LX. THE INFLUENCE OF DIET AND MANAGEMENT OF THE COW UPON THE DEPOSITION OF CALCIUM IN RATS RECEIVING A DAILY RATION OF THE MILK IN THEIR DIET.

BY MARGARET AVERIL BOAS (*Grocers' Company's Research Scholar*)
AND HARRIETTE CHICK.

From the Department of Experimental Pathology, Lister Institute.

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INTRODUCTION.

THERE has been much difference of opinion as to whether cow's milk possesses any specific anti-rachitic properties and, if so, to what extent these may vary in degree according to the diet of the cow and the season of the year. The discovery of Mellanby [1919, 1921] showing the anti-rachitic value of certain animal fats for dogs and the correspondence between these values and their content of vitamin A [McCollum and co-workers, 1913, 1915, 1916] was generally interpreted to mean that vitamin A, the principle promoting growth and preventing xerophthalmia in rats, was also concerned with the calcification of bone and the prevention of rickets in the growing animal. Of the foodstuffs investigated, milk, butter and cream were among the richer sources of vitamin A, and that the content of this principle depends upon the diet of the cow has been abundantly substantiated. Kennedy and Dutcher [1922] showed that milk from cows receiving pasture food in summer was richer in vitamin A than milk from the same herd in winter upon a diet of dry fodder and hay, and that in winter the content of vitamin A could be further reduced by substituting timothy hay for alfalfa hay. Drummond, Coward and Watson [1921] demonstrated an increase in vitamin A in the butter prepared from the milk of cows when the herd was put out to grass in May, and a decrease later in the summer, in July, when the green food failed owing to drought, and oil-cake was re-introduced into the diet. These results have been confirmed by further investigations of Drummond and his colleagues [1924].

Recent work of McCollum and his co-workers [1922, 1, 2], however, points to the conclusion that the organic factor in diet concerned with deposition of calcium in the bones of growing animals is distinct from vitamin A, although in general the two food factors follow a similar distribution in nature and possess similar physical and chemical properties. A quantitative study of the resistance to heat oxidation of these two principles, as contained in cod-liver oil, has been made by Goldblatt and Zilva [1923], and the results are in support of this view. Certain investigators, for example Zucker and Barnett [1923], go further and maintain that the distribution of vitamin A and the anti-

rachitic organic factor is by no means so similar as has been supposed, that specific anti-rachitic properties cannot be demonstrated in milk or butter fat and that cod-liver oil, and probably also egg-yolk [see Hess, 1923], are substances apart, with a specific action upon calcium metabolism. A similar view, based on clinical grounds, has been expressed by Findlay [1922] and also by Galbraith [1923], and several observations are recorded in the older literature [Steinitz, 1903; Rothberg, 1907; Freund, 1909; Orgler, 1912] where addition of extra milk-fat to the diet of young infants actually lowered the retention of calcium in contradistinction to the effect of cod-liver oil. Many of these experiments were continued over too short a period after the change in diet was made, to yield very trustworthy results, and against the conclusions of these workers must be set the recent observations of Korenchevsky [1922], Korenchevsky and Carr [1923] and of Goldblatt and Soames [1923], who have shown that rats receiving a rickets-producing diet, can be protected by a butter or milk ration or by unheated caseinogen, although the amounts required are much greater than in the case of cod-liver oil.

OBJECT OF THE PRESENT EXPERIMENTS.

The present experiments were made in connection with a research undertaken by Dr E. M. Luce in this Institute, the object of which was to study both the growth-promoting and anti-rachitic value of the milk given by the same cow under different conditions of diet and management. This research, of which a résumé has already appeared [1924], will shortly be published in detail. The criteria adopted by Dr Luce were growth, calcium content of the bones and prevention of rickets in young growing rats receiving the milk as an addition to a diet deficient in fat-soluble vitamins. Samples of the milk thus investigated were handed to us for parallel tests, in which the influence upon calcium and phosphorus retention was directly measured by metabolic experiments. The animals used were also rats which received small quantities of the milk as a supplement to a diet deficient in fat-soluble vitamins but adequate in other respects, including vitamins B and C.

METHODS.

The method employed has been described in detail in the preceding paper by Boas [1924]. Each sample of the milk was tested by an experiment with two cages, each containing two young rats of the same sex, of about 40 g. weight at the beginning of the observation. The daily ration of milk was 5 cc. per rat, and was fed separately to each animal. The basal diet was the — A diet described by Boas, except that an attempt was made to correct the defects due to egg-albumin when employed as a sole source of protein. During the last two weeks of Exp. 1, fresh spinach was added to the diet in a ration of 1 g. daily per rat. McClendon and Shuck [1923] and also Goldblatt and Zilva [1923] were unable to demonstrate anti-rachitic properties in spinach, when administered to rats in amounts up to 5 g. daily.

In Exps. II and III, 10 % of the egg-albumin in the diet was replaced by heated, inactivated, caseinogen.

Each cage was provided with a control, containing two rats of the same litter and sex, and receiving a diet exactly similar to the above, except that the daily ration of fresh milk was replaced by 5 cc. of a reconstituted standard dried milk (prepared to contain 3.6 % fat, 3.3 % protein and 6.0 % carbohydrate) and in addition three drops (0.073 g.) of cod-liver oil daily were given to each rat. This provided a constant standard diet, complete in every respect, for comparison.

An account of the cow's diet and management has already been given by Luce, but is briefly recapitulated here for purposes of convenience.

Previous to the experiment the cow had been out at grass but was removed in July, 1922, and placed in a ploughed paddock upon a diet poor in fat-soluble vitamins, consisting daily of white maize meal, 3 lbs. 12 oz.; gluten meal, 3 lbs.; crushed oats and barley, 3 lbs.; mangolds, 50 lbs.; chaff, 18 lbs.; barley straw *ad libitum*. Four months later, on November 12th, the cow was placed in a completely dark stall without any change in diet until May 24th, 1923. A calf was born on January 27th. Milk sample xiv was taken during the last week of this period and was the pooled morning's milk of seven consecutive days.

Fresh green food, fresh meadow grass and clover, was then introduced into the cow's food, and by June 4th 1 cwt. per day was given and formed the entire diet, the animal remaining in the dark stall as before. Milk sample xvi was the pooled morning's milk of seven days at the end of this period which lasted for two months. On August 1st, 1923, the cow was put out to grass. The weather was very fine and sunny during the early part of August. Milk sample xviii was taken in the middle of October, after eleven weeks of exclusive pasture feeding.

The milking was done with great care and extreme cleanliness, sterilised vessels being used. The milk was bottled, the air in the bottles displaced by carbon dioxide and the milk was then pasteurised at 70° for 20 minutes. The carbon dioxide was used to obviate risk of vitamin oxidation at this temperature. The milk was stored in a refrigerator room and was found to keep good for several months.

The three samples of milk tested had the following composition as regards content of calcium, phosphorus and fat.

Milk sample	Fat %	Ca %	P %
XIV	3.85	0.120	0.101
XVI	3.90	0.114	0.079
XVIII	5.10	0.122	0.082

Each observation lasted 5-7 weeks. During the first week the young rats were kept in the metabolism cages and were fed the appropriate diet, but no analyses were made. The observation proper began at the end of this preliminary period, when it was assumed that any disturbance caused by change of diet and environment would be over.

Table I. *Balance sheet for one week, showing Calcium and Phosphorus retained by two female rats aged eight weeks: mean weight at beginning of week, 61 g.; at end, 66 g. (Exp. III, Cage 1. 4th week.)*

Food offered	Intake				Output		
	% Ca in the food	% P in the food	Ca in g.	P in g.		Ca in g.	P in g.
150 g. diet sample H	0.0309	0.1367	0.0464	0.2051	In urine, faeces and uneaten food	0.9226	0.9540
25 g. " J	0.0301	0.1331	0.0075	0.0333			
2.3996 g. CaCO ₃	40	—	0.9598	—			
4.4003 g. K ₂ HPO ₄	—	17.64	—	0.7761	Uneaten food, 39.42 g. (calc.)	0.2284	0.2290
72.25 g. (70 cc.) milk sample XVI	0.1221	0.0831	0.0882	0.0600			
		Total	1.1019	1.0745			
Uneaten diet	0.0309	0.1367	0.2284	0.2290			
24.09 g. dry wt = 39.42 g. wet wt							
		Real Intake	0.8735	0.8455	Real Output	0.6942	0.7250
		Total retention	= Ca 0.1793		P 0.1205		
		"	per rat = Ca 0.0896		P 0.0602		

In Table I is set out an example of the method used, showing the detail of the analyses made upon one of the four cages during the 4th week of Exp. III. The cage was Cage 1 and contained two female rats of mean weight 61 g. at the beginning, and 66 g. at the end, of the week, each receiving 5 cc. (5.1 g.) daily of Milk Sample XVIII.

RESULTS.

Exp. I. Milk sample XIV. Cow receiving dry fodder in a dark stall. (Table II and Fig. 1.)

This observation, made with eight young rats, four of each sex and all from the same litter, lasted for six weeks. In Table II the calcium and phosphorus retention of the four rats, two male and two female, receiving milk XIV is averaged and compared with that of the four rats on the standard diet, with supplement of dried milk and cod-liver oil. The result is quite clear. In both cases the average amount of calcium and phosphorus retained per week diminishes with increasing age of the rat, but is consistently lower in case of the rats receiving milk XIV; in the 6th week of the experiment these rats actually show a negative balance. The total Ca retention per rat over the whole period was 34 % lower than that of the control rats, while the P retention was decreased by only 11 %.

In the curves in Fig. 1 the successive increments of Ca and P retained per rat per week, are plotted against time and illustrate how the discrepancy between the two sets of rats increases as they grow older. The curves of average increase in body weight in the two cases show no significant difference in growth between the animals of corresponding sex on the two diets, the growth in all cases being within normal limits.

There were no obvious differences post mortem, between the rats of the two series. In each animal the femora, tibiae, and fibulae were dissected.

the water content was determined and the proportion of calcium estimated by the method of Aron and Sebauer [1910]. The results showed that the control rats had slightly heavier bones, with more total Ca and a higher percentage but the differences are not perhaps very striking. The figures obtained for percentage of calcium, reckoned on dry weight of the bones (see Table IV) might be regarded as within normal limits in the experimental as well as in the control rats [see Korenchevsky, 1922].

Table II. *Calcium and Phosphorus retention in two male and two female rats on — A diet receiving 5 cc. daily of milk sample XIV, compared with two male and two female rats on — A diet with addition of 5 cc. standard reconstituted dried milk and three drops (0.073 g.) cod-liver oil daily. (All eight rats from the same litter.)*

Week of experiment	Four control rats on standard diet			Four rats receiving milk XIV		
	Mean body weight, g. (beginning of week)	Ca retained, g. per rat	P retained, g. per rat	Mean body weight, g. (beginning of week)	Ca retained, g. per rat	P retained, g. per rat
1	45	0.1877	0.1890	44	0.1376	0.1302
2	71	0.1533	0.1223	62	0.1024 ¹	0.0784 ¹
3	78	0.0633 ¹	0.0712 ¹	68	0.0825 ¹	0.0711 ¹
4	88	0.1396	0.1077	81	0.0852	0.1272
5	97	0.1075 ¹	0.0790	86	0.0946 ¹	0.0851
6	109	0.0835	0.0893	100	0.0147	0.0940
End of 6th week	119	—	—	111	—	—
	Totals	0.7349	0.6585		0.4876	0.5860

¹ Figures calculated from results obtained with two rats only.

Fig. 1. Exp. I. Curves showing growth in weight (B), and retention of calcium (D) and phosphorus (F) in young rats on — A diet, receiving daily 5 cc. of milk sample XIV (cow on dry fodder in dark stall). Curves A, C and E refer to growth in weight and retention of calcium and phosphorus respectively, of control rats from the same litter. Each curve represents average figures for four rats.

*Exp. II. Milk sample XVI. Cow receiving fresh green fodder in a dark stall.
(Table III and Fig. 2.)*

The results of this experiment were similar to those of Exp. I. During the four weeks of the experiment the average total Ca retention of the female rats receiving milk XVI (Cage 1) was 0.3962 g. or nearly 20 % lower than that of the corresponding rats (Cage 3) on standard diet 0.4880 g. In case of the male rats (Cage 5), the retention was diminished by more than 40 % in comparison with the standard (Cage 7). The P retention was less affected, as was also the case in Exp. I, the decrease compared with corresponding control rats being 7 % in case of females and 19 % in case of males. The rate of growth in both series was almost identical.

In this experiment and in Exp. III, the uneaten diet was measured and the difference between this and the total food offered, appears in Tables III and V under the heading Intake. The figures show that an adequate amount

Fig. 2, Exp. II. Curves showing growth in weight (B) and retention of calcium (D) and phosphorus (F) in young rats on -A diet, receiving, daily, 5 cc. milk sample XVI (cow on fresh green food in dark stall).

Curves A, C and E refer to growth in weight and retention of calcium and phosphorus of control rats from the same litter. Each curve represents average figures for four rats.

of both Ca and P was ingested. The defect in the -A diet supplemented by milk XVI is therefore shown to be a failure to induce retention of calcium and, to a less degree, of phosphorus also. In the latter case the figures obtained are much nearer those of the control rats. The question of the ratio of Ca retained to P retained, presents points of some interest and is treated separately in a later section (p. 443).

The analysis of dissected bones, femora, tibiae and fibulae, at the end of experiment, showed a definite small diminution in Ca, for the experimental as compared with the control animals.

Fig. 2 shows curves of average total Ca and P retention in experimental and control animals as well as the average increase in body weight during the period of experiment.

Table III. *Calcium and Phosphorus retention in two male and two female rats on — A diet receiving 5 cc. daily of milk XVI, compared with that of two male and two female rats on — A diet with daily addition of 5 cc. standard reconstituted dried milk and three drops (0.073 g.) cod-liver oil. (All eight rats from the same litter.)*

Week of experiment	Four control rats on standard diet										Four rats receiving milk XVI									
	Cage 3 ♀					Cage 7 ♂					Cage 1 ♀					Cage 5 ♂				
	Ca, g. per rat		P, g. per rat		Mean body wt, g. Beginning of week	Ca, g. per rat		P, g. per rat		Mean body wt, g. Beginning of week	Ca, g. per rat		P, g. per rat		Mean body wt, g. Beginning of week	Ca, g. per rat		P, g. per rat		Mean body wt, g. Beginning of week
	In- take	Reten- tion	In- take	Reten- tion		In- take	Reten- tion	In- take	Reten- tion		In- take	Reten- tion	In- take	Reten- tion		In- take	Reten- tion	In- take	Reten- tion	
1	0.3992	0.1359	0.4187	0.1277	53	0.4220	0.1661	0.4444	0.1308	50	0.4330	0.1387	0.4499	0.1195	55	0.4291	0.1363	0.4530	0.1118	55
2	0.4441	0.1121	0.4443	0.0998	72	0.4224	0.1400	0.4213	0.0893	69	0.4800	0.1207	0.4727	0.0935	71	0.4519	0.1149	0.4472	0.0907	71
3	0.4307	0.1500	0.4159	0.1042	78	0.4086	0.1484	0.3952	0.1009	76	0.4865	0.1224	0.4245	0.1033	83	0.4950	0.1255	0.4738	0.0867	83
4	0.3681	0.0900	0.3865	0.0504	91	0.4325	0.1421	0.4555	0.0925	89	0.3775	0.0145	0.3883	0.0395	98	0.3955	0.0400	0.4086	0.0442	98
End of 4th wk					100					93					104					104
Totals	0.4880		0.3821			0.5066		0.4135			0.3963		0.3558			0.3367		0.3334		

Table V. *Calcium and phosphorus retention in two male and two female rats on — A diet receiving 5 cc. daily milk sample XVIII compared with that of two male and two female rats receiving — A diet and in addition daily 5 cc. standard reconstituted dried milk and three drops (0.073 g.) cod-liver oil. (The four female rats and the four male rats were from two different litters.)*

Week of experiment	Four rats on standard diet										Four rats receiving milk XVIII																					
	Cage 3 ♀					Cage 7 ♂					Cage 1 ♀					Cage 5 ♂																
	Ca, g. per rat		P, g. per rat		Mean body wt, g.	Ca, g. per rat		P, g. per rat		Mean body wt, g.	Ca, g. per rat		P, g. per rat		Mean body wt, g.	Ca, g. per rat		P, g. per rat		Mean body wt, g.												
	In- take	Reten- tion	In- take	Reten- tion		In- take	Reten- tion	In- take	Reten- tion		In- take	Reten- tion	In- take	Reten- tion		In- take	Reten- tion															
1	—	—	—	—	47	0.3960	0.1010	0.4068	0.0960	—	—	—	—	—	42	0.4143	0.1305	0.4185	0.1197													
2	—	—	—	—	62	0.4876	0.1214	0.4798	0.1107	—	—	—	—	—	61	0.4327	0.0905	0.4159	0.0768													
3	0.3965	0.0978	0.3951	0.0667	77	0.4511	0.1320	0.4481	0.0864	56	0.4091	0.0956	0.3952	0.0671	70	0.4482	0.1195	0.4378	0.0828													
4	0.4354	0.0945	0.4326	0.0647	82	0.4879	0.1066	0.4867	0.0680	61	0.4368	0.0897	0.4230	0.0603	77	0.4952	0.1128	0.4813	0.0738													
5	0.3978	0.0744	0.3974	0.0541	89	0.4845	0.1015	0.4319	0.0621	66	0.3983	0.0558	0.3865	0.0381	85	0.4687	0.0684	0.4579	0.0466													
6	0.3856	0.0481	0.3820	0.0303	98	0.3915	0.0605	0.3879	0.0355	72	0.3479	0.0475	0.3338	0.0188	90	0.4099	0.0642	0.3968	0.0174													
	81				99					72					92																	
Total	0.3148				0.2158				0.6230				0.4587				0.2886				0.1843				0.5859				0.4171			
	(Four weeks)								(Six weeks)								(Four weeks)								(Six weeks)							

The "Intake" given in Tables III and V is exclusive of 0.043 g. Ca and 0.030 g. P received weekly per rat in the milk ration.

Table IV. *Proportion of Calcium in bones (two femora, two tibiae and two fibulae) of the rats at the conclusion of Exps. I and II.*

Exp. No.	Cage No.	Sex	Nature of experiment	Rat No.	Wt of bone (fresh) g.	Dried at 110°	Ca. found g.	% water in bone	% Ca		
									In fresh bone	In dried bone	In dried bone mean value
I	1	♀	Milk sample xiv	19	1.3082	0.7325	0.1416	44.01	10.82	19.32	19.66
				21	1.3335	0.7346	0.1503	44.91	11.27	19.99	
		♂	"	23	1.3641	0.7821	0.1524	42.67	11.18	19.48	19.29
				25	1.3206	0.7450	0.1422	43.94	10.71	19.09	
I	3	♀	Control	20	1.5283	0.8966	0.1837	41.34	12.03	20.49	20.46
				22	1.4057	0.8042	0.1642	42.81	11.68	20.42	
		♂	"	24	1.5400	0.8684	0.2037	43.61	13.23	23.46	22.32
				26	1.5984	0.8724	0.1847	45.41	11.56	21.17	
II	1	♀	Milk sample xvi	31	1.1062	0.5784	0.1093	47.71	9.88	18.90	19.08
				33	1.2480	0.6785	0.1306	45.63	10.47	19.25	
		♂	"	35	1.2126	0.6330	0.1085	47.80	8.95	17.14	17.82
				37	1.4617	0.7783	0.1440	46.76	9.85	18.50	
II	3	♀	Control	30	1.1122	0.5938	0.1176	46.61	10.58	19.81	20.08
				32	1.2700	0.6969	0.1418	44.10	11.16	20.34	
		♂	"	34	1.2784	0.6993	0.1334	47.65	10.44	19.94	19.64
				36	1.3128	0.7053	0.1364	46.27	10.39	19.34	

Exp. III. Milk sample xviii. Cow pasture-fed, August and September.
(Table V and Fig. 3.)

The result of this experiment forms a marked contrast with that of the two preceding ones, for, when the — A diet was supplemented with this sample of milk, it showed no significant inferiority to the same diet supplemented with cod-liver oil, as regards power of inducing calcium deposition.

Fig. 3, Exp. III. Curves showing growth in weight (B), and retention of calcium (D) and phosphorus (F) in young rats, receiving daily 5 cc. milk sample xviii (cow at pasture). Curves A, C and E refer to growth in weight, and retention of calcium and phosphorus respectively, of control rats from the same litter. Each curve represents average figures for two male rats.

ERRATUM

p. 441, Table VII, *should read*

Group II:

III

Receiving milk XVIII

Period 1

—

Period 2

1.45

Period 3

1.81

Table VI. *Distribution of calcium and phosphorus in skeleton and soft tissues respectively, in seven rats at the end of Exp. III.*
Age of the rats, 11 weeks.

No. of cage	Nature of experiment	Sex	Rat no.	Body weight, g.		Weight of skeleton, g.	Weight of soft tissues (by difference), g.	Ca found, g.		P found, g.		Total Ca, g.		Total P, g.		Ratio Ca : P		% in soft tissues		% in skeleton dried at 100°		% in skeleton of total	
				Gross	Minus tary canal	air dry	dried at 110°	in soft tissues	in skeleton	in soft tissues	in skeleton	Ca, g.	P, g.	whole body	in soft tissues	Ca	P	Ca	P	Ca	P	Ca	P
3	Control	♀	40	77.4	68.1	3.2143	2.8503	64.9	0.0423	0.7072	0.1463	0.3382	0.7495	0.4845	1.547	2.091	0.289	0.07	0.23	24.81	11.87	94.3	69.8
		♀	42	84.8	74.3	4.2175	3.2959	70.1	0.0581	0.8244	0.1628	0.3985	0.8825	0.5613	1.572	2.089	0.357	0.08	0.23	25.01	12.09	93.4	71.0
7	Control	♂	46	96.7	81.7	5.0089	3.7434	76.69	0.0495	0.8854	0.1609	0.4312	0.9349	0.5921	1.579	2.053	0.308	0.06	0.21	23.66	11.52	94.7	72.8
1	Milk xviii	♀	39	70.9	59.4	3.6876	3.1037	55.7	0.0556	0.7420	0.1353	0.3611	0.7976	0.4964	1.607	2.055	0.411	0.10	0.24	23.90	11.63	93.0	72.7
		♀	41	73.0	62.4	3.9034	2.8023	58.5	0.0479	0.7002	0.1387	0.3430	0.7481	0.4817	1.554	2.042	0.345	0.08	0.24	24.99	12.24	93.6	71.2
5	Milk xviii	♂	43	87.1	77.3	3.8597	3.2513	73.4	0.0550	0.7978	0.1619	0.3784	0.8528	0.5403	1.578	2.158	0.340	0.08	0.22	24.54	11.64	93.6	70.0
		♂	45	97.3	85.6	4.6548	3.6563	80.9	0.0989	0.8558	0.1875	0.4285	0.9547	0.6160	1.550	1.997	0.528	0.12	0.23	23.41	11.72	89.7	69.5
Mean values																1.570	2.066	0.368				93.2	71.0

Table VII. *Showing ratio of calcium : phosphorus retained in the successive fortnightly periods of Exps. I, II and III.*

No. of experiment	No. of cage	Sex	Description	Period 1	Period 2	Period 3	No. of weeks	For whole period of experiment, ratio Ca/P
				1st and 2nd week ratio Ca/P	3rd and 4th week ratio Ca/P	5th and 6th week ratio Ca/P		
Group I:								
I	1 and 5	♂ and ♀	Receiving milk xiv	1.15	0.85	0.45	6	0.83
II	1	♀	"	1.21	0.96	—	4	1.114
II	5	♂	"	1.24	0.65	—	4	1.01
Group II:								
I	7	♂	Control on standard diet	1.14	1.06	1.27	6	1.15
II	3	♀	"	1.09	1.55	—	4	1.28
II	7	♂	"	1.39	1.50	—	4	1.44
III	1	♀	Receiving milk xviii	—	1.81	1.45	4	1.56
III	3	♀	Control on standard diet	—	1.46	1.45	6	1.46
III	5	♂	Receiving milk xviii	1.13	1.48	2.07	4	1.41
III	7	♂	Control on standard diet	1.08	1.55	1.66	6	1.36

This was also true as regards phosphorus retention in case of the male rats but in case of the female rats those receiving milk XVIII retained about 15 % less phosphorus than the controls, during the four weeks period of observation.

At the end of the experiment for each rat in the series an estimation was made of the Ca and P content of the whole animal, separate determinations being made for the soft parts and the skeleton. No significant difference between the two series was found, either in the amounts estimated or when these were reckoned per 100 g. body weight or in the ratio of calcium to phosphorus (see Table VI) in the skeleton and soft tissues respectively.

The separation of the skeleton was carried out in the way described by Donaldson [1919]. After removal of the alimentary canal the animal was weighed and the skin and organs were placed in a crucible together with the larger muscles. The carcase was then digested with about 200 cc. of a 2 % solution of Hudson's soap powder, the vessel containing the mixture being heated in a bath of boiling water 2-4 hours or until the bones could easily be separated with forceps—a lengthy business. The sample of soap powder used was free from P and contained only the slightest trace of Ca in the amount used.

After washing, the skeleton was dried in air, weighed, and then dried at 110° to determine water content. The dried bones were ashed in a crucible, the ash extracted with hydrochloric acid, and Ca and P determinations made in the ordinary way by McCrudden's and Neumann's methods. The dissolved portion of the digest, together with the washings of the skeleton, were evaporated to dryness and the residue added to the crucible containing the skin and other soft tissues and the whole was ashed together.

In order to see whether this method of separation affected the amounts or proportion of Ca and P found, results obtained by this method were compared with those got by analysis of the fresh dissected bone. This was done in the case of two corresponding rats, viz. No. 45 (male, cage 5) receiving milk XVIII, and No. 46 (male, cage 7) on standard diet. The bones selected for analysis were the femur and tibia. The results showed a consistent loss of about 5 % in the amounts of calcium and phosphorus found in the boiled bones. Part of this difference, at any rate, can be accounted for in the small amounts of ligaments and cartilage adhering to the dissected bones, from which the boiled bones were entirely free.

Calcium and phosphorus in femur and tibia analysed (i) after dissection in the fresh condition, (ii) after boiling with 2 % soap powder solution.

Rat No.	Method	Weight of bones dried at 110° g.	Calcium		Phosphorus		Ratio Ca/P
			g.	%	g.	%	
45 (milk XVIII)	Dissected fresh	0.3451	0.0667	18.47	0.0344	9.96	1.94
	Boiled	0.2907	0.0638	22.96	0.0316	10.84	2.02
46 ¹ (standard diet)	Dissected fresh	0.3379 ¹	0.0668	19.76	0.0340	10.06	1.97
	Boiled	0.2720 ¹	0.0636	23.38	0.0322	11.84	1.97

¹ Fibula also included.

DISCUSSION.

The result of the metabolism experiments described above show clearly that young rats receiving a diet deficient in vitamin A (and in anti-rachitic properties) with a small daily addition of milk may show either a normal or defective retention of calcium and phosphorus according to the quality of the milk, this in turn depending on the diet and management of the cow.

When the cow was kept in a dark stall and received either dry fodder or fresh green food the milk was found to be defective in calcium-depositing properties, while under the influence of pasture-feeding, a combination of green food, sunshine and open air, she yielded a milk which given in a small daily ration induced normal calcium retention and calcification of the bones. We are inclined to attribute this difference in quality between the samples of milk to the effect of sunlight upon the cow. These results in the main confirm those obtained by Luce [1924] employing other methods. Her experiments showed in addition that the vitamin A (growth-promoting) value and the anti-rachitic value of the milk were similarly affected by changes in the treatment of the cow.

From the present series no information is obtainable as to the growth-promoting value of the sample of milk tested. The rats were not subjected to any preliminary period on — A diet without supplement, as in the methods adopted by Luce, and their reserves, together with the small daily ration of milk, sufficed to carry them over the six weeks experiment without any failure in growth. This was an essential feature in these experiments. If there had been a marked difference in the rate of growth of the animals receiving the samples of milk under investigation and of the control animals receiving cod-liver oil, differences found in the retention of calcium and phosphorus could have been attributed to this fact and need not necessarily have indicated a failure to deposit these substances. If, however, a striking difference occurred in the amounts of calcium retained with no significant difference in the rate of growth, then this difference must be due to faulty calcium metabolism.

This work is open to the criticism that a single metabolism experiment with each sample of milk provides too slender evidence for such generalisations as are made above. A long period of observation was found necessary in order to obtain trustworthy results and the labour involved in experiments lasting 5–7 weeks proved so great that it was found impracticable to duplicate them.

It might be thought that the higher content of fat in milk sample XVIII accounted for its superiority in inducing calcium deposition. The results obtained by Luce, however, showed that the proportion of fat in any sample bore no necessary relation either to the growth-promoting or anti-rachitic properties. One of the milks (sample VIII) investigated by her, of lowest value in these respects, had the highest percentage of fat found, 5.85 %.

THE CALCIUM AND PHOSPHORUS METABOLISM OF NORMAL ANIMALS AND THE EFFECT OF DEFICIENCY OF FAT-SOLUBLE VITAMINS UPON THE RATIO OF CALCIUM TO PHOSPHORUS RETAINED.

In the last column of Table VII is collected the ratio of total calcium to total phosphorus retained in Exps. I, II and III. In case of normal animals, that is the control animals in all three experiments and the animals receiving the pasture-fed milk in Exp. III, this figure lies between 1.23 and 1.56. These values are comparable with those observed previously in normal young rats

by one of us, and given in the preceding paper [Boas, 1924]: 1.24, 1.77, 1.85, mean value 1.65. In case of the rats receiving milk of poorer quality, however, in Exps. I and II, lower values were obtained: 0.85, 1.01, 1.11. That this difference is a real and significant one will become apparent when other facts have been considered.

Several workers have determined the ratio of total calcium to total phosphorus in the body of the young rat. Korenchevsky and Carr [1923] in a series of observations upon the constitution of the new-born rat, found the ratio of Ca to P equal to 1.00 in the whole fresh body minus the alimentary canal. McCann and Barnett [1922] made complete analyses of the calcium and phosphorus content of skeleton and soft tissues in case of both normal and rachitic rats. The mean value of the ratio of total Ca/total P for normal young rats, reckoned from their figures, varied between 1.38 and 1.65. In case of rachitic rats this ratio remained within normal limits when the growth of the rats had been limited, but in cases developing rickets with fair increase in body weight, the ratio was much reduced in value and varied between 1.06 and 1.33. From Buckner and Peter's [1922] figures the ratio total Ca/total P is 1.78 for male and 1.86 for female rats respectively at two weeks old. These results are at variance with those of other observers, being much too high in comparison with the figure of Korenchevsky and Carr and with those obtained by other workers for other young mammals. Bunge [1874] found this ratio to be 1.36 in a rabbit 14 days old, 1.47 in a dog 4 days old and 1.39 in a cat 19 days old. Abderhalden [1898, 1899] determined the ratio in the young rabbit and guinea-pig and found it to correspond with the ratio in which these elements were presented in the milk. Thus in the guinea-pig the Ca/P ratio was 1.25, in the milk 1.37; in the rabbit 1.36 and in rabbits' milk 1.46.

In the seven rats of Exp. III, which were completely analysed (see Table VI) and which are regarded as normal, the average Ca/P ratio for the whole body was 1.57. The age of these rats was 11 weeks and the average weight, minus the alimentary canal, was 73 g. After comparing this value with that obtained for the new born animal by Korenchevsky and Carr, it seemed of interest to find out whether this, presumably, gradual alteration in the relative content of calcium and phosphorus in the body, taking place in the early weeks of life was accompanied by a similar variation in the ratio of Ca/P retained, or whether this ratio remained constant during the successive weeks of the metabolism experiments. Table VII shows the ratio of Ca to P retained, calculated for successive fortnightly periods in Exps. I, II and III. It will be seen that the results of the observations fall into two groups. The first group concerns the rats fed with samples of milk of poor quality in Exps. I and II. The second group includes the observations upon the control animals in all three experiments and those on the rats receiving "pasture-fed milk" in Exp. III. In both groups the figure for the ratio Ca/P retained in the first two weeks, if one figure is excepted, is much the same and varies

between 1.08 and 1.24, but, whereas in the second group this ratio rises steadily as the animals grow older, in the first group it falls as consistently. This difference is what one would expect. Since the skeleton contains over 90 % of the total Ca of the body but only about 70 % of the total P, any failure in bone formation will naturally be more apparent in the amount of Ca retained than of P. As a result the value of the ratio Ca/P retained will fall more and more below the normal. The curves in Figs. 1 and 2 show that the defective retention of calcium is becoming more serious with increase of time, and that with increasing growth of the animal the skeleton is gradually becoming impoverished. It is noticeable that analysis of the bones does not at this stage show a very marked diminution in calcium content (see Table IV), although the figures obtained were consistently below those obtained in case of the control animals. The duration of the experiments, 5-7 weeks, was probably too short and shows rather the early stages of the gradual process of impoverishment. This observation confirms the conclusion arrived at by Schabad [1910] on the basis of his work with infants, that the early stages of rickets are to be detected only by a defective calcium balance in metabolism, and that changes in the skeleton, which can be diagnosed clinically, occur later.

The reason why the ratio of Ca/P in the animal's body should normally increase in case of young growing rats, at any rate up to the age of 11 weeks, is not very clear. The results obtained by Donaldson [1919] show that the growth of the skeleton is roughly proportional to the growth of the whole body during the early weeks of a rat's life. McClendon [1922] who noted an increase in the ratio Ca/P with increasing age, suggests that this is due to a relatively greater proportion of calcium carbonate being deposited in the bones as the animal grows older. According to Hoppe Seyler [1891], bone can be represented by a formula similar to that of apatite $\text{Ca}_{10}\text{F}_2(\text{PO}_4)_6$ in which the F_2 is replaced by CO_3 . This formula $\text{Ca}_{10}\text{CO}_3(\text{PO}_4)_6$ has a Ca/P ratio of 2.152, whereas in $\text{Ca}_3(\text{PO}_4)_2$ the Ca/P ratio is 1.965. In case of the rats of 11 weeks old of which analyses are given in Table VI, the Ca/P ratio of the skeleton varied between 1.997 and 2.158, giving a mean value of 2.066. This figure agrees very well with the formula for bone suggested by Gabriel [1894], $\text{Ca}_3(\text{PO}_4)_2 + \text{Ca}_5\text{HP}_3\text{O}_{13}$; it lies between those calculated respectively for $\text{Ca}_3(\text{PO}_4)_2$ and for the "apatite" formula and suggests that in normal rats of this age the composition of the bone may be in a state of transition. The proportion of uncalcified material in the skeleton will normally tend to decrease as the animal grows up, and this fact will also tend to make the ratio Ca/P in the bones increase with increasing age.

SUMMARY.

1. Experiments on calcium and phosphorus retention in young growing rats were used to investigate the power of various samples of milk to influence calcium deposition. The milk was fed to the rats in daily rations of 5 cc. and

was added to a basal diet arranged to be complete in all respects, but deficient in fat-soluble vitamins.

2. Three samples of milk were investigated, all obtained from the same cow, (1) after six months on a diet of dry fodder in a dark stall, (2) after two months on a diet of green food in a dark stall, and (3) after two months on pasture in summer.

3. Rats receiving samples (1) and (2) showed defective calcium retention as compared with control rats on the same basal diet supplemented with cod-liver oil and an equivalent ration of dried milk. The retention of phosphorus was also affected, but to a less degree. In these animals the ratio of calcium retained, to phosphorus retained, was found to decrease steadily during the early weeks of life whereas in normal animals this ratio shows a steady increase.

4. Rats receiving sample (3) showed no significant difference in calcium retention as compared with the control animals. Complete analyses of the bodies of these rats, aged 11 to 12 weeks, were made to show the distribution of calcium and phosphorus and the ratio of these substances to one another in the skeleton and soft tissues. The results obtained were practically identical for animals of both series.

5. The influence of cod-liver oil upon calcium deposition is not to be attributed to specific properties peculiar to that substance, but to its high content of fat-soluble vitamins, as a similar effect upon calcium deposition can be demonstrated in the case of cow's milk, the degree depending on the diet of the cow and the amount of sunlight to which she is exposed.

REFERENCES.

- Abderhalden (1898). *Z. physiol. Chem.* **26**, 498.
 — (1899). *Z. physiol. Chem.* **27**, 356.
 Aron and Sebauer (1910). *Abderhalden's Handb. der biochem. Arbeitsmethoden*, **1**, 413.
 Boas (1924). *Biochem. J.* **18**, 425.
 Buckner and Peter (1922). *J. Biol. Chem.* **54**, 5.
 Bunge (1874). *Z. Biol.* **10**, 295.
 Donaldson (1919). *Amer. J. Anat.* **26**, 237.
 Drummond and Channon (1924). *J. Soc. Chem. Ind.* **43**, 312.
 Drummond, Coward and Watson (1921). *Biochem. J.* **15**, 540.
 Findlay (1922). *Brit. Med. J.* **ii**, 846.
 Freund (1909). *Eregeb. inn. Med. Kinderheilk.* **3**, 139.
 Gabriel (1894). *Z. physiol. Chem.* **18**, 257.
 Galbraith (1923). *Quart. J. Med.* **64**, 321.
 Goldblatt and Soames (1923). *Biochem. J.* **17**, 622.
 Goldblatt and Silva (1923). *Lancet*, **ii**, 647.
 Hess (1923). *Proc. Soc. Exp. Biol. Med.* **20**, 369.
 Hoppe Seyler (1891). Quoted by Halliburton, *Text Book of Chemical Physiology and Pathology* (London, Longmans Green & Co.)
 Kennedy and Dutcher (1922). *J. Biol. Chem.* **50**, 339.
 Korenchevsky (1922). *Med. Res. Council, Special Report Series*, No. 71.

- Korenchevsky and Carr (1923). *Biochem. J.* **17**, 187, 597.
- Luce (1924). *Lancet*, i. (In the Press.)
- McCann and Barnett (1922). *J. Biol. Chem.* **54**, 203.
- McClendon (1922). *Proc. Soc. Exp. Biol. Med.* **19**, 7, 356.
- McClendon and Shuck (1923). *Proc. Soc. Exp. Biol. Med.* **20**, 288.
- McCollum and Davis (1913). *J. Biol. Chem.* **15**, 167.
- (1915). *J. Biol. Chem.* **23**, 181, 231.
- McCollum, Simmonds and Pitz (1916). *Amer. J. Physiol.* **41**, 361.
- McCollum, Simmonds, Shipley and Park (1922, 1). *J. Biol. Chem.* **50**, 5.
- McCollum, Simmonds, Becker and Shipley (1922, 2). *J. Biol. Chem.* **53**, 293.
- Mellanby (1919). *J. Physiol.* **52**, *Proc.* xi, liii.
- (1921). *Med. Res. Council, Special Report Series*, No. 61.
- Orgler (1912). *Ergeb. inn. Med. Kinderheilk.* **8**, 142.
- Rothberg (1907). *Jahrb. Kinderheilk.* **66**, 69.
- Schabad (1910). *Arch. Kinderheilk.* **52**, 47.
- Steinitz (1903). *Jahrb. Kinderheilk.* **57**, 689.
- Zucker and Barnett (1923). *Proc. Exp. Biol. Med.* **20**, 375.