days).												
Exp. 47 (7 rats)	– 21·3	+ 70·9	+ 41·2	+ 1·3	+ 12·4	+ 13·7	+ 1·9	– 9·5	– 7·8	+ 11·2	– 12·3	– 1·2
„ 45 (12 rats)	– 16·7	+ 81·3	+ 55·6	– 12·6	+ 45·4	+ 28·9	– 3·3	+ 17·1	+ 13·9	– 6·4	+ 10·0	+ 3·4
„ 44 (7 rats)	– 14·6	+ 39·0	+ 23·4	– 7·8	+ 27·3	+ 19·6	– 8·9	+ 19·8	+ 11·5	– 4·4	+ 9·6	+ 6·5
Average of exps. 47, 45 and 44	– 17·5	+ 63·7	+ 40·1	– 6·3	+ 28·3	+ 20·7	– 3·4	+ 9·1	+ 5·9	+ 0·1	+ 2·4	+ 2·9

On −A diet + 5 cc. FM the macroscopical picture showed a marked difference as compared with −A diet: the rats were considerably better nourished, the skeleton was either normal or, if there were calluses after spontaneous fractures, they were solitary cases, and were far smaller than those in rats on −A diet alone or with OM. On −A diet + 5 cc. HM the macroscopical changes in rats were of a medium character between those of rats fed on OM and FM, being more like the latter. The chemical changes shown in Tables VII–XI are summarised in Table XII. It will be seen from

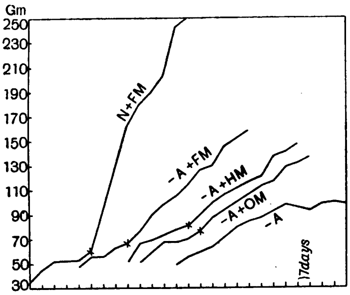

Fig. 3. Weight curves of average rats (males) from experiments 44, 45 and 47. Up to the point marked × rats were kept on −A alone. The diet after that is shown above each curve.

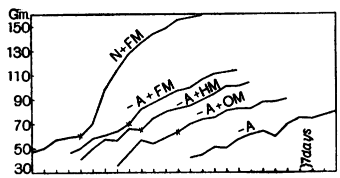

Fig. 4. Weight curves of average rats (females) from experiments 44, 45 and 47. Up to the point marked × rats were kept on −A alone. The diet after that is shown above each curve.

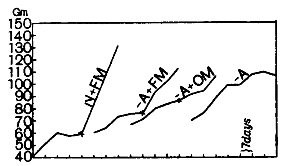

Fig. 5. Weight curves of average rats (males) from experiments 43 and 48. Up to the point marked × rats were kept on −A alone. The diet after that is shown above each curve.

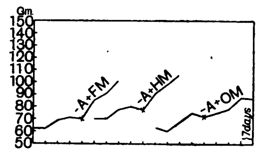

Fig. 6. Weight curves of average rats (females) from experiments 43 and 48. Up to the point marked × rats were kept on −A alone. The diet after that is shown above each curve.

this table that after milk was given for 20–26 days, these changes were only slight, being far more marked after a period of about two months. That is to say, in order for milk to produce a favourable effect, it is necessary that it should be ingested for a considerable period of time. On the whole, the most marked improvement in the chemical composition of the skeleton was observed on giving FM, followed by HM. The addition of OM to −A diet either produced no improvement whatever in the chemical composition of the skeleton or had only a very slight effect. In any case, the addition of 5 cc. milk could not have caused such an improvement in the skeleton in the course of 66 days, as was produced by butter *plus* cod-liver oil in N diet.

On histological examination, the most marked improvement in rickets was observed in the case of a diet of FM or HM. On OM this improvement was usually (though not always) observed, but frequently it was less marked than in the case of FM.

Thus in this series of experiments likewise, the macroscopic, microscopic, and chemical examinations of the skeleton coincided with the data obtained in investigating the growth and weight of animals.

SUMMARY.

In our experiments the rats grew and developed normally, and had a normal skeleton, on a diet of milk *ad libitum* with the addition of the requisite amount of calories in the form of carbohydrates. They were, however, not very fertile and, if they did bear young, the latter became anaemic and cachetic on a milk diet. Though the calcium content of the skeleton of the young was *normal, histologically* there was *osteoporosis*, and even one of the features of rickets (enlargement of the cartilage).

The dilution of heated milk with a 17 % solution of sugar resulted in the composition of the skeleton being worse than that of rats fed on smaller quantities of milk, but with the addition of calories in the form of starch paste. The addition of even a small quantity of milk (5 cc.) to −A diet produced an improvement in rickets caused in rats by a diet deficient in the fat-soluble factor. Such a favourable action of milk was slightly decreased by heating the milk for seven hours at 100° C., and was decreased in a marked degree after the aeration of the milk at 98°·8 C. for the same period of time.

Thus these experiments show that oxidation of milk also reduces its anti-rachitic and growth-promoting properties, as was observed by Hopkins [1920] and Mellanby [1921] in the case of butter. The experiments with larger doses of milk (15 to 50 cc. per diem) have however shown that the oxidation of milk produces *only a partial*, and not total disintegration of the fat-soluble factor, and that in such larger doses oxidised milk produced nearly the same or identical growth of the rats and calcification of the skeleton as was caused by fresh milk. The addition of cod-liver oil to large doses of oxidised milk in most cases had no effect on the results, or if it did affect them, they were only slightly improved.

Thus, in our experiments we failed to obtain such a great disintegration of the fat-soluble factor as to produce rickets in rats fed on an abundant milk diet. No doubt if the oxidation had continued for a longer period, or had taken place at a higher temperature, the results would have been different.

In conclusion, it is necessary to note certain peculiarities obtained on oxidising the fat-soluble factor in milk, as compared with the results of oxidising this factor in butter or cod-liver oil.

Hopkins [1920], Drummond and Coward [1920], and Zilva [1920] showed that the growth-promoting properties of butter are easily disintegrated by oxidation. Mellanby found that oxidation likewise disintegrates the anti-rachitic properties of butter, but not of cod-liver oil. McCollum, Simmonds, Becker and Shipley [1922] found that on oxidising cod-liver oil for 12–20 hours at the temperature of boiling water, its anti-rachitic properties may be preserved, while its capacity of curing xerophthalmia is lost. On these grounds the American authors have come to the conclusion that the so-called "fat-soluble vitamin A" contains two separate vitamins: (1) an "anti-rachitic vitamin," which is disintegrated with difficulty by oxidation, and (2) "vitamin A" itself, far more easily disintegrated by oxidation, and preserving animals from xerophthalmia.

In our experiments with oxidised milk, the following facts should be noted:

(1) Apparently the fat soluble factor is disintegrated by oxidation with greater difficulty in milk than has been observed in the case of butter. This peculiarity may be to a certain extent explained by the fact that in making butter from milk only part of the growth-promoting factor, present in milk, is transferred to the butter. Thus, according to Drummond, Coward and Watson [1921, p. 544]: "Fresh milk from cows fed on green pasture will, in nearly all cases, induce a resumption of growth in rats fed on a diet deficient in vitamin A, when given in a daily supplement of 2 cc. [cf. Hopkins, 1912, 1920]. This amount of milk may be regarded as supplying approximately 0·1 g. of fat per day. In order to induce the same amount of growth by adding a supplement of butter, it is necessary to give about 0·2–0·3 g. per day."

Certain hypotheses may be advanced with regard to this. For instance, McCollum [1917] thinks that water solutions of the non-lipoid constituents of milk contain this substance (vitamin A), indeed, in considerable amount. His experiments lead him to believe that approximately half of the fat-soluble dietary essential is present in the fat and half in the non-fat portion of the milk. Another hypothesis supposes a partial loss of the fat-soluble factor to occur during the churning of the butter (perhaps due to oxidation). If in milk there is a larger amount of fat-soluble factor than in butter, we assume, that to destroy this factor in milk will take longer and be more difficult.

(2) In our not numerous experiments the mere heating of milk at 100° C. for seven hours almost invariably slightly decreased the growth-promoting and anti-rachitic properties of milk (a phenomenon observable on adding 5 cc. HM to −A diet). This cannot be explained by the separation of lime salts in heating the milk[1]. An investigation of the Ca content in OM did not show any essential alteration in it. Therefore our experiments do not support the hypotheses of Daniels and Loughlin of the decrease in the calcium content of milk as a result of boiling or heating the latter (provided the milk is given to the rats after having been shaken). Finally it may be possible that by heating the milk the calcium compounds are altered to those less able to be assimilated by the organism. However, such a small amount of calcium as is found in 5 cc. of milk and added to −A diet could not influence the composition of the skeleton.

It is known, that merely heating butter or cod-liver oil has no effect on the content of fat-soluble factor in either [Osborne and Mendel, 1915; Hopkins, 1920; Mellanby, 1921]. Therefore, if subsequent and more numerous experi-

[1] The milk was shaken thoroughly before being given to the rats or taken for analyses.

ments confirm the fact noticed by us, it may be found that milk contains, besides a fat-soluble factor, another special factor which to some extent is disintegrated by heating.

(3) In our experiments we did not observe any considerable decrease in the growth-promoting properties of milk, without simultaneous loss of its anti-rachitic properties. Almost invariably there was a parallel decrease in the above-mentioned properties after oxidation. This fact does not agree with McCollum's observations on the relative decrease after oxidation of anti-xerophthalmic and anti-rachitic vitamins in cod-liver oil, though it is in accordance with Mellanby's experiment with butter referred to above.

At present it is difficult to explain this peculiarity, and it is only possible to form conjectures:

(*a*) Taking the point of view of McCollum and co-workers, we may assume that butter contains a small amount of the anti-rachitic factor, while its vitamin A content is large. The great degree of oxidation required to disintegrate the large amount of vitamin A may at the same time disintegrate a small quantity of the anti-rachitic vitamin, *e.g.* in butter. Possibly, the enormous amount of anti-rachitic vitamin contained in cod-liver oil has no time to be inactivated by the degree of oxidation which is sufficient to destroy the anti-xerophthalmic and growth-promoting properties.

(*b*) It is possible that the presence of milk fat in whey, in the form of an emulsion, alters the conditions of the oxidation of the fat-soluble factor.

(*c*) In the same way, this fact may, to a certain extent, be explained by the existence of a special factor in milk, different from the fat-soluble factor.

CONCLUSIONS.

(1) When rats are fed on fresh, heated or oxidised milk *ad libitum*, supplemented by carbohydrates, the structure of their skeleton hardly differs from the normal, either chemically or histologically. When oxidised milk is used only a slight degree of osteoporosis in the bones is observed.

(2) Fresh winter milk, as supplied to us by a London Dairy, even to the amount of 5 cc., causes a considerable improvement in rickets induced in rats by −A diet, and a renewal of growth that has been inhibited.

(3) Heating the milk for seven hours at 98·8–100° C., with or without aeration, produces no essential change in its calcium content.

(4) When milk is oxidised at 98°·8 C. for seven hours, its growth-promoting and anti-rachitic properties are obviously decreased, as is seen from experiments with small doses (5 cc.) of milk.

(5) Nevertheless, these properties are so far retained that, on the daily ingestion of 15 cc. and more of oxidised milk by rats for a period up to 132 days, the rats hardly differ, in growth and skeletal development, from those fed on fresh milk.

(6) Apparently, the deficiency of the cow's food in the fat-soluble factor induces a far greater impoverishment of the milk in that factor than the addition of 1 % of peroxide of hydrogen to the milk, and subsequent aeration for seven hours at 98°·8 C.

(7) Heating the milk for seven hours at 100° C. slightly decreases the growth-promoting and anti-rachitic properties of the milk.

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