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Chemical and Histological Changes in the Femurs of Chicks Fed Lysine-Deficient Diets[1]

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Lysine deficiency has been known to result in negative nitrogen balance in man (Albanese et al., '41); in loss of weight (Kligler and Krehl, '50, '52), retarded epiphyseal growth (Haggar et al., '55), decreased hematocrit and hemoglobin values (Gillespie et al., '45) in the rat and retarded growth and changes in the feather coloring in the growing chick (Patrick, '53; Klain et al., '57). Bavetta et al. ('54, '55) reported pathological changes in the teeth and bones of rats due to lysine or tryptophan deficiency and Fischer ('48) found that lysine deficiency resulted in diminished growth of osteoclasts in tissue cultures. Rohdenburg ('58) studied the effect of lysine deficiency on the chemical composition of the tibias of albino rats finding an increase in fat and a decrease in the water content of the bones; no further studies were carried out to determine the cause of these findings.

The present study was conducted to determine the effect of lysine deficiency on the chemical and histological composition of the long bones of New Hampshire chicks.

MATERIALS AND METHODS

One-day-old New Hampshire male chicks were distributed by weight among the experimental groups of each trial. They were fed a basal ration containing the following per 100 gm: sesame oil meal, 40.0; ground yellow corn, 56.7; minerals,[2] 3.0; cod liver oil, 0.3; and 5 ml of a vitamin solution containing per milliliter: inositol, 10; vitamin K, 5; choline chloride, 160; *p*-aminobenzoic acid, 1; niacin, 10; riboflavin, 2; pyridoxine·HCl, 2; thiamine·HCl, 2; Ca pantothenate, 6; biotin, 0.04; folic acid, 0.2; and vitamin B_{12}, 0.003 mg. This basal ration, containing 20% of crude protein, was calculated to contribute 66% of the total lysine requirement for the chick at the 20% protein level (Grau, '48). In the experimental rations L-lysine hydrochloride was added in place of an equivalent weight of corn bringing the total lysine content of the diet to 74, 83 and 100% of the suggested requirement.[3] The birds were kept in cages with raised screen bottoms and feed and water were supplied ad libitum. The various experiments are described under Results.

The birds were weighed every week and 4 or 5 from each experimental group were taken at weekly intervals, bled by heart puncture for hemoglobin and hematocrit determinations, sacrificed and both femurs dissected, cleaned, weighed and their length determined with a caliper. In some experiments the livers were also removed, weighed and prepared for chemical analysis.

One of the femurs was fixed in 10% formaldehyde for histological studies and the other was used for chemical analysis. For moisture determinations, the bones were dried in an oven at 100°C for 18 hours, cooled and weighed. Fat was determined by first boiling the dry bones in 95% ethyl alcohol under reflux for 24 hours and then extracting in a Soxhlet extractor with ethyl ether for the same period of time. After drying they were again weighed and the fat content calculated by difference. The bones were then ashed at 500°C for 12 hours and the calcium and phosphorus content in the ash determined, using the

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[2] In per cent: Ca carbonate, 1.0; bone meal, 1.0; salt, 1.0; and trace elements.
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standard AOAC ('50) methods. In one experiment protein was determined by the macroKjeldahl procedure using selenous acid as the catalyst. All data were calculated on a fresh basis. When liver fat was determined, the vacuum-oven dried, homogenized tissue was extracted with ethyl ether in a continuous extracting apparatus for 6 hours. Hemoglobin was determined in whole blood by the cyanmethemoglobin method (Cannan, '58), and hematocrit by the micro method of McGovern et al. ('55).

For the histological studies, the bone previously decalcified in an ionic bone decalcifier, was sectioned in three parts: a cross section at the middle part of the diaphysis and two longitudinal frontal sections passing through each of the epiphyseal cartilage plates at the level of the zone of endochondral ossification of the femur. Histological sections cut at 6 mμ were stained with hematoxylin-eosin and phosphotungstic acid hematoxylin (PTA Mallory stain). In addition to routine observations the following measurements were made on the histological sections: (1) percentage of myeloid tissue in the bone marrow; (2) thickness of the compact bone at the middle part of the diaphysis; and (3) thickness of the area of endochondral ossification.

For the determination of the percentage of the myeloid tissue, the cross section at the middle part of the diaphysis was used. A microscopic grid, containing 100 squares, was used and the percentage of fat and myeloid tissue was determined in 10 areas with a total count of one-thousand squares. The thickness of the cortical bone at the middle of the diaphysis was measured microscopically at 4 opposite sites (3, 6, 9, and 12, clockwise) and the average calculated. The thickness of the endochondral ossification areas was also microscopically measured at 6 points, three on each side of the middle line and the average calculated. Special care was taken to measure the cartilage plate from the area where the hyaline cartilage starts to undergo degenerative changes (hypertrophic cartilage cells and vacuolization) to the beginning of the spicule formation.

RESULTS

Experiment 1. One-day-old New Hampshire chicks were fed a commercial concentrate containing 20% of crude protein. The fat content of the livers and femurs was determined weekly in 5 birds from each group. This experiment was carried out to give an idea of the normal fat content in the two tissues studied. The bone and liver fat data of normal birds from hatching time to 9 weeks of age are shown in figure 1. The percentage of liver fat decreased rapidly after the first day of age and remained fairly constant through-

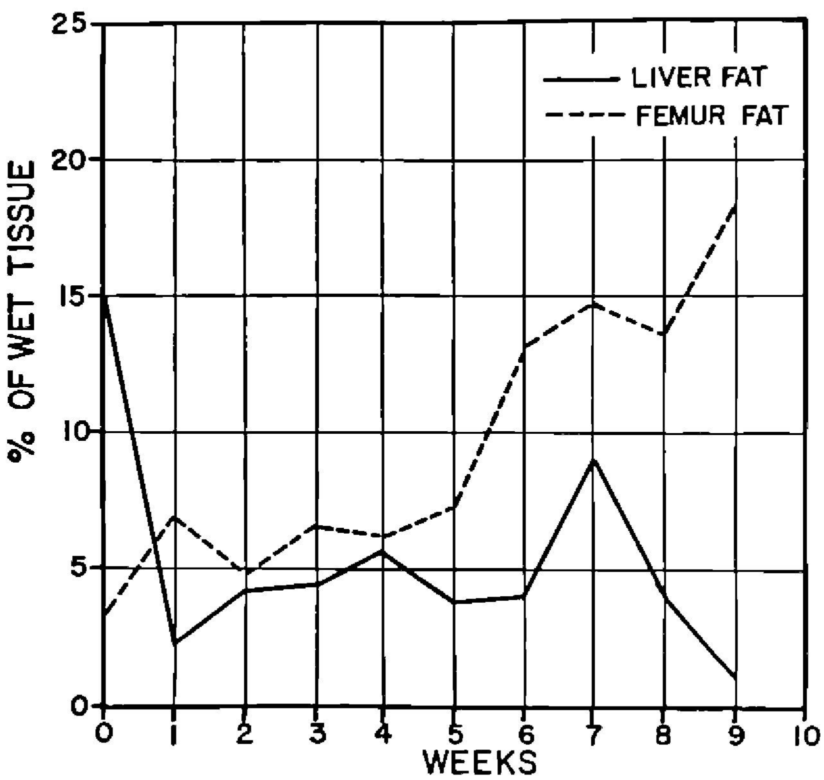

Fig. 1 Femur and liver fat of New Hampshire chicks.

out the experimental period; bone fat increased steadily with increasing age.

Experiment 2. Two groups of chicks were used; one group was fed the control diet with 66% of the lysine requirement, and the other group received the same ration supplemented with L-lysine hydrochloride to provide the full amino acid requirement for the chick. Liver fat was not affected by the lysine deficiency, but bone fat increased and the water content decreased significantly as shown in table 1. These figures represent the average of 5 chicks from each group.

Experiment 3. In order to determine whether other constituents of the bone changed as a result of the lysine deficiency, two experiments were carried out using experimental rations containing 66, 74, 83, and 100% of the lysine requirement. In figure 2 are shown the combined data for body weight in experiments 3 and 4 at the different levels of lysine fed; better weights were obtained with increasing levels of lysine.

In experiment 3 one femur from each of the 4 chicks sampled per group was used for histological studies and the other for chemical analysis of water, fat and ash content. Calcium and phosphorus content of the ash was also determined but the measurement of protein was not feasible. As shown in table 2, the absolute weight of the femur and its length increased with increasing levels of lysine. When the weight of the femur was expressed as percentage of body weight there was no change. This was expected, since the body weight decreased with decreasing levels of lysine. Chemical analysis showed that, as lysine content of the ration increased, bone moisture content also increased, but bone fat decreased. The differences were statistically significant at the 1% level. The percentage of ash and its calcium and phosphorus content remained constant under the different treatments.

Experiment 4. Growth data from this experiment have already been mentioned. In addition, ash content was determined in the right femur, protein in the left femur and moisture and fat in both bones from each of the 4 chicks sampled per group. Data in table 3 show the same inverse relationship between moisture and fat content which was observed in experiment 3. Ash content did not change significantly but the protein content was lower ($P < 0.05$) in the bones of chicks fed low levels of lysine. Hemoglobin and hematocrit values (table 4) also tended to correlate with the lysine level of the ration. As the lysine in the ration increased, there was a corresponding rise in hematocrit and hemoglobin values. These changes were statistically significant at the 1% level for hemoglobin and the 5% level for hematocrit.

Histological findings. The percentage of myeloid tissue increased ($P < 0.01$) with the increase of lysine in the ration, this difference being more striking during the second and third weeks (table 5, column 3).

A microphotograph of the cross section of the middle of the diaphysis in 4 chicks (one from each of the 4 experimental groups during the third week of treatment) indicates that the amount of myeloid tissue increased and fat decreased with higher levels of lysine (figs. 4–7). These findings are consistent with the

TABLE 1

Liver and femur fat in the New Hampshire chicks fed two levels of lysine

Age in weeks	66% of lysine requirement in diet			100% of lysine requirement in diet		
	Liver fat % Wet tissue	% Fresh femur		Liver fat % Wet tissue	% Fresh femur	
		Water	Fat		Water	Fat
1	6.0	46.5	17.8	4.8	58.7	8.2
2	2.3	54.3	15.8	5.1	61.8	5.0
3	2.9	43.4	21.7	3.0	62.5	5.1
4	2.8	50.4	18.8	3.0	57.4	6.6
5	3.2	52.9	14.2	2.7	57.2	7.1
6	2.6	49.1	12.5	2.9	59.8	7.3
7	2.6	52.1	10.8	2.7	56.1	9.8

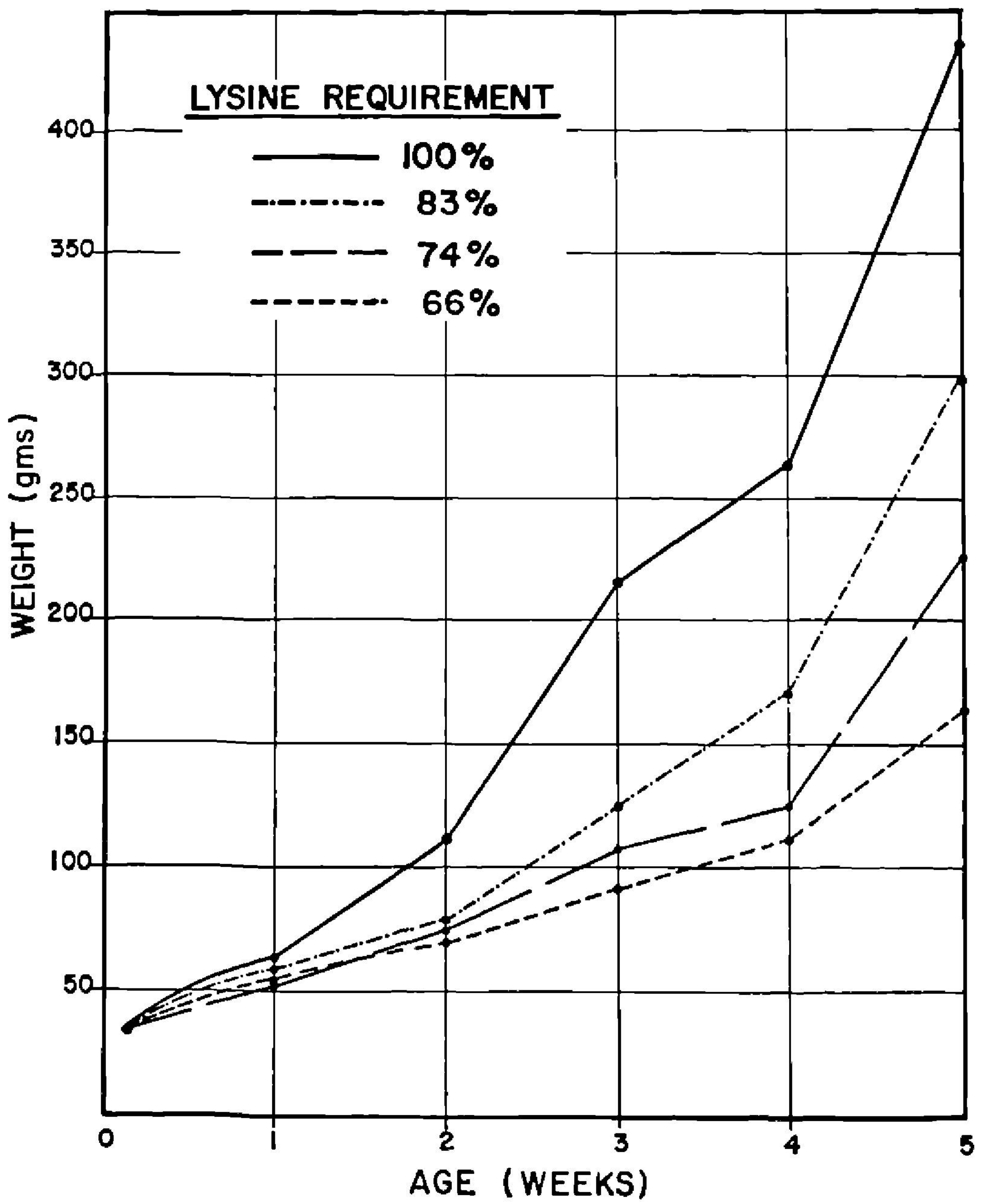

Fig. 2 Weight of chicks fed different levels of lysine (averages for two experiments, 8 chicks/week/diet).

chemical findings as summarized in figure 3. The curve on the left shows the total fat content of the bone as determined by chemical methods and the one on the right the percentage of estimated myeloid tissue in the bone marrow. The fat increase in the bones of the birds fed diets low in lysine was due to fat replacing myeloid tissue.

The 4th column of table 5 presents, in microns, the average thickness of the compact bone at the middle part of the diaphysis. These figures demonstrate that the bone increased in length and thickness in direct proportion to the lysine level of the ration and the body size of the animals. In plate 2 (figs. 8–11) is shown a cross section of the diaphysis of 4 chicks, one from each group during the last week of treatment. There was an increased thickness of the cortical bone as the lysine level of the ration increased. In the last column of table 5 data is given on the thickness of the epiphyseal cartilage plate which confirm the retarded endochondral ossification in lysine deficient animals, previously reported by Haggar et al. ('55).

DISCUSSION

Pérez ('55) demonstrated a growth retardation and retarded bone maturation in children from lower socioeconomic groups of Central America. These changes appear not only in children suffering from severe protein malnutrition (kwashiorkor), but also in apparently healthy children of the same socioeconomic background. The protein intake of these children is low, mainly of vegetable origin (Flores et al., '60) and presumably deficient in lysine and trypto-

TABLE 2

Chemical composition of femurs of New Hampshire chicks (experiment 3)

Age	Lysine in diet, % of requirement	Av. body weight	Femur weight	Femur weight	Femur length	Water	% of fresh femur		% of ash	
							Ash	Fat	Calcium	Phosphorus
		gm	*gm*	*% of body weight*	*cm*					
1 Day	—	34	0.1926	0.58	2.45	68.2	9.1	3.6	29.4	18.8
1 Week	66	50	0.2538	0.51	2.68	55.9	10.9	13.1	33.0	19.0
	74	48	0.2426	0.50	2.66	54.5	9.8	14.3	32.8	18.8
	83	54	0.2289	0.42	2.57	53.5	11.9	11.3	33.0	20.0
	100	57	0.2614	0.45	2.61	55.8	10.7	10.7	32.7	18.8
2 Weeks	66	63	0.3676	0.59	3.03	55.2	11.8	15.8	33.3	16.8
	74	70	0.3671	0.52	2.96	56.0	12.4	11.4	33.3	17.3
	83	78	0.4226	0.54	3.12	55.1	12.7	11.0	33.1	17.3
	100	112	0.5909	0.52	3.26	60.9	12.2	6.1	33.4	17.0
3 Weeks	66	89	0.4699	0.53	3.33	51.9	11.3	17.1	34.1	17.4
	74	94	0.5146	0.54	3.37	54.2	12.2	12.9	32.9	17.7
	83	114	0.6587	0.57	3.74	53.9	12.8	12.6	33.3	17.2
	100	179	1.1072	0.62	3.97	61.2	11.8	4.9	33.6	16.9
4 Weeks	66	104	0.6801	0.65	3.79	51.4	13.5	16.2	34.0	17.2
	74	122	0.7924	0.66	3.88	52.7	13.3	14.2	34.1	17.3
	83	168	1.0656	0.63	4.19	53.9	13.7	10.7	34.6	17.4
	100	275	1.8053	0.65	4.64	58.3	13.3	6.9	34.4	18.2
5 Weeks	66	134	0.8312	0.62	4.18	52.8	14.1	14.8	33.0	18.2
	74	152	0.9854	0.64	4.35	55.1	13.8	11.7	33.9	18.5
	83	217	1.3670	0.62	4.68	55.7	13.7	10.0	34.8	18.3
	100	398	2.7842	0.70	5.58	57.7	13.2	8.8	33.6	17.3

TABLE 3

Chemical composition of femurs of New Hampshire chicks (experiment 4)

Age in weeks	Lysine in diet, % of requirement	Av. body weight	Femur weight	Femur weight	% of fresh femur			
					Water	Ash	Fat	Protein
		gm	*gm*	*% of body weight*				
1	66	56	0.2732	0.48	60.3	9.2	7.2	18.5
	74	56	0.2696	0.48	60.2	11.3	6.6	17.9
	83	61	0.3055	0.49	59.8	11.4	7.1	18.3
	100	68	0.3356	0.50	60.0	10.7	6.7	18.0
2	66	74	0.4104	0.53	51.2	13.5	17.1	15.1
	74	80	0.4466	0.54	55.2	12.0	13.3	16.1
	83	105	0.5706	0.56	58.1	11.6	10.3	16.1
	100	113	0.5943	0.52	60.7	11.6	6.9	16.9
3	66	93	0.5906	0.64	54.7	11.7	14.4	14.4
	74	123	0.6957	0.56	56.7	14.1	8.3	15.4
	83	156	0.9128	0.58	58.0	12.6	9.0	15.3
	100	253	1.5874	0.62	61.2	12.4	4.8	16.7
4	66	119	0.7120	0.59	47.0	13.3	20.5	15.2
	74	129	0.7406	0.57	49.3	13.6	18.2	15.0
	83	173	1.3441	0.63	56.1	13.3	10.1	15.8
	100	252	1.7278	0.68	59.9	12.3	6.8	16.7
5	66	194	1.3149	0.67	48.5	14.2	18.2	15.3
	74	298	2.0003	0.67	51.0	14.0	15.6	15.8
	83	379	2.8427	0.75	54.5	13.0	11.6	16.6
	100	477	3.6443	0.74	54.7	12.3	12.3	15.7

TABLE 4

Hemoglobin and hematocrit values of chicks fed different levels of lysine (experiment 4)

Age in weeks	Lysine in diet, % of requirement			
	66	74	83	100
	Hemoglobin gm/100 ml			
3	7.5 (4)[1]	5.9 (3)	7.7 (2)	9.4 (2)
4	7.7 (3)	7.2 (3)	8.5 (3)	8.9 (4)
5	7.8 (4)	7.8 (4)	8.7 (2)	8.9 (3)
	Hematocrit, %			
3	27.2 (4)	23.7 (3)	29.5 (2)	33.0 (2)
4	28.0 (3)	26.0 (4)	32.0 (3)	31.7 (4)
5	28.0 (4)	29.0 (4)	29.5 (2)	30.7 (3)

[1] Figures in parentheses indicate number of chicks sampled.

phan. Another characteristic of kwashiorkor is an anemia which is normocytic or slightly macrocytic unless iron deficiency is also present; the bone marrow in these cases has been found to be hypoplastic. The anemia responds favorably to the administration of diets high in good quality protein (Scrimshaw et al., '56).

Although these signs may be due to the total protein deficiency, the possibility that lysine deficiency may play an important role in their development cannot be overlooked. It was considered important, therefore, to determine the effect of the deficiency of this amino acid on the bone growth and chemical composition, as well as on the histological changes observed in the bone marrow of experimental chicks. From the experimental data obtained here, the following conclusions can be drawn: deficiency of lysine in chicks resulted in depressed total body and bone growth and in decreased hematocrit and hemoglobin values. Although it might be suspected that these changes could be attributed to negative nitrogen balance and not to lysine

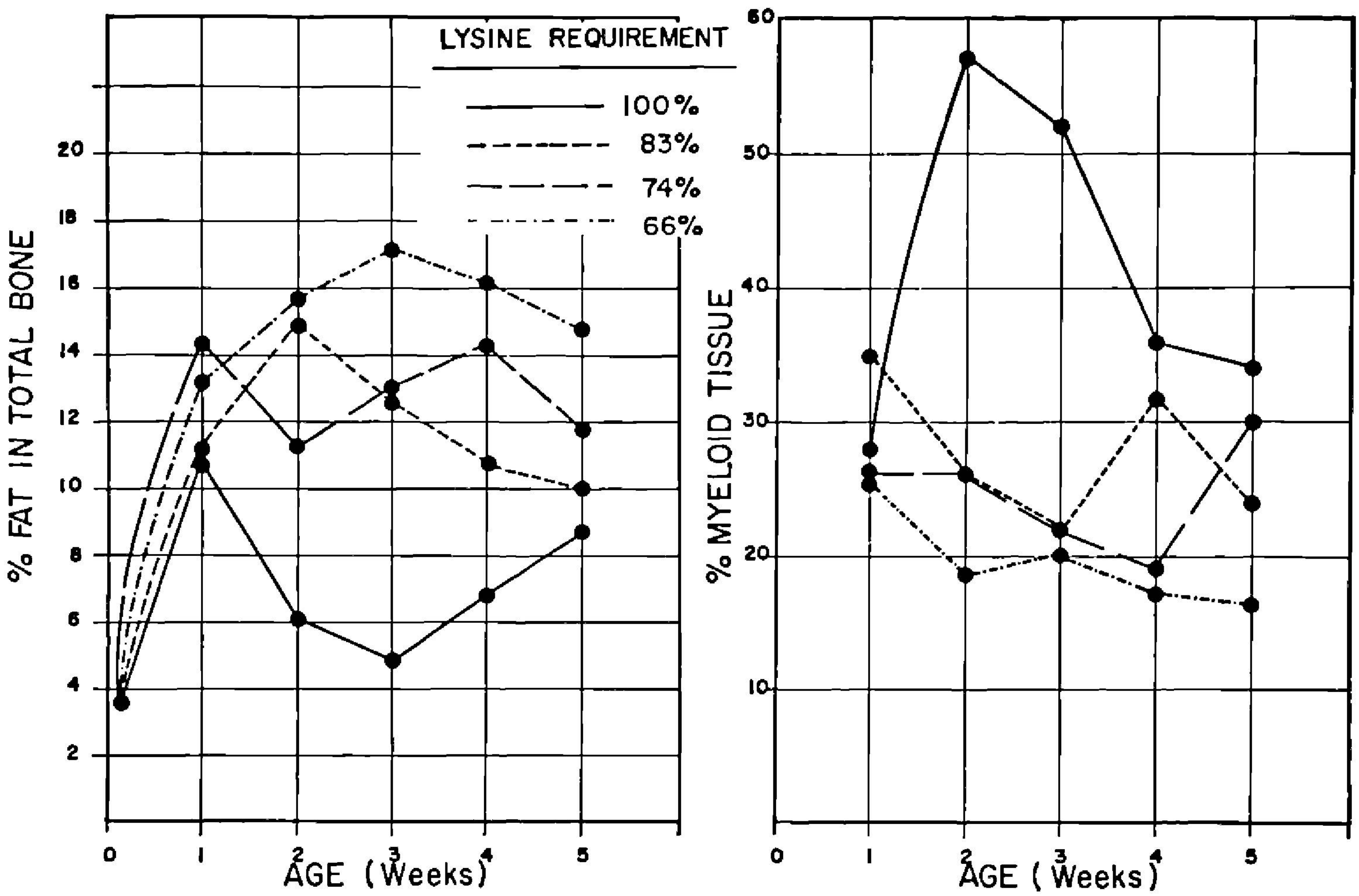

Fig. 3 Fat in total bone and myeloid tissue of chicks fed different levels of lysine (4 chicks/week/diet).

TABLE 5

Percentage of myeloid tissue and thickness of compact bone and epiphyseal plate of bones of New Hampshire chicks (experiment 3)

Age in weeks	Lysine in diet, % of requirement	% of myeloid tissue[1]	Thickness of compact bone	Thickness of epiphyseal plate
			mμ	mμ
1	66	26	182	1251
	74	26	159	731
	83	35	208	940
	100	28	224	1562
2	66	18	282	1124
	74	26	249	1085
	83	26	253	1229
	100	57	452	1857
3	66	20	272	1046
	74	22	292	927
	83	22	403	1518
	100	52	684	2388
4	66	17	286	773
	74	19	341	1124
	83	32	443	1750
	100	36	681	2292
5	66	16	325	1133
	74	30	340	1168
	83	24	450	1526
	100	34	663	1754

[1] The difference to 100% represents fatty tissue.

deficiency *per se*, the deficiency of lysine was not enough to cause negative nitrogen balance; instead, the animals in all of the experimental groups showed an increased body weight throughout the experimental periods (fig. 1).

The changes in the bone marrow appeared to be due to adipose tissue replacing shrinking amounts of myeloid tissue with a corresponding increase in total fat content of the bone and a decrease in its water and protein content. The resulting reduced activity of the bone marrow would eventually produce the low hemoglobin and hematocrit values reported in this and other studies (Gillespie et al., '45). Since the histological architecture of the bone was well preserved and its relative mineral composition did not change appreciably, it appears that lysine deficiency, *per se*, in an otherwise complete diet, did not interfere with either absorption or deposition of calcium salts.

In the animals fed 100% of the lysine requirement in experiment 3, the increase in percentage of myeloid tissue observed in the second and third weeks, and the decrease in older chicks could be due to yellow bone marrow replacing normal bone marrow. In the deficient chicks, these changes were not observed since the myeloid tissue is hypoplastic. The same argument applies to the epiphyseal plate which was thicker during the third and 4th weeks in the birds fed their complete lysine requirement.

It has been reported that the deficiency of lysine results in fatty livers in the rat (Singal et al., '53); however, these results were obtained with diets of very low protein content. In the present chick studies, despite the low levels of lysine fed, no fatty livers were observed due probably to the high protein content of the experimental rations used.

SUMMARY

The effect of lysine deficiency on the chemical and histological composition of the femurs of New Hampshire chicks, fed rations containing different levels of lysine, was studied. As the lysine level of the rations decreased there was a corresponding increase of fat in the femur and a decrease in its water content and in total body weight. Total protein content of the femur was reduced, but the percentage of ash and its calcium and phosphorus content did not change under the various treatments. Hemoglobin and hematocrit values were lower in those animals receiving low levels of lysine in the diet.

The histological studies showed that, as the lysine level of the rations decreased, there was a corresponding increase in the fatty tissue and a decrease in the myeloid tissue of the bone marrow. The thickness of the compact bone and that of the epiphyseal junction increased as the lysine level of the ration increased. The possible relationship of these findings to certain characteristics of severe protein malnutrition in children (kwashiorkor) was discussed.

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PLATE 1

EXPLANATION OF FIGURES

4 Fatty and myeloid tissues of the bone marrow of the femur of a chick fed 66% of its lysine requirement.

5 Fatty and myeloid tissues of the bone marrow of the femur of a chick fed 74% of its lysine requirement.

6 Fatty and myeloid tissues of the bone marrow of the femur of a chick fed 83% of its lysine requirement.

7 Fatty and myeloid tissues of the bone marrow of the femur of a chick fed 100% of its lysine requirement.

Bone marrow of the femur of a three-week-old chick fed, (4) 66, (5) 74, (6) 83, and (7) 100% of its lysine requirement. Observe the increase in myeloid tissue when the lysine level of the ration was raised. PTA Mallory stain. × 125.

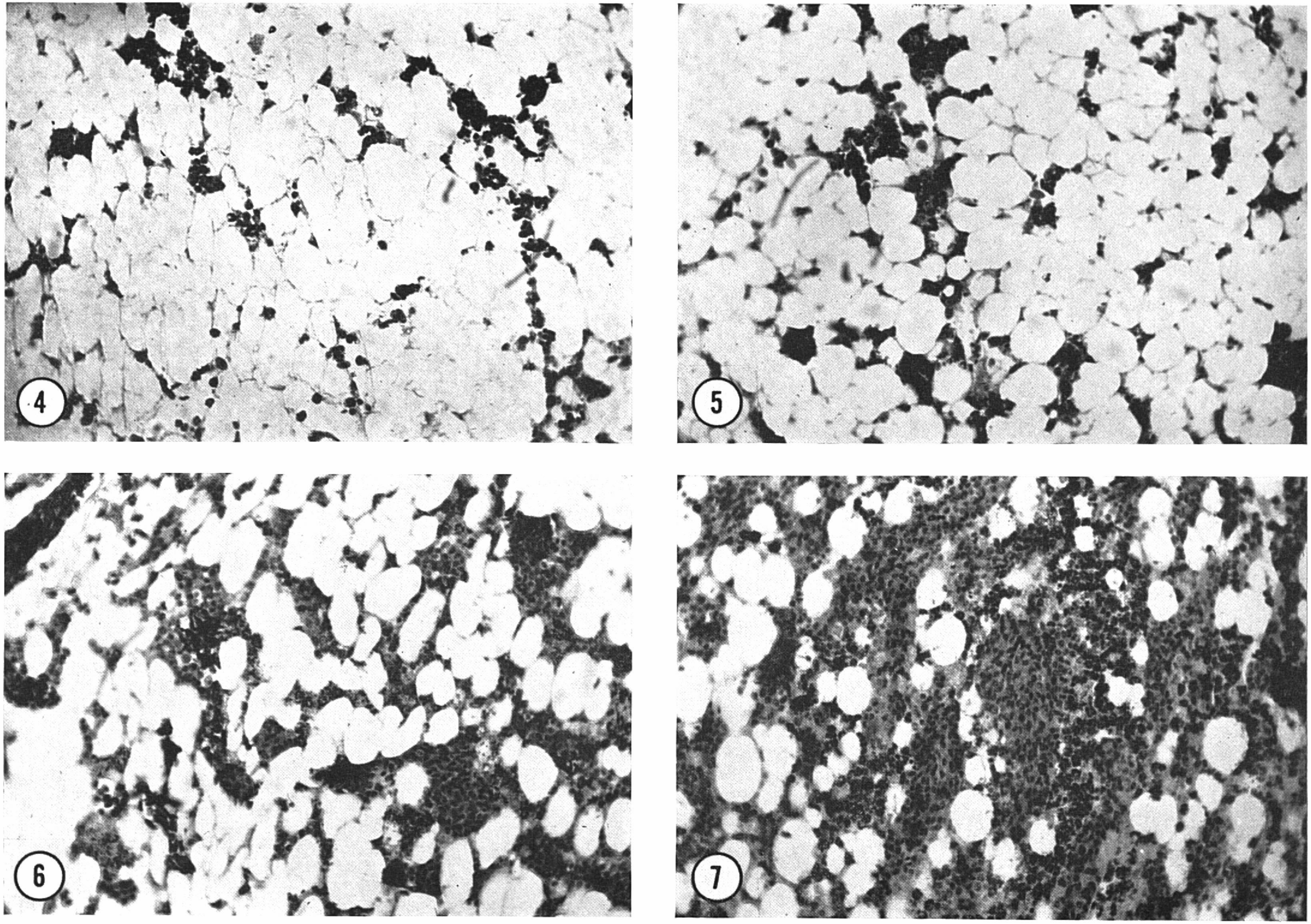
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PLATE 2

EXPLANATION OF FIGURES

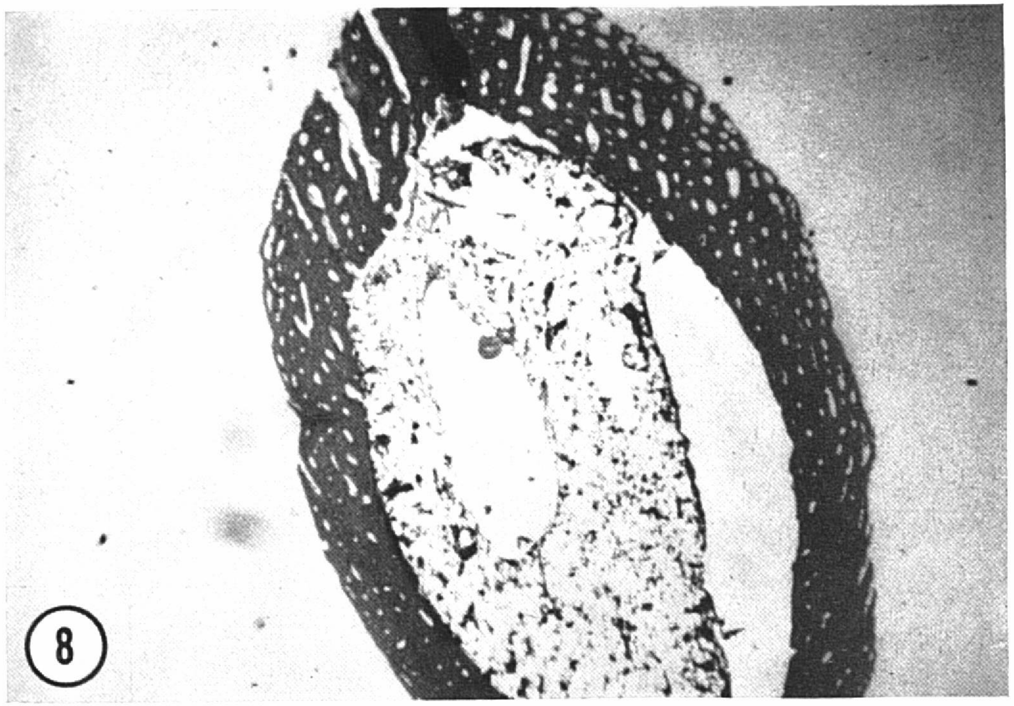
8 Cross section of the diaphysis of the femur of a chick fed 66% of its lysine requirement showing the compact bone and the bone marrow.

9 Cross section of the diaphysis of the femur of a chick fed 74% of its lysine requirement showing the compact bone and the bone marrow.

10 Cross section of the diaphysis of the femur of a chick fed 83% of its lysine requirement showing the compact bone and the bone marrow.

11 Cross section of the diaphysis of the femur of a chick fed 100% of its lysine requirement showing the compact bone and the bone marrow.

Cross section of the diaphysis of the femur of a 5-week-old chick fed, (8) 66, (9) 74, (10) 83 and (11) 100% of its lysine requirement. Note the increased thickness of the cortical bone when the lysine level of the ration was raised. PTA. Mallory stain. × 40.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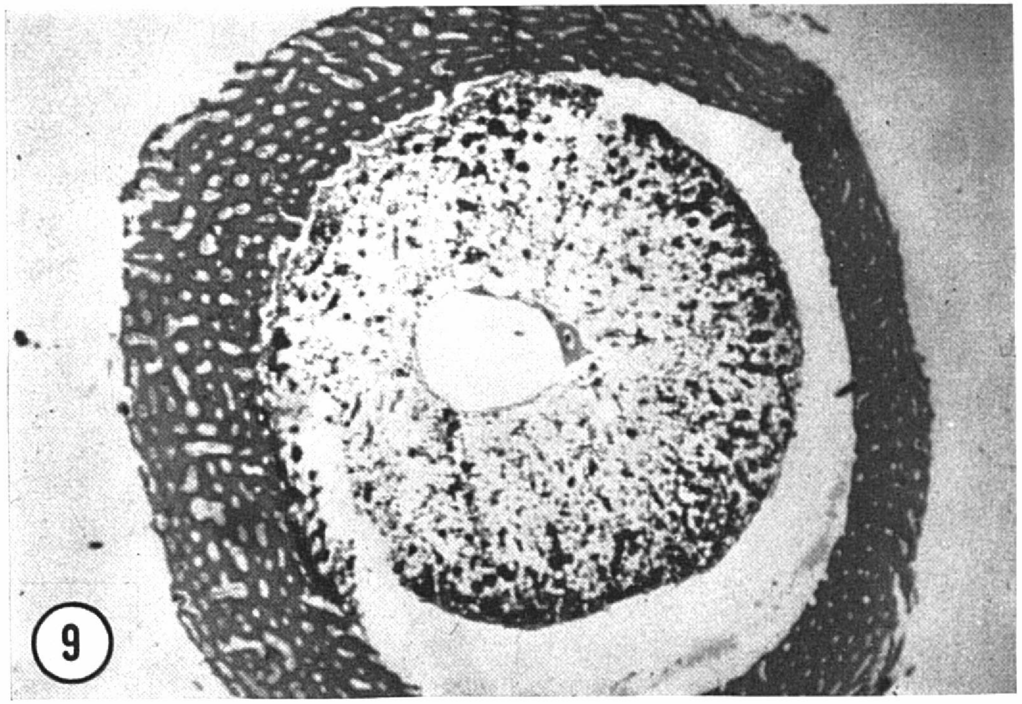
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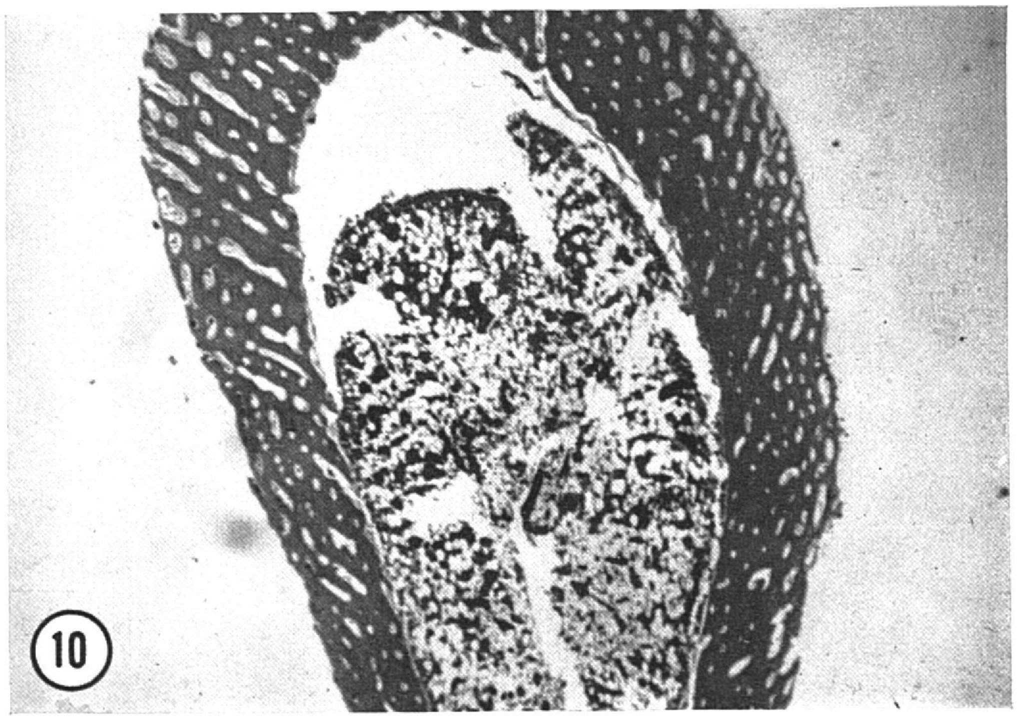
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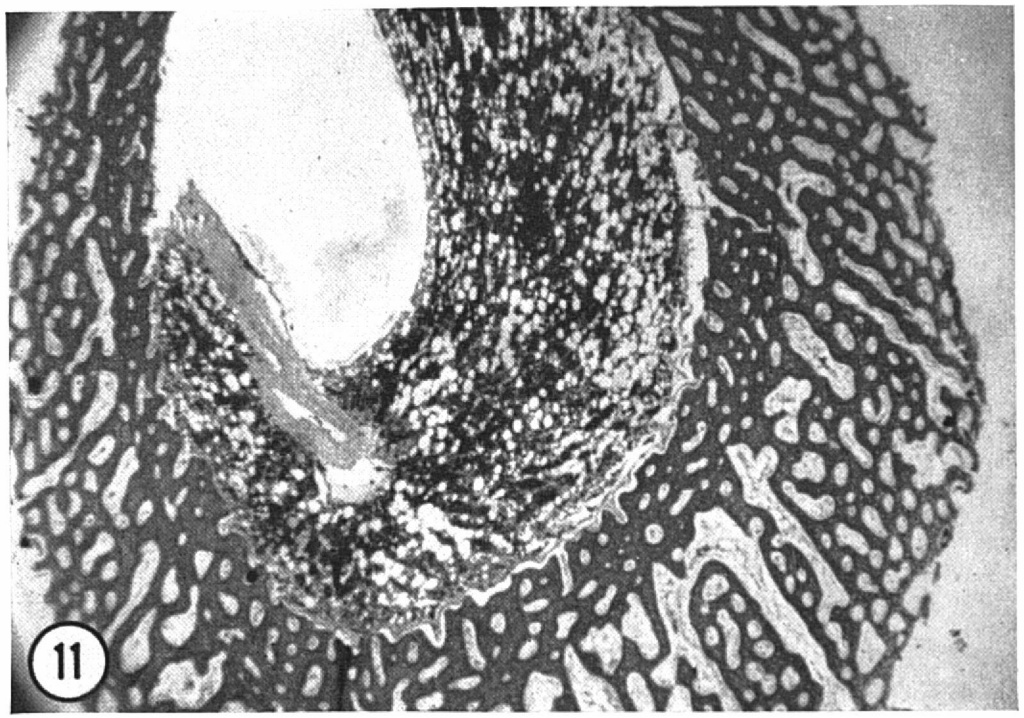
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