Experimental Studies of the A Vitamin

Some Clinical and Anatomic Effects in
Puppies and Rats

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SOME CLINICAL AND ANATOMIC EFFECTS IN PUPPIES AND RATS *

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Recent experiments on the A vitamin show sufficient variation in the results obtained to make this a most inviting field for study. Mellanby [1] states that this accessory food factor is definitely associated with rickets. McCollum,[2] Drummond [3] and others [4] have shown that its absence causes xerophthalmia or keratomalacia and certain bone changes. Hess [5] claims that the bone findings obtained with deficient diets are not typically rachitic. Further reference to the literature is unnecessary, as Sherman [6] and others [7] have covered it thoroughly.

We shall not attempt at this time to prove that a diet deficient in the A vitamin causes rickets, osteoporosis or allied conditions. Our findings are given with the hope that they may contribute in some measure to the ultimate solution of this subject.

1. Mellanby, Edward: Experimental Investigation on Rickets, Lancet **1**: 407 (March 15) 1919.

2. McCollum, E. V.: The Newer Knowledge of Nutrition, New York, the Macmillan Company, 1918.

3. Drummond, J. C.: Researches on the Fat Soluble Accessory Substance, Biochem. J. **13**: 95, 1919.

4. Osborne, T. B., and Mendel, L. B.: Nutritive Factors in Animal Tissues. II, J. Biol. Chem. **34**: 17 (April) 1918; ibid.: A Critique of Experiments with Diets Free from Fat-Soluble Vitamin, J. Biol. Chem. **45**: 277 (Jan.) 1921. Shipley, P. G.; Park, E. A.; McCollum, E. V.; Simmonds, Nina, and Parsons, H. T.: Studies on Experimental Rickets. II. The Effect of Cod Liver Oil Administered to Rats with Experimental Rickets, J. Biol. Chem. **45**: 343 (Jan.) 1921.

5. Hess, A. F.; McCann, G. F., and Pappenheimer, A. M.: Experimental Rickets in Rats. II. The Failure of Rats to Develop Rickets on a Diet Deficient in Vitamin A, J. Biol. Chem. **47**: 395 (July) 1921.

6. Sherman, H. C.: The Vitamins, Physiol. Rev. **1**: 598-630 (Oct.) 1921.

7. Blunt, Katherine, and Chi Chi Wong: The Present Status of Vitamins, J. Home Econ. **12**: 1, 1920. Moore, C. U.: Ten Years' Progress in Children's Diets; Vitamins, Northwest Med. **20**: 246 (Sept.) 1921.

Doubtless many thousand experiments will be necessary before workers in different laboratories, though using standardized diets and technic, will secure identical biologic results.

PURPOSE OF EXPERIMENTS

The purpose of these experiments was (1) to ascertain in our own laboratory the effects of diets deficient in the A vitamin on growth and development of the animal as a whole and on certain glands and organs in particular; (2) to observe if possible any clinical differences during the test period; (3) to determine the relative economy with which normal and deficient animals metabolize their food, by comparison of gain in weight with caloric intake, and finally, (4) to study both grossly and minutely some effects on bone formation.

Criticism has been made of some results published of dietary studies on the A vitamin, because animals from different litters have been used, or because the animals have been killed at different ages. The nutritive ratios of diets have been criticized because of too great variation in the relative amounts of protein or carbohydrates permitted by such dietary provisions as the giving of bread ad libitum. In our work, therefore, we have endeavored to eliminate these variables as far as possible.

A litter of nine albino rats, 1 month old, weighing from 25 to 35 gm. each, was divided into three groups. The animals were placed in a basement room near a large window which admitted an abundance of both light and air. The experiment extended over a period of four months, from February 11 to July 7, 1920, or 120 days. Two deaths occurred during the experimental period; one rat in the lard group was omitted from our results because it died at the end of ten days. A second rat in this same group died on the ninety-sixth day.

DIETS OF THE THREE GROUPS

The diets of the three groups were chemically equal, as in each case we used the so-called "superior" diet of Osborne and Mendel,[8] which is chemically and

8. Osborne, T. B., and Mendel, L. B.: The Choice Between Adequate and Inadequate Diets as Made by Rats, J. Biol. Chem. 35:19 (July) 1918.

biologically well-balanced. The only difference in the diets of the three groups was in the kind of fat used. To the control animals (Group 1), the A vitamin was provided in the form of butter. In the Pacific Northwest, this probably has a fairly constant ratio of fat-soluble vitamin, as our cows secure green grass and fresh vegetables throughout the entire year. Groups 2 and 3 received diets lacking in the A vitamin, as their fat was in the form of linseed oil and lard respectively.

The casein was Merck's as purchased; the starch was ordinary commercial corn starch. The proportions of the elements used are given in Table 1.

The salts were supplied in an organic state in the casein free milk. This was tedious to prepare, as fresh milk was coagulated and filtered. The filtrate was then evaporated to dryness and reduced to a

TABLE 1.—DIETS OF RATS

| Adequate	Per-centage	Deficient	Per-centage
Casein	18	Casein	18
Casein free milk (dried)	28	Casein free milk (dried)	28
Starch	29	Starch	29
Butter fat	25	Linseed oil (or lard)	25
Orange juice (1 c.c. daily)		Orange juice (1 c.c. daily)	

powder. The diets were prepared with exacting precision in the biochemistry laboratory under the supervision of Dr. H. D. Haskins. Growth curves of the rats are presented in Figure 1.

There were no very remarkable variations in the growth curves of the rats. The first two groups contained two males and one female each, so that from the standpoint of sex they are comparable. The final weighings showed that the average weight in Group 1 was 154 gm., or 28 per cent. more than the average in Group 2.

COMPARISONS OF RATS

Skeletal comparisons of all the rats were made by the roentgen ray, by chemical determinations of calcium and phosphorus, and by microscopic sections. To secure an accurate roentgen-ray picture of all the animals at the same time so that the technic would be absolutely identical, it was necessary to anesthetize them. They were then placed on a single plate. The technic used was: distance, 28 inches (70 cm.), milli-

amperes, 20, spark gap, 1¾ inches (4.3 cm.), time four seconds. The results are shown in Figure 2. These four rats are taken from the center of the large roentgenogram of all the rats. It is noteworthy that the bones of the butter group show a greatly increased deposition of calcium when compared with the bones of those receiving their fat in the form of linseed oil. This is particularly noticeable in the forepaws and in the femurs of the two groups.
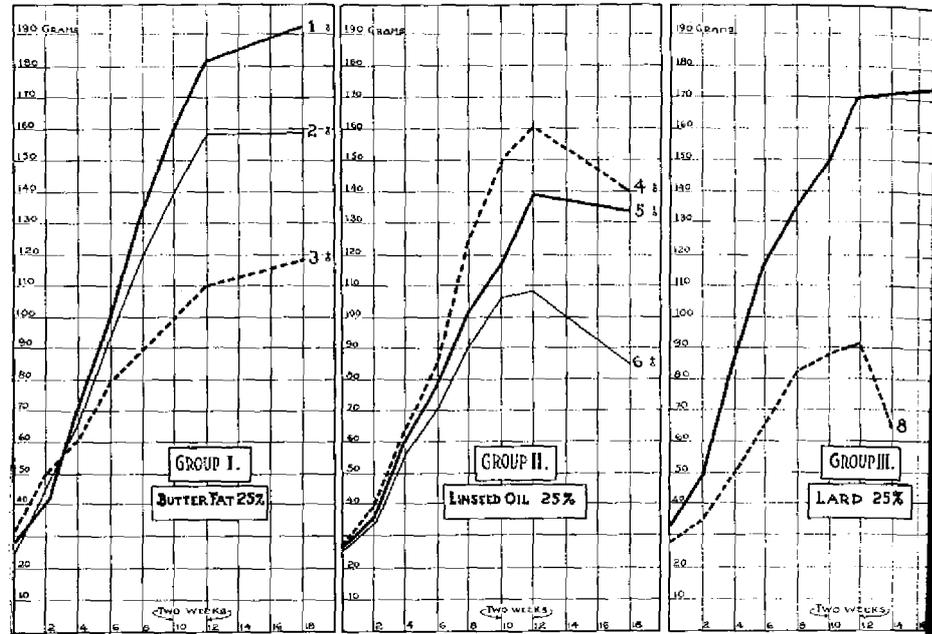
GROWTH CURVES OF RATS ON GOOD DIET (GROUP I) AND POOR DIETS (GROUPS II AND III)

Fig. 1.—Growth curves of rats on good diet (Group I) and poor diets (Groups II and III).

The calcium and phosphorus contents of the bones of the various rats were estimated by a nephelometric method.[9] Sixteen estimations were made representing two from the bones of each animal. The long bones from the extremities and the flat bones from the jaw and skull were treated as separate samples. The phos-

9. Lyman, H.: A Nephelometric Method of Estimating Calcium of the Blood, J. Biol. Chem. 29: 169 (March) 1917.

phorus pentoxid was determined by titrating with a solution of uranium. The results as presented in Table 2 show that, although the roentgen ray indicated a marked difference between the bones of the two groups, chemical estimates are indeterminate so far as this small number of animals is concerned.

The microscopic sections made will be referred to in connection with those from the puppies.
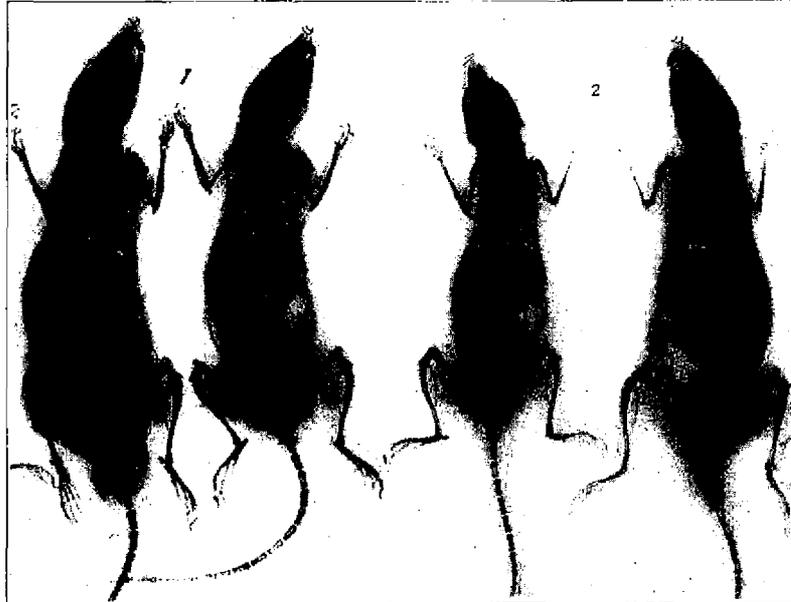

Fig. 2.—Roentgenographic appearance of rats. The two rats on the left are from the group receiving butter, while the other two received an equal amount of linseed oil. The butter group rats are larger and their bones, especially forepaws and femurs, show a greater content of calcium than those of the other group.

These rats at no time developel xerophthalmia or other eye symptoms. Whether casein as purchased contains sufficient fat-soluble vitamin to affect it is now under investigation. Hess [5] found that clinical ophthalmia occurred infrequently even on half the orange juice which our rats received. It is possible that the casein-free milk and the abundance of sunlight had some effect.

The experiments of Daniels and Laughlin [10] on rats indicate that when such a large percentage of lard is given, growth and reproduction are practically normal. Drummond [11] has shown that lard from grass-fed pigs contains appreciable amounts of fat-soluble A. No one has yet demonstrated this vitamin in linseed oil. Therefore, if Group 2 received any, it must have been from a source other than the fat element.

TABLE 2.—CALCIUM AND PHOSPHORUS PENTOXID ESTIMATES IN BONES OF RATS

Group	Number	Bones Used	Calcium per Gram of Bone Mg.	Phosphorus Pentoxid per Gram of Bone, Mg.
Butter	1	Femur	42.5	
		Jaw and skull	38.7	28.6
	2	Femur	42.5	
		Jaw and skull	40.0	31.2
	3	Femur	45.0	
		Jaw and skull	41.2	32.8
Linseed Oil	4	Femur	51.2	
		Jaw and skull	48.7	32.1
	5	Femur	42.5	
		Jaw and skull	37.5	32.8
	6	Femur	41.2	
		Jaw and skull	41.0	32.8
Lard	7	Femur	57.5	
		Jaw and skull	46.0	29.6
	8	Femur	41.2	
		Jaw and skull	45.0	26.8

DOG EXPERIMENTS

In our dog experiments, we used eight out of a litter of nine half-breed English bull pups, born Oct. 13, 1920. At the age of 3 weeks, they were taken from their mother and placed in the laboratory. For five weeks, they were fed on a bread and milk diet, with the addition of iron. They were under close observation during this period and were weighed semiweekly. When 59 days old, they were separated into two groups of four each; one group was used as controls and the other as tests. In the division of the animals, the test group was given the advantage of the better specimens from the standpoint of

10. Daniels, A. L., and Laughlin, R.: Note on the Fat-Soluble Growth-Promoting Substance in Lard and Cottonseed Oil, J. Biol. Chem. 42: 359 (July) 1920.

11. Drummond, Golding, Zilva and Coward: The Nutritive Value of Lard, Biochem. J. 14: 742, 1920.

growth, health and strength. Likewise, this group contained two of the three males. The total weight of the test animals was somewhat greater than that of the controls. The controls, therefore, started the experiment under a considerable handicap, a fact which should be held in mind in our consideration of the ultimate results.

The diets used were identical for both control and test animals, except as to the form of fat given. The controls received butter; the tests, Crisco. The proportions of the different elements used are shown in Table 3.

To insure the maintenance of these percentages throughout the entire period of observation, the various elements were thoroughly mixed, none being given separately except the orange juice and water.

TABLE 3.—DIET OF DOGS

	Percentage
Protein	18
Butter fat (or Crisco)	15
Carbohydrate	62
Salt mixture	5
Orange juice (5 c.c. daily)	

White wheaten bread was selected as the main constituent of the diet. Rice starch was added to bring the carbohydrate up to 62 per cent. The liquid factor was milk serum, that portion of the milk remaining after precipitation of the casein by the action of rennet and removal of the curd by filtration. To bring the protein content to approximately 18 per cent., Merck's casein was used. This was first washed three times with ten volumes of alcohol, and similarly treated with ether. The butter fat was prepared by centrifuging melted butter to remove the proteins. Crisco for the test animals was used as purchased. The salt portion included that in the milk serum and was brought up to 5 per cent. by the addition of iron and ammonium citrate, calcium lactate, and acid calcium phosphate, in the proportions recommended by McCollum [12] and used by Mellanby.[13]

In preparing the diet, a thick paste was made by heating the rice starch and casein with a little milk

12. McCollum and Davis: Nutrition with Purified Food Substances, Jour. Biol. Chem. 20: 161, 1915.
13. Mellanby, Edward: Report of the Medical Research Committee, 1918, p. 16.

serum. The melted fat was then added, and an emulsion obtained which was then further diluted with the balance of the milk serum. The bread was broken into small pieces and thoroughly stirred into the mixture. Each dog was fed separately from a vessel which was weighed before and after the meal. For caloric computations a record was kept in grams of the daily consumption of each animal. Each group was kept in a cage four feet square, in a well-lighted

Fig. 3.—Appearance of Dog C 2 after forty-eight days on an adequate diet. Compare with Figure 8.

and ventilated laboratory. Twice daily, the dogs were allowed to exercise for periods of from fifteen to twenty minutes each. Each day they were watched for any symptoms referable to the faulty diet. The question was raised as to which symptoms, nervous, muscular or bony, would be evidenced first.

After twenty-nine days (Jan. 9, 1921), on the diet outlined above, one of the test animals showed very definite "thick headedness" executing many uncalled-for movements and showing a slight unsteadiness of

gait. The following day, three of the tests showed excessive lacrimation and slight clumsiness of movement as though the sight were impaired or muscles weak. On the thirty-second day, all the test animals showed an uncertainty and indefiniteness in the execution of movements. One (Test 4) exhibited great uneasiness and excessive expenditure of effort without reason, e. g., chasing his tail almost indefinitely. When anyone entered the room, the test animals would bark restlessly, and pace backward and forward in the cage as long as observed (one-half hour); whereas the

Fig. 4.—Appearance of Dog T 2 after forty-eight days on diet lacking the A vitamin. Note the emaciation and the deformity of the forelegs compared with Dog C 2. Compare with Figure 9.

control animals were normal in every way and after greeting one with a bark and tail wagging, would soon lie down. The test animals remained more puppy-like in mentality and behavior throughout the experiment.

During the fifth week, the tests showed less eagerness for their food and became more fussy about it than the controls. One of the tests (Test 3) seemed unable to walk in a straight direction, always circling to the left. Another (Test 4) evidenced persistent uncertainty of movement, with much useless effort.

The sixth week, the test animals ate poorly and ceased growing. It was hoped that by concentrating the diet more food would be consumed, so one-half only of the milk serum was incorporated in the food as usual, the remainder being given as a drink. The trial was a failure as they seemed unable to masticate the food, perhaps from a lack of saliva. The food clove to the roofs of their mouths. The controls, on the
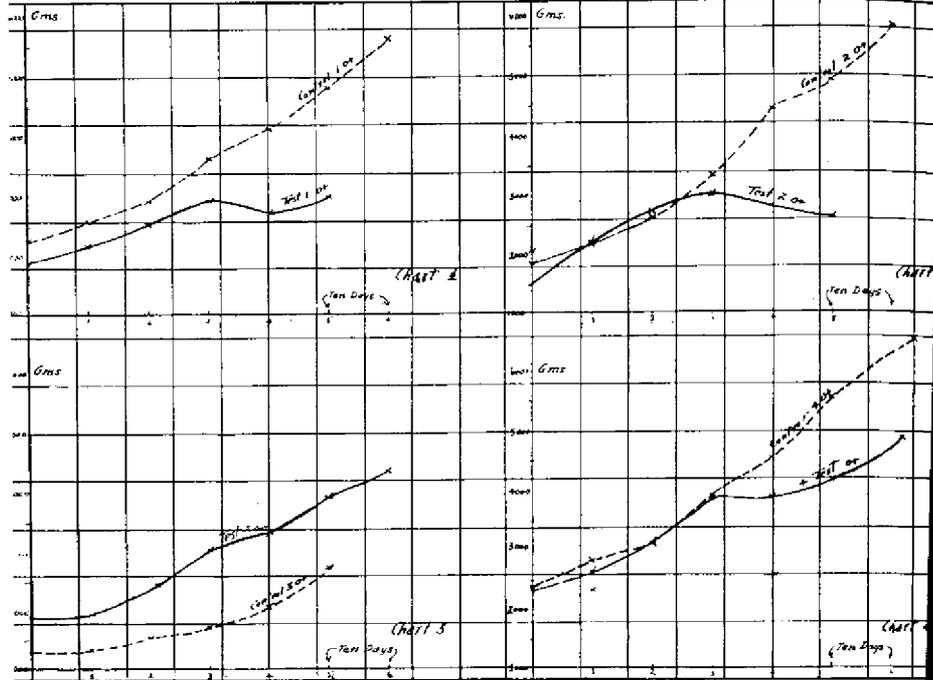
Fig. 5.—Growth curves of puppies.

other hand, ate the concentrated mixture with their usual ease and eagerness.

Up to the sixth week, none of the animals exhibited any intestinal trouble. On the forty-fifth day, the tests developed a diarrhea, one vomited considerably, and one commenced a dry, hacking cough. On the forty-eighth day, photographs were made of all the dogs. Control 2 and Test 2 are here reproduced (Figs. 3 and 4). The great emaciation, roughened coat and

deformed legs of the test animal were in marked contrast to the healthy appearance of the control specimen. On the fiftieth day, marked tenesmus accompanied the diarrhea. The control animals continued in fine condition, but the tests became so ill that it was necessary to add 200 gm. of meat to their
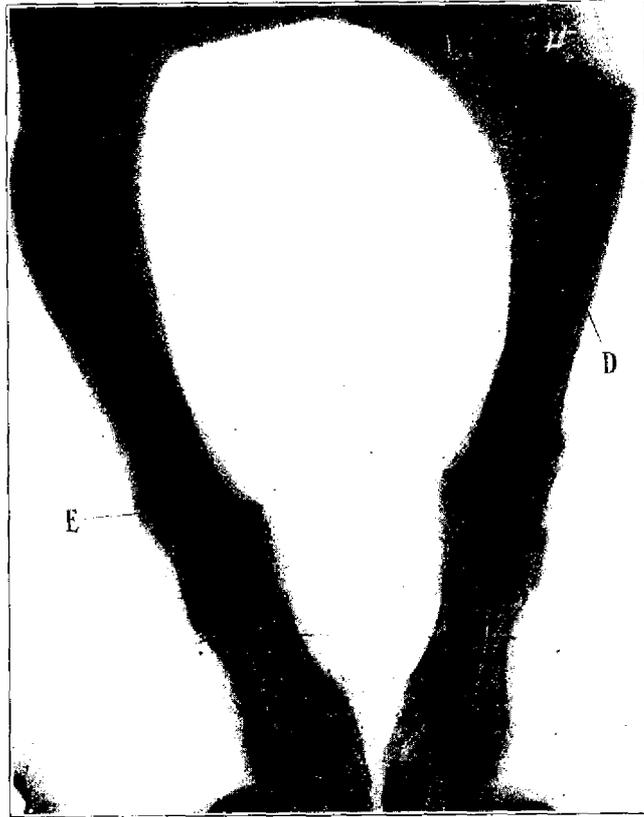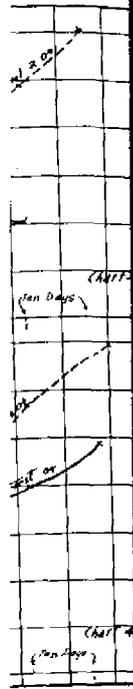

Fig. 6.—Forelegs of Dog C 1, receiving butter. At *E* the head of the bone is well outlined, showing good calcification. Compare with *e*, Figure 7. The bony cortex at *D* is several times thicker than at *d* in Figure 7.

diet. Two were also given whole milk in addition. This they refused, however, and, on the fifty-sixth day, one of the test animals died. Although this one had coughed continuously for several days, the

necropsy revealed no lung lesion. One of the controls was killed the same day, in order that the postmortem findings might be comparable. On the fifty-ninth day, all the remaining animals were killed.

At different times during the experimental period, blood examinations were made, including red, white
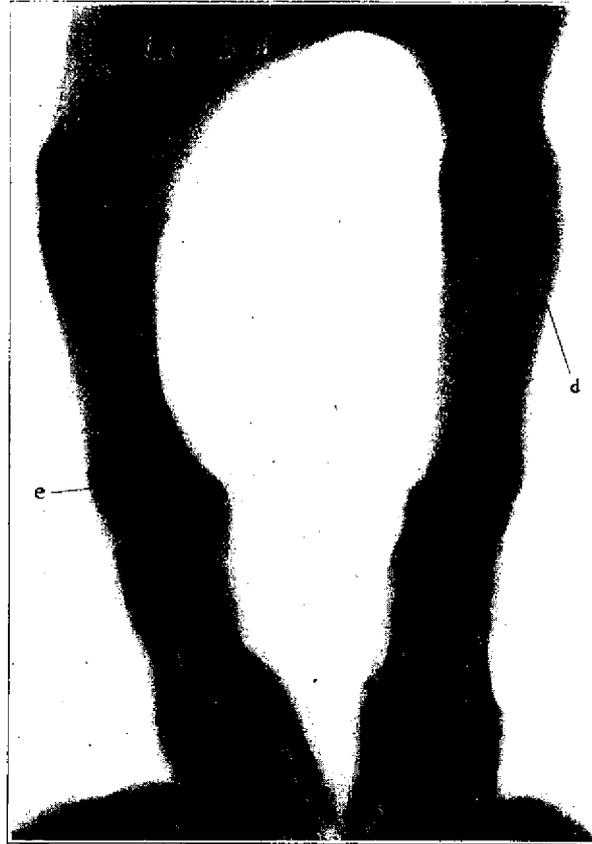
Fig. 7.—Forelegs of Dog T 4, receiving Crisco. Note translucence of epiphysis at e, and wide distance between bone ends at all joints compared with Figure 6.

and differential counts. In both groups, these were within normal limits for dogs.

The growth curves (Fig. 5) of Controls 1, 2 and 4 show a steady and consistent gain in weight. Con-

trol 3 was in the poorest condition of all at the beginning of the experiment, and did not overcome this initial handicap and begin to gain in weight until the experiment was half over.

The test animals gained well for the first thirty-two days. Then Tests 1 and 2 began to lose and never rallied. Test 3 gained fairly well.

FOOD CONSUMPTION

The food consumption of the two groups showed remarkable differences. It has been stated that an
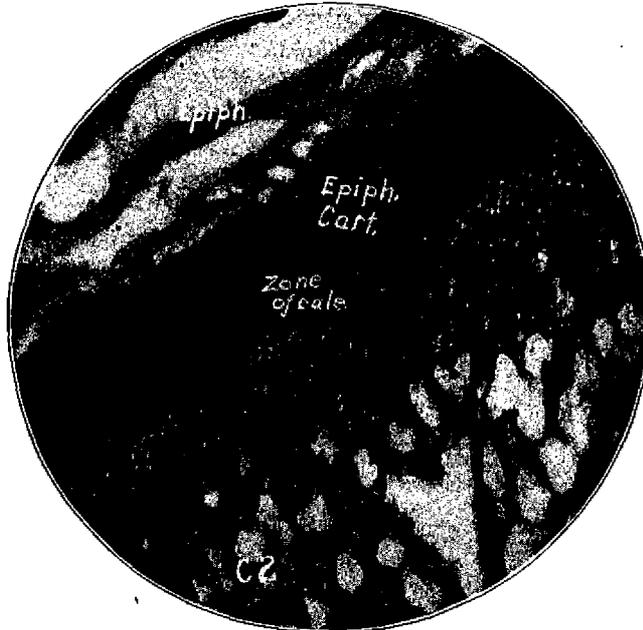
Fig. 8.—Longitudinal section of distal end of femur of Control 2: epiphyseal cartilage, with a well defined zone of calcification.

inadequate diet prevents growth because of its effect on the appetite. Careful computations of the caloric intake per kilogram consumed by each dog daily for the entire period are shown in Table 4.

An average of these shows that the control dogs took only 0.7 of a calory per kilogram more than the tests. In proportion to their daily weight, therefore,

the tests consumed nearly as much as the controls. However, the ability of the two groups to metabolize the food eaten shows a marked contrast.

The economy of an adequate diet is well shown by Table 5.

The total weight of the control group at the beginning was 7,102 gm.; of the test, 7,159 gm. The net gain daily was 55 gm. for the controls and 34 for the tests. The net gain daily per kilogram initial weight was 56 per cent. greater from the adequate diet.

TABLE 4.—CALORIES PER KILOGRAM CONSUMED DAILY BY EACH DOG

Dog	Control, Calories	Test, Calories
1	15.2	15.1
2	16.1	16.1
3	15.1	14.3
4	16.4	14.5
Average	15.7	15.0

TABLE 5.—ECONOMY OF AN ADEQUATE DIET *

	Controls, Gm.	Test, Gm.
Initial weight of group	7,102	7,159
Net gain daily	55.57	34.54
	(233 days)	(229 days)
Net gain daily per kilogram of initial weight	7.74	4.82

Gain in Weight in Respect of Caloric Intake

Calories Consumed	Calories	Calories
1. Daily	528.5	425.5
	(233 days)	(229 days)
2. Daily per kilogram of initial weight	73.5	59.4
3. Per gram net gain	9.5	12.32

* For every gram gained, the test puppies consumed 30 per cent. more calories than the control.

The calories consumed daily per kilogram of initial weight were 73.5 for the controls, and 59.4 for the tests, while the calories consumed per gram of net gain was 9.5 as compared with 12.32. For every gram gained, the test puppies consumed 30 per cent more food than the control. It would seem from these figures that the A vitamin is to the animate machine what oil is to the inanimate. It insures more economical operation and prevents premature senility.

COMPARISON OF DOGS

Roentgenograms of the forelegs of each animal were made on the forty-eighth day. If we choose two animals of approximately the same weight at the time, Control 1 and Test 4, we will find a marked difference in their bones (as shown in Figs. 6 and 7). The cone-shaped head of the ulna was well formed in Control 1 and was absent in Test 4. This last agrees with the one published by Mellanby as typically rachitic.

Fig. 9.—Longitudinal section of distal end of femur of Dog T 2: thin epiphyseal plate and its relation to the epiphyseal and diaphyseal trabeculae.

Not all the dogs showed such marked differences, but a comparison of all the controls shows that they tend more toward the type of Control 1 and the tests toward Test 4. However, we recognize that the findings in so small a group are perhaps partly accidental.

A comparison of some of the glands and organs showed fairly uniform differences. Before excision, the animals were anesthetized and bled to death so as

to have the organs as ischemic as possible. The weight coefficients for the organs of each animal are shown in Table 6 and the averages are given in Table 7.

It will be noted that there is an atrophy or under-development of the thymus, spleen and pancreas, and an hypertrophy of the thyroid and suprarenals.

In both our rats and our puppies, microscopic sections of the bones were made. Those of the puppies illustrate characteristic findings.

TABLE 6.—WEIGHT COEFFICIENTS* OF VARIOUS GLANDS

	Controls				Tests			
	1	2	3	4	1	2	3	4
Thymus	0.231	0.343	0.249	0.255	0.0105	0.0846	0.0825	0.154
Thyroid	0.0266	0.0263	0.0217	0.0249	0.0300	0.0401	0.0403	0.0390
Suprarenal	0.0187	0.0182	0.0262	0.0169	0.0293	0.0342	0.0173	0.0196
Spleen	0.1786	0.2004	0.1920	0.175	0.116	0.150	0.019	0.113
Pancreas	0.341	0.311	0.418	0.233	0.171	......	0.369	0.243
Ovaries	0.0078	......	0.0209	......	0.0283	......	......	0.0163
Testes	......	0.0342	......	......	......	0.0495	0.0401	......

* Weight Coefficient $= \dfrac{\text{Weight of Gland}}{\text{Weight of Animal}}$

TABLE 7.—AVERAGE WEIGHT COEFFICIENTS OF VARIOUS GLANDS

Gland	Coefficients		Percentage of Test to Control
	Control (4 Dogs)	Test (4 Dogs)	
Thymus	0.269	0.0829	31
Thyroid	0.0249	0.0399	160
Suprarenal	0.0187	0.0251	134
Spleen	0.1862	0.124	67
Pancreas	0.3256	0.261	80

Histologic examinations of the epiphyses of the long bones show a narrow zone of cartilage in most cases. This does not, however, resemble the great thickening and irregularity of the cartilage with the lack of calcification, which is considered character-istic of rickets. Photomicrographs of the bones of Control 2 (Fig. 8) show an epiphyseal plate of medium thickness and a well-defined zone of calcification. On the other hand, Test 2 (Fig. 9) shows a thin epiphyseal plate and a very thin zone of calcification. This cor-responds to the findings of Hess [5] in rats, and may be

due to an osteoporosis or to a lack of osteogenesis. The sections of Test 4 are more nearly characteristic of typical rickets than those of any other animal. A cross section of the epiphyseal plate showed thick areas undulating with thinner areas. There was marked preparation for calcification, but very little calcification occurring. Both the epiphysis and the diaphysis show osteoporosis. Test 4 shows one of

Fig. 10.—Section from thickened portion of epiphyseal plate from distal end of femur of Test 4. The thickened places in the epiphyseal plate and the meager calcification are fairly characteristic of typical rickets. Both the epiphysis and the diaphysis show osteoporosis.

the thickened places but no well-defined zone of calcification (Fig. 10).[14]

14. Within the marrow cavities in T_4 and to a lesser extent in one of the controls (C 2) was found the mycelia and sporangia of a mold (apparently related to aspergillus). As far as could be determined, this could not have occurred postmortem because the bones had been immediately fixed in 10 per cent. formaldehyd and had been kept continuously in this until embedded in celloidin for sectioning. This infection may be entirely incidental. It is mentioned with the hope that others will report its presence if found so that its true meaning may be ascertained.

CONCLUSIONS

1. Our rats receiving a diet deficient in the A vitamin showed by the roentgen ray a lessened amount of calcium in the bones.

2. In our puppies, the slower development on a deficient diet was not due primarily to the effect on appetite, because the test animals consumed per kilogram practically as many calories as the controls.

3. In this litter, an adequate diet gives a food-consumption economy of 30 per cent. as determined by the calories required for each weight-unit of increase.

4. Clinically the deficient diet caused early involvement of the nervous and muscular systems followed by digestive disturbances, malnutrition and bony deformities.

5. A comparison of various organs of the test and control groups by means of their weight coefficients shows hypertrophy of the thyroid and suprarenals and an atrophy or a lack of development of the thymus, spleen and pancreas.

6. The deposition of calcium was greater and more normal on the adequate diet as shown by both roentgen ray and histologic examinations.

7. In general, butter contains a factor which is superior to hydrogenated fats and oils in its effect on metabolism and bone formation.

Corbett Building.