XXXV. A STUDY OF THE RELATION OF THE QUANTITY OF FAT-SOLUBLE ORGANIC FACTOR IN THE DIET TO THE DEGREE OF CALCIFICATION OF THE BONES AND THE DEVELOPMENT OF EXPERIMENTAL RICKETS IN RATS.

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INTRODUCTION.

As a result of the pioneer work of Mellanby [1918, 1919, 1920, 1921] on experimental rickets in the dog, it was thought at first that this disease might prove to be a deficiency one, caused, like scurvy and beri-beri, by the lack of a single organic factor from the diet, in this case the fat-soluble organic factor called vitamin A or fat-soluble A. Later the work of Korenchevsky on experimental rickets in the rat tended to confirm Mellanby's early findings and conclusions. In his first publication Korenchevsky states that the deficiency of vitamin A in the diet of rats "produces in some rats a picture resembling slight rickets, and typical rickets in some young rats whose parents had been fed on a diet deficient in vitamin A during conception, pregnancy and lactation." Deficiency of fat-soluble A, or of some other unidentified organic factor very closely associated with it, is now recognised as one of the important factors in the etiology of experimental rickets in dogs and rats. But, to date, most investigators of this subject who believe in the dietetic origin of the disease are of the opinion that the deficiency of this organic factor cannot be the sole cause of the disease. Some authors [Hess and Shipley and collaborators, McCollum and Simmonds, 1921] are very emphatic that a deficiency of fat-soluble A alone in a diet otherwise complete does not produce experimental rickets in rats but merely interferes with osteogenesis and causes the development only of osteoporosis. Similar conclusions were reached by Zilva, Golding, Drummond and Coward [1921], Tozer [1921] and Mackay [1921] as the result of studies on the effects of vitamin A deficiency alone in the diet of pigs, guinea-pigs and cats.

In his first communication on the subject Korenchevsky [1921] refers to

the fat-soluble organic factor as vitamin A, which is the term usually applied to the growth-promoting, anti-xerophthalmic, fat-soluble, organic factor. But in a monograph [1922] recently published he refers to it as the "anti-rachitic organic factor," without admitting or denying its identity with the growth-promoting factor. The work of McCollum, Simmonds, Becker and Shipley [1922] on the nature of the fat-soluble organic factor tends to show that the fat-soluble growth-promoting or anti-xerophthalmic factor and the anti-rachitic or calcium-deposition-promoting factor are either two distinct parts of the one substance or two separate substances very similar in their distribution and many of their properties. In the present communication, since a coincident deficiency of both factors was of necessity dealt with in the investigation, the inclusive term fat-soluble organic factor or simply organic factor will be used when referring to both, and the words growth-promoting or anti-rachitic will be prefixed to the term where specific reference is made to one or other of the factors.

At the time this investigation was started most of the results referred to above had not yet been published. The study was undertaken with a view to determine whether, as Mellanby found in the case of the dog, a deficiency of the organic factor alone in the diet can also produce rickets in rats, and if so, whether a relation exists between the quantity of this factor in the diet and the degree of calcification or severity of the pathological lesion of the bones.

EXPERIMENTAL.

PART I.

In this investigation the rats employed were bred in the Institute from animals fed on fresh cow's milk, white bread, oats and bran, green cabbage leaves (all *ad libitum*) and occasionally some red meat. The milk and cabbage leaf were the main source of the fat-soluble organic factor in this diet. The experiments were carried out during the latter part of autumn and during the winter of 1921, so that the milk was "winter milk," and probably not very rich in the organic factor. Judging from the rapidity with which the offspring from rats kept on the above diet showed the effects of deficiency of the fat-soluble organic factor in their diet, one must conclude that their ante-natal (and post-natal up to the time of weaning) supply of that factor was perhaps adequate but by no means abundant. On this account the same experiments were repeated with rats whose parents had received a larger supply of the organic factor before impregnation and during pregnancy and lactation. This was supplied to the parent animals in the form of cod-liver oil. The results of those experiments are being reported in Part III of this communication. All the rats were kept in a well-lighted and ventilated room, but away from the action of direct sunlight. The windows, which were kept closed, were of very thick plate glass.

Diets. The synthetic diet adopted for the experiments consisted of the following ingredients:

"Casein" (commercial)	20 g.	Marmite	5 g.
Starch (wheat)	50 g.	Orange juice	5 cc.
Fat	15 g.	Distilled water	50 cc.
Salt mixture ¹	5 g.		

This was kneaded into the form of a very thick paste, kept in tightly stoppered glass jars in a refrigerator, and was made fresh about twice a week.

It has been shown by Shipley, Park, McCollum and Simmonds [1921] that a diet which contains 3 % of butter, provided that diet is adequate in all other respects, especially the quality and quantity of its protein and the quantity and proportion of its inorganic elements, supplies an amount of the fat-soluble organic factor sufficient to permit of normal growth, reproduction and normal calcification of the bones of rats. For the normal control diet of these experiments the synthetic diet mentioned above in which the entire amount of fat consisted of fresh butter was adopted. This diet contained 15 g. of butter per 100 g. of dry constituents or 10 % of the wet paste as administered to the rats. This diet will be referred to as diet *A*.

In order to grade the amount of organic factor, yet retain exactly the same proportion of the various constituents, an ingredient had to be used which contained a moderate amount of this factor and which could be purified of it. In this connection advantage was taken of the work of Osborne and Mendel [1915] and of some unpublished work of Zilva (to whom thanks is due for the suggestion) who found that unpurified commercial "casein," when it forms 18 % of a diet otherwise complete, contains sufficient fat-soluble organic factor to permit of moderate growth at first and later slight gradual increase or maintenance of weight over a long period. But the weight curve is never normal and reproduction occurs but rarely. However, the development of pathological lesions in the bones of animals on this diet has not been reported. By using varying proportions of unpurified and inactivated² casein, the content of organic factor in the synthetic diet mentioned was graded without altering the quantity of the ingredient. Five diets differing only in their content of

¹ This is normal salt mixture No. 185 employed by McCollum [1917], the only difference being that the magnesium sulphate used was not anhydrous so that the amount of this salt was altered in order to keep the proportion of magnesium the same.

Salt mixture 185.

Sodium chloride	...	...	43.5
Magnesium sulphate	...	...	137.4
Sodium acid phosphate	...	...	87.2
Potassium phosphate (basic)	...	...	239.8
Calcium diacid phosphate	...	...	135.7
Calcium lactate	...	...	326.8
Ferric citrate	...	...	29.6
			<hr/> 1000.0

² Inactivated casein free from traces of the growth-promoting fat-soluble organic factor detectable by the biological method of Zilva and Miura [1921] was obtained by the heating and oxidation method [see Hopkins, 1920].

unpurified commercial casein, and therefore in their content of organic factor, were thus made. The fat used for these diets was hardened cotton seed oil free from organic factor.

Diet	% unpurified casein	% inactivated casein
<i>B</i>	20	0
<i>C</i>	15	5
<i>D</i>	10	10
<i>E</i>	5	15
<i>F</i>	0	20

In this way a rough gradation of the amount of fat-soluble organic factor was obtained. The difference between diets *A* and *B* in respect of their content of organic factor is obviously great, though how great, it is difficult to say. It was impossible to obtain sufficient animals from the same litter to place on diets with increasing amounts of organic factor so as to breach this gap between diets *A* and *B*.

In all six diets used in these experiments vitamin B (antineuritic) was supplied in the form of marmite and vitamin C (antiscorbutic) in orange juice¹.

The ash of these diets is alkaline. McCollum, Simmonds, Shipley and Park [1922] came to the conclusion that the reaction of the diet did not affect its rickets-producing qualities, but in a recent communication, Zucker, Barnett and Johnson [1923], have shown that for the diets they used, an alkaline ash made the diet more rickets-producing, and that by changing the reaction of a moderately rickets-producing diet to acid, they could convert it into a non-rickets-producing diet. What the effect of changing the reaction of the above diets would have been cannot be stated in this communication.

The amount of calcium in the above diets (*B*, *C*, *D*, *E* and *F*) was estimated by the McCrudden method and was found to be 0.258 g. per 100 g. of the wet paste (as administered to the animals). The normal diet (*A*) contained 0.285 g. per 100 g. of wet paste, slightly more than the others on account of the calcium contained in the butter included in that diet. The amount of phosphorus (estimated by Neumann's method) was 0.548 g. per 100 g. of the wet paste, and was practically the same for all the diets. Estimated for the diets without water the percentage of calcium was approximately 0.4 g. and phosphorus 0.8 g. per 100 g. of diet. When compared with the amount of calcium in the diets employed by McCollum and his collaborators, 0.64 g. per 100 g. of "dry" diet (as administered to the rats) and by Hess and Sherman and their respective associates, 0.48 g. to 0.55 g. per 100 g. of dry diet, the amount of this element used in the above diets is apparently low. Also, instead of the more usual calcium-phosphorus ratio of about two to one (the proportion by weight of these two elements in normal bone) the proportion of calcium to phosphorus in the above diets was approximately

¹ Since these experiments were begun it has been shown by Osborne and Mendel [1922] that orange juice contains also a certain amount of the growth-promoting fat-soluble organic factor. In a few tests by the biological method this was confirmed but the effect of the amounts contained in the diets was found to be very weak. However, in subsequent experiments (Parts II and III) decitrated lemon juice, apparently free from fat-soluble organic factor, was employed as the source of vitamin C.

one to two. Korenchevsky [1922], who employed the same salt mixture and in the same proportion as used in these experiments when studying the effect on rats of complete deficiency of "vitamin A" in the diet, came to the conclusion that the calcium content of the diet was probably low, but in the absence of definite knowledge of what is the optimal amount, and since this must vary with the amount of anti-rachitic factor in the diet, he decided that in "experiments designed to test the influence of varying amounts of anti-rachitic factor, the most striking results may be expected to arise when the content of calcium in the diet is not excessive." Nevertheless, as will be seen later on, although the amount of calcium in the diet was apparently low, yet in the presence of an adequate amount of the fat-soluble organic factor (diet *A*) normal calcification of the bones occurred and rickets did not develop.

In order, therefore, to ascertain that a double deficiency (calcium and organic factor) was not being dealt with, other experiments were performed using diets with increased amounts of calcium, and the results of these are reported in Part II of this communication. It may be stated in short that those experiments demonstrated that the amount of calcium used here was adequate, but by no means excessive and probably not optimal.

Twelve series of rats, every series consisting of six animals from the same litter were employed. Unfortunately, it was not possible to have all the animals of a series of the same sex. In all quantitative work of this kind, for proper comparison, it is best to have all the representatives of a series of the same sex, for it has been the experience of most investigators that males and females differ in their growth on normal diet and in their response to deficiencies in the diet. The twelve litters varied in age from 24 to 41 days at the time special feeding was begun. In the tables, the families are numbered I to XII. All the series were not killed at the same time because of uncertainty about the optimal period necessary for the manifestation of a pathological lesion. Six series were killed after seven weeks of special feeding, and the remainder at intervals up to fourteen weeks, only one series being allowed to remain on the special diets for so long a period (see litter XII in Table I). From every series a representative was placed on every one of the six diets *A* to *F*. Food was given *ad libitum*. The animals were fed daily and the amount consumed the previous day was recorded. The rats on normal diet "*A*" ate more than those on the various deficient diets, and their ration kept increasing as they grew older. But those on the deficient diets *B* to *F* ate larger portions during the first five weeks of the experimental period.

The average amount eaten during the entire experimental period by all the animals on the various diets was as follows:

Average amount consumed daily	
Diet	g.
<i>A</i>	14.3
<i>B</i>	11.2
<i>C</i>	9.7
<i>D</i>	9.9
<i>E</i>	9.3
<i>F</i>	9.4

The above figures do not apply to individual rats because in these experiments three animals on the same diet were kept in one cage. In the experiments reported in Parts II and III the exact amount eaten by every rat is known because they were kept in separate cages.

Growth on the different diets.

On normal diet *A* the rats grew well, though not quite as well as animals whose parents had received cod liver oil during pregnancy and lactation. Towards the latter part of the experimental period two of the animals (R. 347 and R. 352), females, did not gain the normal weekly amount. In those cases this was correlated by loss of appetite which developed for some unknown reason. The rats on diets *B* and *C* for the most part continued to gain steadily though slowly, but did not reach the normal figure. In a few cases, after from seven to eight weeks on these diets, the rats ceased to gain and only maintained their weight. On diets *D* and *E* the animals grew slowly and most of them reached their maximum weight in from five to seven weeks. A few continued to gain a little every week until they were killed. Those that did cease to gain were able to maintain approximately their maximum weight. The animals on diet *F* (completely deficient in fat-soluble organic factor¹) reached their maximum weight in from three-and-a-half to six weeks, depending upon the age and weight of the rat when it was placed on this diet. The older and heavier the rat when special feeding was begun, the more resistant to the deficient diet. All maintained approximately their maximum weight up to the time they were killed. Any indication of impending great loss of weight in one rat (loss of appetite or persistent slight decline of weight) was a signal to kill the whole series to which it belonged in order to avoid the complication of osteoporosis which starvation and loss of weight initiate in bones.

It can be seen in Chart 1 that the average maximum weight of the six series showed a gradation corresponding roughly with the gradation in the amount of organic factor in the diet. As mentioned before, the number of males and females in every group varied considerably so that the curve is not as striking as the indications are that it would have been, had all the animals been of the same sex. At *D* (Chart 1) this point is well illustrated. There is a rise in the curve although the content of the fat-soluble organic factor was less in this diet than in the one preceding it. But of the 12 animals on diet *D*, seven were male, while of the same number on diet *C*, only two were of that sex. Males generally reach a greater weight and gain more rapidly than females whether on normal or on deficient diets. This illustrates very well the fact that in all quantitative nutrition experiments, it is important for purposes of comparison to use animals of one sex in every series.

¹ Though diet *F* is referred to as being "completely" deficient in the organic factor, yet it is realised that undetectable traces were most likely present.

Chart 1. Average final weight of the rats on the different diets.

Chart 2. Average dry weight of bones (femur, tibia and fibula) of the rats on the different diets.

Chart 3. Average amount of calcium in the bones of the rats on the various diets.

Chart 4. Average percentage of calcium per wet weight of bones.

Gross autopsy findings.

Although, as indicated in a previous part of this paper, the "store" of the fat-soluble organic factor in the rats used in these experiments was probably not very great at the time special feeding was begun, and although the calcium content of the experimental diets was probably not optimal, yet the control animals on diet *A* were, judging from gross appearances, quite normal. The fur was smooth and glossy, the eyes showed no abnormalities and the teeth in most cases presented the normal light yellow translucent appearance, though in a few rats there was a slight degree of opacity. The bodies seemed well proportioned, but no measurements were made. They were well nourished.

Chart 5. Average percentage of calcium per dry weight of bones.

There was an abundant deposition of fat in the normal regions, and the musculature was well developed and of good colour. The chest was full and rounded. The costal portion of the ribs was of the natural width and thickness for the size of the animals and, to the touch, appeared very well calcified. There were no deformities or fractures of any of the bones. The costochondral junction of the ribs was not enlarged, and the line of demarcation between cartilage and bone at the costochondral junctions was straight and very clearly defined. There was no epiphyseal enlargement of the long bones.

The animals on diet *B* differed grossly from the controls of the same litters on diet *A* in that they were smaller, lighter, and not as well nourished, though

better in all those respects than the rats on diets *C*, *D*, *E* and *F*, all poorer than *B* in their content of organic factor. The costal portion of the ribs was not as wide, as thick or as firm as that of the rats on diet *A*. In two rats (R. 367 and R. 369) several of the ribs showed a spontaneous fracture in the costal portion, but in no case was there any obvious deformity or enlargement at the costochondral junction of the ribs, or epiphyseal enlargement of the long bones. The shafts of the long bones were more readily broken than those of the normal rats.

The general appearance of the rats on diet *C* was similar to that of the animals on diet *B*, though they averaged a little smaller and lighter and were not quite so well nourished as the latter. The only striking difference was in the number of rats which had fractured ribs and the number of fractured ribs in those rats in which they occurred. The fractures occurred in four rats, affected from two to four ribs, and in one case there were several fractures in three ribs. In several rats there was slight enlargement of the costochondral junctions and the costochondral line was slightly curved and not quite sharply defined. The costal portion of the ribs and the shaft of the long bones were slightly more brittle than in those on diet *B*.

On diet *D* fractures were less frequent than in the rats on the foregoing diet. They occurred in two cases and were not multiple. There were no other striking differences between the animals on these two diets.

The rats on diet *E* presented a gross appearance similar in most respects to that of the rats on diet *F* to be described in detail below. They were slightly heavier, however, and a shade better nourished than those on diet *F*, but fractures occurred in the same number of cases (50 %), affected more ribs and were more often multiple.

The appearance of the rats on diet *F* was strikingly different from that of the animals on diet *A* from the same litters. They were very much smaller and lighter (about half or less) and were poorly nourished. The coat was rough and dull, and the front teeth in all cases were white and very opaque. This opacity of the teeth was always one of the first signs to develop as a result of deficient fat-soluble organic factor in the diet. All the animals on the above diets, with the exception of *A*, showed this to a more or less marked degree at the time they were killed. In no case did xerophthalmia develop. The chest in most of these animals was poorly developed, presenting the appearance of a tall cone with narrow base and, in many cases, sunken wall. Only in a few instances (rats R. 411, 413 and 415) were the costochondral junctions of the ribs greatly enlarged. In some there was no enlargement at all, while in the remainder this was only from slight to moderate. In 50 % of the animals on this diet fractures were present in many ribs, and in some the fractures were multiple. In both of these respects the condition was not quite as bad in the rats on diet *F* as in those on diet *E*. In the rats on both of these diets (*E* and *F*) the fracture was surrounded by a large, white, rather firm callus. The bony portion of the ribs varied in thickness and width

in the different animals, being very narrow and thin in some, and of moderate width and thickness in others, but in no case were they even approximately normal, with the exception of rat 418, which was placed on the deficient diet when aged 41 days and weighing 60 g. The costal portion of the ribs could be bent and broken with ease compared with the normal, and the same held true in most instances for the shaft of the long bones. In a few rats the lower epiphysis of the radius was obviously enlarged. This was also the case in some of the animals on diet *E*. In no case was there any evident deformity or fracture of the shaft of the long bones which could be recognised at autopsy. Histologically, however, one infraction of a radius from a rat on diet *E* was discovered.

The internal organs of the rats on the various diets showed no obvious gross pathology. In a few rats on diets *E* and *F* slight enlargement of the spleen was noted.

Histology.

Technique. From every animal, three or more ribs, one radius and one humerus were taken for histological examination. These were first fixed in formalin (10 % in normal saline) for 48 hours, and then subjected to slow decalcification in Mueller's fluid. In a few cases it was possible to cut the bones without previous decalcification. The latter, when sectioned, were treated with silver nitrate (the von Kossa method) and counterstained with alum, carmine and eosin. The sections of the bones which were partly decalcified in Mueller's fluid were stained with haematoxylin and eosin.

The microscopical appearance of the ribs and long bones of the animals on diet *A* was in most cases quite normal within the limits of natural variation. But in two rats (R. 347 and R. 352) a slight degree of osteoporosis complicated the picture. Both of these animals were gaining very little weight during the last two weeks of the experimental period. (For illustration of normal rib see Pl. V, figs. 1 and 2.)

In no rat on the various deficient diets (*B*, *C*, *D*, *E* and *F*) was the histology of the bones absolutely normal. But in a number of rats on the less deficient diets, especially diet *B*, the microscopical appearance of the bones was found to be nearly normal, and they are classified as such under the heading of diagnosis in Table I. These differed from the normal in that the cortex was thinner, the bone marrow cavity wider and the trabeculae of the primary spongiosa were fewer, thinner and less regular in their arrangement. But there were no cartilagenous changes and cortex, trabeculae and provisional zone of calcification were normally calcified. Thus a slight degree of osteoporosis complicated the otherwise normal picture.

Where a definite increase of cortical osteoid was present, without any abnormal changes in the cartilage, a diagnosis of osteomalacia was made, the degree varying with the amount of osteoid (see Plate V, fig. 4). It is realised that morphologically this cannot be distinguished from some cases of very late rickets, and that some authors now use as the only criterion for the

Table I.

No. of rat	Sex	No. of litter	Age (in days)		Diet	Days on diet		Weight (g.)		Gain in g.	In bones			Histological diagnosis
			Initial	Final		Initial	Final	Initial	Final		% H ₂ O	% Ca wet	% Ca dry	
R. 347	♀	I	32	81	A	49	38	110	72		43.66	11.60	20.33	Nearly normal (very slight osteoporosis)
R. 350	♂	II	34	83	A	49	47	177	130		40.95	12.52	21.20	Normal
R. 351	♀	III	30	79	A	49	41	177	136		40.53	12.00	20.18	"
R. 354	♀	IV	26	74	A	48	23	125	102		42.49	11.03	19.31	"
R. 356	♀	V	27	75	A	48	35	162	127		44.82	11.39	20.65	"
R. 357	♂	VI	24	72	A	48	21	137	116		41.83	12.03	20.69	"
R. 348	♀	VII	32	94	A	62	48	183	135		40.73	12.33	21.00	"
R. 352	♀	VIII	33	94	A	61	42	113	71		41.29	12.10	20.61	Nearly normal (very slight osteoporosis)
R. 353	♀	IX	25	86	A	61	22	165	143		41.96	11.83	20.39	Normal
R. 355	♀	X	26	88	A	62	25	126	101		40.40	11.86	19.91	"
R. 349	♂	XI	34	122	A	88	47	200	153		33.96	14.19	21.32	"
R. 358	♂	XII	41	137	A	96	61	247	186		33.94	13.95	21.56	"
Averages											40.54	12.23	20.59	
R. 359	♀	I	32	81	B	49	50	104	54		42.09	10.47	18.05	Nearly normal
R. 362	♀	II	34	83	B	49	42	93	51		43.96	10.91	19.47	"
R. 363	♀	III	30	79	B	49	33	103	70		45.92	10.34	19.17	"
R. 366	♀	IV	26	—	B	—	—	—	—		—	—	—	"
R. 368	♀	V	27	75	B	48	38	124	86		51.49	7.92	16.34	Died. Enteritis. Osteoporosis
R. 369	♀	VI	24	72	B	48	25	81	56		52.25	7.40	14.77	Moderate osteoporosis and very slight osteomalacia
R. 360	♀	VII	32	94	B	62	44	148	104		43.15	10.85	19.09	Moderate osteoporosis, slight osteomalacia
R. 364	♀	VIII	33	94	B	61	43	125	82		43.62	10.28	18.44	Nearly normal
R. 365	♀	IX	25	—	B	—	—	—	—		—	—	—	Very slight osteomalacia
R. 367	♀	X	26	88	B	62	27	91	64		45.93	9.49	17.56	Died. Enteritis. Severe osteoporosis
R. 361	♀	XI	34	122	B	88	42	96	54		41.13	11.15	18.95	Slight osteomalacia
R. 370	♀	XII	41	137	B	96	58	133	75		37.88	12.95	20.58	Nearly normal
Averages											44.74	10.17	18.24	"
R. 371	♂	I	32	81	C	49	48	93	45		44.40	8.83	15.89	Slight osteomalacia
R. 374	♀	II	34	83	C	49	46	84	38		51.34	8.04	16.53	Moderate osteomalacia and slight osteoporosis
R. 375	♀	III	30	79	C	49	43	93	50		42.99	9.13	16.00	Very slight osteomalacia
R. 378	♀	IV	26	74	C	48	23	77	54		50.05	6.54	13.10	Moderate rickets
R. 380	♀	V	27	75	C	48	22	63	41		50.06	8.32	16.66	Very slight osteomalacia and osteoporosis
R. 381	♀	VI	24	72	C	48	22	70	48		53.25	6.58	14.09	Slight rickets and moderate osteoporosis
R. 372	♀	VII	32	94	C	62	43	108	65		36.50	10.62	17.76	osteomalacia
R. 376	♀	VIII	33	94	C	61	36	82	46		41.09	9.74	16.59	Moderate osteoporosis and slight osteomalacia
R. 377	♀	IX	25	86	C	61	25	65	40		48.33	6.28	12.16	"
R. 379	♀	X	26	88	C	62	26	82	56		46.18	8.98	16.74	Very slight osteomalacia
R. 373	♀	XI	34	122	C	88	48	108	60		38.04	11.05	17.84	Nearly normal
R. 382	♀	XII	41	137	C	96	54	127	73		40.74	11.25	19.00	"
Averages											45.26	8.78	16.03	"

R. 383	♂	I	D	32	81	49	43	105	62	48-38	7-63	14-79	Moderate rickets and osteoporosis
R. 386	♂	II	D	34	83	49	50	110	60	44-09	9-55	16-88	Slight osteomalacia and osteoporosis
R. 387	♂	III	D	30	79	49	38	84	46	42-66	9-86	17-20	Moderate osteoporosis
R. 390	♀	IV	D	26	74	48	25	62	37	53-73	5-60	12-15	" rickets
R. 392	♀	V	D	27	75	48	33	80	47	50-75	8-21	16-68	Slight osteoporosis
R. 393	♀	VI	D	24	72	48	23	69	46	45-23	7-15	14-37	Moderate rickets and osteoporosis
R. 394	♀	VII	D	32	94	62	47	80	33	40-97	8-31	13-92	Very slight osteomalacia and osteoporosis
R. 388	♀	VIII	D	33	94	61	40	104	64	45-38	8-32	15-24	" rickets and moderate osteoporosis
R. 389	♀	IX	D	25	86	61	21	48	27	49-77	6-12	12-19	Moderate rickets and slight osteoporosis
R. 391	♀	X	D	26	88	62	30	96	66	48-46	7-64	14-83	" osteomalacia
R. 385	♀	XI	D	34	122	88	47	150	103	39-95	11-56	19-93	Nearly normal
R. 394	♀	XII	D	41	137	96	60	145	85	41-01	10-36	17-56	Slight osteoporosis
Averages										45-86	8-36	15-47	
R. 395	♂	I	E	32	81	49	53	84	31	44-84	7-62	13-82	Moderate osteomalacia
R. 398	♂	II	E	34	83	49	40	74	34	48-95	9-01	17-58	Slight osteomalacia
R. 399	♂	III	E	30	79	49	49	111	62	48-59	8-25	16-08	Moderate osteomalacia and slight osteoporosis
R. 402	♂	IV	E	26	74	48	25	52	27	54-46	4-88	10-69	" rickets and osteoporosis
R. 404	♂	V	E	27	75	48	33	82	49	52-67	6-22	13-14	Slight osteomalacia
R. 405	♂	VI	E	24	72	48	20	49	29	55-70	4-98	11-58	Severe rickets
R. 396	♂	VII	E	32	94	62	50	101	51	44-40	9-10	16-51	Moderate osteomalacia
R. 400	♂	VIII	E	33	94	61	42	82	40	49-27	7-89	15-73	" rickets
R. 401	♂	IX	E	25	86	61	28	68	40	47-38	6-59	12-52	"
R. 403	♂	X	E	26	—	—	—	—	—	—	—	—	Killed and eaten
R. 397	♂	XI	E	34	122	88	42	99	57	47-19	9-25	17-52	Slight osteoporosis
R. 406	♂	XII	E	41	137	96	57	120	63	41-69	12-20	20-92	Nearly normal
Averages										48-65	7-81	15-09	
R. 407	♂	I	F	32	81	49	43	66	23	44-37	6-89	12-44	Very slight rickets and osteoporosis
R. 410	♂	II	F	34	83	49	38	75	37	45-63	9-50	17-37	Slight osteoporosis
R. 411	♂	III	F	30	79	49	44	109	65	48-85	7-52	14-69	Moderate rickets
R. 414	♀	IV	F	26	—	—	—	—	—	—	—	—	Killed and eaten
R. 416	♀	V	F	27	75	48	33	63	30	48-80	6-33	12-37	Slight osteomalacia and moderate osteoporosis
R. 417	♀	VI	F	24	72	48	23	54	31	46-41	6-85	12-87	Very slight rickets and moderate osteoporosis
R. 408	♀	VII	F	32	62	30	—	—	—	—	—	—	Slight osteomalacia and moderate osteoporosis
R. 412	♀	VIII	F	33	94	61	49	62	13	44-25	7-46	13-11	Moderate osteoporosis
R. 413	♀	IX	F	25	86	61	27	58	31	53-27	5-80	12-44	Severe rickets
R. 415	♀	X	F	26	88	62	25	62	37	52-77	7-15	15-10	Moderate rickets
R. 409	♀	XI	F	34	122	88	43	77	34	49-05	8-19	16-00	Slight osteoporosis and very slight osteo- malacia
R. 418	♀	XII	F	41	137	96	60	125	65	44-06	10-85	19-28	Slight osteoporosis
Averages										47-75	7-65	14-56	

diagnosis of rickets, a definite increase in the amount of osteoid tissue regardless of the presence or absence of changes in the cartilage.

If, in addition to the increase of endosteal, periosteal and peritrabecular osteoid, pathological changes were also present in the epiphyseal cartilage or in that at the costochondral junctions, then a diagnosis of rickets was made, the degree depending "arbitrarily" upon the severity of the lesions. These lesions were the increased proliferation, disorganisation and imperfect calcification of the cartilage at the zone of provisional calcification, the presence and character of a rachitic metaphysis and the amount of osteoid. In some cases there were fractures, the callus of which consisted mainly of osteoid, poorly calcified or uncalcified cartilage and fibrous connective tissue (see Pl. VI, figs. 5-8 for varying degrees of rickets).

Both osteomalacia and rickets occurred in the rats on diets *C*, *D*, *E* and *F*. Both were in many cases complicated by some degree of osteoporosis and this is indicated under the heading of diagnosis in Table I.

The following table (Table II) shows a comparison of the number of normal, nearly normal, and pathological cases which occurred on the various diets. In this table they are classified according to the predominating lesion present, and in the case of osteomalacia and of rickets regard is taken of the severity of the condition.

Table II.

Diet	Total No. of Rats	Normal	Nearly normal	Osteo- porosis	Osteomalacia			Rickets			
					Very slight	Slight	Moderate	Very slight	Slight	Moderate	Severe
<i>A</i>	12	10	2	0	0	0	0	0	0	0	0
<i>B</i>	12	0	6	4	1	1	0	0	0	0	0
<i>C</i>	12	0	2	2	3	2	1	0	1	1	0
<i>D</i>	12	0	1	3	1	1	1	1	0	4	0
<i>E</i>	11	0	1	1	0	2	3	0	0	3	1
<i>F</i>	11	0	0	5	0	1	0	2	0	2	1

It is seen in the above table that rickets and osteomalacia were most frequent in the rats on diets *D*, *E* and *F* and that the two severe cases of rickets occurred in rats R. 405 and R. 413, on diets *E* and *F* respectively.

In the rats on diet *F*, pure osteoporosis occurred more frequently than in those on the other diets. Most investigators who have studied the pathology of the bones of rats on a diet completely deficient in fat-soluble organic factor have either killed the rats after they had been on the diet for a very long time, when, in many instances, they were losing weight very rapidly, or studied the bones of rats that had died after a long period on such a diet. It is probable that this fact accounts for the divergent results obtained and consequently for the difference of opinion held by investigators of the effect of deprivation of the fat-soluble organic factor alone from the diet of rats.

Analysis of bones for calcium content.

The calcium content of the bones was estimated by Aron's method [Aron and Sebauer, 1910]. Femur, tibia and fibula were chosen for analysis. These

were mechanically cleaned of all soft tissue with the exception of the ligaments round, and the cartilage and ligaments within, the knee-joint. The patella was removed. Each hind leg was analysed separately, but the percentage of calcium and of water corresponded so closely in the duplicate estimations that in Table I only the average of the two is given (see also Charts 2-5). The following table (Table III) gives the average figures for all the rats on the various diets.

Table III.

Diet	% Ca per wet weight of bones	% Ca per dry weight of bones	Deficiency of Ca as % of normal		% H ₂ O in bones	Excess of H ₂ O as % of normal
			Wet bones	Dry bones		
<i>A</i>	12.23	20.59	—	—	40.54	—
<i>B</i>	10.17	18.24	16.8	11.4	44.74	10.3
<i>C</i>	8.78	16.03	28.2	22.1	45.26	11.6
<i>D</i>	8.36	15.47	31.7	24.8	45.86	13.1
<i>E</i>	7.81	15.09	36.2	26.7	48.65	20.0
<i>F</i>	7.65	14.56	37.5	29.3	47.75	17.7

Table III shows a striking difference between the percentage of calcium in the bones of rats on diet *A* and that of the remaining five diets which differ from it only in the fact that their content of fat-soluble organic factor is less. In this table also a definite gradation of the average percentage of calcium in the bones is noted which corresponds with the gradation in the amount of fat-soluble organic factor in the diets (see Chart 5). The differences are not nearly as great between the remaining diets in respect either of calcium content of the bones or organic factor content of the diets. But there is as much or more difference between the calcium content of the bones of rats on diets *F* and *B* as between those on diets *B* and *A*. However, in the individual series (see Table I) this regular gradation is not always seen. The fact that the animals of a series were not always of the same sex probably interfered with the regularity of the gradation in calcium content, as it did in the case of the growth curves mentioned above.

When the amount of fat-soluble organic factor in the diet is adequate, the percentage of calcium deposited in the bones of rats of approximately the same age is remarkably uniform regardless of the weight of the animals, which may vary within fairly wide limits. At the age of about four months a rat on normal diet reaches its maximum degree of calcification.

On the other hand the rats on diets deficient in fat-soluble organic factor, especially those on diets *E* and *F*, showed very great variation in the calcium content of the bones, without any very definite relation to their final weight but with some relation to the age at which they were put on the deficient diets. The older and heavier the animal at the time it was put on the deficient diet the more likely it was to have a relatively high calcium content especially if not kept on the diet very long. (Compare rat R. 418 with R. 409 both on diet *F*, and R. 406 with R. 397 both on diet *E*.) The corresponding rats from

the same litters on the other diets more rich in organic factor showed less difference in their calcium content, despite the difference in the initial age, and, on the normal diet (*A*), rat R. 349 actually had a higher percentage of calcium in its bones than rat R. 358 despite the fact that the initial and final age of the former was less than that of the latter (see Table I).

The water content of the bones of rats on diet *A* was from 33 % to 42 % of the wet weight of the bones. In the animals on diet *F* the water content of the bones varied from 44 % to 53 %. The rats on diets *B*, *C*, *D* and *E* also showed a definite increase in the percentage of water. The water content of the bones increased as the amount of organic factor in the diet decreased (see Table II).

SUMMARY AND DISCUSSION.

A study has been made of the relation of the quantity of fat-soluble organic factor in a diet to the degree of calcium deposition in the bones and to the development of experimental rickets in rats kept away from direct sunlight.

By grading the amount of fat-soluble organic factor in the diet and leaving all other constituents constant, there resulted a corresponding gradation in the percentage of calcium deposited in the bones. It is, therefore, evident that the organic factor plays an important role in the mechanism for the deposition of calcium in bones, but how it acts is not elucidated by this investigation.

Korenchevsky found that with his "—A diet," which corresponds to diet *F* described above, he produced in some young rats a picture resembling rickets. This is confirmed by the results of the above investigation. What is more, it is shown that complete absence of the fat-soluble organic factor from the synthetic diet used is not necessary in order to initiate in the bones of some young rats changes which bear a definite resemblance to rickets or osteomalacia. In fact more cases of rickets and osteomalacia occurred in the rats on diets *D* and *E* than on diet *F*, which was the most deficient in the organic factor. This is perhaps best explained by the fact that on the latter diet complicating osteoporosis was more frequent and more severe, and this masked or prevented manifestation of the rachitic lesion.

Whenever the effect of the diminution or complete absence of only one of several variable factors is being investigated it is necessary that the others should be present in at least optimal quantity if not in excess. The three substances in the diet which, so far, have been considered to bear an important relation to the development of experimental rickets in rats are fat-soluble organic factor, calcium and phosphorus. Compared with the percentage used by other investigators the quantity of phosphorus in the above diets was certainly optimal. But the same cannot be said of calcium, for it was present in appreciably lower amounts than that recommended as the optimum by other investigators. McCollum and his collaborators, for the type

Table IV.

No. of rat	Sex	No. of litter	Age (in days)			Days on diet	Average amount of food eaten daily	Weight (g.)		Gain in g.	In bones				Histological diagnosis
			Diet	Initial	Final			Initial	Final		% H ₂ O	% Ca wet	% Ca dry		
G. 370	♂	xiii	F	27	83	56	10.4	42	94	52	46-95	7.83	14.80	Very slight rickets and slight osteoporosis	
G. 371	♂	xiii	F	27	83	56	10.4	39	79	40	53-25	6.43	13.69	Moderate rickets	
										Averages	50-10	7.13	14.24		
G. 372	♂	xiii	F 1	27	83	56	10.2	42	87	45	51-83	7.59	15.77	Very slight rickets and osteoporosis	
G. 373	♂	xiii	F 1	27	83	56	10.2	38	90	52	51-64	6.92	14.32	Moderate rickets	
										Averages	51-73	7.25	15.04		
G. 374	♂	xiii	F 2	27	83	56	11.6	42	95	53	48-10	9.81	18.90	Moderate osteoporosis	
G. 375	♂	xiii	F 2	27	83	56	11.6	34	112	78	45-94	9.25	17.11	Slight osteoporosis	
										Averages	47-02	9.53	18.00		
G. 376	♂	xiv	F	23	79	56	9.7	41	71	30	50-05	6.78	13.71	Slight rickets	
G. 378	♀	xiv	F	23	79	56	9.7	43	76	33	48-80	6.77	13.23	Moderate rickets	
										Averages	49-42	6.77	13.47		
G. 379	♀	xiv	F 1	23	79	56	9.4	41	80	39	44-14	8.12	14.60	Slight rickets	
G. 380	♀	xiv	F 1	23	79	56	9.4	39	87	48	48-97	7.58	14.87	Moderate rickets	
										Averages	46-69	7.85	14.73		
G. 381	♂	xiv	F 2	23	79	56	10.1	41	100	59	44-12	8.20	14.68	Slight rickets	
G. 382	♀	xiv	F 2	23	79	56	10.1	36	82	46	45-95	8.30	15.36	Moderate osteomalacia	
										Averages	45-03	8.25	15-02		
G. 339	♀	xv	F	27	78	51	9.4	31	56	25	54-30	6.53	14-28	Moderate osteomalacia	
G. 340	♀	xv	F	27	68	41	9.4	33	54	21	48-20	5.72	11-50	Slight rickets and osteoporosis	
G. 341	♂	xv	F	27	78	51	10.5	38	93	55	48-19	7.73	14-92	Slight osteoporosis	
										Averages	50-23	6.66	13-56		
G. 342	♀	xv	F 1	27	78	51	11.7	34	87	53	43-83	8.90	15-84	Slight osteomalacia	
G. 343	♀	xv	F 1	27	68	41	11.7	31	65	24	51-34	7.32	15-16	" rickets	
G. 344	♂	xv	F 1	27	78	51	14.7	35	89	54	45-05	8.19	14-90	" osteomalacia	
										Averages	46-74	8.13	15-30		

of diets used by them, recommend 0.64 g. per 100 g. of dry substance as the optimum for calcium, while in diets *A* to *F* used in this investigation the amount of calcium was 0.258 g. per 100 g. of wet paste or 0.42 g. per 100 g. of diet without water or orange juice. How close this quantity of calcium is to the optimal amount for the type of diet used cannot be stated, and without definite knowledge of the amount of this element absorbed from the diet by rats, cannot be correctly estimated. Therefore, an investigation was made of the effect of increasing the quantity of calcium in the diet most deficient in organic factor (diet *F*) used in the above experiments, and the result is reported in Part II of this communication.

PART II.

The effect of increasing the calcium content of diet F.

In order to ascertain whether, in the experiments described in Part I, one was dealing with a deficiency of calcium as well as of fat-soluble organic factor, the amount of calcium was increased in diet *F* (the diet most deficient in organic factor) and the effect on the bones of rats of the administration of this diet enriched in calcium content was investigated. To diet *F*, as used in the experiments of Part I, calcium carbonate was added so that two new diets were made, one with double and the other with four times the quantity of calcium contained in diet *F*, which will be referred to as diet *F* 1 and *F* 2 respectively.

Diet	Calcium per 100 g. of wet diet	Phosphorus per 100 g. of wet diet	Proportion of Ca to P
<i>F</i>	0.258	0.538	0.479 : 1
<i>F</i> 1	0.538	0.538	1 : 1
<i>F</i> 2	1.076	0.538	2 : 1

Experiments.

Series I. Of a litter of six rats similar in origin to those used in Part I, two were placed on diet *F* and the same number on each of the new diets, *F* 1 and *F* 2, containing increased amounts of calcium. They were kept on these diets for eight weeks. The results of chemical analysis and histological examination of the bones are given in Table IV.

Series II. Another litter of six rats was divided equally between the three diets. The results are summarised in Table IV.

Series III. Three rats of a litter of six were placed on diet *F* and three on *F* 1. The details of the experiment are given in Table IV.

The following is a comparison of the *average* percentage of water and of calcium in the bones of all the rats on the three diets.

Table V.

Diet	Average % Ca in wet bones	Average % Ca in dry bones	Average % H ₂ O in bones
<i>F</i>	6.95	13.85	50.39
<i>F</i> 1	7.54	14.89	49.21
<i>F</i> 2	8.89	16.51	46.02

An appreciable increase is noted in the average percentage of calcium per wet and dry weight of the bones, and a decrease in the average percentage of water in the bones of rats on diets *F* 1 and *F* 2 as compared with *F*. The highest average calcium content and the lowest water content was in the rats on diet *F* 2 which contained the greatest amount of calcium. But the differences between the three groups were not very great, and in all the rats the calcium content of the bones was very far below the normal, which, for rats of that age, should be about 13 % per wet weight and 21 % per dry weight. The water content of the bones should be about 38 % for normal rats of that age. Therefore, in all the rats of the three series the percentage of water in the bones is definitely increased.

The only gross differences between the rats on diets *F* 1 and *F* 2 and those on diet *F* were that the former averaged slightly larger and heavier (though their nourishment was no better) and fractures occurred less frequently. In the ribs of only one case on diet *F* 2 did fractures occur, in three on diet *F* 1 and in five on diet *F*.

Histologically, the differences between the bones of the three types of rats were not very great. In the main, the bones resembled those described for diet *F* in Part I of this communication. However, although the three types were distinctly abnormal, the bone lesions varying from pure osteoporosis to slight rickets (see Table IV) yet, in the main, the pathological condition was most severe in the rats on diet *F* and least in those on diet *F* 2. There was but little to choose between those on diets *F* and *F* 1 although the latter contained double the quantity of calcium, an amount practically equal to that considered optimal by McCollum and his collaborators.

SUMMARY OF PART II.

Although the increase of the amount of calcium in diet *F* so that it was optimal (diet *F* 1) and even excessive (diet *F* 2) resulted in a corresponding but not very great average increase of calcium deposit in the bones, yet it did not prevent the development of pathological lesions in the bones similar to those described for diet *F* in Part I of this investigation. From the above results one must conclude that the amount of calcium in diet *F* was adequate to protect against the development of pathological changes in the bones provided the other constituents of that diet were present in adequate amounts. Therefore, since the only known dietetic deficiency in diets *F*, *F* 1 and *F* 2 was the fat-soluble organic factor, the above results serve as further proof that a diet deficient in the fat-soluble organic factor alone and otherwise complete, when administered to rats without exposing them to direct sunlight or any artificial substitutes for it, can initiate in the bones of some young rats a pathological condition resembling rickets or osteomalacia.

PART III.

The effect of increasing the pre-experimental store of fat-soluble organic factor upon the degree of calcification and development of the bones of rats on diets similar to those used in Part I.

Coward, Lush and Palmer [1923], have shown that a rat stores an appreciable amount of the growth-promoting anti-xerophthalmic organic factor of its diet in at least one of its organs, the liver. The storage of this factor in the body of the rat has also been inferred from the indirect proof furnished by studies of the growth of rats from different litters on a diet completely deficient in the fat-soluble growth-promoting factor. The tremendous variation in the growth curves led to the conclusion that the store of this factor in the young depends upon the amount of it in the mother's milk and therefore, in the diet of the mother. It was suggested in Part I of this communication that the store of fat-soluble organic factor in the rats employed in those experiments was not very great at the time special feeding was begun, and that it was possibly a combined effect of slight deficiency of this factor during the pre-experimental period and the greater deficiency of it during the period of special feeding which was responsible for the poor calcification and the pathology of the bones.

Having determined (Part II) that the mere increase of the amount of calcium in the most deficient diet (*F*) employed in the experiments of Part I did not prevent the development of pathological changes in the bones, it was considered important to investigate the influence of the pre-natal and post-natal (up to the time the animals were put on the special diets) supply of the fat-soluble organic factor upon the effects produced by the administration of diets *B*, *C*, *D*, *E* and *F* described in Part I.

In order to do this, litters were bred from rats to whose general diet (similar to that of the parent rats described in Part I) was added a paste containing cod-liver oil as the extra source of the fat-soluble organic factor. This will be referred to as the "cod-liver oil breeding diet" and was constituted as follows:

Cod-liver oil breeding diet:

Commercial "casein"	20 g.	Tricalcium phosphate	4.8 g.
Starch	60 g.	Calcium lactate	0.3 g.
Cod-liver oil	5 g.	Potassium phosphate	0.6 g.
Yeast	6 g.	Water	67 cc.
Sodium chloride	0.3 g.		

Of this paste about 10 g. was given daily to every breeding rat prior to and during pregnancy, and the ration was increased up to about 25 g. during the period of lactation. As a result, these breeding rats, both male and female, were in excellent condition, reproduction occurred frequently, and the effect

on the young was very striking. At the age of from 18 to 21 days most of these young rats were as heavy as animals aged 25 to 28 days whose parents were on the same general diet but without the addition of cod-liver oil breeding paste. The young probably ate some of this special breeding paste for four or five days prior to the time they were put on experiment. With four litters of these specially bred rats, every litter consisting of seven females, the experiments described in Part I were repeated.

In order to give the extreme test to the prophylactic action of cod-liver oil in the parents' diet on the prevention of bone pathology in the young placed after weaning on the various diets deficient in organic factor (*B-F*), the rats were put on these special diets at the age of three weeks, much earlier than in the experiments of Part I. In the latter, the rats varied from 24 to 41 days, the average age at the time they were put on the special diets being 30 days.

The only changes were the substitution of decitrated lemon juice for the orange juice in all the diets, and the addition of a seventh diet, another normal control diet, containing 5 g. of cod-liver oil which replaced 5 g. of cotton-seed oil in diet *B*, but was otherwise similarly constituted. This diet will be referred to as diet "*AA*."

Diet "AA":

Starch (wheat)	50 g.	Salt mixture (No. 185)	5 g.
Unpurified "casein" (commercial)	20 g.	Lemon juice (decitrated)	5 cc.
Cod-liver oil	5 g.	Marmite	5 g.
Hardened cotton-seed oil	10 g.	Water (distilled)	50 cc.

From every litter a rat was placed on every one of the seven diets. The four litters were three weeks old at the time they were placed on the special diets, and they were all kept on them for nine weeks. This is a slightly longer period than was used in the experiments of Part I, but these rats felt the deficiency of the organic factor in the various diets so much less than those of the first investigation, that it was deemed advisable to allow them a longer period on the special diets in order to give the deficiency of the organic factor enough time to act.

The control rats on normal diets *AA* and *A* grew very well indeed, those on diet *A*, which contained butter, growing as well and looking as normal as those on diet *AA* which contained cod-liver oil.

The effect of the good reserve store of the fat-soluble organic factor was very noticeable, for all the rats on the various deficient diets, even those on the most deficient (diet *F*), gained weight more quickly, continued to gain for a longer period, reached a higher maximum weight and appeared more nearly normal than the rats on the corresponding diets in the experiments described in Part I. However, there was a definite difference between the growth of the rats on diets *AA* and *A* and that of the rats on the first deficient diet (*B*). But, unlike the results in the first investigation, very little difference

was noted in the growth and general condition of the rats on the five deficient diets. The gradation in the average maximum weight of the rats corresponding with the amount of organic factor in the diet which was noted in Part I did not manifest itself at the end of the experimental period. In fact, in a few instances the rats on diet *F* reached a maximum weight equal to that of some on diet *B*. The average final weight and average maximum gain differed but little in the rats on the five deficient diets but all were from 20 to 30 % less than the normal in those respects (see Table VI).

Macroscopical findings.

At autopsy, the rats on diets *AA* and *A* presented no gross abnormalities and were better nourished and developed than the rats on diet *A* in the first investigation. The butter used for diet *A* in the experiments of Part I was winter butter whereas summer butter was used in this investigation. The rats on the diet containing summer butter were similar in all respects to those receiving cod-liver oil in their food. Of course it should be noted that relatively much more butter than cod-liver oil was used, but the amount of cod-liver oil in diet *AA* was optimal.

The rats on the various deficient diets were not as large and not nearly as well nourished as the ones on the normal diets. Those on diets *B* and *C* from the same litters differed but little from one another, but on each of those diets the representatives from the four different litters did vary somewhat in their gross appearance. The teeth of some of these rats were slightly opaque, and the bony portions of the ribs and the shafts of the long bones were thinner and more brittle than normal. In one rat on diet *B* (G. 49) and one on diet *C* (G. 50) both from the same litter, there was very slight enlargement of the costochondral junctions. There were no fractures of any of the bones.

Two of the rats on diet *D* unfortunately died early in the experiment of acute enteritis. The remaining two (G. 31 and G. 97) were similar to those which showed the least abnormality on diets *B* and *C*.

Slight enlargement of the costochondral junction of the ribs was noticed in rat G. 32 on diet *E*, while the same enlargement, and fractures of several of the ribs were seen in rat G. 66. The bony portion of the ribs and the shaft of the long bones were quite brittle in these two cases. But the remaining two rats, with the exception of slight brittleness of the bones, showed no gross abnormality.

Two of the rats on diet *F* (G. 33 and G. 67) from the same litters as those on diet *E* which showed gross evidence of pathology of the bones, had moderate enlargement of the costochondral junctions and numerous fractures of the ribs, but rats G. 53 and G. 99 showed no gross evidence of disease of the bones except slightly increased fragility. In all the rats on diets *E* and *F* the teeth were moderately opaque, but the opacity was greatest in those rats which showed macroscopical lesions of the bones. It should be noted here that the

Table VI.

No. of rat	Sex	No. of litter	Diet	Age (in days)		Days on diet	Average amount of food eaten daily g.	Weight (g.)		Gain in g.	In bones			Histological diagnosis
				Initial	Final			Initial	Final		% H ₂ O	% Ca wet	% Ca dry	
G. 27	♀	XVI	AA	21	88	67	16.0	20	155	135	39.39	13.80	22.86	Normal
G. 47	♀	XVII	AA	21	84	63	15.5	24	168	144	35.99	14.18	22.16	"
G. 61	♀	XVIII	AA	22	85	63	13.9	26	133	107	37.70	13.23	21.24	"
G. 93	♂	XIX	AA	22	84	62	16.7	23	154	131	36.96	13.48	22.78	"
								Averages			37.51	13.67	22.24	
G. 28	♀	XVI	A	21	88	67	14.1	19	136	117	36.87	14.20	22.48	"
G. 48	♀	XVII	A	21	84	63	15.7	26	158	132	36.93	13.98	22.17	"
G. 62	♀	XVIII	A	22	85	63	15.5	27	171	144	39.02	13.73	22.52	"
G. 94	♂	XIX	A	22	84	62	15.9	22	134	112	39.59	13.82	22.87	"
								Averages			38.10	13.93	22.51	
G. 29	♀	XVI	B	21	88	67	14.6	20	128	108	40.33	12.37	20.73	Nearly normal
G. 49	♀	XVII	B	21	84	63	12.5	24	121	97	45.69	10.62	19.52	Very slight rickets (healing)
G. 63	♀	XVIII	B	22	85	63	11.0	31	101	70	40.95	10.43	17.87	Slight osteomalacia
G. 95	♀	XIX	B	22	84	62	15.5	22	123	101	38.70	12.18	19.87	Nearly normal
								Averages			41.41	11.40	19.44	
G. 30	♀	XVI	C	21	88	67	14.0	18	108	90	40.11	11.82	19.75	Nearly normal
G. 50	♀	XVII	C	21	84	63	13.1	23	127	104	43.60	10.70	19.13	Slight rickets
G. 64	♀	XVIII	C	22	85	63	13.1	29	112	83	41.90	10.17	17.49	Moderate osteomalacia
G. 96	♀	XIX	C	22	84	62	14.5	24	109	85	41.91	11.89	20.47	Nearly normal
								Averages			41.88	11.14	19.21	
G. 31	♀	XVI	D	21	88	67	10.4	20	77	57	44.01	9.16	16.36	Nearly normal
G. 51	♀	XVII	D	21	32	11	—	23	21	-2	—	—	—	Died. Enteritis. Osteoporosis severe
G. 65	♀	XVIII	D	22	62	40	—	25	57	32	—	—	—	Died. Enteritis. Slight rickets, moderate osteoporosis
G. 97	♀	XIX	D	22	84	62	16.0	25	137	112	40.55	11.98	20.17	Nearly normal
								Averages			42.28	10.57	18.26	
G. 32	♀	XVI	E	21	88	67	16.3	20	110	90	40.82	12.0	20.28	Slight osteomalacia
G. 52	♀	XVII	E	21	84	63	12.9	24	97	73	41.82	10.95	18.82	Moderate osteoporosis
G. 66	♀	XVIII	E	22	85	63	9.9	23	78	55	47.16	8.52	16.12	Very slight rickets and osteoporosis
G. 98	♀	XIX	E	22	84	62	14.1	26	116	90	26.99	12.27	19.45	Nearly normal
								Averages			41.68	10.93	18.66	
G. 33	♀	XVI	F	21	88	67	14.7	19	96	77	42.95	9.82	17.21	Slight osteomalacia and osteoporosis
G. 53	♀	XVII	F	21	84	63	14.6	25	117	92	44.75	10.89	19.26	Slight osteoporosis.
G. 67	♀	XVIII	F	22	85	63	12.3	28	96	68	42.75	9.53	16.64	Slight rickets and osteoporosis
G. 99	♀	XIX	F	22	84	62	17.1	27	124	97	36.84	12.14	19.68	Nearly normal
								Averages			41.82	10.61	18.19	

gross lesions above described which manifested themselves in the rats on some of the deficient diets occurred mainly in those from the same two litters XVII and XVIII.

Histology.

The bones of the control rats on diets *AA* and *A* were normal in every respect and no differences were noted between the bones of rats that received butter and those that received cod-liver oil in their food.

On diet *B*, the bones of rats G. 29 and G. 95 from litters XVI and XIX respectively, were practically normal. The trabeculae of the primary spongiosa were not quite as large or as numerous and were not arranged as orderly as those of the perfectly normal bones, but the cartilage and cortex were normal. However, rat G. 49 from litter XVII had very slight rickets and rat G. 63 from litter XVIII slight osteomalacia.

The rats which had diet *C* were similar to those on diet *B* from the corresponding litters.

Of the two rats on diet *D* which died early, one (G. 51) showed severe osteoporosis, and the other (G. 65) rickets and moderate osteoporosis. Rats G. 31 and G. 97 were practically normal, the histological picture being similar to that described for the bones of rats on diet *B* from the same litters.

In the bones of the rats on diets *E* and *F* osteoporosis was a more frequent complicating feature. All the animals on these two diets showed it in some degree (see Pl. V, fig. 3). One case of very slight rickets (G. 66) occurred among the rats on diet *E*, and one of slight rickets (G. 67) among those on diet *F*.

Chemical analysis.

As before, femur, tibia and fibula of both hind limbs were analysed for calcium content. Table VI shows that the bones of the rats on the control diets *AA* and *A* contained the normal amount of water and of calcium for rats of that age. No difference was found between the rats on the diet containing butter and those on the one containing cod-liver oil. In fact, on the average, the bones of rats on the butter diet calcified slightly better than those of rats on the cod-liver oil diet. The average calcium content of these control animals was slightly higher than that of the control rats on diet *A* in the experiments of Part I. Two facts probably explain this difference, (a) the greater store of the fat-soluble organic factor in the rats of the last experiments as compared with the first at the time they were placed on the special diets and (b) the fact that the butter used in the experiments of Part I was winter butter. Both undoubtedly played a part.

Contrary to what occurred in the experiments reported in Part I, no definite gradation was found in the percentage of calcium in the bones of rats on the various deficient diets. The bones of rats on diets *B* and *C* had practically the same percentage of calcium and of water, while those of the rats on diets *D*, *E* and *F* differed but little in their percentage of calcium and of

water. However, by dividing the seven diets into three groups, some indication of grading can be seen, the average percentage of water in the bones increasing, and that of calcium decreasing slightly as the content of the diet in fat-soluble organic factor diminished. As in the experiments of Part I, the greatest difference was between the normal and the first group of diets deficient in organic factor.

Table VII.

Diets	Average % Ca per wet weight of bones	Average % Ca per dry weight of bones	Deficiency of Ca as % of normal		Average % H ₂ O in bones	Excess of H ₂ O as % of normal
			wet bones	dry bones		
<i>AA</i> and <i>A</i>	13.79	22.35	—	—	37.80	—
<i>B</i> and <i>C</i>	11.27	19.32	18.28	13.56	41.64	10.15
<i>D, E</i> and <i>F</i>	10.70	18.37	22.41	17.81	41.93	10.92

SUMMARY OF PART III.

In order to determine whether a deficiency in the store of the fat-soluble organic factor in the body of the rats prior to the experimental period played a part in the results obtained in the experiments reported in Part I, the same experiments were repeated with rats whose parents had received cod-liver oil, before and during pregnancy, and the entire period of lactation.

The young grew better during the period of lactation, and later felt the effect of the deficient diets much less than the rats of Part I experiments, whose parents did not receive the special breeding paste containing cod-liver oil.

The average percentage of calcium in the bones of the rats on all the diets was higher and the percentage of water lower than on the corresponding diets in the experiments of Part I. There was very little difference between the percentages of calcium and of water in the bones of the rats on the various deficient diets, the gradation in those respects, so marked in Part I experiments, being very slight and apparent only when the diets were divided into three main groups *AA* and *A*; *B* and *C*; *D, E* and *F*. It would seem that the store of organic factor which these rats possessed at the time they were put on the special diets was able to make up in great part for its deficiency in those diets.

Despite the increased store of the fat-soluble organic factor which these rats presumably had at the time they were put on the special diets, one or two rats on all the deficient diets developed either slight osteomalacia or slight rickets. Those rats that developed a pathological condition of the bones were all from the same two litters xvii and xviii.

PART IV.

Finally an investigation was made of the effect of increasing the calcium content of *F* diet, using rats bred from parents whose food contained an abundance of fat-soluble organic factor.

For this a litter of nine rats was employed bred from a mother who, in addition to the ordinary diet of fresh milk, white bread, oats, bran and green cabbage leaf, received daily for many months prior to and during the entire period of pregnancy and lactation, 10 g. of a paste containing fresh summer butter.

This will be referred to as "butter breeding paste" and was constituted as follows:

Butter breeding paste:

"Casein" (commercial)	20 g.	Calcium lactate	0.3 g.
Starch (wheat)	50 g.	Acid potassium phosphate	0.6 g.
Butter	15 g.	Yeast	6.0 g.
Sodium chloride	0.3 g.	Water	67 cc.
Tricalcium phosphate	0.8 g.		

The rats were only three weeks old, averaged 31 g. in weight, and were in excellent condition when they were put on the special diet.

By the addition of calcium in the form of calcium carbonate to diet *F*, diets *F* 1 and *F* 2 were made, the former containing double and the latter four times the quantity of calcium in the original diet *F*. These are identical with diets *F* 1 and *F* 2 used in Part II. Also, by removing some of the calcium lactate from salt mixture No. 185 and replacing this by powdered agar the calcium content of diet *F* was reduced by about one-fourth. This will be referred to as diet *F* 3. The purpose of the latter was to determine whether the calcium content of diet *F* is very near the lower limit.

Diet	Per 100 g. wet diet		Proportion of Ca : P	
	Amount of Ca	Amount of P	Ca	P
	g.	g.		
<i>F</i> 3	0.193	0.538	0.375	1.0
<i>F</i>	0.258	0.538	0.479	1.0
<i>F</i> 1	0.538	0.538	1.0	1.0
<i>F</i> 2	1.076	0.538	2.0	1.0

The rats were kept on these diets for eight weeks. All gained weight about equally, those on the diet containing the least amount of calcium (*F* 3) reaching a final weight as high as those of the corresponding sex on the remaining three diets. None reached the normal weight for rats of that age, but all grew better than those on diet *F* in Part I (whose parents were on the ordinary normal diet) (see Table I) and as well as those on diet *F* in Part III (whose parents were on the ordinary normal diet + cod-liver oil breeding paste (see Table VI)). At autopsy, no gross abnormalities of the bones were noted except slightly increased brittleness. In that respect the bones of rats on diet *F* 3 were slightly worse than the others, but no spontaneous fractures occurred in any of the rats. Histologically, no appreciable differences were found between the rats on diets *F*, *F* 1 and *F* 2, their bones being practically normal and showing only a very slight degree of osteoporosis. The bones of

Table VIII.

No. of rat	Sex	No. of litter	Age (in days)			Days on diet	Average amount of food eaten daily	Weight (g.)		Gain in g.	In bones			Histological diagnosis
			Diet	Initial	Final			Initial	Final		% H ₂ O	% Ca wet	% Ca dry	
G. 398	♂	xx	F 3	22	77	55	12.5	33	132	99	38.77	11.92	19.47	Slight osteoporosis
G. 399	♀	xx	F 3	22	77	55	12.5	31	107	76	39.55	12.15	20.12	Nearly normal
G. 400	♂	xx	F 3	22	77	55	13.1	31	97	66	40.05	10.23	17.09	Moderate osteoporosis
										Averages	39.45	11.43	18.89	
G. 401	♂	xx	F	22	77	55	14.5	33	116	83	39.88	11.85	19.72	Nearly normal
G. 402	♀	xx	F	22	77	55	14.5	30	117	87	38.75	13.29	21.21	"
										Averages	39.31	12.57	20.46	
G. 403	♂	xx	F 1	22	77	55	13.2	31	139	108	40.65	12.09	20.80	Nearly normal
G. 404	♀	xx	F 1	22	77	55	13.2	30	92	62	38.50	12.12	19.71	"
										Averages	39.57	12.10	20.25	
G. 405	♂	xx	F 2	22	77	55	11.4	31	108	77	39.80	12.32	20.49	Nearly normal
G. 406	♀	xx	F 2	22	77	55	11.4	28	83	55	36.87	12.40	19.65	Slight osteoporosis
										Averages	38.33	12.36	20.07	

the rats on diet *F* 3 were more osteoporotic than the others. But no histological signs of rickets or of osteomalacia were present in the bones of any of the rats. The percentage of calcium and of water was practically the same in the bones of all the rats (see Table VIII), but the calcium was slightly less and the water slightly more than normal for rats of that age. Those on diet *F* 3 contained on the average a little less calcium than the others, which corresponds with the histological results.

Table IX.

Diet	In bones		
	Average % H ₂ O	Average % Ca wet	Average % Ca dry
<i>F</i> 3	39.45	11.43	18.89
<i>F</i>	39.31	12.57	20.46
<i>F</i> 1	39.57	12.10	20.25
<i>F</i> 2	38.33	12.36	20.07

SUMMARY OF PART IV.

When rats containing a large store of fat-soluble organic factor are used, increasing (double and four times) the calcium content of diet *F*, *i.e.* 0.258 g. per 100 g. wet diet, does not increase the percentage of calcium deposited in the bones. But a decrease of the calcium content of diet *F* by about 25 % (diet *F* 3) shows a corresponding slight decrease in the percentage of calcium deposited in the bones.

The percentage of calcium in diet *F* (used in Part I and Part II experiments) is adequate, but it is rather close to the lower limit of requirement.

FINAL CONCLUSIONS.

1. When rats are used which have only a *small* "store" of fat-soluble organic factor (rats bred from parents on the ordinary normal diet), grading the content of that factor in their diet results in a corresponding gradation of the percentage of calcium in their bones. Histologically, some of the rats on the diets very deficient in fat-soluble organic factor show slight to severe osteomalacia or rickets (Part I).

2. When rats are used which have a *large* "store" of fat-soluble organic factor (bred from parents who, in addition to the ordinary normal diet, received the cod-liver oil breeding paste), grading the content of that factor in their diet results in only the slightest gradation of the percentage of calcium in the bones. Even in some of these well-bred rats, on the diets deficient in organic factor, very slight osteomalacia or rickets may develop (Part III).

3. In the absence of exposure of the animals to direct sunlight or artificial substitutes for it, the fat-soluble organic factor in the diet, by itself, plays an important rôle in the mechanism for the deposition of calcium in the bones of rats. The reduction below a certain level of the quantity of fat-soluble organic factor in an otherwise complete diet results in a diminished calcium deposit in the bones.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

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Goldblatt, p. 325. In the legend of Fig. 8. *for* "Moderate rickets"
read "Severe rickets and slight osteoporosis."

4. A normal diet containing 10 % (per wet weight) of fresh summer butter, proved as good as one containing an optimal quantity (3.6 %) of cod-liver oil, in causing normal growth and the normal development and calcification of the bones of rats.

5. The calcium content of the diets, although rather close to the lower limit, is adequate, but probably not optimal and certainly not excessive (Part II and Part IV).

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EXPLANATION OF FIGURES.

The figures in Plates V and VI are from the rats of these experiments, and were chosen to illustrate the diagnoses given in the tables.

Abbreviations:

<i>Co.</i>	Calcified cortical bone.	<i>Z.P.C.</i>	Zone of provisional calcification.
<i>Sp.</i>	Spongiosa.	<i>H.C.</i>	Hypertrophic cartilage cells.
<i>O.</i>	Osteoid.	<i>B.M.</i>	Bone marrow.
<i>P.</i>	Periosteum.	<i>Met.</i>	Metaphysis.
<i>Z.C.P.C.</i>	Zone of proliferous cartilage.		

Fig. 1. Rat R. 350 (Part I). Costochondral junction of rib. Diet A. Normal Rat. Age 83 days. Weight 177 g. Normal calcification of cortical bone, trabeculae and zone of provisional calcification. Trabeculae of primary spongiosa numerous and regularly arranged. Very little osteoid. Normal rib. Haem.-eosin. Magn. $\times 32$.

Fig. 2. Normal rat. G. 27 (Part III). Rib. Description same as Fig. 1. Haem.-eosin. Magn. $\times 32$.

Fig. 3. Rat G. 52 (Part III, Table VI). Rib. Thin cortex. Very few trabeculae in primary spongiosa. Intense calcification of *Z.P.C.* Very little osteoid. Moderate osteoporosis. Haem.-eosin. Magn. $\times 32$.

Fig. 4. Rat G. 63 (Part III, Table VI). Rib. Note increase of osteoid. Zone of provisional calcification normally calcified. Slight osteomalacia. Haem. and eosin. Magn. $\times 32$.

Fig. 5. Rat G. 49 (Part III, Table VI). Note increase in size of zone of provisional calcification, the cells nearest the spongiosa showing fair calcification of their matrix. Increase of cortical osteoid and that surrounding the trabeculae of the spongiosa. Very slight (healing?) rickets. Haem. and eosin. Magn. $\times 32$.

Fig. 6. Rat G. 69 (Part I, Table I). Rib. Greater width, and imperfect calcification of zone of provisional calcification than in Fig. 5, rat G. 49. Also slightly more osteoid than in the previous figure. But cortex is relatively thinner and trabeculae fewer. Slight rickets and osteoporosis. Haem. and eosin. Magn. $\times 32$.

Fig. 7. Rat R. 389 (Part I, Table I). Rib. Note even greater width of zone of provisional calcification than in Fig. 6. Very imperfect calcification and some disorganisation of it by the ingrowth of blood vessels from the marrow. Relatively large amount of osteoid. Moderate rickets. Haem. and eosin. Magn. $\times 32$.

Fig. 8. Rat R. 413 (Part I, Table I). Rib. Note the very great width and imperfect calcification and disorganisation of the zone of provisional calcification. Relatively very large amount of cortical osteoid. Infraction of cortical bone, with deformity. Moderate rickets. Haem. and eosin. Magn. $\times 32$.

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