V I T A M I N S T U D I E S I N T H E L A M B

I. Riboflavin Deficiency and Requirement

II. Vitamin E Deficiency and Estimated Requirement

by

Rudolf Culik

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in Partial Fulfillment of the requirements for the Degree of

D O C T O R O F P H I L O S O P H Y

Department of Animal Husbandry

1951

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Affectionately dedicated

to

my deceased

Mother and Father

293159

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I. Riboflavin Deficiency and Requirement
II. Vitamin E Deficiency and Estimated Requirement

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AN ABSTRACT

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in Partial Fulfillment of the requirements
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D O C T O R O F P H I L O S O P H Y

Department of Animal Husbandry

1951

Approved R. H. Nelson
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VITAMIN STUDIES IN THE LAMB

Part I. Riboflavin Deficiency and Requirement

A purified liquid diet (synthetic milk) was successfully used to raise lambs two to eleven days old. This ration was made up to contain 16 per cent solids and was composed of (on dry matter basis) casein 30%; glucose 28%; lactose 10%; lard 10%; salt mixture 6%. In addition the ration contained all of the fat and water soluble vitamins. Eight lambs fed this ration grew normally with an average daily gain 0.44 lb.

A group of seven lambs were fed the riboflavin deficient synthetic diet. At about the fourth to sixth day on the deficient diet the lambs developed diarrhea and ceased to gain in weight. For the next six to seven days no gain or loss was noted. Shortly after, the lambs lost their appetites completely, showed symptoms of pneumonia and died. Usually the lambs did not live beyond the twentieth day of the experimental period. In two lambs showing symptoms of riboflavin deficiency, including pneumonia, were rapidly alleviated and cured with administration of crystalline riboflavin.

Pathological studies of the riboflavin deficient lambs showed lung involvement with bilateral, progressive pneumonia, general fatty degeneration and necrosis of the liver, fatty degeneration of the kidney and congestion and fatty degeneration of the adrenals.

The growth response of ten lambs on five different levels of riboflavin (5, 50, 150, 250 and 350 micrograms per pound of body weight per day) was directly proportional to the level of

riboflavin administered. The two highest levels of riboflavin administered proved to be adequate to support good health and to promote normal growth of the young lamb in the preruminant state. It was observed that the requirement for riboflavin in the young lamb was somewhere between 250-350 micrograms per pound of body weight per day.

Part II. Vitamin E Deficiency and Estimated Requirement

A diet similar to that in Part I was used to study vitamin E deficiency in 51 young suckling lambs. The source of fat in the diet of the first group was commercial lard. The deficiency symptoms appeared in 30-75 days. In the second and third groups molecular distilled lard, very low in Vitamin E, was used and the symptoms were observed in 20-55 days. The symptoms of vitamin E deficiency were characterized by disturbances in locomotion, stiffness, paralysis, pneumonia and sudden death. Blood analysis showed low tocopherol levels. Post mortem examinations revealed dystrophy of the skeletal and cardiac muscle. The lesions in the heart were invariably most pronounced in the right ventricle. Microscopic examinations of the tissues showed degeneration and necrosis of the skeletal and cardiac muscle, together with fatty degeneration of the liver and kidney. Addition of 100 mg of alpha-tocopherol to the diet every other day protected 12 positive control lambs from muscular dystrophy. An initial dose of 500 mg of alpha-tocopherol and 100 mg administered every other day to eight depleted lambs alleviated the symptoms in 3-5 days.

Microscopic examination of the tissues from lambs treated for 30-40 days revealed almost complete regeneration of the muscles. While this experimental work was in progress, the symptoms and degenerative changes of the musculature of lambs affected with so-called "Stiff-Lamb" disease under field conditions were also studied. The condition known as "Stiff-Lamb" disease as observed in the field appeared to be identical in every respect with nutritional muscular dystrophy produced on vitamin E deficient diet.

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Part I.

RIBOFLAVIN DEFICIENCY AND REQUIREMENT

INTRODUCTION

Since many of the infectious and epidemic diseases of domestic animals here in the United States have been eradicated or fairly well controlled, more and more emphasis is being placed on studies of nutritional diseases or diseases suspected to be of nutritional origin.

Before the problem of nutritional diseases could be attacked it was essential that basic studies in animal nutrition such as the indispensability and quantitative requirements of certain nutrients be determined. By means of semi-purified and purified diets developed during the last twenty years, essential nutrients of most animals and quantitative requirements of some animals have been studied and established.

Practically no information is available concerning the requirements of the suckling lamb for specific nutrients. Venkatachalam *et al.* (1949) found that the high mortality of lambs during the suckling period was dependent on both environmental and hereditary factors. Bell (1947) reported "poor mothering and lack of milk" of the ewe as a significant if not the major limiting factor in sheep production. Therefore it seemed desirable to study the essential nutrients and nutritional requirements of the young suckling lamb and possibly correlate this high mortality with nutrition.

In relation to its birth weight and compared with the calf the lamb grows more rapidly; therefore requires extra nutrients to meet its needs. Lambs normally double their birth weight in about 14 days while the calf requires 47 days (Pearson et al., 1946). Since milk from the dam is the only constituent of the diet of both young lambs and calves for at least the first few weeks of life Pearson studied the composition of colostrum and milk of both cows and ewes. The colostrum and milk of the ewe was found richer in riboflavin, thiamine, nicotinic acid and pantothenic acid than the colostrum and milk of the cow. Riboflavin levels for the ewe were found two to three times higher than in the colostrum and milk of the cow. Riboflavin was then thought to be of a special significance in the diet of a young suckling lamb and the present investigation was undertaken to determine the indispensability and quantitative requirements of this vitamin for young suckling lambs fed a purified liquid diet (synthetic milk).

OBJECT

The object of this investigation was threefold. First, to determine whether a liquid purified diet could be prepared in the laboratory which would support normal growth and health of the young lamb. Second, to study the effects of riboflavin deficiency by the use of the same diet when riboflavin was omitted. Third, to determine the quantitative requirement of riboflavin for the young suckling lamb.

REVIEW OF LITERATURE

Rats

As early as 1928, before the existence of riboflavin as a vitamin was definitely established, Chick and Roscoe (1928) used rats for the biological assay of vitamin B_2. Rats on a vitamin B_2 free diet lost appetite, grew slowly and developed dermatitis characterized by loss of hair especially from the eyelids. Inflamed and later bloody nostrils, ears and paws were also characteristic of this deficiency. Small amounts of vitamin B_2 prevented or cured the syndrome and restored normal growth.

Sherman and Derbigny (1932) studied vitamin G (B_2) in relation to the composition of the diet particularly to protein levels. Rats on a low protein diet (6 per cent) developed the deficiency symptoms much earlier and more severely than rats on a high protein diet (18 per cent).

Day and Langston (1934) produced cataract in addition to the already established symptoms of vitamin G (B_2) deficiency in the rat in nearly 100 per cent of albino rats fed vitamin G (B_2) free diet.

In a further study of cataract in the rat as a characteristic symptom of flavin deficiency Day *et al.* (1937) fed rats a diet deficient in all the then known B vitamins but supplemented with rice polish extract and additional amounts

of lactoflavin. This diet protected the animals from alopecia and cataract while the litter mates on the same diet without lactoflavin developed the characteristic symptoms of deficiency. By intramuscular injection of 120 gamma of crystalline riboflavin twice weekly Day et al. (1938) were able to arrest and clear up the cataract in most of the affected animals. In the same year Day and Darby (1938) studied the riboflavin requirement of the rat by growth response. These authors established the minimum level of riboflavin for normal growth as 120 gamma of riboflavin per week.

Bessey and Wolbach (1939) observed that rats on a riboflavin deficient diet exhibited in addition to loss of appetite, poor growth and alopecia a new syndrome; vascularization of the cornea characterized by radial ingrowth of capillaries from the limbus vessels and turbidity due to leucocytic infiltration. Supplementation of the diet with small amounts of riboflavin cleared the turbidity in few days and the ingrowth of vessels disappeared in a few weeks.

Axelrod et al. (1940) studied the mode and site of action of riboflavin. Analysis of the liver and kidney of riboflavin deficient rats showed a decrease in riboflavin and d-amino acid oxidase content. Administration of riboflavin promptly restored the riboflavin levels in both organs as well as the d-amino acid oxidase level of the kidneys and raised the level of d-amino acid oxidase of the liver.

The following year in further studies on riboflavin and its role in respiratory enzymes Axelrod and Elvehjem (1941) showed a definite decrease of xanthine oxidase level in the tissues of riboflavin deficient animals.

Shaw and Phillips (1941) kept rats on a riboflavin deficient diet very high in fat content. Rats on this diet developed neuropathological changes of the main peripheral nerve trunks similar to those observed in chicks.

Sullivan and Nicholls (1941) described typical, uncomplicated symptoms of riboflavin deficiency in the rat. Among the first symptoms were loss of appetite, retarded growth and later loss of weight and emaciation. Skin changes developed in about 5-6 weeks. The hair was uneven, unthrifty and crusted with a dark reddish brown substance. Hair was lost from the eyelids and later progressive alopecia affected most of the body. Nostrils, ears and paws were bloody and swollen.

Microscopically, the epidermis was atrophied and showed hyperkeratosis without inflammation. The sebaceous glands were swollen and later atrophied. The lips became erythematous and swollen. If the animal was not treated at this stage of deficiency death would follow.

Mannering and Elvehjem (1944) studied the influence of the composition of the diet on riboflavin requirement of the rat. Dextrin and starch showed sparing action on riboflavin while sucrose did not. Lactose especially spared riboflavin

as measured by growth response and excretion of riboflavin in the feces. Lactose, dextrin and starch evidently served as very good substrates for bacteria and promoted intensive intestinal synthesis of this vitamin. High fat diets accelerated and produced more severe deficiency symptoms than diets low in fat.

Limited information is available on riboflavin essentiality and requirement for adult rats. Warkany and Schraffenberger (1944) kept breeding stock rats on low riboflavin diet. Despite the fact that the mothers did not show any typical symptoms of chronic riboflavin deficiency a number of young were born with various congenital malformations and defects of the skeleton.

Influence of the diet, particularly the effects of protein and fat on riboflavin requirement were studied again by Czaczkes and Guggenheim (1946). These authors confirmed the earlier findings of Shaw and Phillips (1941) that a low fat diet would spare riboflavin but did not agree with Sherman and Derbigny (1932) that a high protein diet has sparing action on riboflavin. On the contrary both high protein and high fat diets decreased the excretion of riboflavin in urine and feces and diminished the riboflavin level in various tissues and organs. Low fat diet showed a definite sparing action on riboflavin while low protein diet did not affect excretion or storage of riboflavin in the rat.

Pig, Calf and Lamb

There is practically no information concerning vitamin B complex requirements for the adult pig, cow and sheep. It has been demonstrated that a few members of the vitamin B complex if not all of them are synthesized in the rumen of the adult ruminating animal or in the intestinal tract of the pig (McElroy and Goss, 1940a, Wegner et al., 1940 and Hunt et al., 1943). Under farm conditions the natural feed stuffs ordinarily contain adequate amounts of the B vitamins to supplement those synthesized in the rumen. However in the young lamb, calf and pig when the rumen is non-functional or intestinal synthesis has not taken place, the amount of B vitamins in the diet is of great importance.

Purified diets used in large animal investigations were developed recently. Hughes (1939) used a purified diet to demonstrate the indispensability of riboflavin and other factors of the vitamin B complex for the young growing pig. Pigs on a deficient diet grew slowly and were unthrifty while pigs on the same diet supplemented with rice bran filtrate made normal gains and stayed in good condition. The author concluded that both thiamine and riboflavin are essential for normal growth and health. The following year the same author (Hughes, 1940a) used the same purified diet and established that the minimum requirement of riboflavin for young growing pigs was 3 milligrams of crystalline riboflavin per 100 pounds of body weight.

Patek et al. (1941) produced and described the symptoms of riboflavin deficiency in the pig. Loss of appetite and retarded growth were followed by corneal opacity, dermatitis, changes in hair and hoofs and eventually death. Addition of riboflavin to the diet alleviated the symptoms rapidly. Wintrobe and associates (1944) in addition to the symptoms described by Patek observed myelin degeneration of the sciatic and brachial nerves, cataract, moderate normocytic anemia and fatty liver and kidney.

Van Poucke and Krider (1946) and Krider et al. (1949) used a highly purified diet to establish the quantitative requirement of riboflavin for growing pigs. Feed efficiency and daily gains closely followed the levels of riboflavin in the diet. The minimum riboflavin intake per day was 1.4 mg per lb of ration. Pigs on diets with less than 1.3 mg did not make normal gains. Feed efficiency was improved 18 per cent when the riboflavin level was increased from 0.25 to 1.50 mg per lb of ration per day.

Mitchell et al. (1950) noticed that the urinary excretion of riboflavin of growing pigs increased in a hot environment, while a cold environment tended to decrease the output.

Wiese and coworkers (1947) first approached the problem of the requirement for specific nutrients of the young suckling calf. These workers developed a purified, liquid diet (synthetic milk) composed of vitamin free casein, lard, cerelose and salts supplemented with wheat germ oil and all known fat

and water soluble vitamins. Calves on this diet made normal daily gains and maintained good health condition. After successfully raising the calves on the purified liquid diet the same group studied the requirement of a number of B vitamins. Among those first studied was riboflavin (Wiese et al., 1947b). Calves fed a riboflavin deficient liquid purified diet developed the first symptoms after about two weeks. These consisted of hyperemia of the buccal mucosa and tongue, lesions in the corners of the mouth, on the lips and around the navel. At about the same time the calves lost their appetite, developed scours and became emaciated. Deficient calves exhibited excessive salivation and lacrimation and progressive loss of hair especially in the region of the abdomen. The navel region became inflamed and irritated. A single injection of 5 mg of crystalline riboflavin improved the condition in one day. The appetite was improved and salivation and lacrimation decreased. However the symptoms reappeared after a few days. A diet supplemented with 5 mg of crystalline riboflavin per day restored the appetite and the calves gained in weight and condition. All the symptoms gradually disappeared. The lesions started to heal and new hair started to grow. The authors did not observe vascularization of the cornea or opacity of the lens. Post mortem examination of the cured animals did not show lesions. The authors concluded that the very young non-ruminating calf required a dietary source of riboflavin.

Warner and Sutton (1948) checked the work of Wiese et al. (1947b) by feeding calves with natural colostrum and milk in which most of the riboflavin had been destroyed by exposure to sunlight or to a mercury vapor lamp. About 96 per cent of the riboflavin in the milk was destroyed.

Riboflavin deficiency symptoms were similar to those observed by Wiese and his group. Post mortem examinations showed a mild catarrhal enteritis, mild edema of the cerebrum and some pathological changes in the kidney. Administration of 2 mg of riboflavin daily to one deficient calf promptly restored the appetite, arrested the diarrhea and in time completely restored a thrifty condition. One calf on the same diet supplemented with 2.99 mg of riboflavin daily throughout the experiment grew normally without showing any disorder or symptom of deficiency.

Very little work has been done on the requirement of the suckling lamb for specific nutrients. Pearson and Schweigert (1947) attempted to increase the level of riboflavin in milk of ewes by supplementing the natural ration with extra crystalline riboflavin. The results showed that the riboflavin level was not greatly affected.

The site of destruction of orally administered riboflavin and pantothenic acid in the sheep was investigated by Olcese and Pearson (1948). They found that both vitamins were very rapidly and effectively destroyed in the gastrointestinal tract.

Other Laboratory Animals

The requirement of riboflavin as an essential dietary factor in the ration has been demonstrated for a number of laboratory animals and man.

Lepkovsky and Jukes (1936), Stokstad and Manning (1938) and Phillips and Engel (1938) produced riboflavin deficiency symptoms in chicks and turkeys. In chicks the deficiency might be acute, characterized by general paralysis and a neuromalacia-like disorder or chronic type characterized by "curled toe" paralysis. Both conditions were accompanied by degenerative changes in the myelin sheaths of the main peripheral nerve trunks, swelling and fragmentation of axis cylinder and possible degeneration of motor end plates and muscle fibers. In turkeys the symptoms consisted of slow growth, diarrhea, emaciation and severe dermatitis.

There was no agreement concerning characteristic symptoms of riboflavin deficiency in the dog. Zimmerman and Burack (1934), Street and Cowgill (1939) and Street et al. (1941) described the symptoms as diarrhea, vomiting, marked muscular weakness and sudden collapse accompanied by a fall in body temperature, fall in respiratory rate and variable heart rate. In chronic riboflavin deficiency the authors observed neurologic abnormalities characterized by degeneration of peripheral nerves and posterior columns of the spinal cord. The authors also observed opacity of the cornea in a few cases.

Potter et al. (1942) produced symptoms of riboflavin deficiency in the dog similar to those observed in the rat; that is, dermatitis. The authors also observed a mild microcytic hypochromic anemia and fatty degeneration of the liver.

Riboflavin was also shown to be essential for the mouse (Jones et al., 1945), cotton rat (McIntire et al., 1944), hamster (Routh and Houchin, 1942), monkey (Waisman, 1944 and Cooperman et al., 1945) and man (Sebrell and Butler, 1938).

EXPERIMENTAL PROCEDURE

Animals Used

Altogether 25 lambs were used. The various treatments and distribution of breeds, sexes, ages and weights when put on the experimental diets are summarized in table 1. Lambs of both mutton and fine wool type representing six different purebred breeds and one crossbred lamb (Suffolk and Hampshire) were obtained from the Experiment Station flock at Michigan State College. The system used in assigning lambs for different treatments consisted of random allotment as the lambs were born during January and February in pens in the clinic and during spring months in the college experimental barns. Lambs of both sexes, single or twins were used without discrimination and the only prerequisite was health and normal condition. The age of the lambs when put on the experimental diet varied from two to eleven days. All lambs received colostrum before being taken from the ewes.

Feeding and Care

Throughout the experiment all the animals were kept in concrete-floored pens. The temperature and humidity was fairly constant. Usually two or three lambs were in one 12 x 4 foot pen and were changed from one pen to another every other day. The pens were then scrubbed and cleaned with hot water and disinfected with a lye solution to elimin-

TABLE 1

DISTRIBUTION OF LAMBS IN THE EXPERIMENTAL GROUPS

Trial	Lamb No	Treatment	Breed	Sex	Age when Put on experiment (Days)	Weight when put on experiment (lbs)
	2	Riboflavin deficient diet	Cotswold	F	2	11.5
	4	"	Oxford	F	3	9.0
	5	"	Cotswold	F	2	10.0
	6	"	Shropshire	M	2	7.2
	7	"	"	M	2	7.0
1	10	Depleted - Treated	Suffolk	M	3	10.2
	11	"	Shropshire	F	3	9.5
	1	Positive control	Rambouillet	M	9	11.0
	3	"	Suffolk x Hampshire	F	3	9.4
	8	"	Rambouillet	M	11	15.0
	9	"	"	M	3	11.5
	12	"	"	M	8	13.0
	13	5 micrograms riboflavin per lb per day	Rambouillet	F	3	9.5
	14	5 "	"	M	10	11.8
	15	50 "	"	F	3	7.0
	16	50 "	Dorset	M	4	7.5
	17	150 "	Rambouillet	M	3	8.5
2	18	150 "	Dorset	M	4	8.0
	19	250 "	Rambouillet	F	5	9.2
	21	350 "	Dorset	F	6	10.0
	23	250 "	"	M	4	7.0
	25	350 "	"	M	3	7.6
	20	Positive control	Rambouillet	M	11	13.1
	22	"	"	F	5	10.6
	24	"	"	F	4	12.8

ate as far as possible complications with outside diseases. Burlap bags filled with a small quantity of wood shavings served for bedding. These were also changed every other day. The lambs were fed from nursing bottles five times a day at 7 and 11 A.M. and 3, 7 and 11 P.M. The milk was kept under refrigeration and an estimated quantity was thoroughly shaken and warmed to about 80° F. prior to feeding. The amount of milk each lamb consumed per feeding was recorded together with observations and remarks regarding health, appetite and general behavior. All the animals were weighed every other day before the morning feeding. The weights were estimated to the tenth of a pound and recorded for further analysis.

Composition and Preparation of Synthetic Milk

The composition and method of preparation of the purified diet used in this study were similar to those described by Wiese et al., (1947b). The salt mixture was similar to that described by Johnson et al. (1948). The compositions are shown in tables 2 and 3. The synthetic milk contained 16 per cent solids which was slightly higher than the average per cent of solids in milk of normal lactating ewes. The B vitamins were dissolved in a 20 per cent alcohol solution in such a proportion that 10 ml of the stock solution supplied the necessary vitamins for one liter of milk. Stock solution of fat soluble vitamins was prepared by fortification of cod liver oil with pure vitamin A and D and by adding vitamin E

TABLE 2

COMPOSITION OF THE PURIFIED DIET

Component	Per cent	Vitamins	Mg. per kg. of Liquid Diet
Casein (Labco)	30	Thiamine	1.4
Glucose	28	Riboflavin	3.0
Lactose	10	Nicotinic acid	4.0
Lard	26	Calcium panto-thenate	5.0
Salt mixture	6	Pyridoxine	2.0
		Pteroyl glutamic acid	0.2
		p-Amino benzoic acid	4.0
		Inositol	40.0
		Biotin	0.04
		Choline	400.0
		Alpha-Tocopherol acetate	2.0
		2-Methyl naphtho-quinone	0.4
		Vitamin A	4000 I.U.
		Vitamin D	500 I.U.

TABLE 3

COMPOSITION OF SALT MIXTURE (5 Kgs.)

Component	Grams
NaCl	594.00
K_2HPO_4	1288.00
$CaHPO_4$	1690.00
Ca lactate	1144.00
$MgSO_4$	177.00
$FeSO_4$	96.50
KI	3.00
$MnSO_4$	5.00
$ZnCl_2$	1.00
$CuSO_4 \cdot 5H_2O$	1.00
$COCl_2$	0.50
	5000.00

and K in such manner that 1 ml of the stock solution was used for one liter of milk. Both stock solutions were prepared in large quantities and kept under refrigeration.

After a few trials the method finally adapted for preparing the milk was as follows. Distilled water (29,500 ml) was measured into an ordinary 10 gallon milk can and heated with steam to 80-85° C. Sodium bicarbonate (82 gms) was dissolved in this water and vitamin free casein (Labco)* was brought in suspension by means of an electric stirrer modified for this purpose. The casein was added slowly with constant rapid stirring which was continued 30 to 45 minutes to obtain a complete dissolution of casein. Glucose (1613 gms) and lactose (576 gms) were then dissolved in this homogeneous suspension. Meanwhile, commercial lard (1498 gms) was heated to 80-85° C on the steam bath and mixed with 36 ml of stock solution of fat soluble vitamins. This lard-fat soluble vitamin solution was slowly added to the casein solution and the stirring accelerated. In this state the solution of casein, sugars and lard was homogenized in a Manton-Gaulin homogenizer** at 3,200-3,500 pounds pressure per square inch.

The salt mixture (345 gms) was dissolved in a part of the already homogenized milk using Waring blendors. Not more

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*The Borden Company, Labco and Vitamin Products Department, 350 Madison Avenue, New York 17, N.Y.

**Manton Gaulin Manufacturing Co., Inc., Everett 49, Mass.

than about 50 gm of salt were added to an appropriate amount of milk in the blendor and agitated for 7 to 10 minutes at full speed to insure a complete dissolution of all minerals. Salt in this form and the appropriate amount of B vitamins stock solution were added to the homogenized milk, stirred again for 2-3 minutes before the milk was bottled into the clean and sterilized gallon bottles. The milk was kept under refrigeration at 42° F except when used for feeding. Synthetic milk prepared and stored under the above conditions kept well for over two weeks. Usually two cans of milk were prepared at one time. This quantity was sufficient for 7 to 10 days.

Blood Studies

To determine the initial and presumably normal blood picture all the lambs were bled as soon as possible and never later than five days after being placed on the experimental diets. The blood was collected every week thereafter and the hemoglobin concentration, total and differential leukocyte counts were determined. About one ml of blood was drawn from the jugular vein into a vial containing a small amount of dry potassium oxalate as anti-coagulant. A blood smear for differential leukocyte count was prepared and stained with Wrights stain. The hemoglobin concentration was determined by "Cenco-Sheared-Sanford-Photelometer"* and

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*The Cenco-Sheared-Sanford Photelometer, Central Scientific Company, Chicago, Illinois.

expressed in grams per 100 ml of blood. The total leukocyte count was determined by using a one per cent solution 0.1 N HCl to which was added one or two drops of gentian violet. A hemocytometer with Neubauer ruling was used for the actual enumeration. The oil immersion objective was used to classify the leukocytes covering as many as possible different areas of the film in order to obtain a reliable count.

Urine Studies

After two weeks on the experimental diet both deficient and control lambs were placed in a $2\frac{1}{2}$' x 4' metabolism cage with a fine wire mesh floor and a 24-hour urine sample collected for riboflavin assay. The urine was collected in a brown glass bottle containing about 5 ml of toluene and acetic acid as preservatives. The 24-hour excretion of urine was measured and immediately assayed in the Agricultural Chemistry laboratory using Lactobacillus casei.

Gross Pathology Studies

Lambs were observed closely every day at each feeding and the observations recorded. Whenever it was evident that the lamb would die in the period between feedings or during the night the animal was sacrificed by exsanguination under ether anesthesia and necropsied. The control lambs were sacrificed in a similar manner. In a few instances the deficient lambs died suddenly and necropsy was performed as soon as possible. As soon as the carcass was opened, the lungs,

liver and heart-blood were cultured for bacteria on blood-agar plates. These were then incubated at 37° C for two or three days and the results recorded. After the organs have been cultured the whole carcass and every organ were carefully examined for gross lesions.

Microscopic Studies

Sections for histopathological studies were taken from the liver, lung, kidney, heart, adrenals, stomach (pylorus) duodenum, jejunum, ileum, ileocecal valve and colon. These tissues were fixed in Zenker's fluid, embedded in parafin, cut at 7 micra and stained with hematoxylin-eosin. Sections from the liver, kidney, heart and adrenals were also fixed in 10 per cent formol-saline solution, cut as frozen sections and stained with Sudan IV. Tissue from the corner of the mouth, brain, sciatic nerve, spinal cord, and eye were prepared for histopathological studies from three deficient and two control lambs.

Riboflavin Requirement

The first trial consisted of five control and seven lambs on the riboflavin deficient diet. Two lambs when depleted and showing advanced symptoms of deficiency were treated and cured with riboflavin.

In the second trial were three control and ten experimental lambs. Calculated from the level of riboflavin in the milk consumed by the control lambs in the first trial the

approximate requirement of riboflavin per pound of body weight per day was 250 to 300 micrograms (table 4). The 10 experimental lambs were divided at random into five subgroups. The levels of riboflavin were set arbitrarily at 5, 50, 150, 250 and 350 micrograms per pound of body weight per day. This was added to the milk consumed at one feeding per day. Solutions of crystalline riboflavin in 20 per cent alcohol were made and the exact amount of riboflavin, calculated from body weight taken every other day, was measured with a pipette and mixed with the milk for the individual lamb just before feeding. Care was exercised that all milk so prepared was consumed, insuring the full intake of riboflavin. If necessary the lamb was forced to drink the amount of milk prepared.

TABLE 4

AVERAGE DAILY RIBOFLAVIN INTAKE OF FIVE CONTROL LAMBS CALCULATED FROM AN AVERAGE DAILY FEED CONSUMPTION AT DIFFERENT BODY WEIGHTS

Body Weight in lbs	Milk Consumed		Micrograms Riboflavin per lb Body Weight per Day
	oz	ml	
10	30	887	266
11	32	946	258
12	36	1064	277
13	38	1124	259
14	41	1212	259
15	44	1301	260
16	50	1478	276
17	57	1685	297
18	57	1685	279
19	58	1715	270
20	59	1745	261
21	57	1685	240
22	56	1656	226
23	56	1656	215
24	57	1685	211

RESULTS

Growth

The gain in body weight and the efficiency of feed utilization of five control lambs, and five lambs on the riboflavin deficient diet are indicated in tables 5 and 6. Table 7 shows the gain in body weight and feed utilization of two lambs during the depletion period and during the period of riboflavin therapy. The individual growth curves of all seven lambs on the experimental diet and one curve representing the average growth of the five control lambs are illustrated in graph 1. A study of table 5 disclosed that the average daily gain of the control lambs over the twenty-eight-day period was 0.44 lb. Over the same period the control lambs required 1.07 lb. of dry matter to produce a one lb gain in body weight and consumed an average of 0.54 lb of dry matter daily. The average daily gain of the five lambs fed the riboflavin deficient diet over a period of 19 days was 0.054 lb and the dry matter required to produce a one lb gain in body weight was 5.47 lbs (Table 6). Since the average survival time of the lambs fed the riboflavin deficient diet was 18 days, a more accurate comparison of the control and deficient groups would necessitate taking into account the growth performance of the control lambs for only the first eighteen days. This is illustrated in

TABLE 5

GROWTH RESPONSE AND FEED UTILIZATION OF CONTROL LAMBS
(3 mg. of riboflavin in kg. liquid diet)

Lamb No	1	3	8	9	12	Average
Age when put on experiment, days	9	3	11	3	8	7
Weight when put on experiment, lbs.	11.0	9.4	15.0	11.5	13.0	11.5
Days on experiment	34	26	25	30	23	28
Total gain, lbs.	13.8	13.0	10.3	14.0	10.0	12.22
Av. daily gain, lbs.	0.40	0.50	0.41	0.46	0.43	0.44
Ttoal dry matter consumed, lbs.	15.0	13.2	12.3	14.74	9.97	13.04
Lbs. dry matter per lb. gain	1.08	1.01	1.19	1.05	0.997	1.07

TABLE 6

GROWTH RESPONSE AND FEED UTILIZATION OF RIBOFLAVIN DEFICIENT LAMBS

Lamb No	2	4	5	6	7	Average
Age when put on experiment, days	2	3	2	2	2	2
Weight when put on experiment, lbs.	11.5	9.0	10.0	7.2	7	8.9
Days on experiment	18	21	18	17	23	19
Total gain, lbs.	1.5	0.8	1.5	0.6	0.5	0.98
Av. daily gain, lbs.	0.083	0.040	0.083	0.035	0.022	0.054
Total dry matter consumed, lbs.	5.57	4.56	5.30	3.30	4.45	4.64
Lbs. dry matter per lb. gain	3.71	5.7	3.53	5.50	8.9	5.47

TABLE 7

RESPONSE TO RIBOFLAVIN THERAPY

Lamb No	10		11	
Age when put on experiment, days	3		3	
Weight when put on experiment, lbs.	10.2		9.5	
Diet fed	Riboflavin Deficient	Riboflavin Therapy	Riboflavin Deficient	Riboflavin Therapy
Days on experiment	19	21	16	14
Total gain, lbs.	0.3	9.2	0.9	7.4
Av. daily gain, lbs.	0.015	0.44	0.056	0.53
Total dry matter consumed, lbs.	4.78	9.82	3.3	6.6
Lbs. dry matter per lb. gain	15.9	1.06	3.7	0.89

Graph 1. Growth curves of control, riboflavin deficient and riboflavin treated lambs.

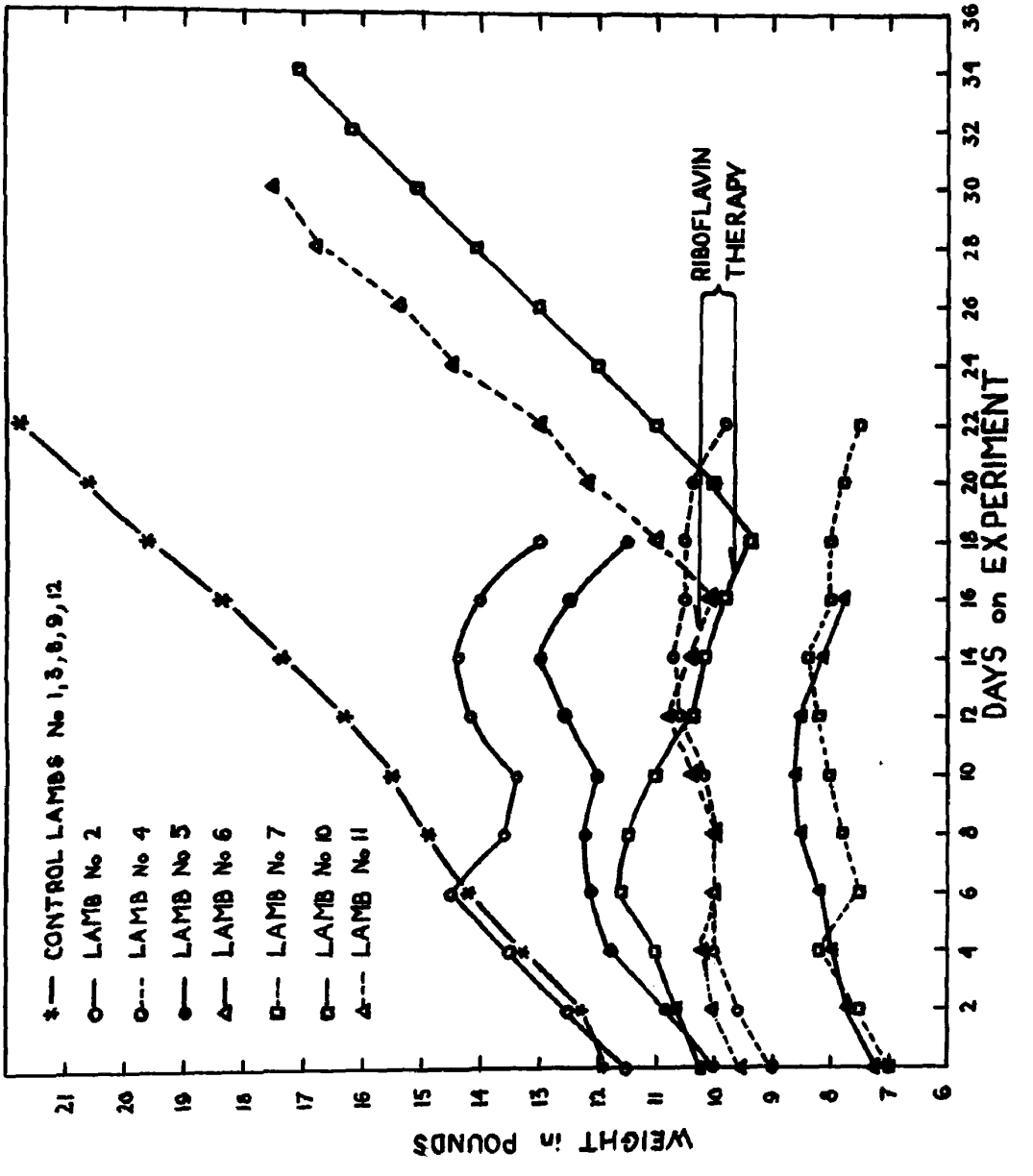

CONTROL LAMBS No 1, 3, 8, 9, 12
LAMB No 2
LAMB No 4
LAMB No 5
LAMB No 6
LAMB No 7
LAMB No 10
LAMB No 11
RIBOFLAVIN THERAPY
WEIGHT in POUNDS
DAYS on EXPERIMENT

table 8. The data from three control lambs from the second trial are also included in this table. The average daily gain of 0.043 lb for the riboflavin deficient lambs as compared to the average daily gain of 0.39 lb for the control group indicated that the young lamb has a definite requirement for riboflavin.

It can be seen from graph 1 that the lambs fed the riboflavin deficient diet grew at a normal rate for about 4 to 6 days. At this time the appetite of the animals declined for about 3 to 4 days while their weights remained the same or slightly decreased. The appetite improved to some extent between the eighth and fourteenth day and the lambs made some gain in body weight. After 12 to 14 days on the experiment the lambs lost their appetites completely, lost weight rapidly, became emaciated and, on the seventeenth to twenty-third day, all deficient lambs were in a moribund condition. Two lambs died, three were sacrificed and two lambs were treated with riboflavin on the sixteenth and nineteenth day on the experimental period (Table 7).

The general condition of the treated lambs improved in hours after the injection of riboflavin and the normal appetite was restored in two days. The animals were gaining weight by the second day and in one week all the symptoms, including emaciation, disappeared. While, during the period of seventeen days on the deficient diet, the average daily gain was 0.035 lb and 9.82 lbs of dry matter was needed to produce one lb of gain; during the seventeen day period

TABLE 8

GROWTH RESPONSE OF THE CONTROL AND DEFICIENT LAMBS FOR THE FIRST EIGHTEEN DAYS OF THE TRIAL

	Control	Riboflavin Deficient
Number animals	8	7
Initial age days	3 - 11	2 - 3
Av. initial weight, lbs	12	9.3
Av. eighteen day weight, lbs*	19.1	10.0
Days on experiment	18	18
Av. daily gain, lbs	0.39	0.043
Av. daily dry matter consumed, lbs	0.41	0.14
Lbs dry matter lb gain	1.18	7.63

*The control lambs were fed the same diet for 23 to 36 days.

of riboflavin treatment, the average daily gain in body weight was 0.48 lb. and only 0.97 lb. of dry matter was required to produce one lb. of weight. The growth response to riboflavin of the two lambs (No. 10, 11) is also illustrated in graph 1.

Symptoms of Deficiency

The growth of the control lambs was uneventful except for occasional mild diarrhea, probably due to overfeeding. The diarrhea soon disappeared without treatment. After about six days the lambs fed the deficient diet developed a severe diarrhea accompanied by loss of appetite. The lambs became unthrifty, depressed and moved slowly. The diarrhea receded after about four to six days and the appetite improved temporarily. After the twelfth day the deficient lambs developed difficulties in breathing which progressively became the typical symptoms of pneumonia characterized by rapid and labored breathing and dullness. The animals refused to eat, became lethargic, would not walk or stand and the condition terminated by a complete collapse and death.

The lambs were observed every day but the oral lesions, excessive salivation and lachrymation, loss of hair and inflammation of the navel area, the typical symptoms of riboflavin deficiency in calves described by Wiese et al. (1947) and Warner and Sutton (1948) were not noted in riboflavin deficient lambs. The principle and only symptoms of riboflavin deficiency in the lamb were loss of appetite, diarrhea, retarded growth,

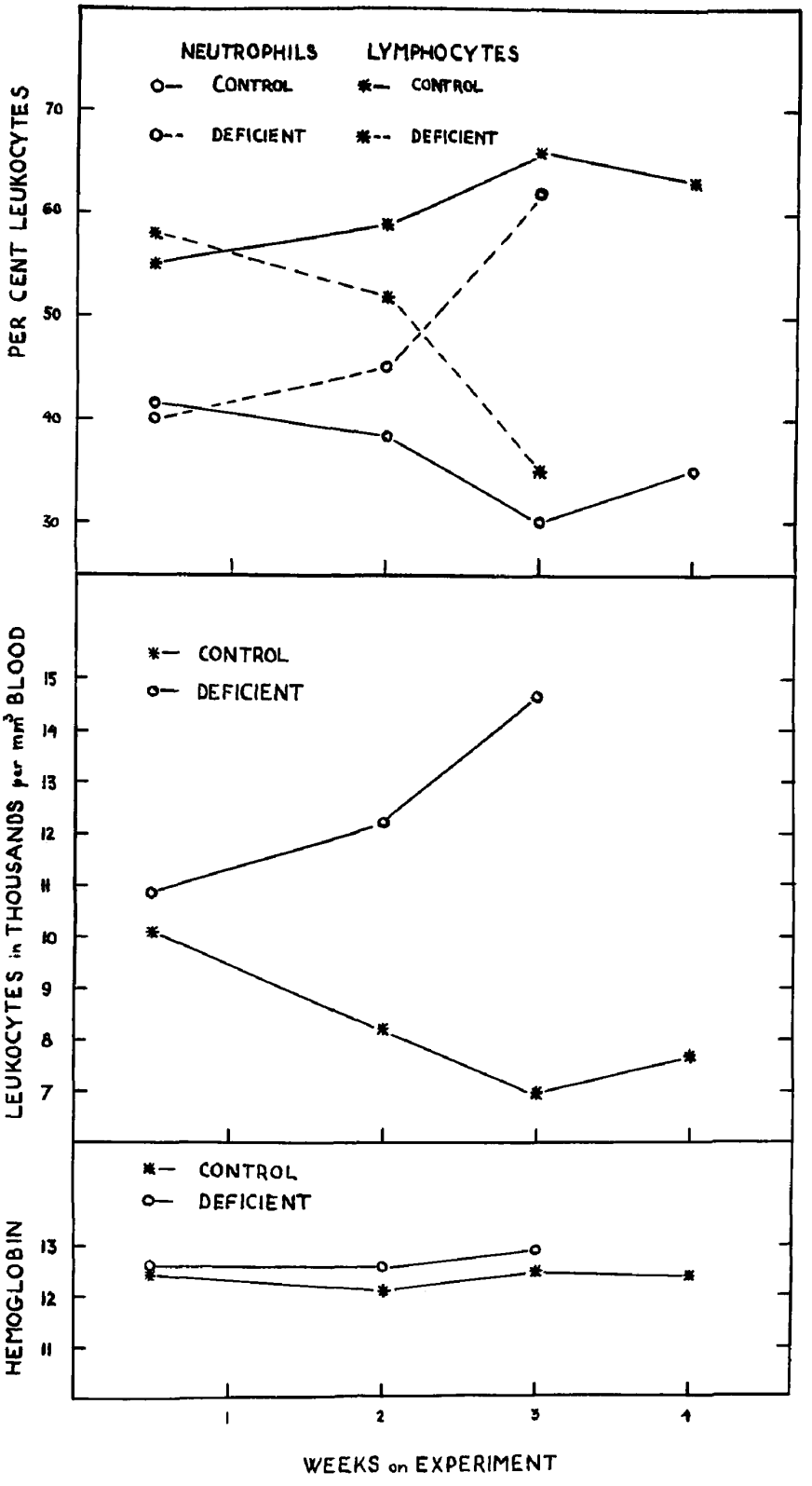
followed by emaciation, dullness, pneumonia and death.

Blood Studies

The results of the initial and weekly blood analyses of the eight control and seven deficient lambs were summarized in table 10 (page 52) and graph 2. The average initial hemoglobin levels of the control and experimental groups were 12.6 and 12.8 gm per 100cc of blood, respectively, with values ranging from 10.8 to 14.2 gm per cent. The levels of hemoglobin remained fairly constant throughout the experiment. The initial leukocyte count per mm^3 and the ratio of polymorphonuclear leukocytes to lymphocytes of both control and deficient lambs showed only slight variation. The values of the leukocytic counts were 10,125 and 10,950 per mm^3, and the ratios of neutrophils to lymphocytes were 42:55 and 46:52.

The most significant difference between the control and riboflavin depleted lambs was found in the leukocyte count. During the experimental period the leukocyte count of the control lambs decreased from the initial 10,125 to 7,700 per mm^3 while the number of leukocytes of the deficient lambs gradually increased and reached 14,650 per mm^3 by the third week. This increase, no doubt, was due to the pneumonia which also was reflected in a reversal of the differential count. During the experiment the neutrophils of the control lambs gradually decreased in favor of the lymphocytes and at the end of the experiment the ratio was 35:63, which is within

Graph 2. Hemoglobin, leukocyte and differential counts of control and riboflavin deficient lambs.


NEUTROPHILS
CONTROL
DEFICIENT
LYMPHOCYTES
CONTROL
DEFICIENT
PER CENT LEUKOCYTES
70
60
50
40
30
CONTROL
DEFICIENT
LEUKOCYTES in THOUSANDS per mm³ BLOOD
15
14
13
12
11
10
9
8
7
CONTROL
DEFICIENT
HEMOGLOBIN
13
12
11
1
2
3
4
WEEKS on EXPERIMENT

normal range for young healthy lambs. However, in the deficient lambs, the ratio reversed and, especially during the final stages of deficiency, the number of neutrophils greatly increased. By the third week, just before the lambs died, were sacrificed or treated, the ratio was 62 polymorphonuclear leukocytes to 35 lymphocytes. Monocytes, eosinophils and basophils showed no changes in numbers.

Urine Studies

It was difficult to make an accurate comparison of the urinary excretion of riboflavin from the control and depleted animals. Only two metabolism cages were available for collection of the 24-hour urine samples. As a result, the assays were staggered over the last two weeks of the experiment which represented two thirds of the total survival period of the deficient group. These circumstances eliminated the possibility of simultaneously comparing the two groups. The average urinary excretion per 24 hours was then calculated by pooling the values of riboflavin excreted from all lambs in a particular group obtained during the two week period. Usually one control and one deficient lamb were selected at random for urine collection in one day. The variation in urinary excretion of riboflavin within the group was rather marked but there was always a definite and striking difference between the values of the control and depleted animals. The average urinary excretion of riboflavin of the eight control lambs was 0.50 mg.

per 24 hours while that of the seven deficient lambs was 0.01 mg per 24 hours.

Gross Pathology

Controls. One of the five control lambs was released from the experiment when about 25 lbs in weight and did not show any signs of ill health. One control lamb died suddenly after two weeks. Post mortem examination revealed that the cause of death was overeating and not directly related to the diet nor to its composition. The carcass was well nourished. The intestinal tract showed severe enteritis which extended from the duodenum to the rectum. The intestinal contents were bloody. Soon after being put on the experimental diet the lamb showed some clinical symptoms of pneumonia. However, at the time of death, no evidence of pneumonia was observed. Cultures prepared from lungs, liver, gall bladder, spleen and heart blood were negative. Three ribs had been broken in early life, probably at lambing, which would explain the labored breathing. The other three control lambs were sacrificed for gross and histopathological studies after three, four and five weeks on the experimental diet. The carcasses were in excellent condition and did not show any gross pathological changes except two broken ribs in one lamb. The lungs, livers, gall bladders and spleens were cultured on blood agar plates and were found negative after a suitable period of incubation.

Riboflavin deficient lambs. Of the seven riboflavin deficient lambs, two died suddenly and necropsies were performed. Three lambs were sacrificed when in a moribund condition as a result of complete depletion of riboflavin. Two lambs were treated with riboflavin for 17 days after a 17 day period of depletion and then sacrificed for study. The gross pathology of the five riboflavin deficient lambs was similar with only minor variation. The principal features were (1) lung involvement with bronchopneumonia, (2) fatty degeneration of the liver and (3) fatty degeneration of the kidney. The lungs of all five depleted animals showed bilateral bronchopneumonia which seemed to be progressive, rapidly spreading from apical to cardic and diaphragmatic lobes of both lungs. There was usually a marked zone of congestion between the pneumonic areas and the normal part of the lung. In one lamb, grayish fibrinous exudate was present between the lungs and the thoracic wall and the lungs adhered to the ribs. When cut and squeezed, the consolidated areas showed frothy mucous exudate in the trachea, bronchi and bronchioles. Cultures prepared from lungs and bronchi yielded pleomorphic gram negative rods resembling Pasteurellae organisms. Post mortem examination of the two riboflavin treated lambs, previously depleted on riboflavin deficient diet, showed "cold pneumonia" in the right apical lobe in both lambs. The line of demarkation between the normal and the pneumonic part was not distinct and resolution was in progress. Cultures prepared from these areas were negative.

Macroscopic examination of the liver from deficient animals showed pathological changes ranging from mild degeneration to necrosis. In three lambs, several necrotic areas were observed in different parts of the liver. Otherwise the changes consisted of generalized fatty infiltration and degeneration characterized by enlargement of the organ, yellowish or brown grayish color, smooth surface, soft consistency and increased friability (Fig. 1). The liver of the two treated animals did not show macroscopic lesions.

The changes in the kidneys of all the riboflavin deficient lambs followed the same pattern. There was a marked fatty degeneration and the cortex was always much more affected than the medulla. The line of demarcation between the cortex and the medulla was very pronounced. The medullary zone was usually congested and slight edema was present in the pelvis. The outer surface of the kidney was of a dull, yellow color and in some instances patchy. When cut, the surface bulged and had a prominent parboiled appearance. In more severe cases the cut surface easily fluctuated on pressure and the streaks running from the outer surface to the pelvis were prominent (Fig. 1). The kidneys of the two riboflavin treated lambs showed a very mild fatty infiltration characterized by a slightly yellow cortex. The medulla and pelvis were normal.

The heart of the riboflavin deficient lambs appeared normal. The ductus arteriosus and foramen ovale of all the lambs wereclosed. Due to the presence of pneumonia in prac-

tically every case, there was an increase of fluid in the pericardial sac.

Grossly, the adrenals were enlarged. The cut surface showed a marked line of demarcation between the cortex and medulla. In most instances hemorrhage was clearly visible. On section the outer surface and the cortex were pale and flabby (Fig. 1). The other organs, including the whole digestive tract, thyroid, eye and spleen, were normal. The same was true in regard to the skeletal, nervous and muscular system.

Microscopic Pathology

Controls. Microscopic studies of the tissues, taken from the control lambs, failed to reveal pathological changes. In two lambs frozen sections from the liver, stained with Sudan IV, showed a mild fatty infiltration which was considered to be a physiological increase of hepatic fat.

Riboflavin deficient lambs. The microscopic changes in the deficient lambs were confined to the liver, kidney and adrenals. The changes in the liver ranged from cloudy swelling to focal necrosis. Neither of these extreme conditions were typical of the deficiency. The most prominent feature, common in all deficient lambs, was diffuse fatty degeneration, affecting most of the hepatic cells in the lobule. Usually the most pronounced fatty degeneration was in the center of the lobule (Fig. 2) while on the periphery the hepatic cells were still

intact or undergoing various degrees of cloudy swelling. One or more fat globules of varying size filled the hepatic cells and pushed the nuclei against one side of the cell wall, depriving the cell of nutrition which resulted in karyolysis, karyorrhexis and pyknosis. In most instances the liver showed acute venous congestion which was generalized and very marked. The sinusoids were greatly distended and occasionally filled with an excessive number of blood cells. Necrosis observed in few instances was focal in character, located usually on the surface of the liver and on the periphery of the lobule. The sinusoids were damaged. The necrotic areas were partly or completely surrounded by hepatic cells undergoing fatty degeneration and cloudy swelling.

The most uniform microscopic lesions were found in the kidney. The cortex showed extensive and diffuse fatty degeneration while the medulla underwent various degrees of cloudy swelling or mild fatty changes. The kidney was markedly congested and hemorrhagic and involved both parenchyma and intestinal tissues. The renal corpuscles, including Bowman capsules and glomeruli, seemed to be normal. In two lambs there was a tendency toward hyaline changes within the glomeruli and convoluted tubules. The basement membrane of the capillary loops seemed to be slightly thickened and the capillaries slightly dilated. The epithelium of the convoluted tubules showed marked cloudy swelling and severe fatty changes (Fig. 3). The cells were swollen, the cytoplasm appeared

granular and projected into the lumen of the tubules. In some tubules the cells probably ruptured and the material formed hyaline casts in the convoluted tubules. These tubules were dilated, the epithelial cells were atrophied and the nuclei degenerated. Frozen sections stained with Sudan IV showed that most of the fat had accumulated in the convoluted tubules (Fig. 4). Some cellular infiltration and proliferation of interstitial tissue in the medulla was observed in one lamb. Lymphocytes were predominant but a few macrophages also were present.

It was difficult to delineate between the physiological and pathological fatty infiltration of the adrenals. Microscopic studies of the frozen sections from the adrenals of the control lambs revealed the presence of fat droplets between the glomerulosa and fasciculata of the cortex. Sections from the deficient lambs showed a slightly increased amount of fat in the same region. In two deficient lambs both the cortex and medulla were infiltrated with fat (Fig. 5, 6). Otherwise the adrenals from the deficient lambs were congested and the sinusoids, especially around the central vein, were dilated to some extent. Both the central vein and adjacent sinusoids were filled with blood. However, the margins of the sinusoids were intact.

Microscopic examination of the heart muscle, intestinal tract, skin from the corner of the mouth, eye, brain, spinal cord, bracenial and sciatic nerves, stained with hematoxylin

eosin and Kultschitzky's hematoxylin solution, failed to reveal pathological changes.

Treated Lambs. Microscopic changes of the liver and kidney from the two riboflavin treated animals, previously depleted of the vitamin, were similar in nature to those of the deficient animals. The liver still showed granularity of the hepatic cells and some fat droplets within the cells. The sinusoids were less distended and there was less congestion throughout the liver. The epithelial cells of the convoluted tubules of the kidney were swollen and in some places the cells were desquamated. Albuminous granular material in the lumen of the tubules was occasionally observed. The glomeruli appeared normal.

Riboflavin Requirement

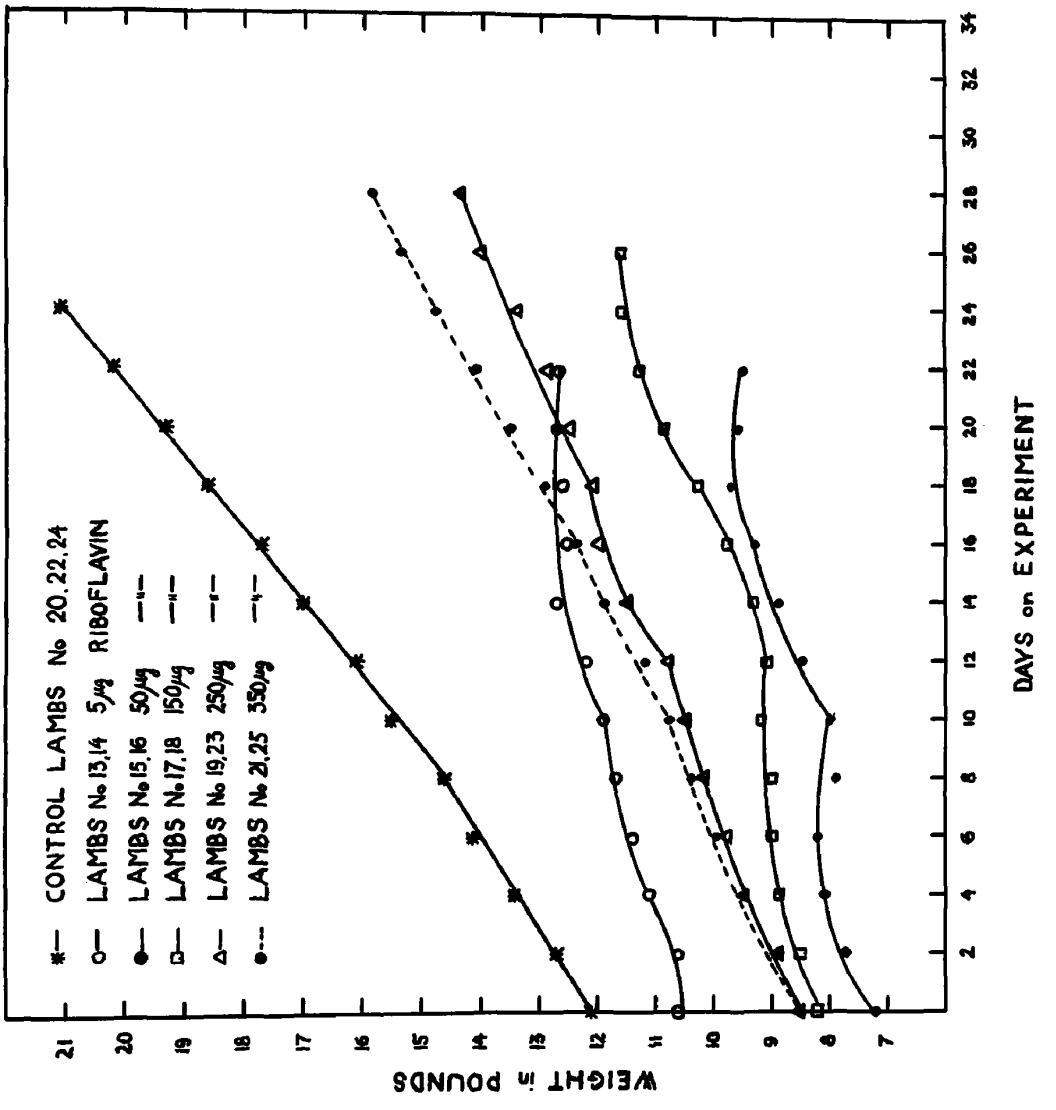
The results of the second trial in regard to growth and feed utilization are shown in table 9 and graph 3. The trial consisted of three positive control and ten lambs on five different levels of riboflavin. The growth curves are averages of the gains in body weight of two lambs in each subgroup while the growth curve of the controls represents three lambs. The three control lambs on the complete purified diet grew normally and the gains in body weight and feed utilization compared with the five control lambs from the first trial. Two control lambs were released from the experiment after 27 and 35 days. The third lamb was sacrificed for gross and histopathological studies.

TABLE 9

GROWTH RESPONSE AND FEED UTILIZATION OF CONTROL LAMBS AND LAMBS ON VARIOUS LEVELS OF RIBOFLAVIN

Lamb No	13	14	15	16	17	18	19	23	21	25	20	22	24
Age when put on experiment, days	3	10	3	4	3	4	5	4	6	3	11	5	4
Weight when put on experiment, lbs.	9.5	11.8	7.0	7.5	8.5	8.0	9.2	7.0	10.0	7.6	13.1	10.6	12.8
Level of riboflavin micrograms/lb/day	5	5	50	50	150	150	250	250	350	350	Control	Control	Control
Days on experiment	21	37	42	25	42	45	33	45	27	45	27	18	35
Total gain	1.0	3.0	4.0	1.1	6.5	4.5	6.0	10.4	7.8	13.4	13.7	5.4	12.0
Av. daily gain	0.047	0.081	0.095	0.045	0.15	0.10	0.18	0.23	0.29	0.32	0.51	0.30	0.34
Total dry matter consumed	5.76	7.90	9.96	4.87	13.45	10.98	8.87	12.58	8.78	12.93	13.7	6.88	16.24
Lbs. dry matter per lb. gain	5.92	2.63	2.49	4.43	2.07	2.44	1.44	1.21	1.26	1.24	1.0	1.27	1.35

Graph 3. Growth curves of control lambs and lambs on various levels of riboflavin.


CONTROL LAMBS No 20.22.24
LAMBS No 13,14 5/µg RIBOFLAVIN
LAMBS No 15,16 50/µg
LAMBS No 17,18 150/µg
LAMBS No 19,23 250/µg
LAMBS No 21,25 350/µg
WEIGHT in POUNDS
DAYS on EXPERIMENT

The post mortem examination did not show lesions. The same was true for the microscopic studies of the tissues from various organs. The four lambs on the two lowest levels of riboflavin (5 and 50 micrograms per lb of body weight per day) resembled the lambs on the riboflavin deficient diet in growth, feed utilization, symptomat. ology, gross and microscopic pathology. The growth was poor, due to the intermittent loss of appetite and diarrhea. One lamb died after 21 days on the experimental diet and the other three animals were necropsied for further studies. Congestion of the lungs and pneumonia were found in two lambs. All four lambs, however, showed mild to severe fatty degeneration of the liver and kidney and enlargement of the adrenals. After 37 days on the experimental diet, the liver and kidney of the lamb on the 5 micrograms riboflavin level showed a severe fatty degeneration, characterized by an intense yellow appearance and mottling of the liver and by prominent streaks and yellow color of the cortex of the kidney. An intense zone of congestion at the junction of the cortex and medulla was also observed. The ileum from the same lamb was thickened and wrinkled in some areas and showed some congestion. The growth response of the lambs on 150, 250 and 350 micrograms of riboflavin was directly proportional to the amount of riboflavin administered to the respective lamb. The average daily gain was 0.125, 0.205 and 0.305 lb. The amount of 2.25, 1.32 and 1.25 lb of dry matter were needed respectively to produce one lb of body weight. The growth

of the lambs on 150 and 250 micrograms was rather slow but the animals showed less diarrhea and retained a fair appetite throughout the experiment. All six lambs remained in good health and fairly good condition. The lambs on 350 micrograms and one lamb on 250 micrograms were released from the experiment and the other three animals were sacrificed.

The lambs on 150 micrograms of riboflavin showed fatty degeneration of the kidney and liver and enlarged adrenals. No pneumonia or congestion of the lung was observed. The results of the weekly blood analyses did not follow any consistent pattern except in the two lambs on the lowest riboflavin level. The leukocyte count of those lambs increased toward the end of the experimental period. The differential count, however, was unchanged. If the growth response, expressed in gain of body weight, was used as a criterion for establishing the riboflavin requirement of the young lamb, it seemed that a level of 350 micrograms per lb of body weight per day was high enough to insure a normal growth or growth comparable with the control animals.

There was no difference between the four lambs on the lowest levels of riboflavin (5 and 50 micrograms/lb/day) and the deficient lambs as far as the microscopic pathology was concerned. One lamb on 50 micrograms of riboflavin actually showed much more pronounced changes in the kidney than any other deficient lamb. The glomeruli were markedly congested and practically all of them were in a state of early hyaline

degeneration (Fig. 7). Hyaline droplets and protein material were escaping from the capillary loops and were concentrated on the side opposite to the vascular pole of the renal corpuscles between the parietal and visceral layers of Bowman's capsule. There was degeneration of the capillary loops and in a few instances the glomeruli were completely atrophied. The liver showed various degrees of cloudy swelling and fatty degeneration.

Of the four lambs on the two highest riboflavin levels (250, 350 micrograms/lb/day), only one (350 micrograms) was sacrificed for further studies. Only slight changes in the liver and kidney were observed. However, these were not considered to be significant. Fair growth and condition and good health suggested that the level was adequate to support the vital processes of the kidney and liver.

DISCUSSION

The growth data and growth curves (Tables 5 and 9, Graphs 1 and 3) representing eight control lambs on a complete purified liquid diet (Tables 2 and 3) for a period of 18 to 35 days indicated that the purified diet (synthetic milk) prepared in the laboratory was adequate in all the nutrients to support normal growth and health of the young lamb. The average daily gain of the control lambs was 0.40 lb. and required 1.17 lbs. of dry matter to produce 1 lb. gain in body weight. Kean and Henning (1949) in a study of the daily gains of 787 lambs from birth to 32-36 pounds in body weight reported an average gain of 0.53 lb. per day.

In spite of the fact that the lambs were fed only five times a day and received a rather limited amount of the ration to avoid overfeeding and the complications resulting from it, the growth of the control lambs compared favorably with the growth of lambs running with ewes and nursing at will. Five of the control lambs were sacrificed at the end of the experiment. Gross and microscopic examination of the carcasses and organs did not show significant changes.

Williams *et al.* (1950) defined the B vitamins as "those organic substances which act catalytically in all living cells and which function nutritionally for at least some of the higher animals". Since the first studies of

riboflavin deficiency in the rat by Chick and Roscoe in 1928 and the establishment of riboflavin constitution and its synthesis in 1935, the function of riboflavin as an essential nutrient for a number of higher animals was studied and established. Riboflavin has been shown to be an essential nutrient for the rat (Chick and Roscoe, 1928; Day and Langston, 1934; Bessey and Wolbach, 1939); mouse (Jones et al., 1945); cotton rat (McIntire et al., 1944); hamster (Routh and Houchin, 1942); monkey (Waisman, 1944); chicks and turkeys (Lepkovsky and Jukes, 1936; Phillips and Engel, 1938); dog (Zimmerman and Burack, 1934; Potter et al., 1942); pig (Hughes, 1939; Patek et al., 1941; Wintrobe et al., 1944; Krider et al., 1949) and calf (Wiese et al., 1947b; Warner and Sutton, 1948). Studies of the symptomathology of riboflavin deficiency in the above mentioned species of animals revealed some features common and characteristic to all. This observation agreed with the generally accepted idea that the B vitamins were required by every living cell of animal tissues and, if not available, the deficiencies would manifest themselves by similar symptoms in different species of animal. The studies of riboflavin deficiency in the lamb, however, revealed that, except for loss of appetite and retarded growth, none of the typical symptoms of aribоflavinosis, such as alopecia erythema and corneal vascularization in the rat and dog, dermatitis in the hamster and swine and cataract in the rat and swine, were observed in the riboflavin defi-

cient lamb. Furthermore, none of the characteristic symptoms of riboflavin deficiency found in the calf (Wiese et al., 1947b) were observed in the riboflavin depleted lamb. Some indication for the explanation of the striking difference in the symptomatology of the riboflavin deficient lambs was suggested by markedly increased riboflavin level in the colostrum and milk of the ewe as compared with colostrum and milk of the cow. Moreover, the rapid growth of the lamb in its early postnatal life would necessitate a different arrangement in regard to riboflavin supply, distribution and availability to different organs and tissues. The presence of pneumonia in all lambs on the riboflavin deficient or restricted diets suggested that the pneumonia was in some way related to riboflavin deficiency. It may be that lung tissue has a higher riboflavin requirement than any other tissue in the body and that the pathological changes of the lung developed before those of the skin and eye. It was difficult to determine if the pathological changes of the liver, kidney and adrenals resulted from a limited intake of the ration and later from general inanition or from the loss of function due to the unavailability of riboflavin itself. The normal appearance of the liver and kidney of the two deficient lambs, treated with riboflavin for 17 days, suggested that the changes were due to riboflavin deficiency rather than to general inanition. In regard to the lungs of these two lambs, the evidence of past pneumonia and the process of healing without any treatment except

riboflavin indicated that this type of pneumonia was somehow related to riboflavin deficiency. Whether the pneumonia produced by a riboflavin deficiency diet in the laboratory has any relationship to the pneumonia occuring under field conditions is not known. Venkatachalam et al. (1949) in a study of the various causes of death in young lambs, found that, out of 483 lambs, pneumonia was responsible for almost one-third of the deaths.

The data for hemoglobin, total and differential leukocyte count of the control and riboflavin deficient lambs were shown in table 10. Contrary to a microcytic hypochromic anemia in dogs, observed by Spector et al. (1943), and a moderate normocytic anemia in swine, reported by Wintrobe et al. (1944), the blood picture of the deficient lamb, in general, was unchanged. The hemoglobin level remained normal and constant throughout the experiment, provided that the values of the first blood determination were considered normal. That the initial blood values represented the normal values of the new born lamb was supported by a higher leukocyte count accompanied by a higher per cent of neutrophils at the beginning of the experiment and by a gradual drop of the leukocytes and neutrophils and an increase of lymphocytes as the lambs became older. This was observed in the control lambs. Decrease of leukocyte count and neutrophils in favor of lymphocytes toward maturity is a normal phenomenonof any

TABLE 10

SUMMARY OF BLOOD ANALYSES
OF THE CONTROL AND RIBOFLAVIN DEFICIENT LAMBS

Days on Expl. Diet when Bled	Control (8 lambs)							Riboflavin Deficient (7 lambs)						
	Hb*	WBC	P	L	M	E	B	Hb	WBC	P	L	M	E	B
1 - 5	12.6	10.125	42	55	2	1	0	12.8	10.950	40	58	1	0	1
5 - 14	12.1	8.250	38	59	2	1	0	12.6	12.250	45	52	2	0	1
14 - 21	12.5	6.950	30	66	1	2	1	12.9	14.650	62	35	1	0	0
21 - 30	12.4	7.700	35	63	0	2	0							

* Hb .... Hemoglobin

WBC .... Leukocytes (white blood cells)

P .... Polymorphonuclear leukocytes (neutrophils)

L .... Lymphocytes

M .... Monocytes

E .... Eosinophils

B .... Basophils

young animal. The increased leukocyte count and the reversal of the differential count (increased per cent of neutrophils) in the riboflavin deficient lamb was explained by the presence of pneumonia. It seemed more probable to relate both the increase of the leukocytes and neutrophils to the infection (acute bronchopneumonia) than to riboflavin deficiency. An increase of leukocytes and especially of neutrophils is a characteristic feature of acute inflammation or infection. The pneumonia observed among the riboflavin deficient lambs was acute, rapidly spreading and terminating in death.

It was rather unfortunate that the relatively short period of riboflavin depletion in the lamb and the limited facilities available to collect the urine samples prevented more thorough study of urinary excretion of riboflavin. These limitations were further aggravated by a great variation in the urinary riboflavin levels within the groups. It might be explained on the basis of varying storage capacity of the vitamin in the lamb before being placed on the deficient diet and, perhaps, by individual abilities of the lamb to economize the limited amount of riboflavin. The variations in the urinary excretion of riboflavin, no doubt, were also greatly influenced by the age of the lamb when taken away from the ewe. The age of the lambs when put on the experiment ranged from two to eleven days. However, the average urinary riboflavin excretion for the control lambs was 0.50 mg per 24 hours, compared with 0.01 mg per 24 hours for the riboflavin

deficient group. The disappearance of riboflavin from the urine of the lambs on the riboflavin deficient diet indicated that the vitamin was not synthesized either in the rumen or intestinal tract or in the tissues. It was interesting to note that the rumen of the control lamb was not developed and evidently was not yet functioning even after 34 days on the liquid purified diet.

It has been pointed out by others that the symptoms, gross and microscopic changes in any vitamin deficiency, seemed to be the result of a diminished function of the body cells and not the result of specific biochemical processes. Very little is known about the biochemical changes occuring in the various tissues of different organs of the animal when on a riboflavin deficient diet. Axelrod et al. (1940, 1941) reported a decreased d-amino acid oxidase activity in the liver and kidney of riboflavin deficient rats and markedly lowered activity of xanthine oxidase of deficient rats measured by oxygen consumption rates. Consistent and rather uniform gross and microscopic lesions of the lung, liver, kidney and adrenals suggested that the tissues of these organs were the first depleted of riboflavin. The cellular dysfunction manifested by the symptomatology, gross and microscopic pathology described, was then a deficiency of one and only one nutritional factor, that is riboflavin, and not compound deficiencies due to poor intake of a supposedly adequate diet. Similar growth response, symptoms, survival time, pathology,

a rather short period of inanition, dramatic response to riboflavin therapy in respect to growth and health and a rather liberal amount of all other B vitamins in the diet suggested that the deficiency, as observed, could have been of riboflavin.

The average daily intake of riboflavin per pound of body weight of the control lambs was around 250 mgm. This was calculated from the 3 mg riboflavin level in one liter of purified milk as shown in table 2. The average intake increased up to 297 micrograms during the first three weeks on the diet. After three weeks the lambs were growing faster and required less dry matter to produce one pound of gain. Consequently, the riboflavin intake, directly dependent on the amount of milk consumed, was decreased.

The average daily gain of the control lambs receiving around 250 mgm of riboflavin per pound of body weight per day, mixed in the milk and fed at each feeding, was 0.40 lb. while the average daily gain of the two lambs on 250 and 350 micrograms of riboflavin, administered once a day, was 0.205 and 0.305 lbs. respectively. The only explanation for this difference in growth response was the mode of vitamin administration. The control lambs were always fed a complete ration. The amount of riboflavin (3 mg per liter of milk) was added to the milk in the laboratory and the control lambs received a part of the daily intake at each feeding. In the lambs on the various levels of riboflavin, the amount

was administered once a day. It was possible then that riboflavin administered with each feeding increased the utilization of the feed which was reflected in faster gains of the control lambs. It was also felt that the level of 3 mg of riboflavin in one liter of milk, set arbitrarily for the control lambs, was adequate to support the health and general condition of the young lamb, and to produce a normal growth and gain in body weight.

SUMMARY

A purified liquid diet (synthetic milk) containing 16 per cent solids, composed of vitamin free casein (30%), glucose (28%), lactose (10%), lard (26%), salt mixture (6%) and all fat and water soluble vitamins, was adequate to support good health and normal growth of two to eleven day old lambs. It was shown that the excretion of the urinary riboflavin in riboflavin deficient lambs was greatly lowered. The hemoglobin concentration was unchanged. The leukocyte count was increased and was reflected in an increased per cent of neutrophils in the differential count.

Riboflavin deficiency in the young lamb was characterized by lowered appetite, intermittent diarrhea, poor growth, emaciation, pneumonia and death. The survival period was 16-21 days.

Pathological studies of the riboflavin deficient lambs revealed lung involvement with bilateral pneumonia, general fatty degeneration and necrosis of the hepatic cells, fatty degeneration of the cortex and medulla of the kidney, congestion and fatty degeneration of the adrenals.

Symptoms of riboflavin deficiency were rapidly alleviated and cured with administration of crystalline riboflavin.

The growth response of ten lambs on five different levels of riboflavin (5, 50, 150, 250 and 350 micrograms per pound of body weight per day) was directly proportional to the level

of riboflavin administered. The two highest levels of riboflavin administered once a day even though comparable to the riboflavin intake of the control lambs, proved to be adequate to support good health and condition of the young lamb but inadequate to promote normal or optimal growth. It was felt that the optimum requirement of the young lamb was somewhere between 250-350 micrograms of riboflavin per pound of body weight per day and that the lamb in the preruminant state had a definite requirement for riboflavin.

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Fig. 1. Lamb No. 4.

Liver, kidneys and adrenal from riboflavin deficient lamb. Note prominent streaks in the kidneys. The adrenal is enlarged and the line of demarkation between the cortex and medulla is lost.

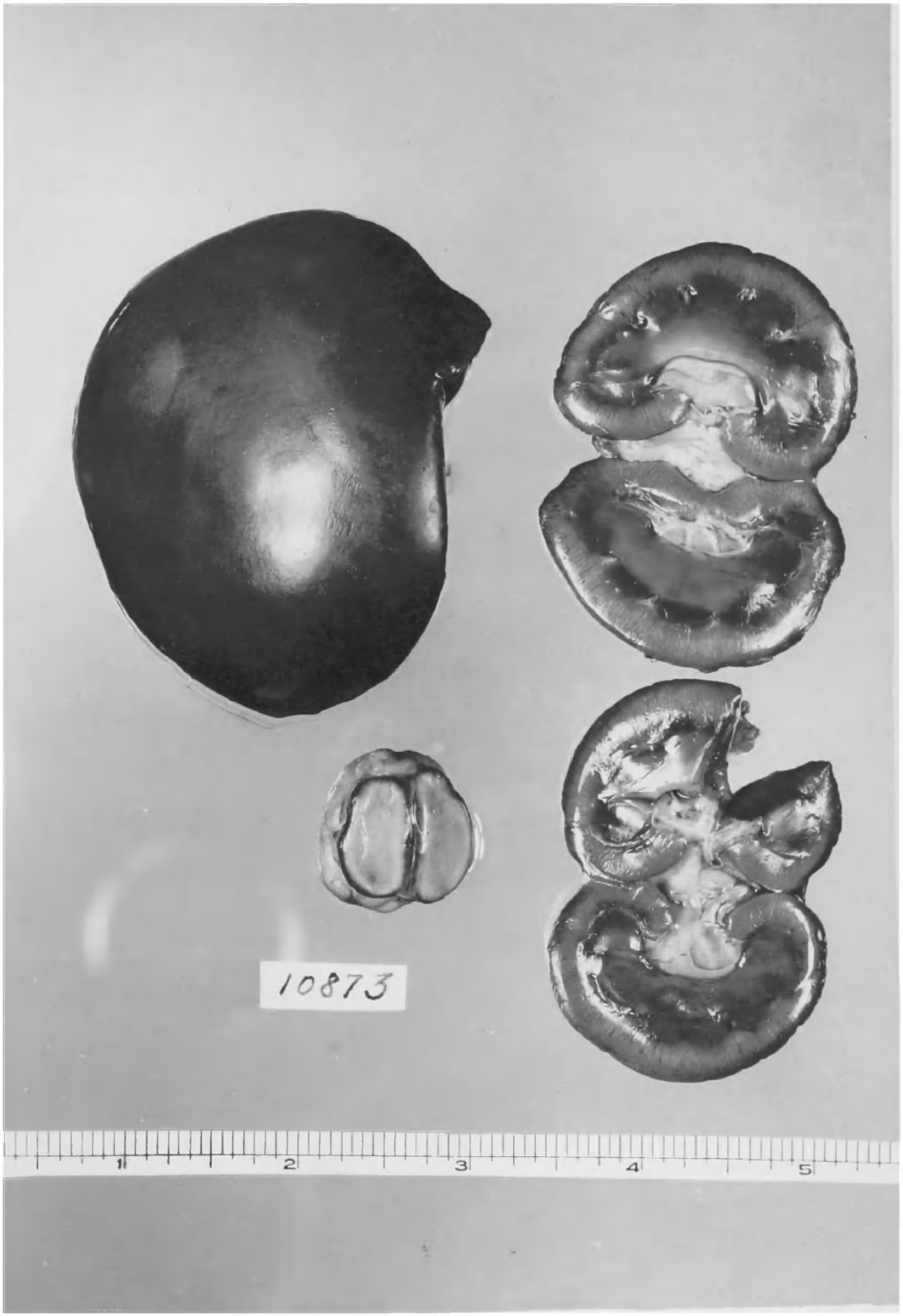
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Fig. 2. Lamb No. 7.

Extensive fatty infiltration of the liver. The hepatic cells are filled with fat globules. The nuclei are pyknotic and the sinusoids, especially close to the central vein, are distended.

Hematoxylin and Eosin stain x 600

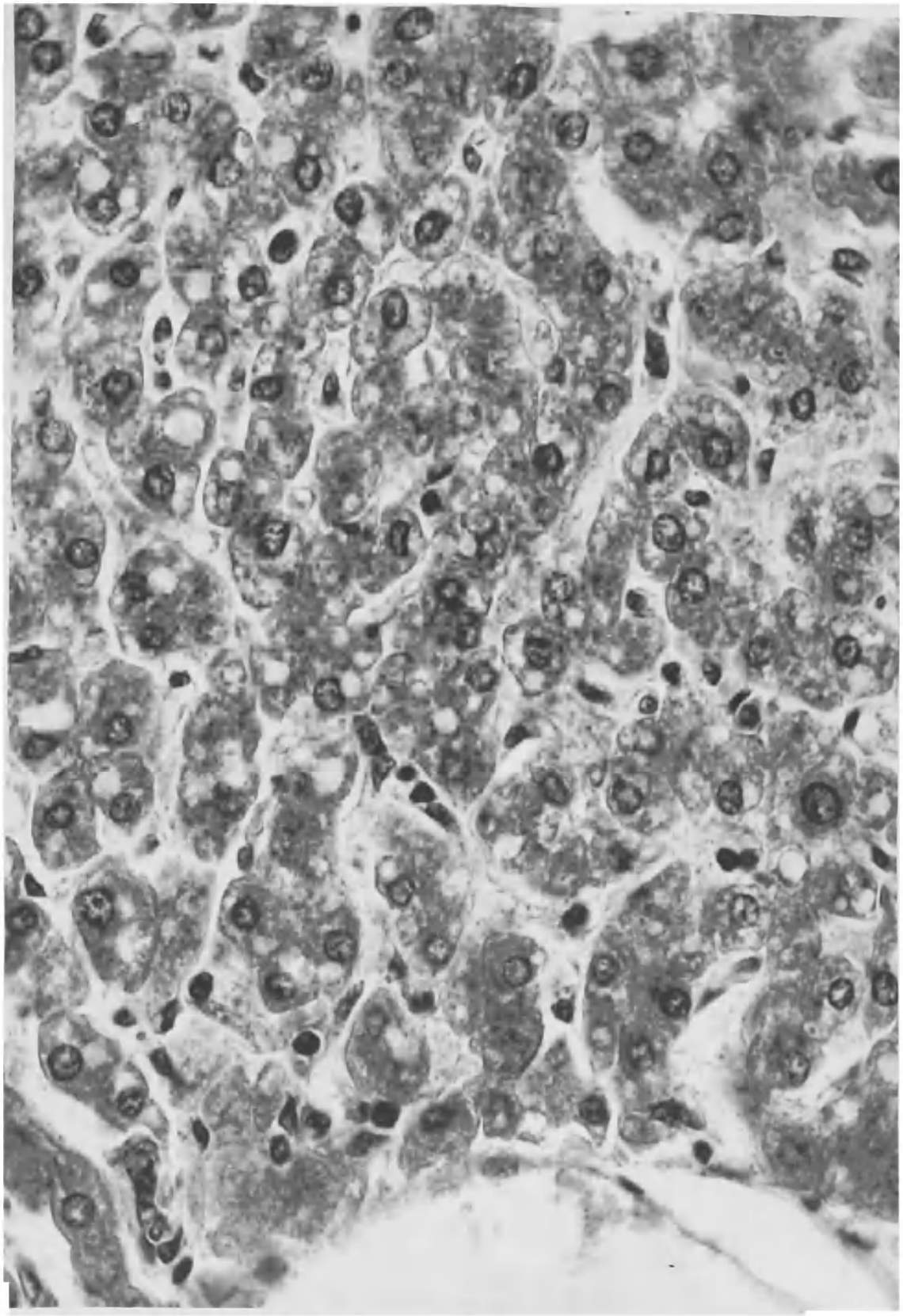


Fig. 3. Lamb No. 2.

Cortex of the kidney showing severe fatty infiltration of convoluted tubules and tendency toward hyaline degeneration in glomeruli. Note the disappearing capillary loops.

Hematoxylin and Eosin stain x 600

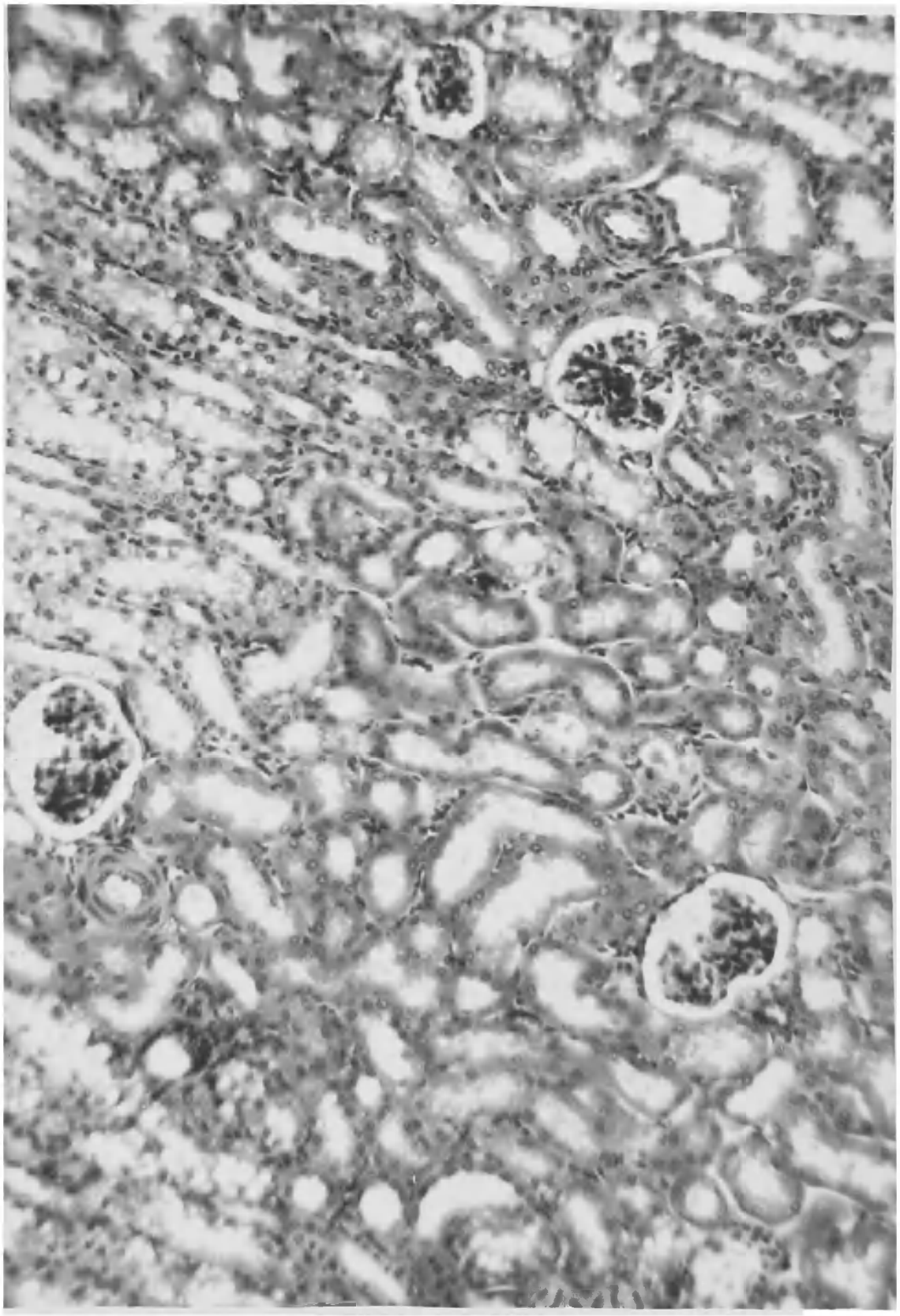


Fig. 4. Lamb No. 2

Distal convoluted tubules with excessive amount of fat globules.

Frozen section, Sudan IV stain x 850

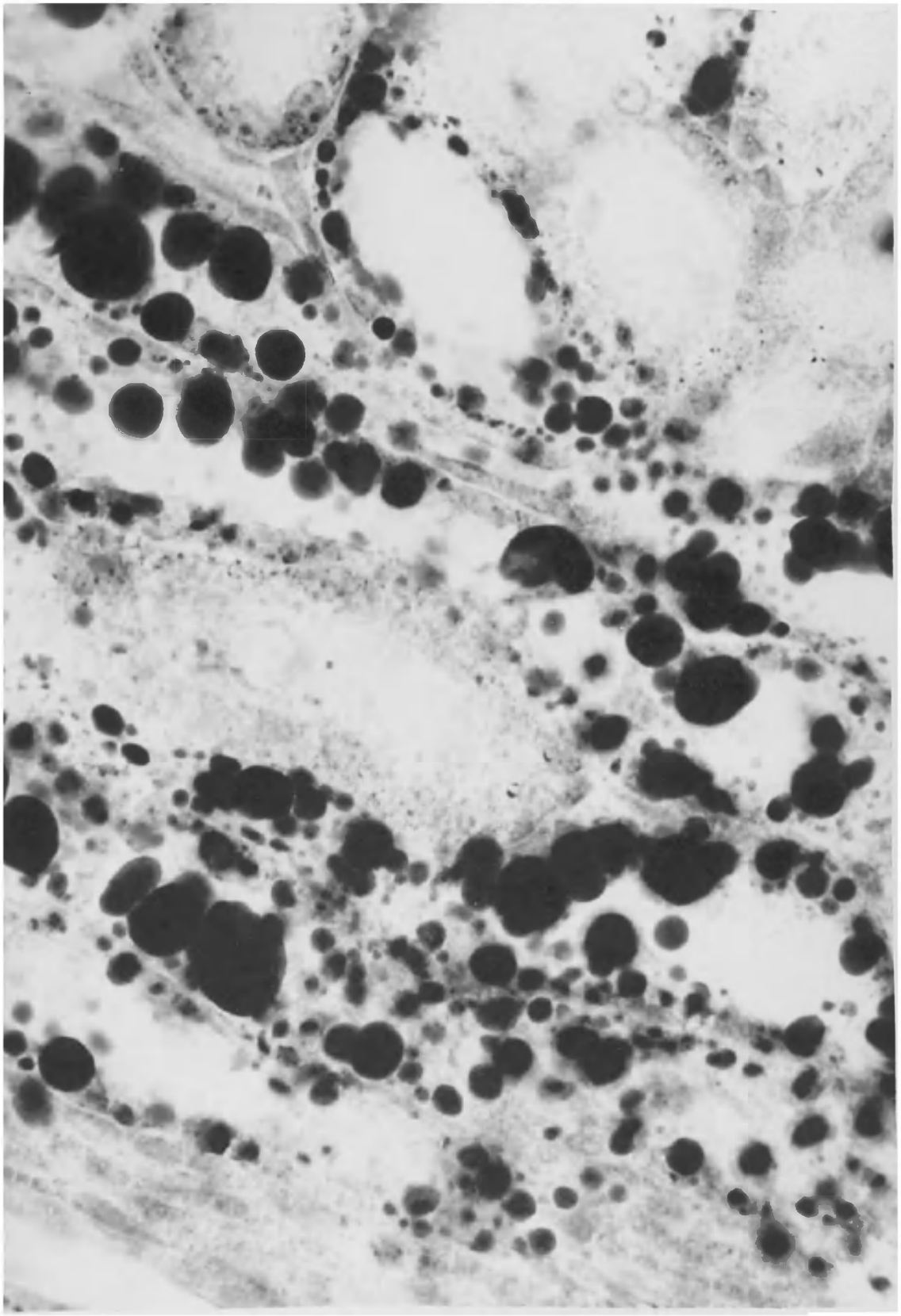


Fig. 5. Lamb No. 5.

Pronounced fatty infiltration of the cortex and medulla of the adrenal. The prominent and rather different fatty infiltration between glomerulosa and fasciculata was considered physiological.

Frozen section, Sudan IV stain x 40

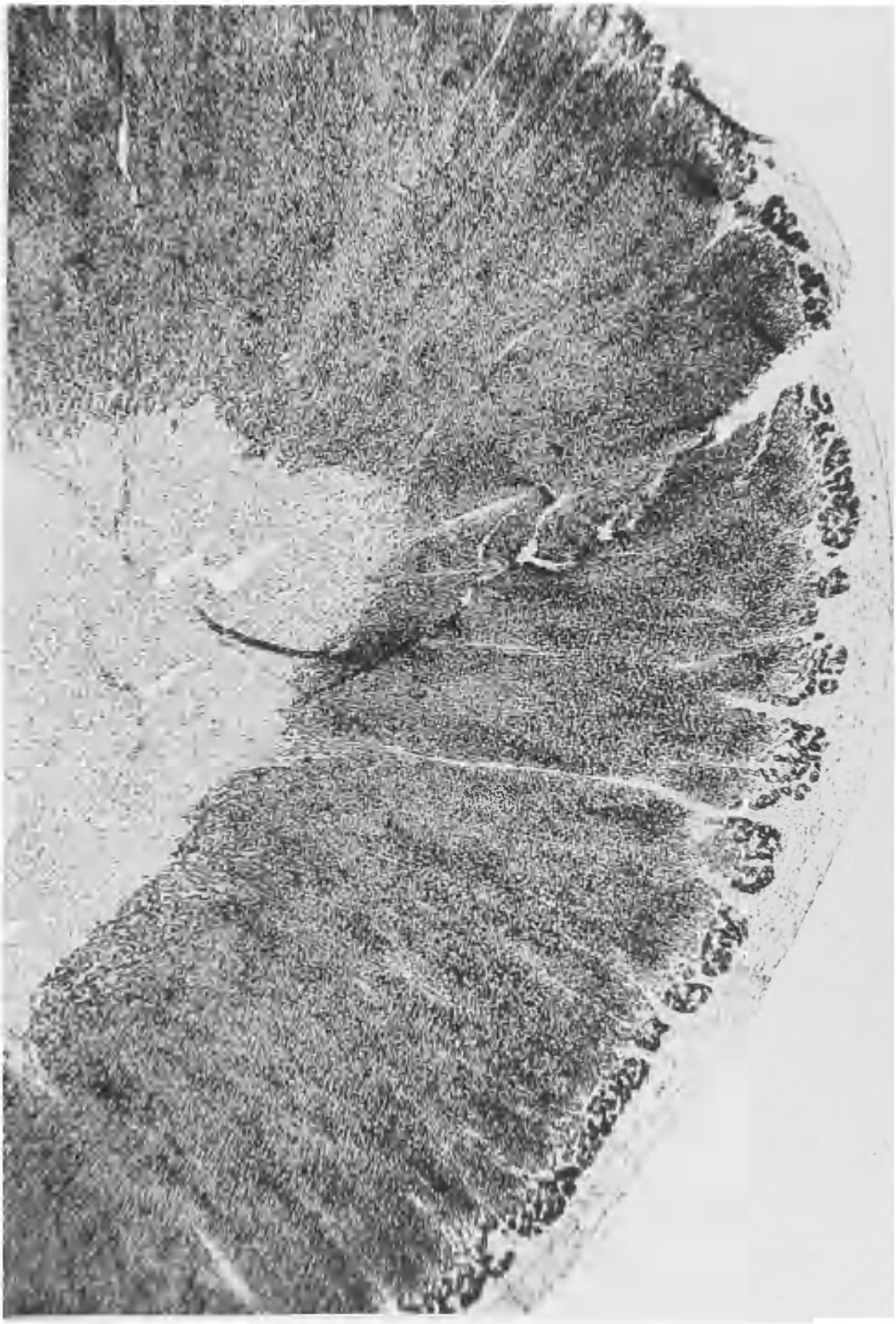


Fig. 6. Lamb No. 5.

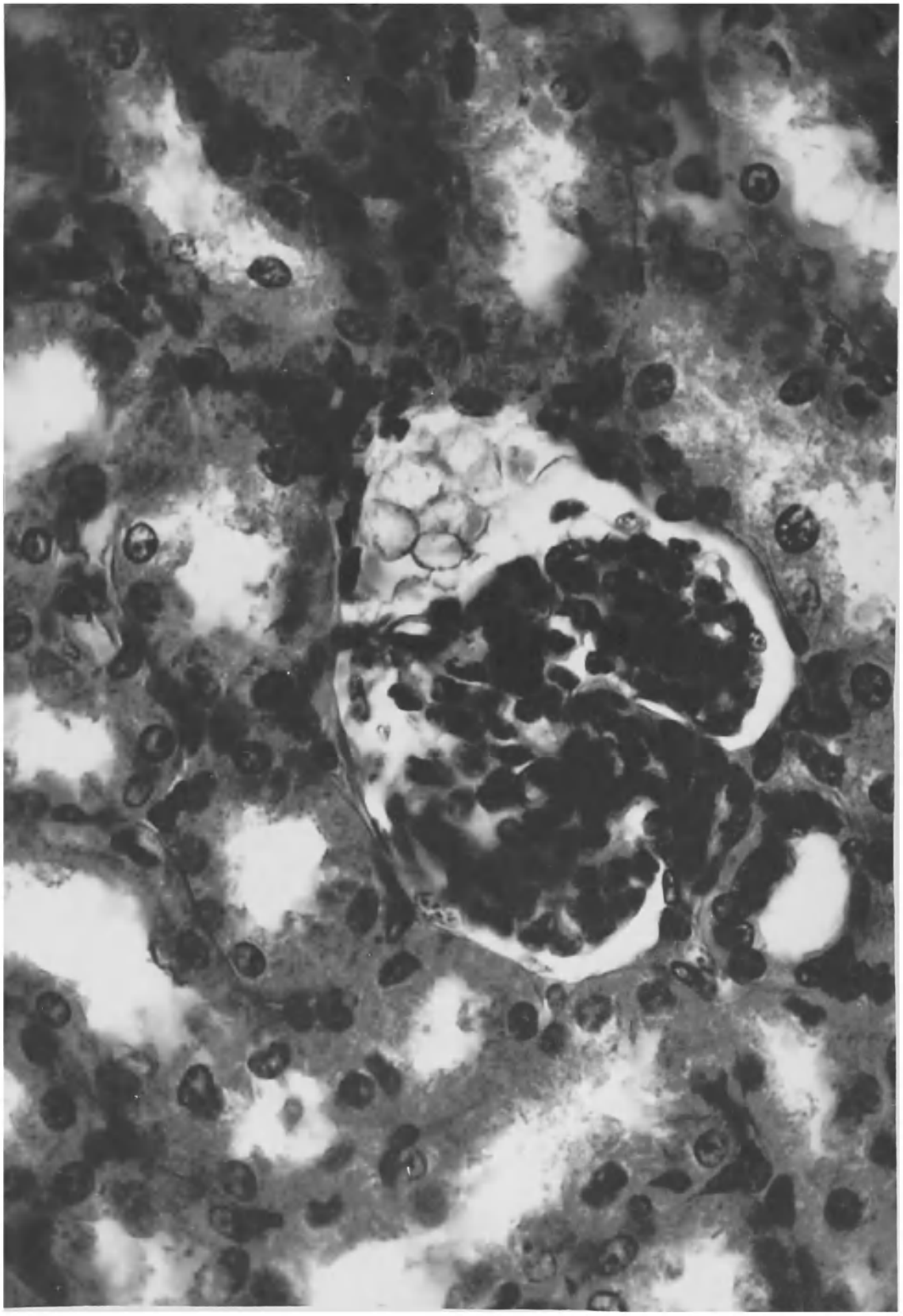
Higher magnification of adrenal showing fatty degeneration. The sinusoids are distended but the borders are intact.

Frozen section, Sudan IV stain x 125

Fig. 7. Lamb No. 10.

Cortex of the kidney showing glomerulus in early stage of hyaline degeneration. The hyaline droplets are being accumulated on one side, while the capillary loop is disappearing. Convoluted tubules show pronounced cloudy swelling.

Hematoxylin and Eosin stain x 850



Part II.

VITAMIN E DEFICIENCY AND ESTIMATED REQUIREMENT

INTRODUCTION

Since 1934 vitamin E deficiency has been suspected to be the cause of so called "Stiff-Lamb" disease. This disease of lambs is characterized by stiffness, paralysis and muscle degeneration similar to the nutritional muscular dystrophy (Zenker's degeneration) produced in laboratory animals on diets low or deficient in vitamin E. However, there was no direct evidence to substantiate the idea that vitamin E deficiency was the primary and the only factor responsible for the disease. In previous work a purified diet in liquid form (synthetic milk) proved to be adequate to support the growth and health of the young lamb and was used to study the riboflavin requirement of lambs. It seemed logical then that using a purified diet, low in vitamin E (tocopherols), the role of this vitamin in animal nutrition and particularly its relationship to muscular dystrophy of lambs could be studied and possibly elucidated.

OBJECT

The object of these studies was a) to produce vitamin E deficiency in the lamb by the use of a liquid purified diet, low or deficient in tocopherols, b) to study and compare the symptoms and lesions of vitamin E deficiency, produced in the laboratory with the symptoms and lesions of the so called "Stiff-Lamb" disease occuring under field conditions, and c) to determine the quantitative requirement of alpha-tocopherol for the young suckling lamb.

REVIEW OF LITERATURE

Nutritional Muscular Dystrophy in Laboratory Animals

Until 1928 vitamin E was thought to be entirely concerned with reproduction. Evans and Burr (1928) observed and described paralysis of young rats suckling mothers kept on a vitamin E deficient diet. At first it was thought that the paralysis was neurogenic in origin.

Goettsch and Pappenheimer (1931), using a semi-purified diet consisting of oats, wheat bran, casein, lard, skimmed milk and salt mixture, produced a dystrophy of the sketetal musculature of the guinea pig and rabbit. The diet was thought to be complete and the skeletal muscle changes were not due to starvation, infection or scurvy. Histopathologically, the lesions of the skeletal muscles were described as Zenker's degeneration or nutritional muscular dystrophy. No heart lesions or changes in the central nervous system were observed.

Madsen *et al.* (1935) produced a dystrophy of skeletal and heart muscle in guinea pigs, rabbits, goats and sheep kept on natural or synthetic diets supplemented with large doses of cod liver oil. The authors suggested that the saponifiable part of the cod liver oil was toxic to the animals and caused the lesions. The following year Madsen

(1936), comparing various sources of fat on a semi-purified diet and the effect of cod liver oil, observed that guinea pigs developed dystrophy of the skeletal muscle sooner on a diet containing lard and cod liver oil than on a diet containing cotton seed oil and vitamin A and D concentrate.

Cummings and Mattill (1931) and Mattill (1938b) did not agree with the toxic theory of cod liver oil. They postulated that the muscle dystrophy was due to vitamin E deficiency which resulted from destruction of vitamin E by oxidative rancidity of cod liver oil and animal fat.

Shimotori et al. (1940) produced, cured and prevented nutritional muscular dystrophy in the guinea pig by supplementing the Madsen et al. (1935) diet with wheat germ, wheat germ oil or alpha-tocopherol. This diet was adequate and complete in all B vitamins. The authors postulated that vitamin E was the specific factor which cured and prevented the muscle degeneration.

Muscular degeneration, similar to that of laboratory animals on a vitamin E low diet, and testicular degeneration characteristic of vitamin E deficiency were observed in dogs and reported by Brinkhouse and Warner (1941). This condition was produced by means of a chronic biliary fistula in a period of seven to nine months. Evidently the bile was essential for vitamin E absorption and utilization.

Telford (1941) studied the histopathology of the degenerated skeletal muscles from rats on a vitamin E deficient

diet. In addition to Zenker's degeneration, the nerve endings were destroyed. On treatment with vitamin E, regeneration of the skeletal muscle was accompanied by regeneration of nerve endings.

The muscular dystrophy of rabbits produced by large doses of cod liver oil was treated with the disodium salt of alpha-tocopherol phosphate administered parenterally and with alpha-tocopherol acetate injected intramuscularly. Eppstein and Morgulis (1942) found the parenteral administration more effective than the ingestion. Creatinuria was reported to be an index of vitamin E deficiency.

Mason (1942) analysed tissues from rats on a low tocopherol diet and found that the heart muscle had proportionally twice as much vitamin E as did the skeletal muscle.

Pappenheimer (1943) in a review on vitamin E emphasized that vitamin E was essential in metabolism of skeletal muscles of all mammals, but did not seem to be essential in metabolism of the nervous system.

Heart lesions in the rabbit similar to Zenker's degeneration of the skeletal muscle, induced by a vitamin E deficient diet, and responsible for sudden death due to myocardial failure were first reported by Houchin and Smith (1944). These authors also observed an increased sensitivity of the depleted animals to pitressin, greater resistance to toxic effects of cardiac glucosides, cardiac dilatation and an increased oxygen consumption.

Gullickson and Calverley (1946) studied the cause of sudden death of cattle in the field and calves on a vitamin E deficient diet. The electrocardiographs prepared during the period of deficiency showed definite changes in the various intervals and potentials with inconsistent deflections. The histopathological examination of the myocardium showed atrophy and scarring of the cardiac muscle fibers and an increased cellular infiltration. Electrocardiographs of rabbits on vitamin E deficient synthetic diet studied by Bragdon and Levine (1949) showed some changes in waves. Microscopic examination of the myocardium revealed foci of necrosis and an inflammatory reaction. The sequence of events was a coagulative necrosis of sarcoplasm with loss of striation, pyknosis and karyorrhexis of muscle nuclei, edema, hemorrhages and infiltration of the areas with monocytes and polymorphonuclear leukocytes. Some calcification was also observed.

Menschik et al. (1949) studied the distribution of fat and fat changes in mice on a diet with and without vitamin E. Animals on vitamin E supplemented diet increased the amount of neutral fat in the body, increased the amount of adipose tissue and showed physiological fatty infiltration throughout the body. Animals on a vitamin E deficient diet soon became lean, neutral fat disappeared from the tissues and was replaced by acid fast lipo-proteic material composed of un-

saturated fatty acids, phospholipids, cholesterol and protein. The authors suggested that vitamin E was involved in fat metabolism.

Tobin (1950) used 2, 10 and 20 per cent of cod liver oil in a diet to produce various degrees of muscle degeneration in mice. Animals on the 2 per cent cod liver oil diet did not show locomotor disturbances or any other visible symptoms of deficiency. Only 15 per cent of the animals revealed slight hyaline degeneration of individual muscle fibers characterized by centrally located nuclei or groups of nuclei arranged in rows or columns. Animals on the 10 per cent cod liver oil developed acute paralysis in 16 to 30 days. Microscopically the muscles were necrotic, showed loss of striation, segmentation and clumping of the sarcoplasm, collapse of sarcolemmal sheats, presence of giant cells and some calcified areas. Regenerative changes were characterized by proliferation of sarcolemmal nuclei, regeneration of sarcoplasm and renewed striation.

Vitamin E deficiency in chicks characterized by cerebellar disorder (encephalomalacia) was produced and described by Pappenheimer and Goettsch (1931) and Jungherr (1949). Ducklings on the same high fat vitamin E deficient diet developed dystrophy of the skeletal muscle (Pappenheimer and Goettsch 1934) while turkeys showed myopathy of the gizzard (Jungherr and Pappenheimer 1937).

Stiff Lamb Disease

The so-called "Stiff-Lamb" disease was first reported and described by Metzger and Hagan (1927). The symptoms were a disturbance in locomotion, stiffness of the hind and fore legs, paralysis and death by starvation due to inability of the lamb to follow the mother. The gross and microscopic lesions of both the skeletal and cardiac muscles were described as noninfectious and noninflammatory in nature.

Jungherr and Welch (1927) described the degenerative changes in the muscle as secondary to coccidiosis. In his next report on "Stiff-Lamb" disease, Welch *et al.* (1929) stated that lambs previously confined to small pens when suddenly put on pasture were especially susceptible to the disease. Severe exercise in pasture was suggested as the cause of the disease.

Umbilical infection as the primary cause of "Stiff-Lamb" or "White Muscle" disease was suggested by Marsch (1929).

Willman *et al.* (1931) produced "stiff lambs" experimentally by feeding pregnant ewes a ration consisting of a grain mixture high in protein and alfalfa or clover hay. A lack of exercise was also considered as a possible etiological factor.

Marsch (1932) explained the whitish lesions of the muscles as deposits of tricalcium phosphate caused by a disturbance of the calcium and phosphorus ratio in the affected lamb.

In 1934 the disease among lambs, calves and heifers was reported from Norway by Slangsvold and Lund-Larsen (1934). The degenerative changes in skeletal and heart muscles were similar to hyaline degeneration with increased deposition of calcium. A balanced ration with adequate amounts of minerals and vitamins was suggested as a preventive measure.

Willman et al. (1934) summarized the four year study on "Stiff-Lamb" disease. The authors consistently were able to produce "stiff lambs" from ewes fed a ration consisting of oats, barley, cull beans and second cutting alfalfa hay. Previously suspected causes of the condition such as exercise of the ewes during pregnancy, heavy feeding of concentrates and creep feeding of the lambs were discarded.

Willman et al. (1935) treated the "stiff lambs" with vitamin C without success, and concluded that vitamin C was not related to the disease.

Lee and Scrivner (1935) studied the histopathology of the disease and found whitish lesions in the kidney similar to those in the skeletal and cardiac muscle. The authors reproduced the condition experimentally in lambs and rabbits by inoculating those animals with bacteria isolated from the heart of a stiff lamb. Chemical analysis of the degenerated tissues showed increased calcium and phosphorus.

To determine if the concentrates or the hay was the

"Stiff-Lamb" producing factor in the ration, Willman et al. (1936) replaced the alfalfa hay by mixed clover and timothy hay. Lambs became stiff as before, proving that the grain mixture and not the hay was the "Stiff-Lamb" factor.

Schofield and Bain (1939) successfully treated a number of "stiff lambs" with phosphoric acid added to milk. Phosphorus deficiency as the cause of the condition was suggested by Wawter and Records (1939). Ewes fed alfalfa hay alone produced "stiff lambs". When rolled oats were fed with alfalfa hay the disease was prevented.

Sholl (1939) reported "Stiff-Lamb" disease in Michigan. The mortality was up to 30 per cent. Blood analyses showed very low creatinine and high non-protein nitrogen values.

Willman et al. (1939) disproved the phosphorus deficiency as the cause of the condition. Added phosphorus to the "Stiff-Lamb" ration failed to prevent the development of symptoms.

Willman et al. (1940) definitely proved and recognized the disease as nutritional in origin. The cause was something other than phosphorus and ascorbic acid deficiency. The authors observed that the incidence of "stiff lambs" was much lower when wheat germ meal was added to the "Stiff-Lamb" ration. The following year Willman et al. (1941) supplemented the "Stiff-Lamb" ration with unextracted wheat germ meal. None of the lambs born from ewes on this ration became stiff while eleven out of twenty-seven lambs from the

ewes without wheat germ meal developed the disease.

Thorp (1942) described "Stiff-Lamb" disease and suggested preventive methods.

Willman et al. (1944, 1945, 1946) suspected vitamin E as a factor responsible for "Stiff-Lamb" disease. The authors prevented the condition either by administration of an oil solution of mixed tocopherols to the ewes before and to their lambs after parturition or by feeding an oil solution of d-l alpha-tocopherol acetate to the lambs immediately after birth. Stiff lambs also responded and were successfully treated by subcutaneous injection of a disodium salt of d-l alpha-tocopherol phosphoric acid ester in an aqueous solution.

Whitting et al. (1949), using the "Stiff-Lamb" producing ration of Willman et al. (1934), compared the tocopherol levels of blood plasma from ewes and lambs and also the colostrum and milk tocopherol levels from the ewes with a group of normal controls. The results indicated a reduction from 15 to 48 per cent in the tocopherol levels of the blood plasma, milk and colostrum in the ewes and lambs on the "Stiff-Lamb" producing ration.

EXPERIMENTAL PROCEDURE

Animals Used

Beginning with the fall of 1950, 51 lambs 3-4 days old were used for this study. Lambs of both sexes of the mutton and fine wool type were obtained from the Experiment Station flock at Michigan State College or purchased from farms in the area. Purebred or crossbred, single or twin lambs were used and the only prerequisite was good health and normal condition. All lambs received colostrum before being taken from the ewes. Eleven out of the 51 lambs died early in the experiment due to causes not related to vitamin E deficiency and were diagnosed as overeating (2 lambs), intestinal obstruction (2 lambs), coccidiosis (1 lamb) and pneumonia (3 lambs). The cause of death in three lambs was not identified.

Feeding and Care

The composition and the method of preparation of the synthetic milk and the care of the animals were described in detail in Part I of this thesis. A few modifications were made however: the lambs were changed from one pen to another every day instead of every other day to prevent any outside disease. Ten per cent of lactose was replaced by 10 per cent glucose to eliminate a possible synthesis of any vitamin in the intestinal tract of the lamb. The lambs were fed four times a day instead of five. The amount of milk

each lamb consumed per feeding was recorded as well as all the observations of symptoms, health, appetite and general behavior. All the lambs were weighed every four days.

The work reported herein consisted of three trials. In the first trial were five positive control and ten experimental lambs. The source of fat in the purified liquid diet was commercial lard purchased locally. After 40 days on this diet two control and three animals on supposedly vitamin E low diet were treated daily for 25 days with 3 ml. of fresh cod liver oil. All lambs were sacrificed when depleted and showing symptoms.

The second trial consisted of four control and six lambs on the deficient diet. The source of fat in this group was molecular distilled lard (low tocopherol animal fat)*, containing less than five micrograms of tocopherols per gram, and especially suitable for experimental work with vitamin E. Two lambs after depletion showed advanced symptoms of deficiency and were treated with alpha-tocopherol. The control and the other four deficient lambs were necropsied for histopathological study.

The third trial comprised three control and twelve lambs on the synthetic milk with distilled lard as the source of fat. Four animals were depleted and sacrificed while the other eight lambs were treated and cured after showing symp-

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*Distillation Products, Inc., Rochester, 13, New York

toms of deficiency.

Blood Studies

The blood studies and the technique of blood collection were similar to those described in Part I. The lambs were bled immediately when put on the purified diet to determine the initial blood picture. On alternate weeks an additional 20 ml. of blood was drawn for analysis of tocopherols. The tocopherol content of the blood plasma was determined by the hydrogenation method of Quife and Biehler (1945). The blood was centrifuged immediately after it was brought to the laboratory and the tocopherol determination was made as soon as possible. At least two determinations were carried out for each sample.

Gross Pathology

Care was exercised to sacrifice the depleted animals whenever possible before dying, in order to avoid possible post mortem changes. However, it was difficult to predict the final stages of deficiency, especially if it was not complicated with pneumonia. In a number of instances death was sudden and the animals displayed no serious signs of ill health other than symptoms of vitamin E deficiency. Necropsy was performed as soon as possible.

All control lambs were sacrificed by exsanguination under ether anesthesia. The autopsy technique was described in Part I. In order to preserve the heart intact for gross

and microscopic studies, the heart blood was not cultured.

Microscopic Studies

Tissues for histopathological study were taken from various muscles of the rear and front legs, from the heart, from the longissimus dorsalis and occasionally from the abdominal muscles, diaphragm, tongue and masseter. The specimens were fixed in Zenker's fluid. Paraffin sections were cut seven micra thick and stained with hematoxylin-eosin. The tissues from the liver, kidney and adrenals were fixed both in Zenker's fluid and 10% formol saline and stained, respectively, with hematoxylin-eosin and Sudan IV. Heart specimens were also fixed in formalin, cut frozen and stained with Sudan IV for fat.

Estimated Alpha-tocopherol Requirement, Treatment and Recovery

The purified vitamin E low diet of the 12 control lambs was supplemented with 100 mg. of alpha-tocopherol administered orally on alternate days. A rather high level of alpha-tocopherol was chosen to eliminate any possible doubt that lesions, if found in the control lambs, were due to a border line level of the vitamin.

Altogether eight depleted lambs were treated with alpha-tocopherol. The animals were depleted until they showed paralysis and were unable to stand. In this condition the eight animals were given an initial dose of 500 mg. of alpha-

tocopherol followed by 100 mg. every other day. The vitamin was diluted in fresh corn oil and administered orally by means of a syringe fitted with a rubber hose. The lambs were sacrificed at various intervals of therapy to study the process of muscle regeneration.

RESULTS

Growth and Health

It was understood from the work of Willman *et al.* (1945, 1946) and from the reports on "stiff lambs" from the field that the appetite of the affected lambs was not greatly depressed, at least in the first stages of deficiency. It was thus felt that the growth rate would not be a major criterion of deficiency and that feeding the lambs four times a day instead of five times was justified. However, the milk consumption per feeding was recorded and the weights of the lambs were taken every four days. Despite this limitation in feeding, the control lambs on both commercial and distilled lard diet grew normally, made moderate daily gains and stayed in good health and condition. The average daily gain of all 12 control lambs was 0.28 lb. over periods on the diet. The control lambs required an average of 1.46 lbs. of dry ration to produce a one pound gain in body weight. This rather low daily gain of the control lambs indicated that feeding the newborn lamb four times a day was inadequate to obtain an optimal growth rate (Kean *et al.* 1949) or at least gains comparable to those reported in Part I.

The lambs on the vitamin E low diet with commercial and distilled lard grew normally, remained healthy and made gains in body weight similar to the controls for a period of

about 28 and 14 days respectively. After this time a majority of the deficient lambs began to show symptoms of pneumonia which was also reflected in an increased leukocyte count. The affected lambs were treated with 100,000 units of procaine penicillin injected intramuscularly in the neck region every other day. None of the lambs fully recovered and this therapy had to be continued until the end of the experiment to keep the animals alive. The other deficient lambs with no complications of pneumonia, despite the existing symptoms of deficiency, retained a very good appetite and remained in good condition until they died suddenly due to heart failure or were sacrificed for further study.

Symptoms of Deficiency

The first noticeable symptoms of vitamin E deficiency in the lambs on the diet containing commercial lard as the source of fat appeared in four to six weeks, while the animals on the molecular distilled lard diet showed the first symptoms in three to four weeks after being placed on the respective rations. The lambs became dull and tired and preferred to remain in a laying position. They showed some difficulty in rising, were reluctant to walk and later were reluctant to cross small obstacles. This was accomplished with difficulty or by falling. Still later a definite stiffness, especially of the hind legs, was noticeable. The animals were hardly able to get up and walk. When assisted to a standing position, they assumed a

characteristic arched back position (Fig. 8). After standing for a short time equilibrium was lost and the animals fell. The hind part of the body was usually the first to go down (Fig. 9). In the later stages the lambs were paralyzed in both hind and front legs and were laid prostrate. Difficulty in swallowing the liquid diet was observed in two lambs. Twenty four out of 28 deficient lambs developed a respiratory distress which later developed into pneumonia. These lambs were immediately treated with penicillin. This was the typical sequence of events during the experiment.

Several animals showed only a mild stiffness and did not develop the sequence of symptoms characteristic of chronic deficiency. These lambs died suddenly without showing any severe stiffness or paralysis. Post mortem examination clearly indicated cardiac failure due to extensive lesions in the heart.

The source of fat in the diet of lambs in the first trial, as mentioned above, was commercial lard. During the experiment it was evident that the amount of vitamin E in commercial lard was sufficient to greatly delay the deficiency symptoms.

Madsen et al. (1935), Pamukcu (1948) and others produced the muscular dystrophy characteristic of vitamin E deficiency in laboratory animals by supplementing the diet with cod liver oil. To check the possibility of inducing

the same muscular dystrophy or speeding up the appearance of vitamin E deficiency symptoms with cod liver oil in lambs, two control and three deficient animals previously on commercial lard diet for 40 days were treated daily for 25 days with 3 ml of fresh cod liver oil. There was no significant difference between the treated and non-treated lambs in regard to the time required to produce the symptoms or the severity of the muscular dystrophy. The amount of cod liver oil administered apparently was too small. The time required to produce symptoms of deficiency on commercial and molecular distilled lard diets and the effect of cod liver oil is indicated in table 11.

Blood Studies

The average hemoglobin value of all 51 lambs determined within five days after being put on the experimental diets was 12.8 gm per 100 ml of blood. The range was 10.4 to 15.9 gm per cent. The average hemoglobin value of the 10 control lambs just before the termination of the experiment decreased slightly and was 12.4 gm per 100 ml of blood. This slight decrease of hemoglobin was not considered significant. The control lambs then maintained an approximately normal hemoglobin level throughout the experiment. In every vitamin E deficient lamb there was a gradual drop of hemoglobin. The average value calculated from the last values available before the deficient lamb died or was sacrificed

TABLE 11

EFFECT OF COMMERCIAL AND MOLECULAR DISTILLED LARD AND COD LIVER OIL ON TIME REQUIRED TO PRODUCE SYMPTOMS OF VITAMIN E DEFICIENCY

Trial	No of Lambs	Diet	Lard Used	Cod Liver Oil Therapy	Days to Produce Deficiency
1	5	Positive control	Commercial	---	No symptoms
	10	E low	Commercial	---	30 - 80
	2*	Positive control	Commercial	3 ml. daily	No symptoms
	3*	E low	Commercial	3 ml. daily	62 - 74
2 and 3	7	Positive control	Molecular distilled	---	No symptoms
	18	E deficient	Molecular distilled	---	20 - 55

* Prior to administration of cod liver oil the lambs were on commercial lard diet for 40 days.

was 10.4 gm per 100 ml blood, excluding three lambs which developed anemia in the final stages of deficiency and showed values of 5.1, 5.6 and 6.5 gm of hemoglobin per 100 ml of blood. The average leukocyte count of all lambs at the beginning of the experiment was 8,100 per mm^3 of blood with the average ratio of neutrophils to lymphocytes 55:43. No significant pattern was exhibited by the leukocyte or differential counts. However, the leukocyte count and the per cent of neutrophils were significantly increased if the deficiency was complicated with pneumonia.

The initial level of tocopherols in the blood plasma was determined on 23 lambs. These determinations were carried out on blood drawn from 0-15 days after the lambs were placed on the experimental diets. The values ranged from 0.040 to 0.377 mg of total tocopherols per 100 ml of blood with an average value 0.179 mg. The results of the blood analysis for total tocopherol of lambs on the commercial and distilled lard diets and lambs treated with cod liver oil and alpha-tocopherol are shown in tables 12 and 13. In every case the values represent at least two analyses for each sample of blood.

The tocopherol level of blood plasma of the control lambs on the commercial lard diet supplemented with 100 mg of alpha-tocopherol every other day significantly increased throughout the experiment, while the control lambs on the molecular distilled lard treated similarly showed a less pro-

TABLE 12

TOCOPHEROL CONTENT OF BLOOD PLASMA IN LAMBS ON COMMERCIAL LARD DIET AND THE EFFECT OF COD LIVER OIL ON THE LEVEL OF PLASMA TOCOPHEROLS

No of Lambs	Diet	Cod Liver Oil Therapy	Days on Experiment 0 - 15	30 - 45	60 - 70
			mg/100 ml	mg/100 ml	mg/100 ml
4	Positive control	---	0.372 0.084	0.485 0.212 0.266 0.266	0.281 sacrificed cod liver oil cod liver oil
			0.228*	0.307*	0.281
8	Vitamin E low	---	0.216 0.115 0.377 0.244 0.159 0.144 0.187	0.141 0.075 0.095 0.097 0.141 0.147 0.141 0.106	sacrificed 0.069 0.119 sacrificed cod liver oil 0.109 sacrificed cod liver oil
			0.206*	0.118*	0.099*
2**	Positive control	3 ml daily		0.266 0.266	0.169 0.156
				0.266*	0.162*
2**	Vitamin E low	3 ml daily		0.147 0.141	0.072 0.072
				0.144*	0.072*

* Average

** Prior to administration of cod liver oil the lambs were on commercial lard diet for 40 days.

TABLE 13

TOCOPHEROL CONTENT OF BLOOD PLASMA IN LAMBS ON MOLECULAR DISTILLED LARD DIET AND THE EFFECT OF ALPHA-TOCOPHEROL THERAPY ON THE LEVEL OF PLASMA TOCOPHEROLS

No of Lambs	Diet	Alpha-tocopherol Therapy	Days on Experiment 0-15	15-25	25-40	40-55	55-65	65-75	75-80
			0.131	0.228	0.162	0.299	0.406	0.238	0.428
			0.070	0.112	0.091	0.187	0.234	0.153	0.175
	Positive		0.059	0.172	0.332	0.159	0.366	0.294	0.241
6	control	---	0.078		0.150		0.148		
					0.131		0.109		
					0.109				
			0.085*	0.171*	0.163*	0.215*	0.252*	0.228*	0.281*
			0.131	0.228	0.147	0.069		0.000	
			0.159	0.084	0.040	0.097		0.069	
			0.159	0.097	0.054	0.084		0.069	
			0.090	0.156	0.065	0.069		0.000	
			0.078	0.097	0.109	0.128		0.084	
	Vitamin E		0.040	0.097	0.050	0.069		0.069	
15	deficient	---	0.127	0.125	0.103	0.097			
			0.116	0.097	0.091	0.069			
				0.084	0.078	0.050			
				0.156	0.091	0.069			
					0.134				
					0.050				
					0.103				
					0.050				
					0.103				
			0.113*	0.122*	0.084*	0.080*		0.049*	
							0.131		0.175
							0.384		0.313
	Vitamin E	Initial dose					0.200		
8**	deficient	500 mg					0.250		
							0.200		
		100 mg every					0.806		
		other day					0.128		
							0.131		
							0.279*		0.244*

* Average ** Prior to alpha-tocopherol therapy the lambs were on vitamin E deficient diet for 40 to 50 days.

nounced increase of blood plasma tocopherol.

Deficient lambs on the commercial lard diet showed a gradual and consistent drop in tocopherols content of blood plasma over the 70 day experimental period. On the diet containing molecular distilled lard, about the same drop in tocopherol content of the plasma occurred in 30 to 35 days. The average tocopherol level of the deficient lambs which survived over 60 days on the molecular distilled diet was 0.049 mg per 100 ml of blood with two lambs showing a complete absence of tocopherol in the blood.

Daily administration of 3 ml cod liver oil for 25 days to two control and three deficient lambs lowered the average tocopherol content of blood plasma respectively from 0.266 to 0.162 mg and from 0.144 to 0.072 mg.

Gross Pathology

Post mortem examination of the control lambs did not reveal significant changes. Three out of 12 control lambs developed signs of pneumonia during the experiment and were treated with procaine penicillin. The lungs of these three lambs showed unilateral "cold pneumonia" confined to the right apical lobe. Gross pathological studies of every vitamin E deficient lamb revealed degeneration of skeletal and cardiac muscle accompanied by pneumonia or lung congestion. The degeneration of skeletal muscles ranged from very mild to severe dystrophy depending on the length of time the particular lamb was on the deficient diet. In the early

stages of deficiency the affected muscles were pale and anemic. Later, the lesions in the skeletal muscles appeared on the surface as grayish or whitish areas or streaks. Usually only a part of the muscle was affected. On cross section the whitish or grayish areas again indicated that not all of the muscle fibers and bundles were involved. The affected muscles were moist and characteristic yellow grayish gelatinous material was present between the muscles and especially around the sciatic and brachial nerves. It was observed that the muscles of both hind and fore legs were affected earliest and showed much more severe degeneration than the muscles of the shoulder, rump, loin or neck. In four severe cases, practically all of the musculature was affected including the diaphragm, intercostal muscles, masseter and the tongue. Examinations of the carcasses showed that both sides of the body were about equally affected.

The most striking feature of the deficient lambs was the characteristic lesions in the heart which, in most instances, were confined or were most pronounced within the right ventricle (Fig. 10). Again, depending on the length of time the animals were on the deficient diet, the degree of degeneration was variable. The lesions were usually in the form of round patches ranging in size from pin point up to 12 mm in diameter (Fig. 11) and occurred singly, in groups or were very diffuse and affected most of the ventricle. The lesions were of yellowish or whitish color and the line of demarcation between

the degenerated and healthy area was very distinct. In the later stages of deficiency under the superficial appearing lesions of the endocardium, degeneration of the myocardium was in progress and, in one extreme case, the degeneration of the cardiac wall reached the epicardium (Fig. 12). In this particular case the lesions were found in both ventricles and atria including the aorta. In four other instances the lesions were observed in the left ventricle and atria. The hearts in general were flabby and the amount of fluid in the pericardial sac was greatly increased. Diffuse subepicardial hemorrhages were observed in a few.

Respiratory distress was one of the earliest symptoms of vitamin E deficiency and later pneumonia was confirmed at necropsy. Twenty four out of thirty deficient animals showed localized unilateral or bilateral pneumonia. In most instances pneumonia was unilateral and only the apical and cardiac lobes were involved. In other cases the once affected parts of the lung were consolidated and in the process of healing.

In only three lambs the pneumonia was active, affected both lungs and was considered a primary cause of death. In all three lambs the thoracic cavity was filled with a large amount of fibrious exudate which adhered to the walls of the thoracic cavity. The lungs were imbedded in this exudate. In 12 control lambs only two showed pneumonia at autopsy. The lungs of the control and deficient lambs were cultured

on blood agar plates and in most instances gram negative pleomorphic organisms resembling Pasteurellae were isolated after[a] two to three day period of incubation.

The changes in the liver ranged from very mild fatty infiltration to focal necrosis. The liver was pale and when cut appeared greasy. In most instances there was general fatty degeneration. In three cases focal necrosis was observed.

The kidneys of the deficient lambs showed general fatty degeneration involving both the cortex and medulla. In a number of lambs the degeneration was pronounced, especially in the cortex which was yellowish in color with white streaks running from the capsule to the medulla.

Most of the adrenals seemed to be normal. A few appeared to be enlarged, showed numerous petichial hemorrhages in the cortex and general fatty degeneration. In three lambs which showed active bronchopneumonia and severe muscular dystrophy the adrenals were damaged to the extent that the line of demarcation between the cortex and medulla had disappeared.

Microscopic Pathology

Gross and microscopic studies of the muscle tissues from various parts of the body indicated that the muscle degeneration was progressing from the hind part of the body toward the fore part. Also the severity of the muscle dystrophy and its extent over various parts of the body was

directly correlated with the length of time the lamb was on the deficient diet. However, the nature of the myodegeneration was about the same in all cases. The chain of events was visualized as hyaline degeneration of the sarcoplasm followed by fragmentation of the affected muscle fibers and coagulation necrosis accompanied by cellular infiltration, proliferation of muscle fiber nuclei and fibroblasts. In the early stages of degeneration only a few muscle fibers were involved while others in the same area were normal (Fig. 13). The affected muscle fibers first appeared to be swollen and later became shrunken in diameter and length, assuming a wavy character and stained darker with Hematoxylin and Eosin (Fig. 14). The interstices of the muscle fibers were filled with cellular material and fluid (Fig. 15). The muscle fibers lost the cross and later the longitudinal striation, and became granular (Fig. 16). The granular sarcoplasm became a homogeneous mass inside the sarcolemma which was still not affected. Finally the hyalinized sarcoplasm fragmented and vacuoles within the sarcolemma were observed (Fig. 17). The nuclei of the muscle fibers either became pyknotic, or had undergone karyorrhexis or karyolysis. A rather mild infiltration of the necrotic areas with polymorphonuclear leukocytes, lymphocytes and macrophages suggested that the injured muscle tissue was initially involved in an inflammatory pattern (Fig. 18). This was before repair and regeneration had commenced. At the same time numerous macrophages and giant cells

invaded the area. The role of giant cells in clearing the debris was evident (Fig. 19). As the muscle fibers disappeared they were replaced with very rapidly proliferating fibroblasts. Some areas were completely devoid of muscle fibers and a typical fibrosis within the muscle was observed. However, there was also ample evidence to show that simultaneous degeneration and regeneration of the muscle was taking place. New muscle fibers characterized by fainter stain and centrally located nuclei arranged in rows were often detected in the muscles of both the deficient and treated lambs (Fig. 20).

The lesions in the cardiac muscle were similar to those in the skeletal musculature. Starting with the endocardium the degeneration progressed into the myocardium (Fig. 21). The line of demarcation between the necrotic and normal areas was rather sharp. The individual muscle fibers showed changes characterized by hyaline degeneration, fragmentation and coagulation necrosis followed by proliferation of fibroblasts (Fig. 22). Increased activity of the muscle nuclei and invasion of the areas with macrophages, neutrophils and lymphocytes were also noted. The giant cells observed in the degenerated skeletal muscle were absent in the cardiac muscle. It seemed that the proliferation of fibroblasts in the areas of degeneration was more intense than in the skeletal muscle. Some areas were almost completely composed of fibroblasts and connective tissue (Fig. 23). Repair was also noticed in the lambs while still on the deficient diet. On many occasions

small, young muscle fibers faintly stained were detected in the areas of fibrosis (Fig. 24). It seemed that these new muscle fibers originated from adjoining normal muscle and that regeneration was progressing from the myocardium toward the endocardium. It was interesting to note that the Purkinje fibers appeared to be normal despite the fact that the immediate areas were necrotic (Fig. 23).

The liver in most instances was congested and showed changes ranging from cloudy swelling to focal necrosis. Frozen sections stained with Sudan IV showed pronounced fatty degeneration which was rather diffuse affecting all of the liver. The central part of the lobule was usually filled with fat globules to a greater extent than the periphery. Occasionally the congestion was very severe in the central veins and the adjoining sinusoids were filled and distended with blood.

In most animals, especially in those with secondary pneumonia, the kidney was congested and the changes ranged from cloudy swelling to a pronounced fatty degeneration (Fig. 25). The proximal convoluted tubules were dilated, and filled to some extent with hyaline material. The renal corpuscles appeared to be more or less normal. The congestion was especially severe in the medulla and some hemorrhages were also noted (Fig. 26).

Microscopic studies of the adrenals did not show consistent changes. In most of the instances the adrenals were

normal or showed slight fatty infiltration. In two or three lambs the adrenals were completely degenerated. The architecture was completely lost and the whole adrenal was a mass of coagulation necrosis.

Treatment and Estimated Alpha-tocopherol Requirement

Eight lambs from the second and third trials showing advanced symptoms of deficiency characterized by inability to get up were treated with alpha-tocopherol. The initial dose was 500 mg, followed by 100 mg every other day administered orally. A definite response was noticed in three to five days. On the fourth and fifth day the animals were able to get up and to move to some extent. Later the animals were able to move freely and did not show symptoms of deficiency. The lambs were sacrificed at intervals during the alpha-tocopherol therapy, namely over a period of 10 to 45 days. This was done to study the process of recovery grossly and the muscle regeneration microscopically. The symptoms disappeared completely in 15 to 20 days. Post mortem examination of the lambs treated with alpha-tocopherol up to about 25 days revealed still visible degeneration of the skeletal and cardiac muscle and some slight changes in the liver and kidney. It was observed, however, that especially after 20 and 25 days of therapy the skeletal muscle degeneration seemed to be less marked. The heart lesions were not as extensive and the patches were smaller in size and of grayish color, less whitish

and ischemic than in progressive degeneration. The borders between the normal and affected areas were also less distinct.

Microscopic examination of the tissues from the skeletal and cardiac muscles revealed repair of the necrotic areas by very active muscle regeneration characterized by increased proliferation of muscle fiber nuclei and fibroblasts and by increased numbers of macrophages and giant cells (Fig. 27). This was especially true in the lambs which had been on therapy 10 and 15 days. The regeneration due to therapy seemed to be the same as that observed while the degeneration was still in progress. The new muscle fibers appeared to be originating from the sarcolemma of the degenerated muscle or from the muscle fiber nuclei which proliferated extensively. The new muscle fibers varied in size, stained faintly with hematoxylin-eosin, displayed various degrees of cross or longitudinal striation and one pole terminated in a round point which probably was the active center of growth (Fig. 28, 29, 30). The nuclei, always numerous, were arranged in rows and were centrally located (Fig. 31). As the therapy continued, more and more new muscle fibers appeared and in lambs treated for 40 and 45 days the regeneration was almost complete except that the increased number of muscle nuclei was still noted (Fig. 32). The same process of new muscle fiber formation was observed in the cardiac muscle. The two lambs treated for 40 and 45 days did not show heart lesions grossly or significant

changes microscopically. Transverse and longitudinal section of skeletal muscles from two positive control lambs were shown in Fig. 33 and 34.

Unfortunately it was impossible in this study to determine the exact quantitative requirement of the young lamb for vitamin E (alpha-tocopherol). The 100 mg of alpha-tocopherol administered orally every other day was set arbitrarily and appeared to be more than adequate. Evidence to substantiate this was a marked increase of the blood tocopherol level in the control lambs over the initial level which could be considered normal for the young lamb. Furthermore, a rather remarkable response to the therapy consisting of 500 mg alpha-tocopherol as an initial dose and 100 mg every other day suggested that 100 mg of alpha-tocopherol was enough to arrest the degenerative changes in the musculature, to clear up the symptoms, initiate muscle regeneration and support normal biological processes in the body.

"Stiff-Lamb" Disease in the Field

While these experimental studies were in progress, many cases of the so-called "Stiff-Lamb" disease were reported by farmers in Michigan. A number of affected lambs, dead or alive, were brought to the laboratory for necropsy and provided an ample opportunity to study and compare the gross and microscopic pathology of both field and experimental cases. Furthermore, the incidence of "stiff-lambs" in the sheep flock at

Michigan State College permitted observation of the symptoms in the field as well as the opportunity for therapy with alpha-tocopherol. The symptoms, gross and microscopic degenerative lesions in the skeletal and cardiac muscle (Fig. 35, 36, 37, 38, 39), and the response to therapy were identical with those produced experimentally using the purified vitamin E deficient diet. The degree and extent of muscle degeneration was variable but in every case both skeletal and cardiac muscles were involved. It was noticed, however, that degeneration of skeletal muscles in the lambs from the field was much more severe and affected larger areas than in the lambs on the experimental diet.

In regard to heart lesions no difference was observed between the experimental and field cases. Again the heart lesions were found usually in the right ventricle (Fig. 40). However in one lamb both the right and left heart was involved (Fig. 41,42). Secondary pneumonia was noticed in every field case which suggested that respiratory distress and pneumonia are characteristic features of nutritional muscular dystrophy occurring under field conditions. As none of the treated and recovered "stiff lambs" was sacrificed, a direct comparison of the muscle regeneration was not available. Judging from the disappearance of the symptoms and from the complete recovery of the lambs in the field, the process of muscle regeneration was no doubt the same.

DISCUSSION

Until 1928 when Evans and Burr developed paralysis in young suckling rats from mothers deprived of vitamin E, this vitamin was thought to be entirely concerned with reproduction. Since then vitamin E deficiency, characterized grossly by locomotor disturbances and microscopically by degeneration of the skeletal and cardiac muscles, has been experimentally produced and described in guinea pigs and rabbits (Goettsch and Pappenheimer 1931), rats (Pappenheimer 1939), dogs (Brinkhouse and Warner 1941) and in mice (Pappenheimer 1942). Vitamin E deficiency in chicks, characterized by encephalomalacia, was described by Pappenheimer (1931) and Jungherr (1949). Ducklings on the same diet developed a general myopathy (Pappenheimer and Goettsch 1934) while turkeys showed myopathy of the gizzard (Jungherr and Pappenheimer 1937).

Degeneration of the skeletal and cardiac muscle in the lamb similar to Zenker's degeneration, produced experimentally in laboratory animals, was first described by Metzger and Hagan (1927). These authors recognized the degenerative changes in the muscles as noninfectious and noninflammatory in nature. Since then many workers in this country and abroad have described a similar condition among lambs and postulated various causes responsible for the disease and suggested a variety of preventive measures.

Willman et al. (1934) produced "stiff lambs" from ewes kept on a specific natural ration and definitely proved the nutritional nature of the disease (Willman et al. 1940).

Morgulis and Spencer (1936), Shimotori et al. (1940), MacKenzie and McCollum (1941) and Willman et al. (1941) reported that wheat germ oil, wheat germ meal, wheat bran and alpha-tocopherol protected guinea pigs, rabbits and lambs from nutritional muscular dystrophy.

Willman et al. (1945, 1946) prevented and cured "stiff lambs" by administration of pure alpha-tocopherol or its derivatives to pregnant ewes or affected lambs immediately after birth.

Whitting et al. (1949) analyzed the blood colostrum and milk for tocopherols from ewes on a vitamin E deficient diet and blood tocopherol level from affected lambs. The values were up to 48 per cent lower when compared with normal control ewes and lambs. In view of these findings it was believed that nutritional muscular dystrophy described as "Stiff-Lamb" disease was due to vitamin E deficiency.

The role of vitamin E in protecting the normal structure of the muscle, and the biochemical changes in regard to the composition and physiology of the muscle fiber due to vitamin E deficiency are unknown. There were, however, some facts which permitted speculation on the possible role of this vitamin in regard to muscle dystrophy. The most striking characteristic of tocopherols is their antioxidant activity.

If tocopherols would retain their antioxidant activity in vivo it could be rightly thought that most of their biological functions would be centered around this property in the metabolism of fat.

Dam (1949) in a series of experiments demonstrated the presence of peroxides in the body fat of chicks and rats reared on a vitamin E low diet and supplemented with 5 per cent cod liver oil. The control animals on the same diet supplemented with 2.5 mg per cent of alpha-tocopherol acetate showed no peroxide value and absence of the deficiency symptoms. He suggested that degeneration of the muscle was primarily due to some product which resulted from the process which the highly unsaturated fatty acids underwent in the cells while vitamin E was lacking. Further evidence of antioxidant activity of tocopherols *in vivo* was demonstrated by Harris *et al.* (1944). Tocopherols exerted a pronounced sparing action on vitamin A in rats fed a vitamin A low diet. Whitting *et al.* (1949) studied the effect of tocopherols on the vitamin A mammary and placental transfer in the sheep, goat and pig. The only increase of vitamin A was noted in the liver of the newborn lamb.

Harris *et al.* (1947) increased butter fat production in dairy cows by supplementing the ration with 500-1,000 mg of alpha-tocopherol.

The sparing or protective action of tocopherols on fat itself was shown by Menschik *et al.* (1949). Mice on a high

vitamin E diet made substantial gains in body weight in the form of fat, while fat of those on the vitamin E free diet was lost and replaced with unsaturated fatty acids, phospholipids and protein.

Roderuck et al. (1949) studied rather extensively the chemical and enzymic alterations in muscles affected by experimental dystrophy. QO_2 values of muscles from vitamin E deficient rabbits, guinea pigs and hamsters were greatly increased. This suggested that alpha-tocopherol was involved in the overall cellular oxidation. The nature of the substrate in the muscle undergoing increased combustion during vitamin E deficiency was unknown. However, decreased creatine content of the muscle, creatinuria and increased creatine in the liver, reported also by Goettsch and Brown (1932) in rabbits, indicated that protein metabolism was involved. Analyses of the muscle tissues for various amino acids showed glutamine to be especially abundant in the normal muscle as free amino acid. The glutamine content of dystrophic muscles was markedly lowered. However, the total non-glutamine amino acid content seemed to be unchanged. Roderuck provided further evidence in support of the idea that protein rather than fat metabolism was involved in the process of muscle degeneration. It is a well known fact that biotin is destroyed in the presence of auto-oxidizing unsaturated fats. If tocopherol acted as an antioxidant in vivo, its absence would promote auto-oxidation and production of unsaturated fatty acids and

peroxides which would destroy biotin. However, both free and total biotin values of normal and dystrophic muscles from hamsters, guinea pigs and rabbits varied within the limits of error. The authors admitted that if fatty acid peroxides were involved in muscle dystrophy the peroxides for some reason did not destroy biotin in vivo. Roderuck, in the course of these studies, also observed a marked decrease in the activity of the succinic dehydrogenase system. This inhibition was explained by Ames and Risley (1949). Alpha-tocopherol precipitated ionic calcium which is an activator of diphosphopyridine nucleotidase. It was interesting to note that all three tocopherols, alpha, gamma and delta phosphates, proved to have the same inhibitory effect and an addition of Ca ions increased the activity of the system to the same extent. The biological activity of the various tocopherols, however, was found to be entirely different.

The blood studies of the vitamin E deficient lambs showed a definite and consistent drop of the hemoglobin concentration throughout the experimental period. Three deficient lambs developed anemia toward the end of the experiment with values a little over 5 gms of hemoglobin per 100 ml blood. Gyorgy and Rose (1949) suggested and substantiated by many experimental studies <u>in vivo</u> and <u>in vitro</u> a protective action of tocopherol against hemolysis. Rats on a tocopherol deficient diet injected with 160 mg per kg of alloxan intraperitoneally

developed a severe hemoglobinuria and hemoglobinemia and died due to almost complete hemolysis. On autopsy the kidneys were completely filled with blood. Supplementation with tocopherol decreased the mortality and completely protected all animals from hemolysis. That alloxan as such and alone was not responsible for the hemolysis was evident from the fact that it disappeared from the blood stream in two to three minutes after the injection. Alloxan was possibly converted into a number of intermediate compounds in the absence of tocopherols. These compounds tested on vitamin E deficient rats showed more or less the same hemolyzing activity. Tocopherol again fully protected the animals from hemolysis. In vitro, studies showed that under appropriate conditions blood cells from vitamin E deficient animals were completely hemolyzed, while those of tocopherol supplemented animals were not affected. This experiment further demonstrated that the protection against hemolysis was a function of the cell and not of the body fluid. The authors stated that anemia was not characteristic of vitamin E deficiency unless under extremely abnormal conditions of metabolism. From the evidence it was concluded that tocopherol was necessary to protect the erythrocyte against hemolysis by its antioxidant effect.

A rather striking feature of experimentally produced vitamin E deficiency was the extremely high incidence of pneumonia. In practically every report of "Stiff-Lamb" disease encountered in the field, pneumonia was mentioned as one of

the symptoms or as a complication which resulted from the disease. All the "stiff lambs" brought to the laboratory for necropsy showed active or passive pneumonia. It was evident that a lack of vitamin E increased the susceptibility for respiratory infection which finally terminated in pneumonia. It was suggested in connection with riboflavin, that the organ with an especially high requirement for the vitamin was affected first. Evidence supporting this was available in the heart lesions but no studies were done in regard to the requirement and storage of tocopherols in the lung. Respiration of biopsied skeletal muscle tissues from vitamin E depleted animals was extensively studied by Houchin (1942) and Houchin and Mattill (1942). The oxygen consumption increased up to 250 per cent. Oral or intravenous administration of alpha-tocopherol considerably decreased the oxygen consumption in one hour and an almost normal respiration was restored in 22 hours. The oxygen consumption was also lowered by 40 per cent when alpha-tocopherol was added to the saline medium with dystrophic muscle slices from rabbits and hamsters.

Kaunitz and Pappenheimer (1943) measured the total oxygen consumption of a group of vitamin E deficient rats and a group supplemented with alpha-tocopherol. The total oxygen consumption of the deficient group was considerably higher. Since skeletal muscles account for nearly half of the body weight, it could be postulated that during vitamin E deficiency the

oxygen requirement is increased greatly. This, no doubt, would result in an extraordinary stress on the lungs and heart and increase the susceptibility to alteration.

The characteristic lesions in the heart were considered pathognomonic of experimentally induced vitamin E deficiency in the lamb. This was to some extent in conflict with observations and reports on the disease occurring under field conditions. Although observed and described, not all workers considered degeneration of heart muscle typical of vitamin E deficiency. Either the lesions were not reported in some instances or were not present at all. In the course of this study it was observed that lambs from the field at necropsy showed much more pronounced degeneration of the skeletal muscles and fewer and less distinct heart lesions than the lambs depleted on tocopherol deficient diet. This reversal was explained by a lack of exercise in lambs kept in small pens under laboratory condition and the excessive exercise of lambs running with ewes. It was quite possible that the skeletal muscles in lambs running freely on pasture, due to increased metabolism, were depleted sooner than in the lambs confined to small pens.

Some explanation for the delayed degeneration of the cardiac muscle as compared with skeletal muscle was reported by Mason (1942). The analyses of the heart and various organs for tocopherol showed that heart tissue from normal or defi-

cient rats contains twice as much of the vitamin as any other tissue including the liver. The author further explained the possible steps leading to the heart lesions and eventual failure. The overall deficiency greatly increased the demand for oxygen and reduced the capacity to work. This consequently caused dilatation and weakness of the organ which increased the susceptibility for pathological changes.

It was of interest to note that loss of appetite, retarded growth, diarrhea and emaciation, the symptoms of most of the vitamin deficiencies, were not observed in vitamin E deficiency produced experimentally or observed in the field. This strongly suggested that the deficiency described was due to lack of vitamin E and vitamin E alone.

SUMMARY AND CONCLUSIONS

Nutritional muscular dystrophy in the lamb was produced by using a liquid, purified vitamin E deficient diet. The source of fat in the diet of the first group was commercial lard while in the second and third groups molecular distilled lard, very low in vitamin E, was used.

The symptoms of vitamin E deficiency were characterized by disturbances in locomotion, stiffness, paralysis, susceptibility to pneumonia and sudden death. Blood analysis showed lowered hemoglobin concentration and low tocopherol levels.

Post mortem examinations showed dystrophy of skeletal and cardiac muscle and fatty degeneration of the liver and kidney. The lesions in the heart were invariably most pronounced in the right ventricle.

Addition of 100 mg alpha-tocopherol to the diet every other day protected 12 positive control lambs from muscular dystrophy and greatly alleviated the symptoms of depleted animals in three to five days. Microscopic studies showed almost complete regeneration of the muscle after 30 days of therapy.

The symptoms, gross and microscopic pathology of the musculature of lambs, affected with "Stiff-Lamb" disease under field conditions were also studied. The symptomathology and

lesions of "stiff lambs" from the field appeared to be identical in every respect with the nutritional muscular dystrophy produced on the purified vitamin E deficient diet.

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Fig. 8. Lamb No. 39.

A typical stance of the lamb in earlier stages of vitamin E deficiency. Note the arched back.

Fig. 9. Lamb No. 34.

Later stage of vitamin E deficiency. The lamb lost equilibrium and fell. The normal appearance of the head suggests no physical pain.

Fig. 10. Lamb No. 12.

Lesions within the right ventricle. Note the complete degeneration of the wall.

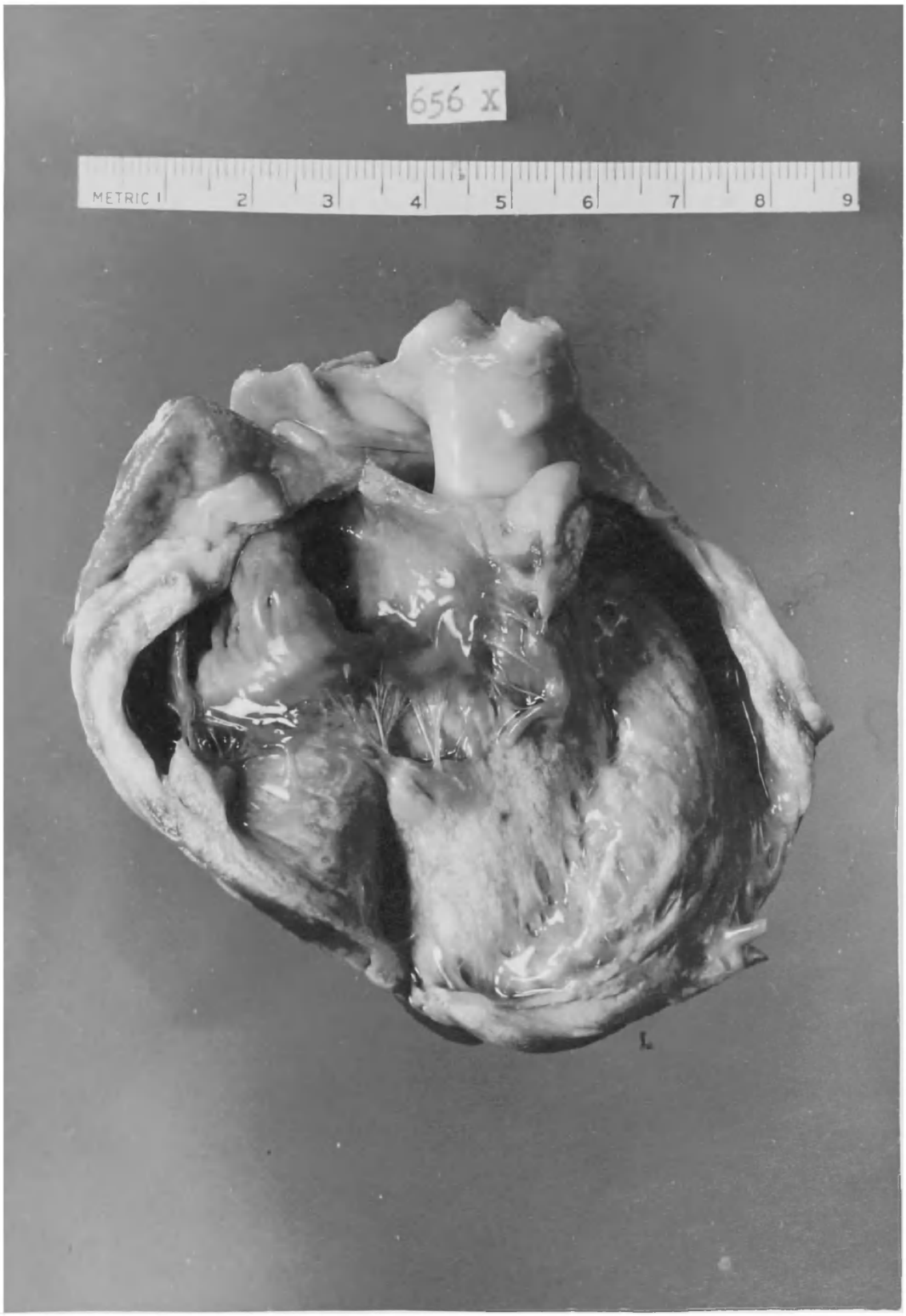
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Fig. 11. Lamb No. 24.

Right ventricle showing extensive Zenker's degeneration. The diameter of the lesion at the apex of the heart was approximately 12 mm.

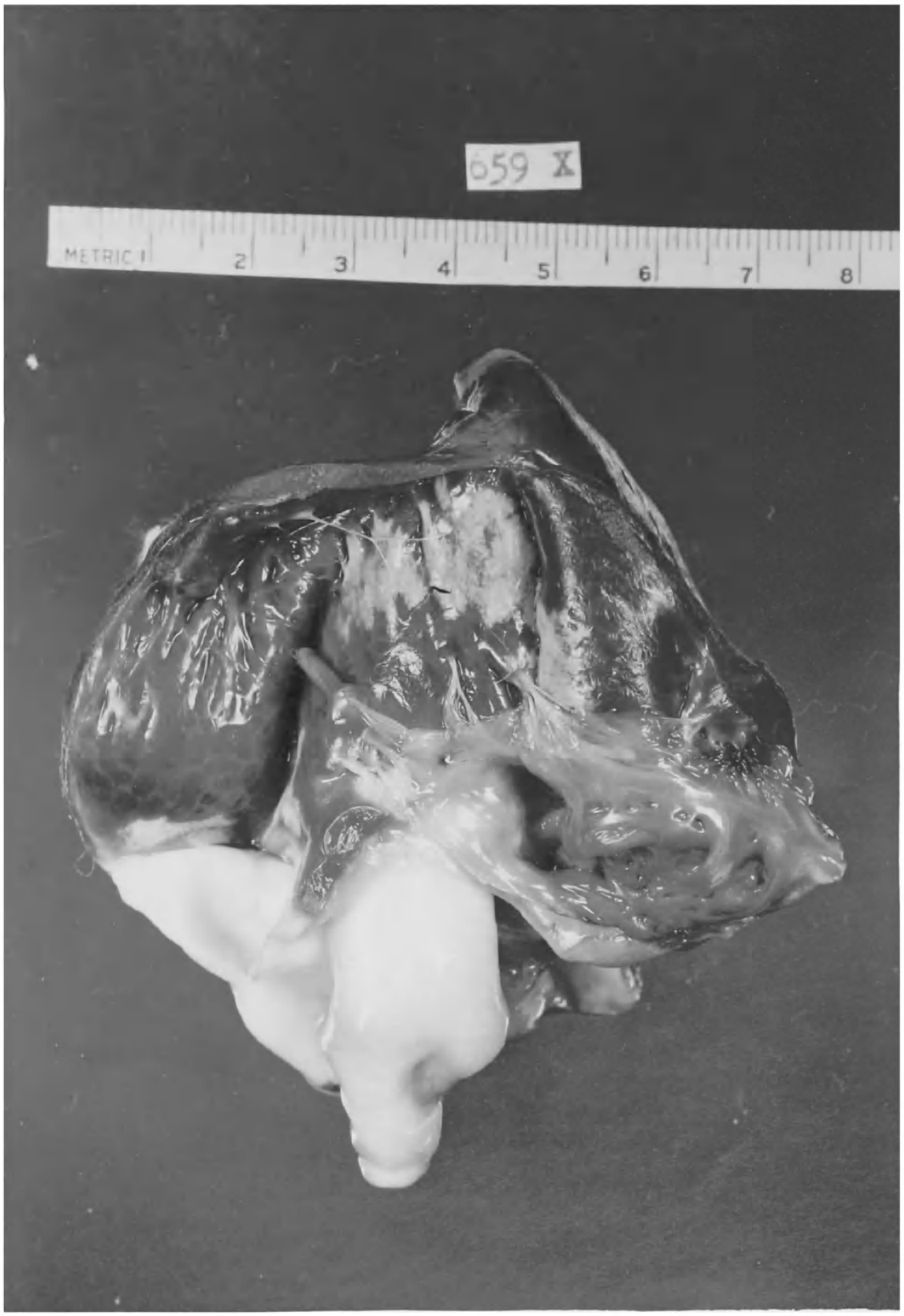
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Fig. 12. Lamb No. 12.

The degeneration involved the whole cardiac wall. Note the whitish lesions on the surface.

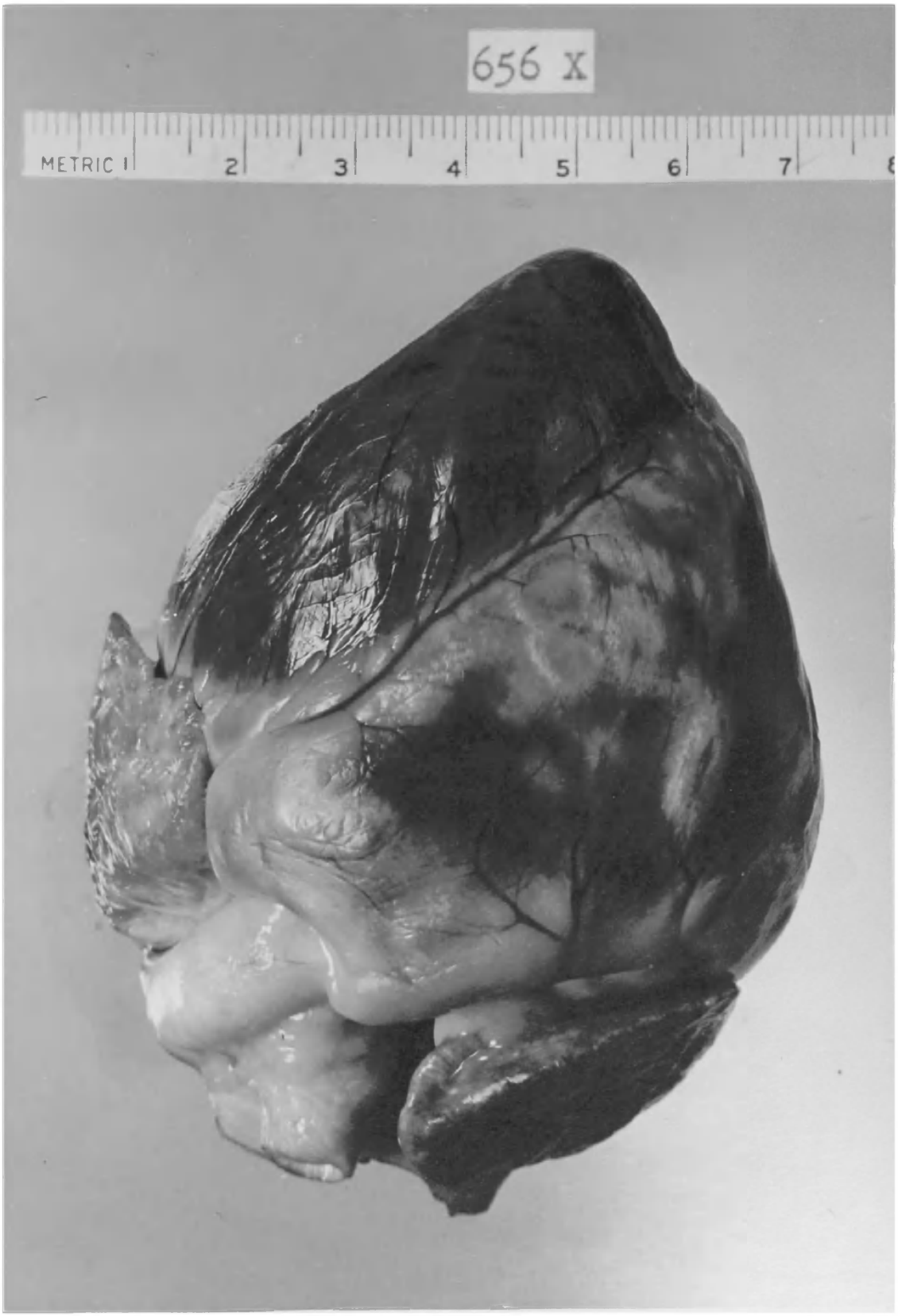
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Fig. 13. Lamb No. 14.

Loss of striation and extensive cellular infiltration within degenerated muscle fibers. Parts of the muscles are intact.

Hematoxylin and Eosin stain x 1150

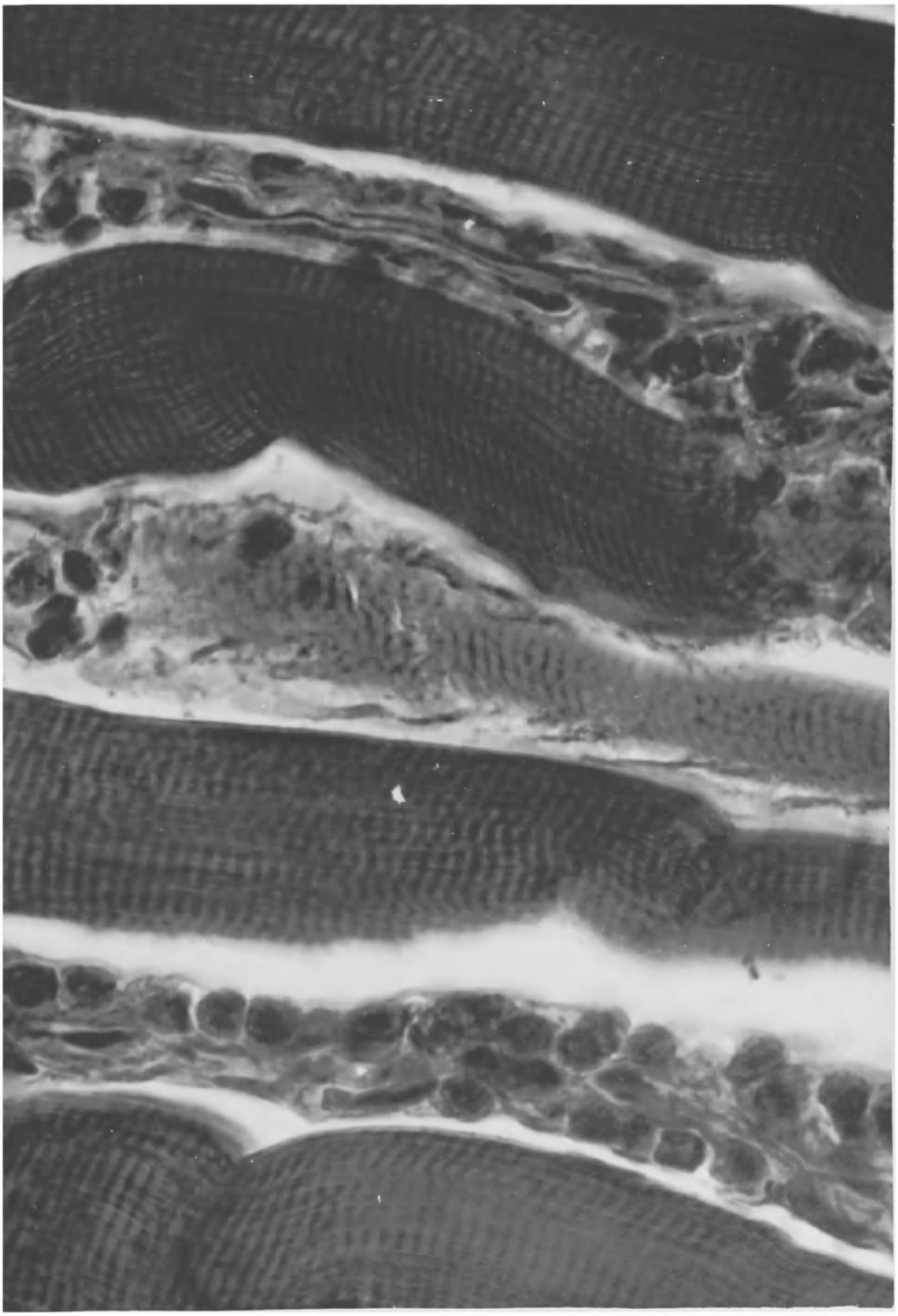

Fig. 14. Lamb No. 6.

The disappearing muscle fibers and proliferation of connective tissue.

Hematoxylin and Eosin stain x 170

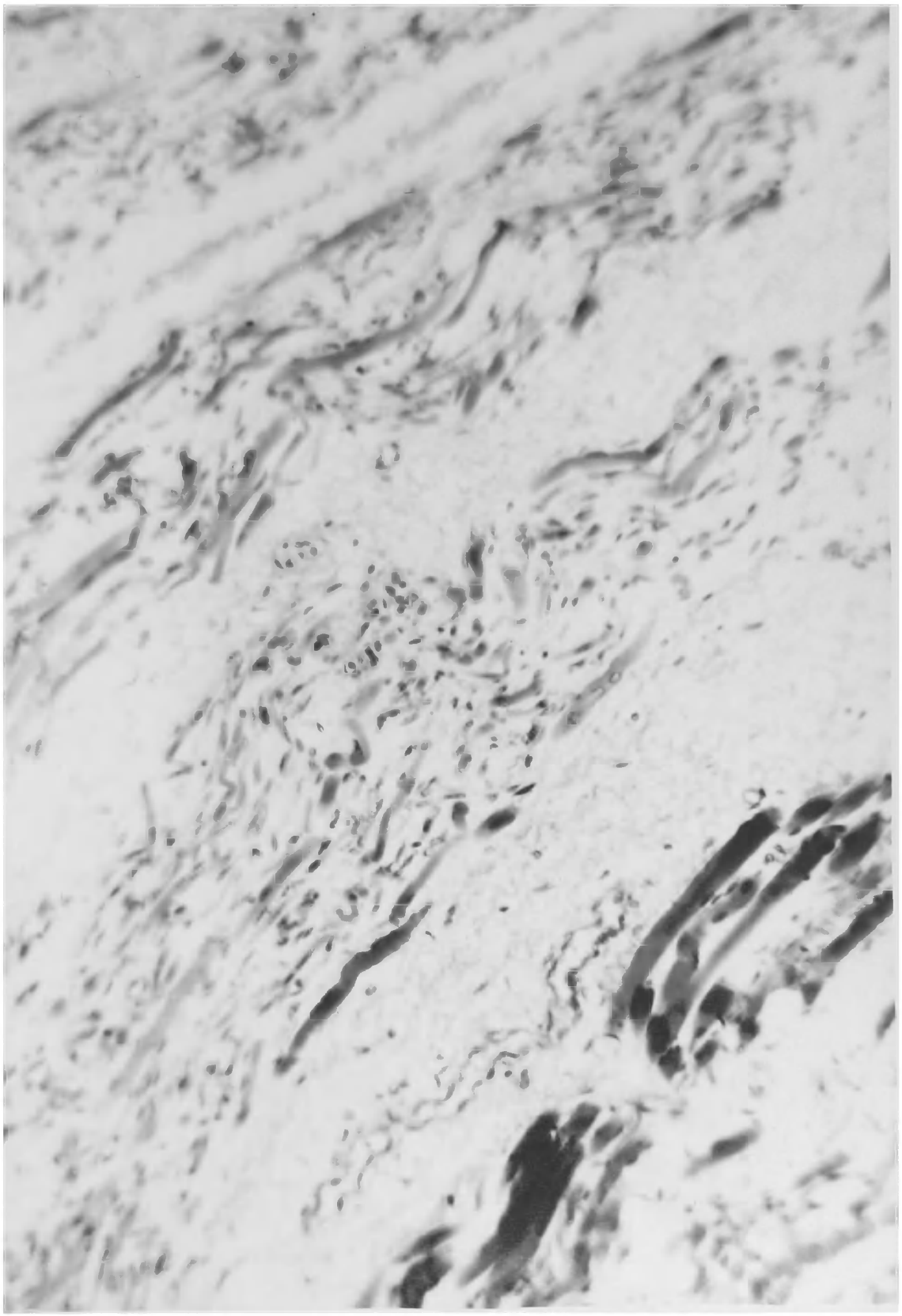

Fig. 15. Lamb No. 18.

Interstitial edema in the skeletal muscle. Fragmentation of the muscle fibers is in progress.

Hematoxylin and Eosin stain x 170

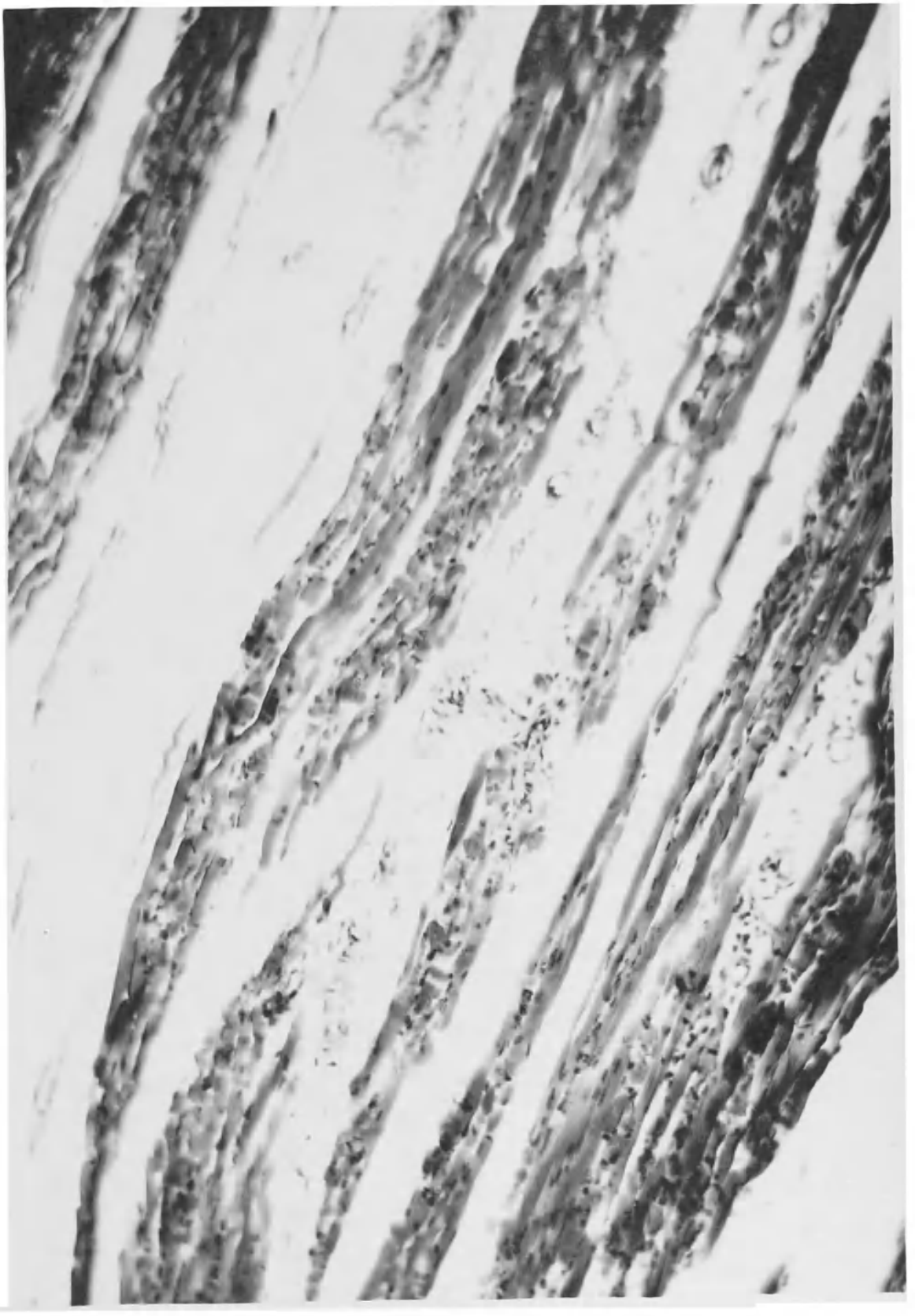

Fig. 16. Lamb No. 26.

Beginning of muscle degeneration. Note the increased granularity of the muscle fibers.

Hematoxylin and Eosin stain x 750

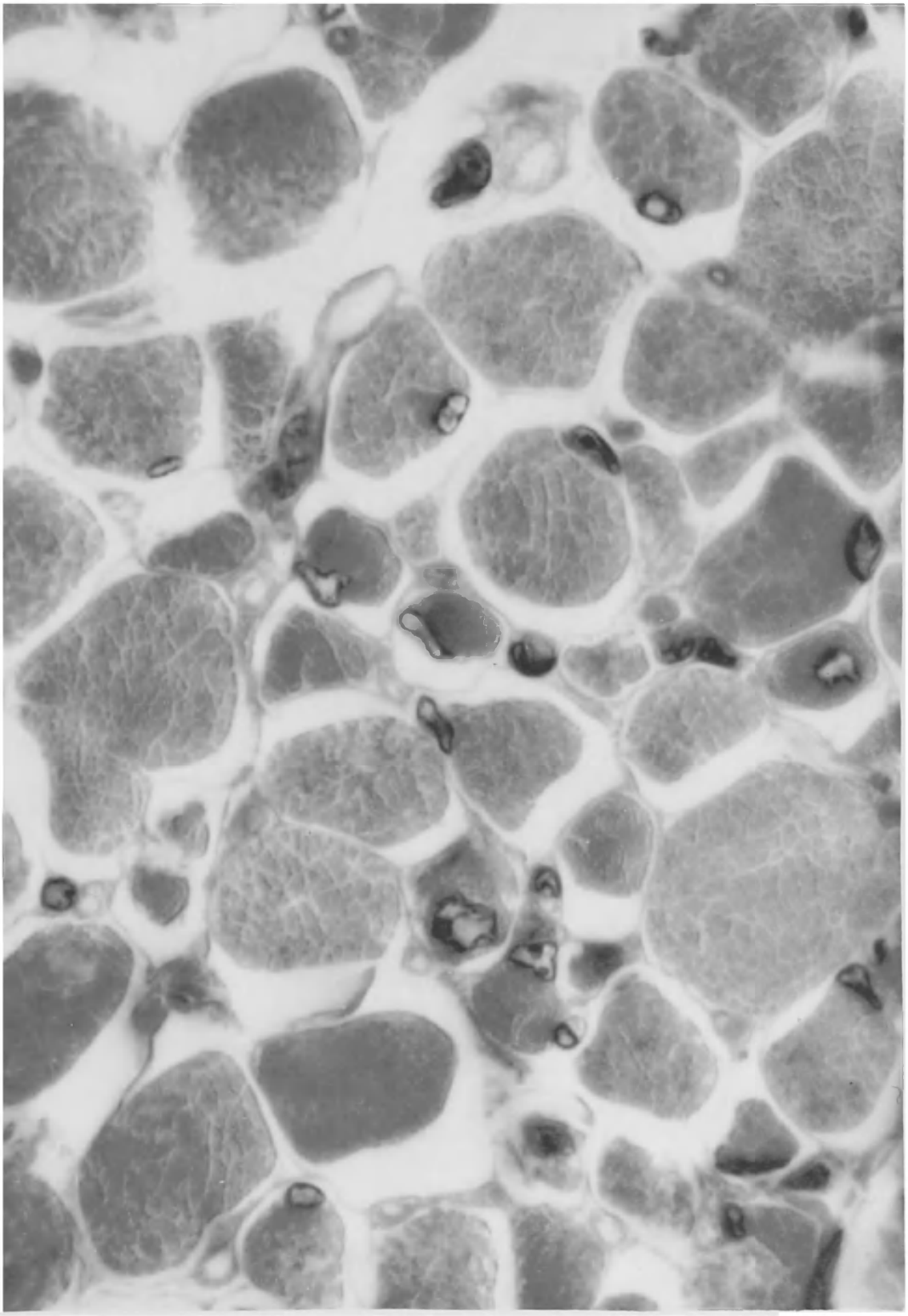

Fig. 17. Lamb No. 11.

Vacuolization of the affected muscle fiber.

Hematoxylin and Eosin stain x 1150

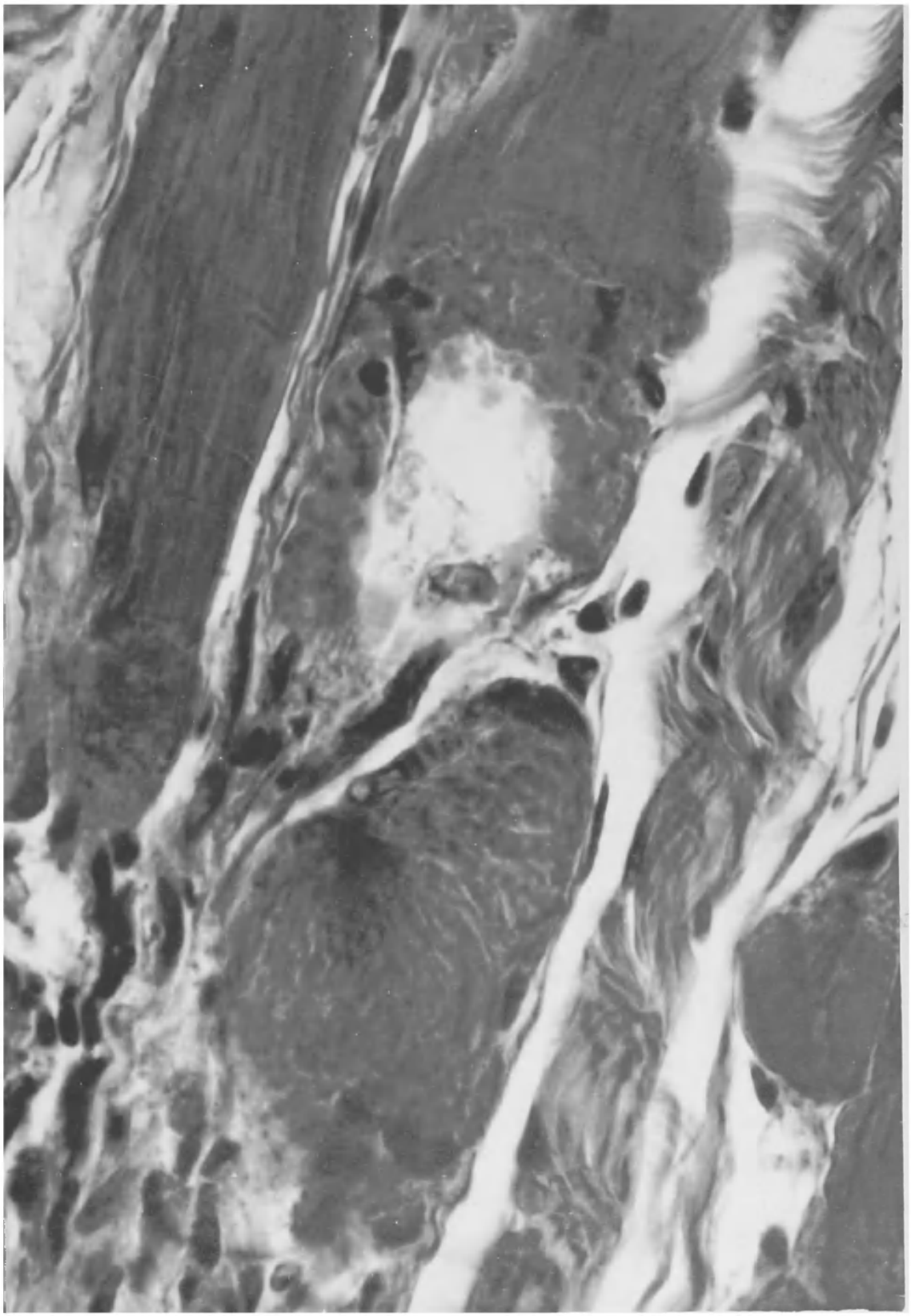

Fig. 18. Lamb No. 14.

Histiocytic infiltration in an area of degeneration and necrosis.

Hematoxylin and Eosin stain x 750

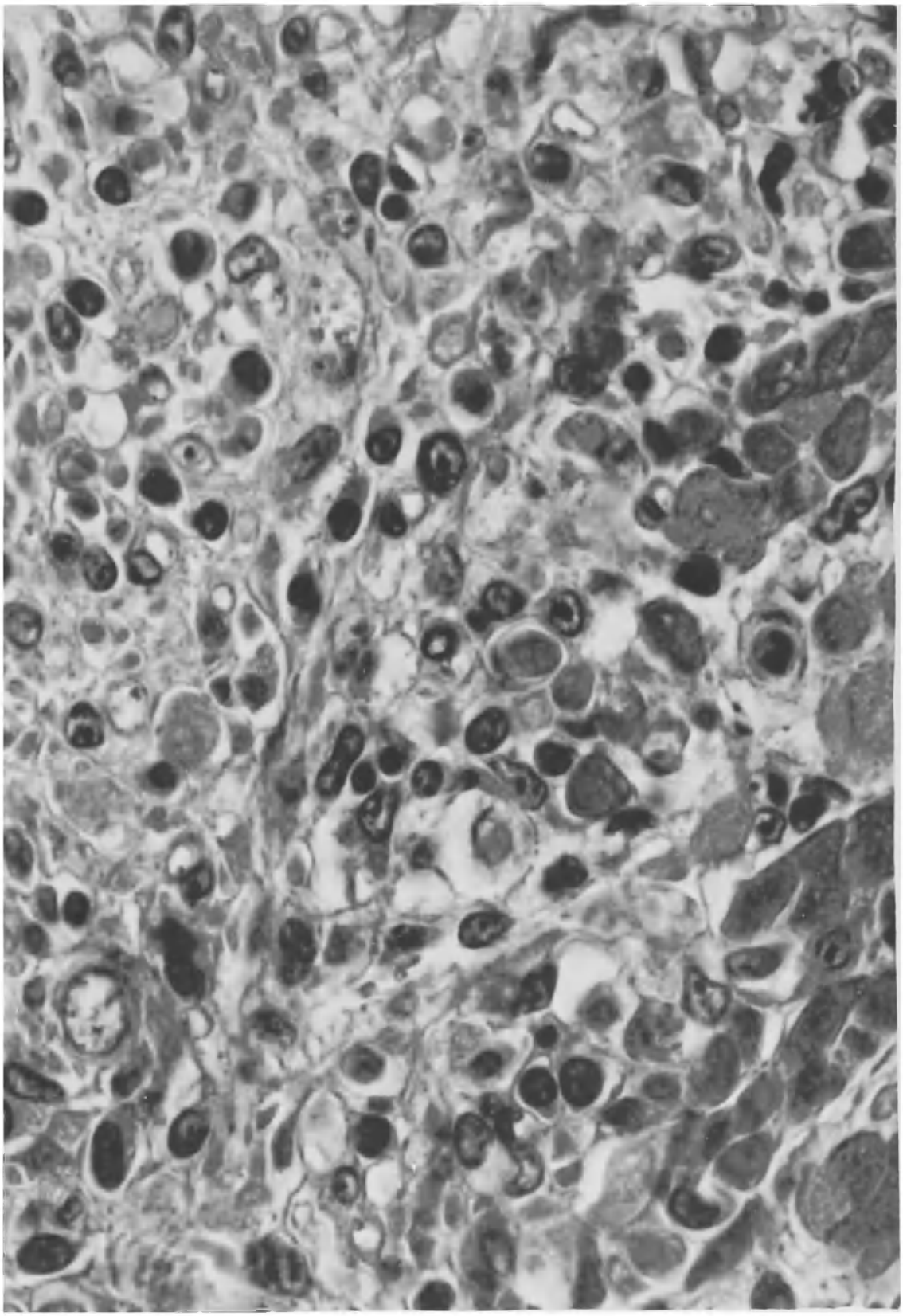

Fig. 19. Lamb No. 8.

Histiocytes and giant cell in the area of degeneration.

Hematoxylin and Eosin stain x 1150

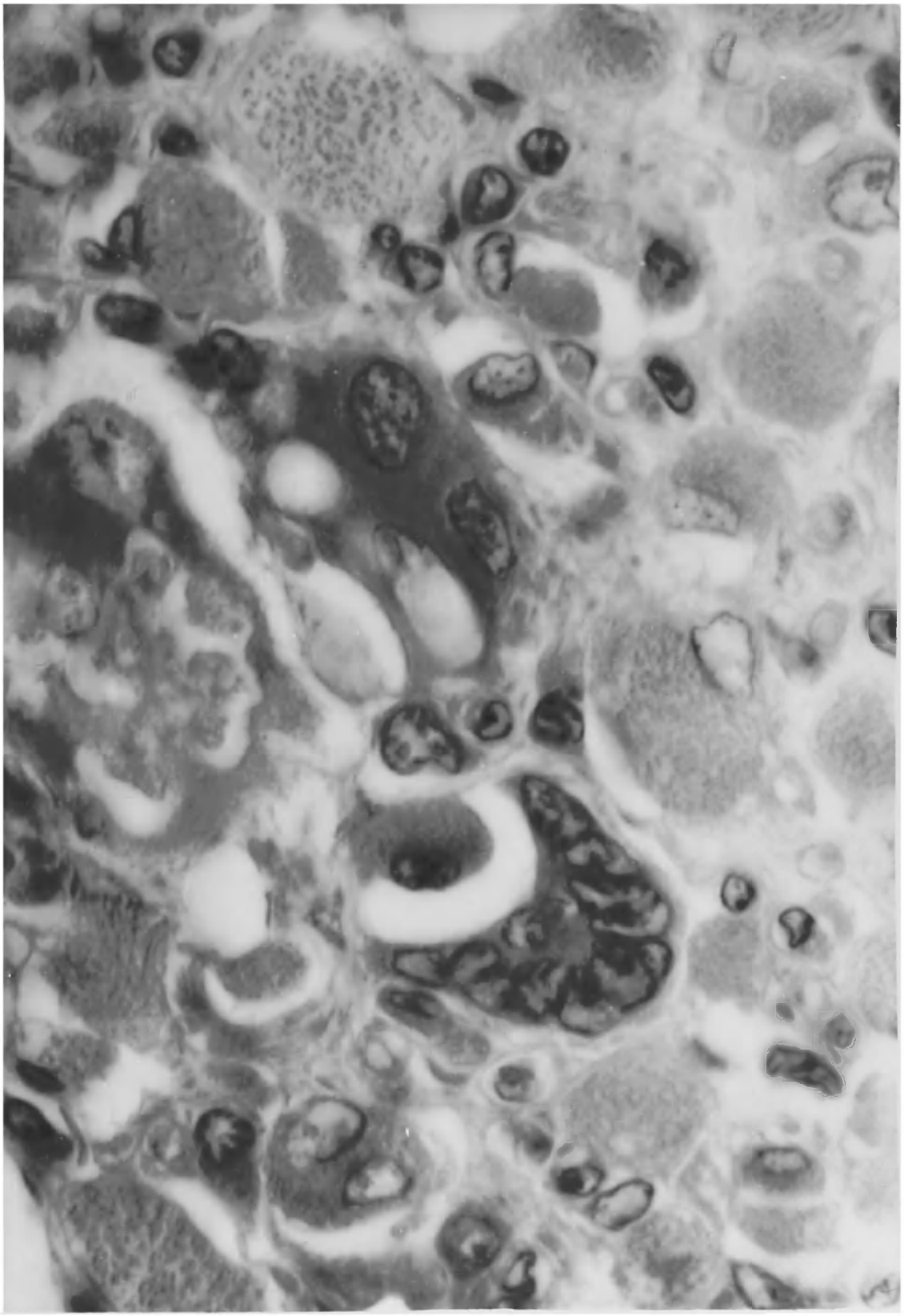

Fig. 20. Lamb No. 17.

Young muscle fibers showing longitudinal striation. The nuclei are located centrally and at the end of the muscle fiber. The lamb was not treated.

Hematoxylin and Eosin stain x 750

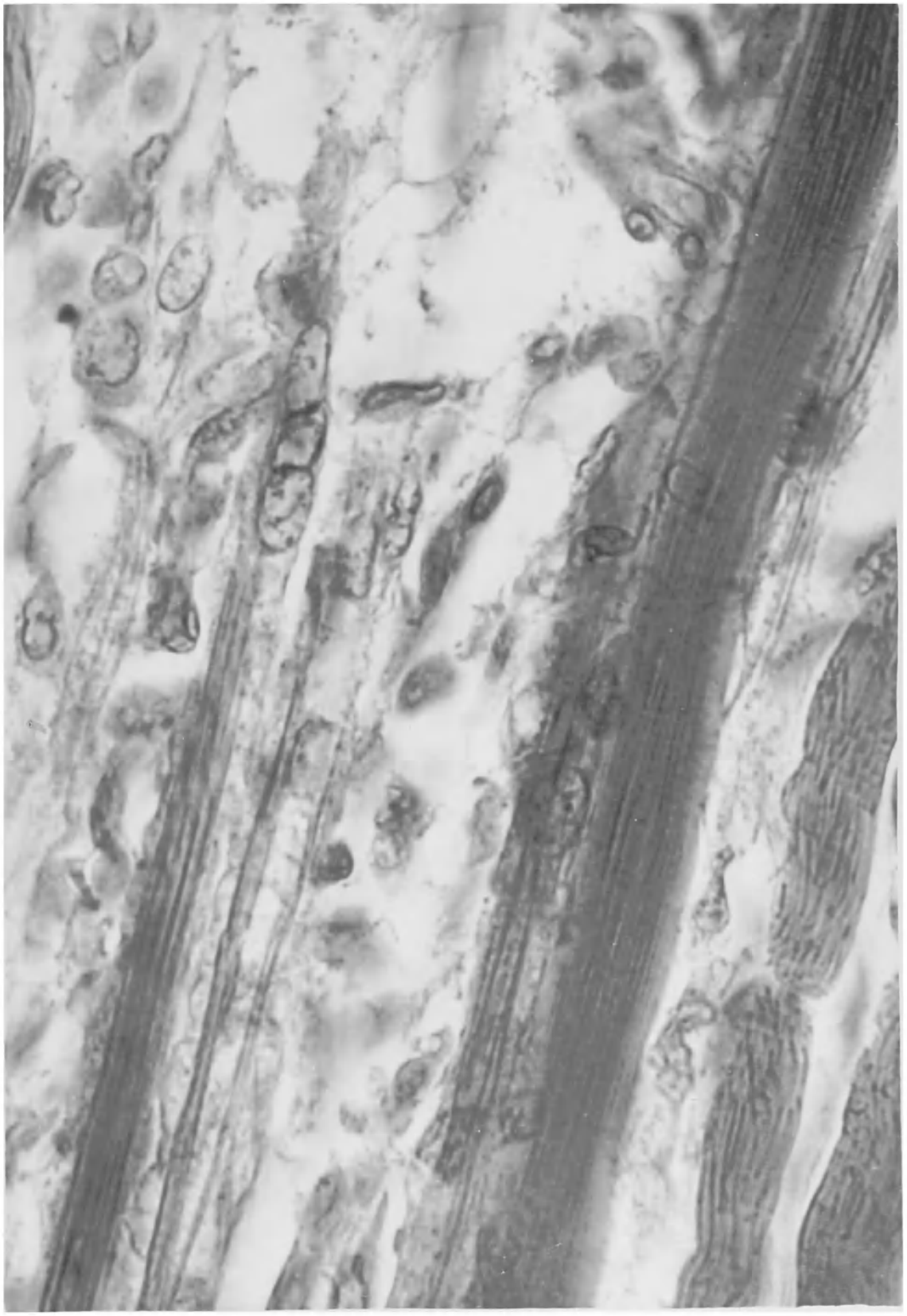

Fig. 21. Lamb No. 12.

Degeneration and necrosis extending from the endocardium into myocardium.

Hematoxylin and Eosin stain x 50

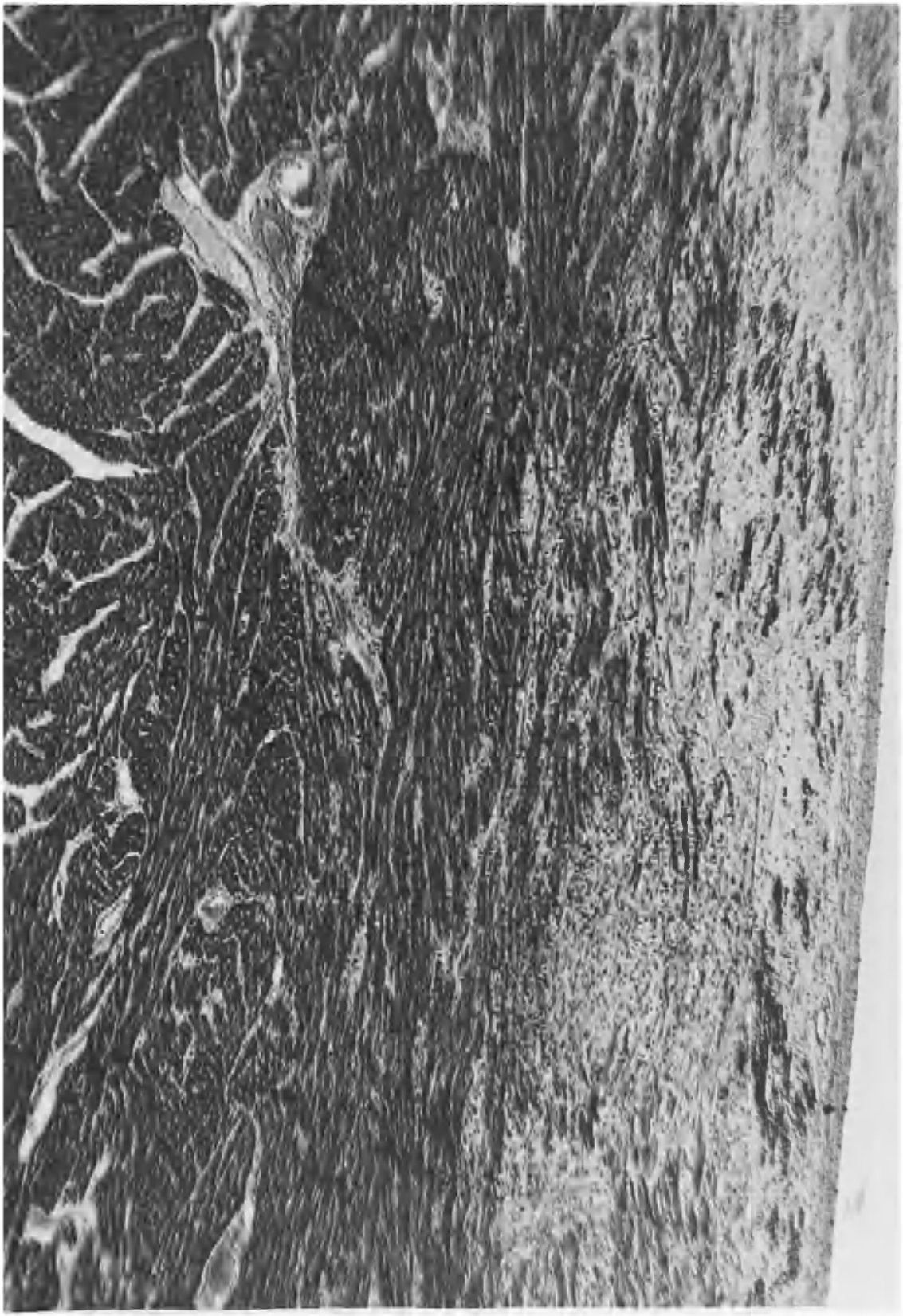

Fig. 22. Lamb No. 19.

Heart muscle showing fragmentation of the fibers and presence of histiocytes. The adjoining blood vessel is congested.

Hematoxylin and Eosin stain x 750

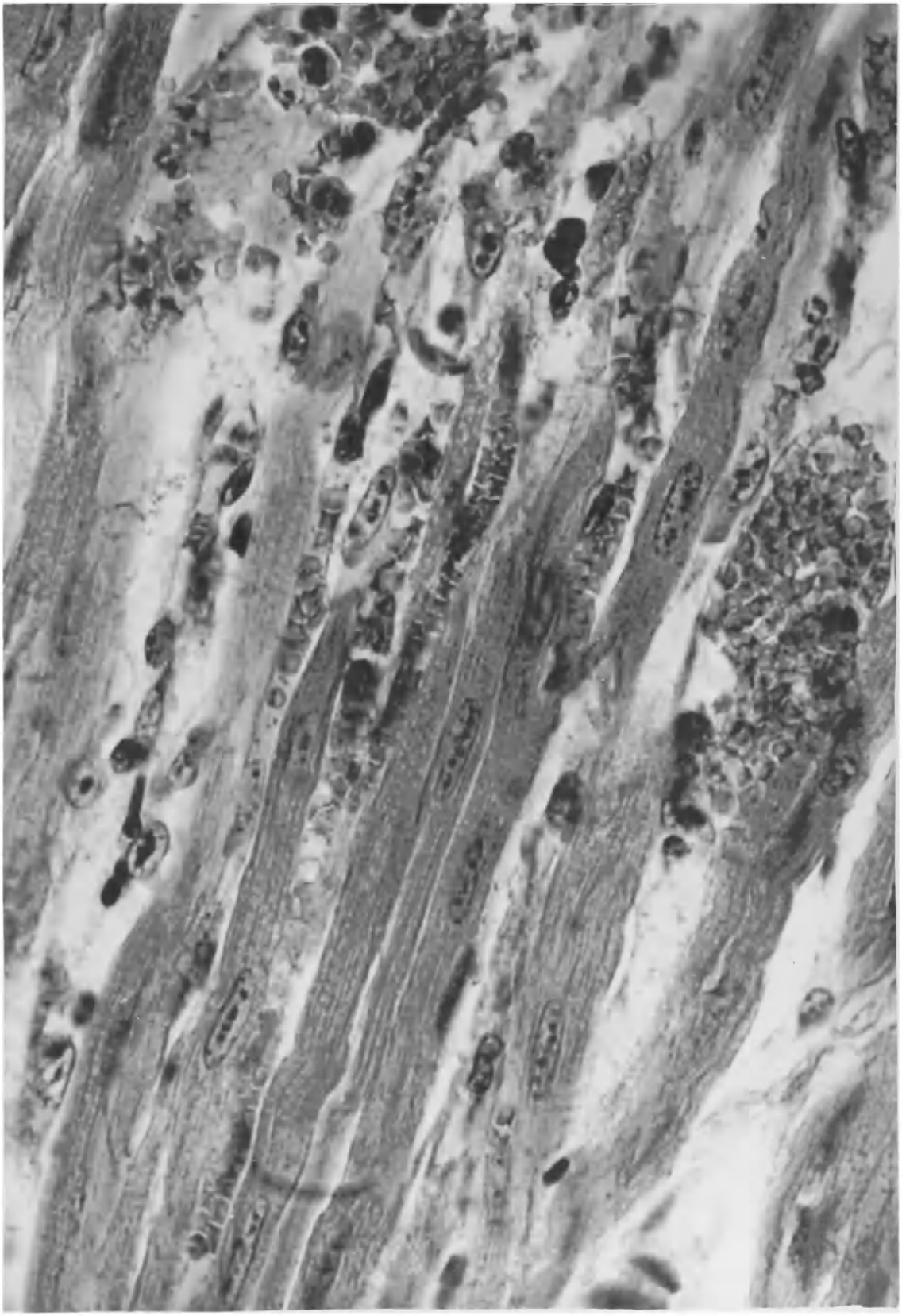

Fig. 23. Lamb No. 12.

Cardiac muscle showing degeneration and necrosis. The Purkinje fibers seem to be normal.

Hematoxylin and Eosin stain x 170

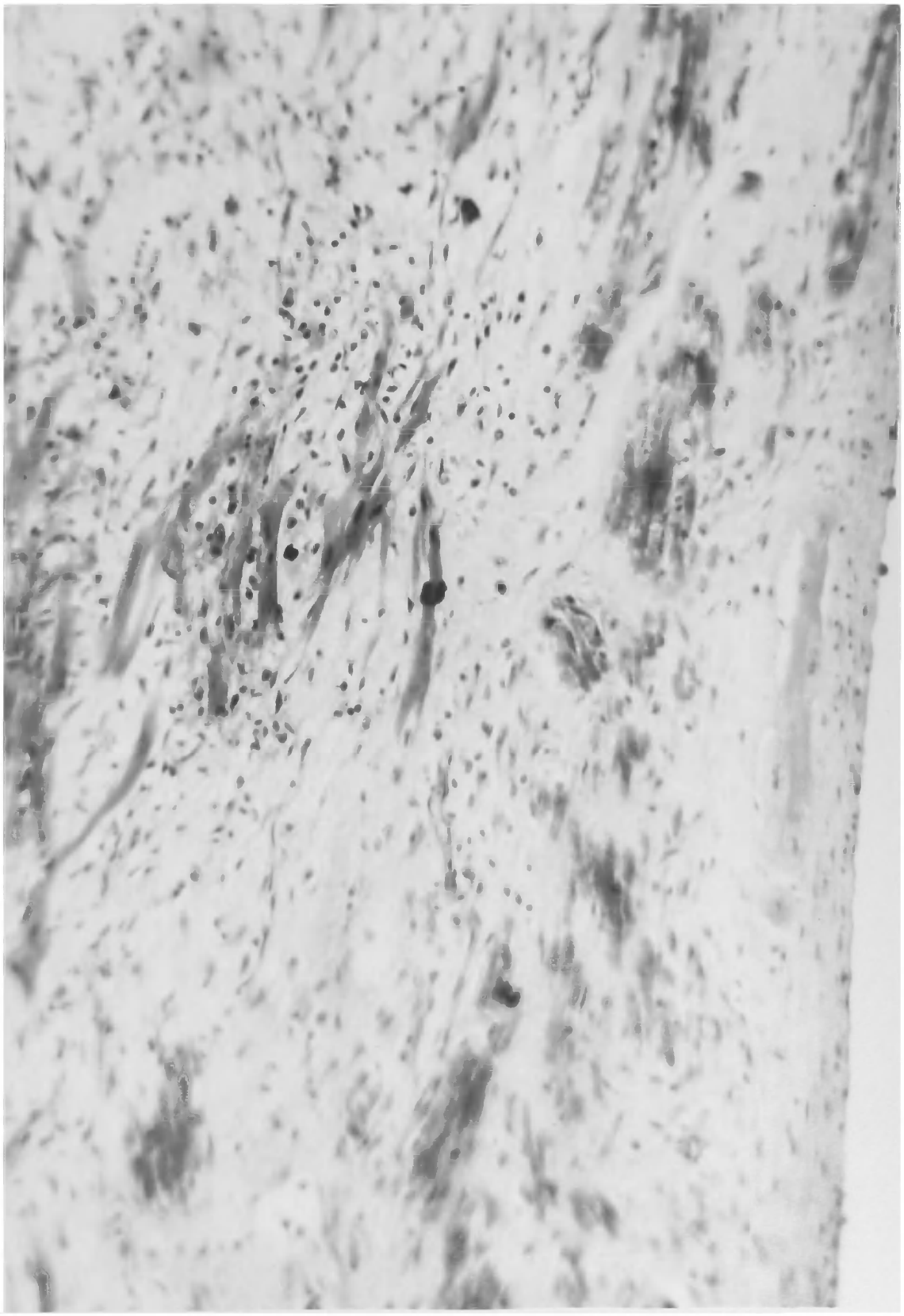

Fig. 24. Lamb No. 47.

Degenerating cardiac muscle. These young muscle strands are similar in appearance to capillaries but the protoplasm is continuous with the muscle fibers.

Hematoxylin and Eosin stain x 750

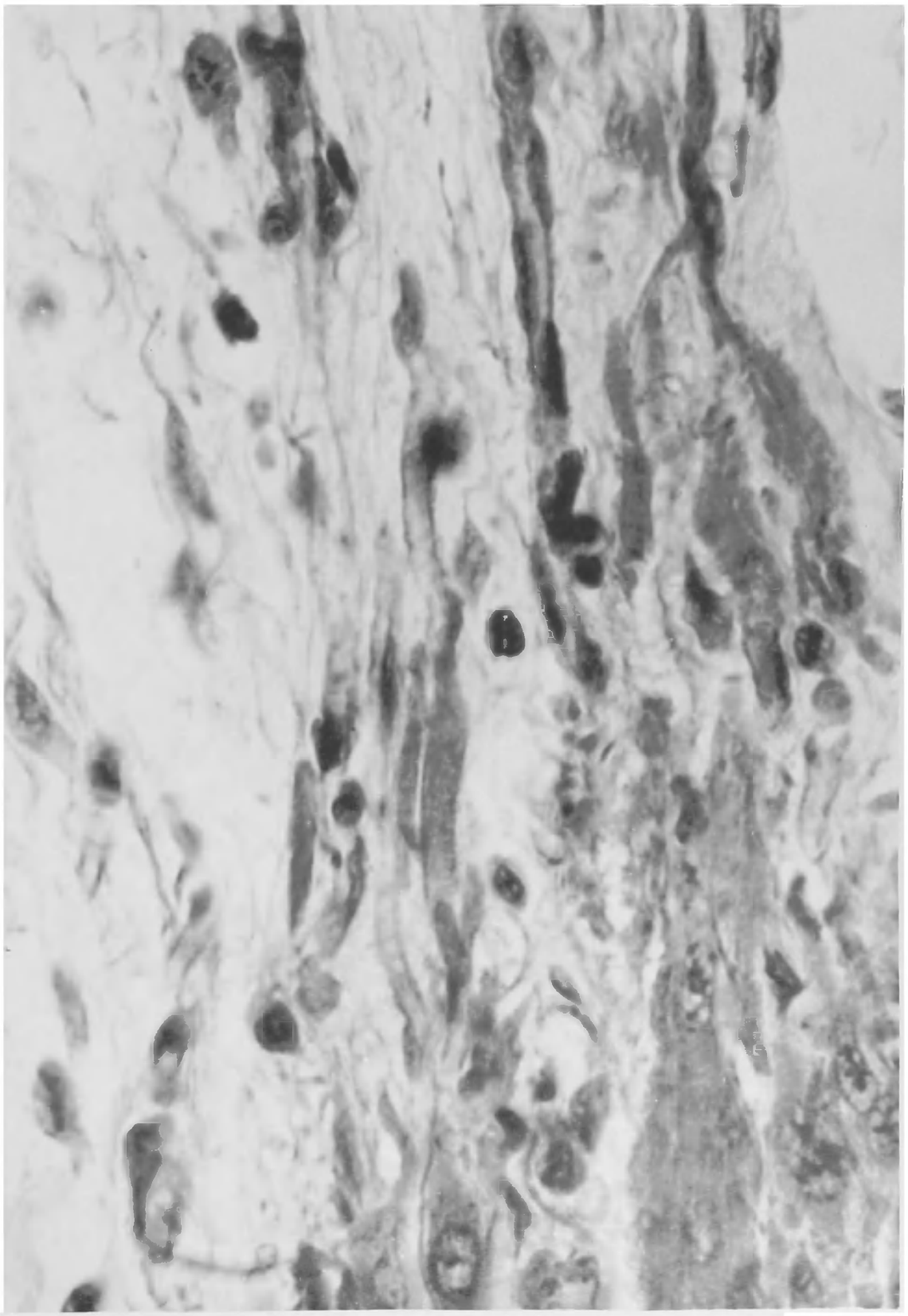

Fig. 25. Lamb No. 43.

Fatty degeneration of the kidney. Cells of the convoluted tubules are filled with fat droplets.

Frozen section Sudan IV stain x 170

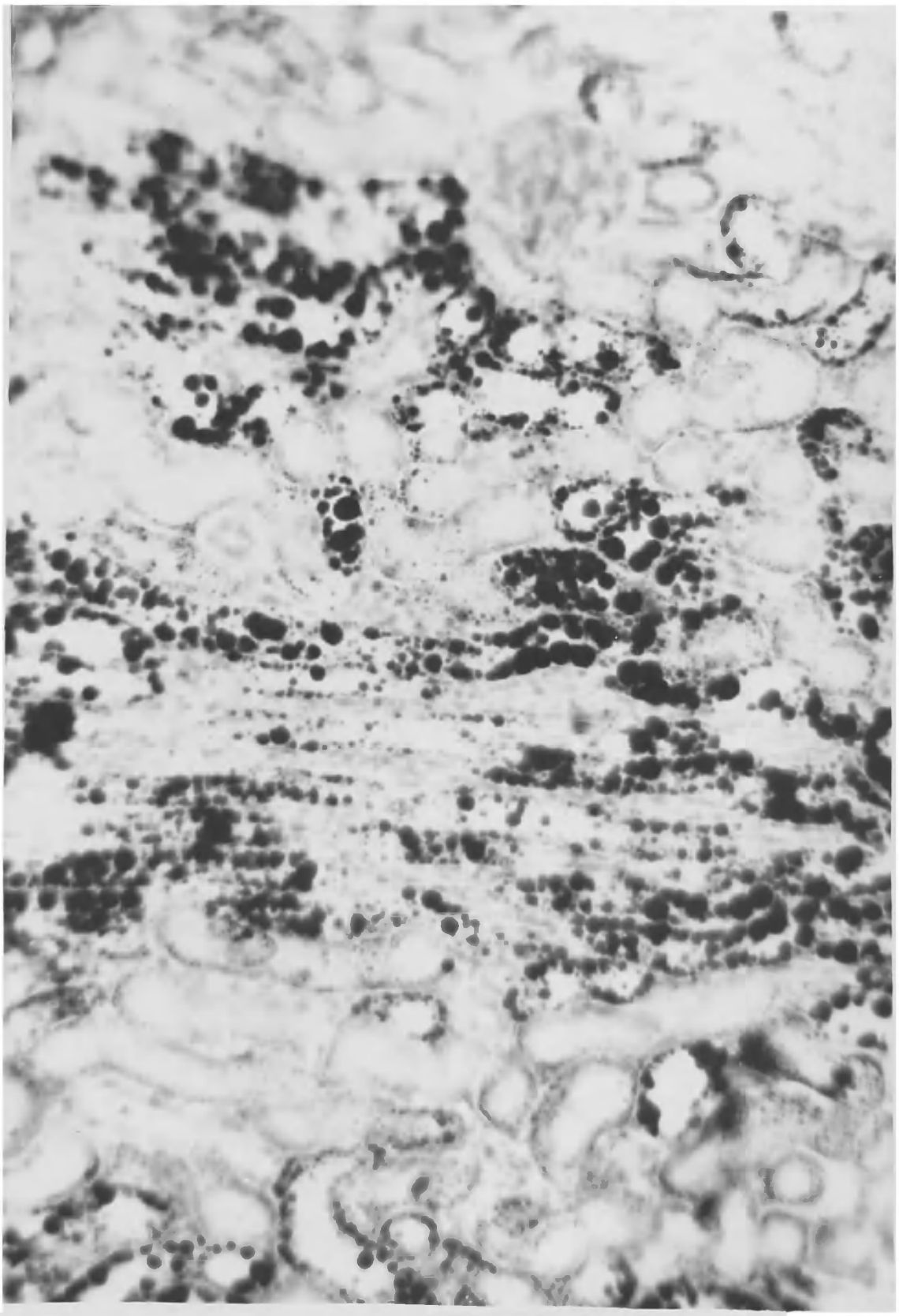

Fig. 26. Lamb No. 27.

Medulla of the kidney showing interstitial edema and congestion of blood vessels.

Hematoxylin and Eosin stain x 600

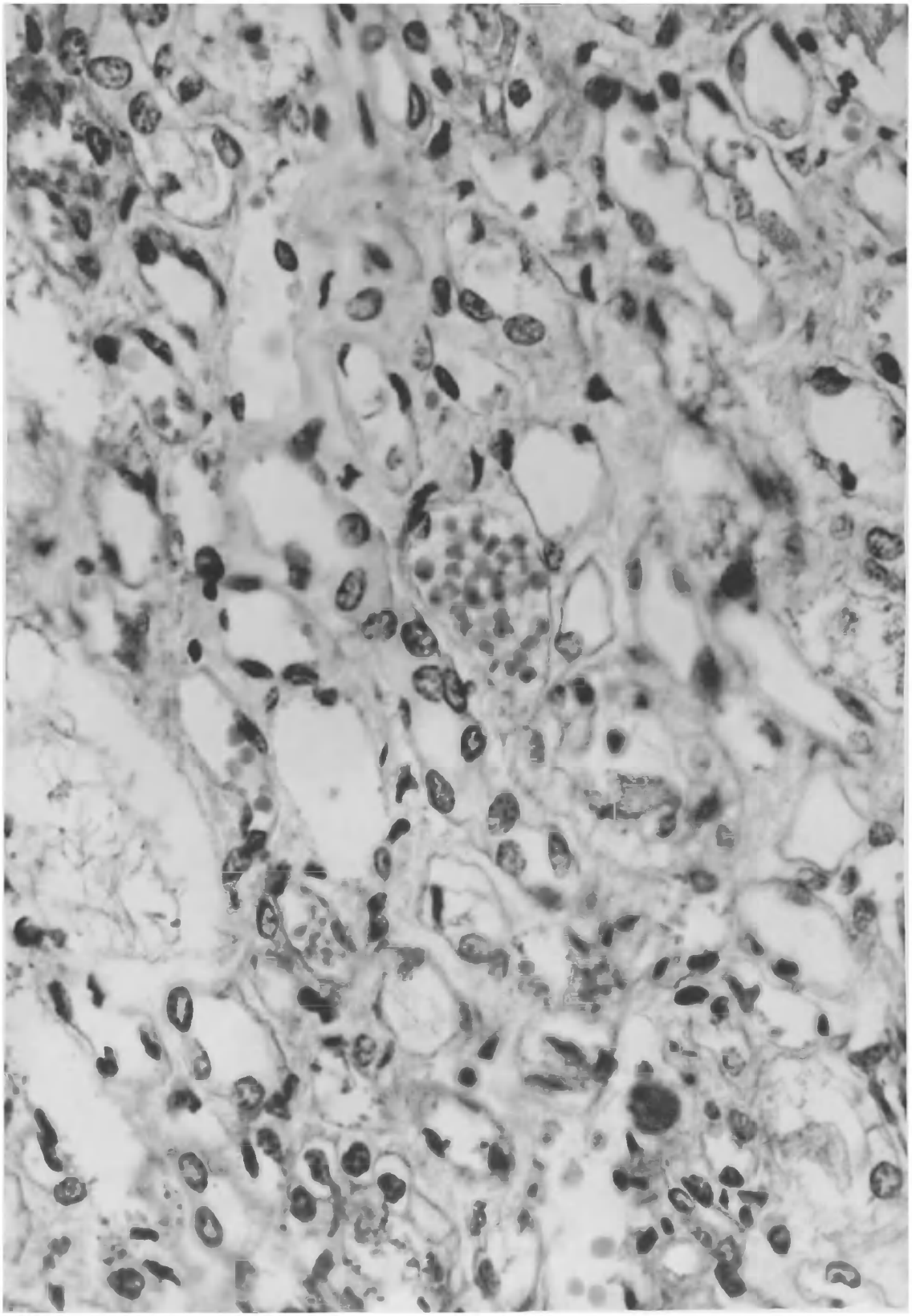

Fig. 27. Lamb No. 39.

Giant cells with engulfed debris. Intensive cellular infiltration is also noted.

Hematoxylin and Eosin stain x 750

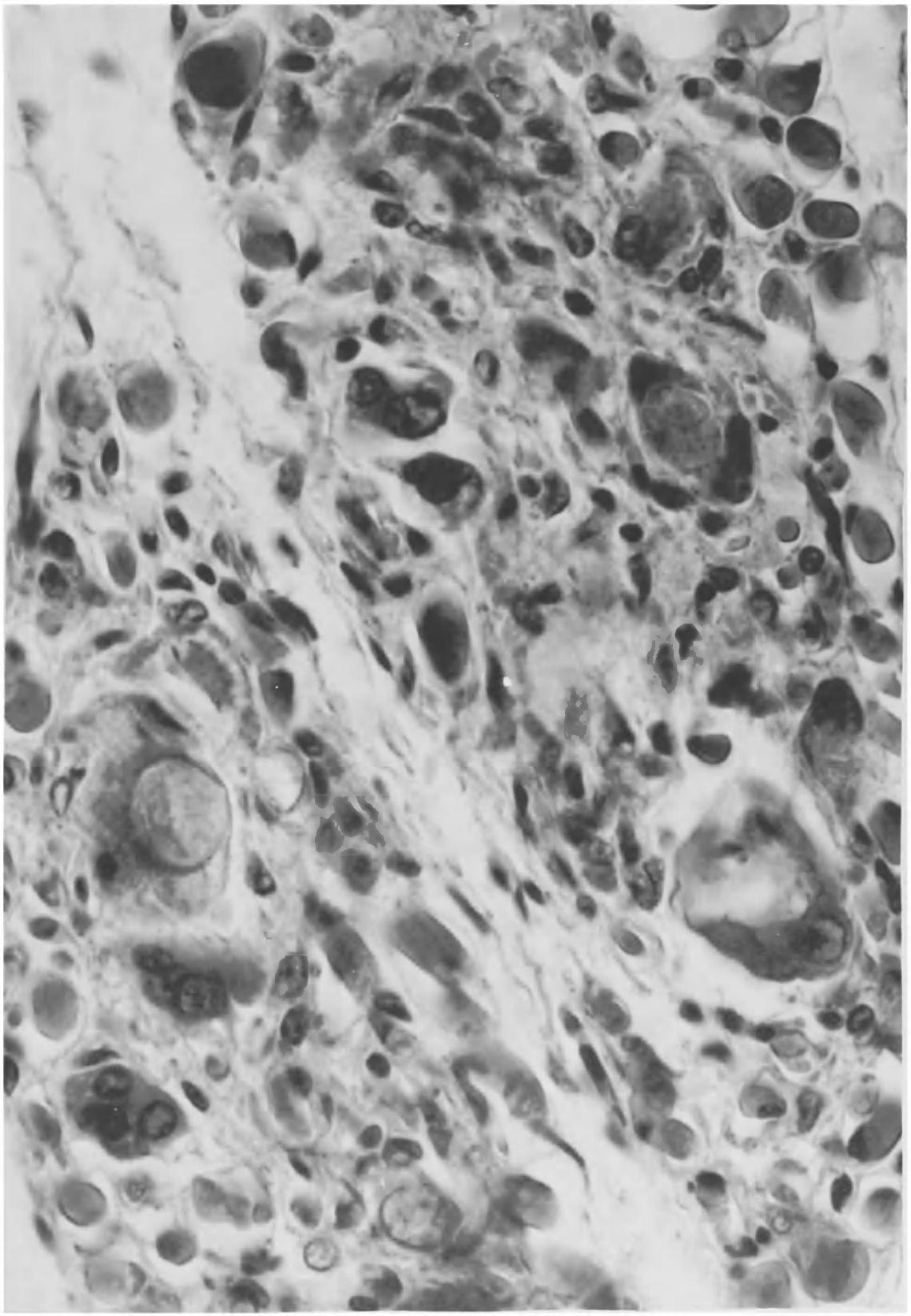

Fig. 28. Lamb No. 34.

Young muscle fibers beginning to show cross striation.

Hematoxylin and Eosin stain x 750

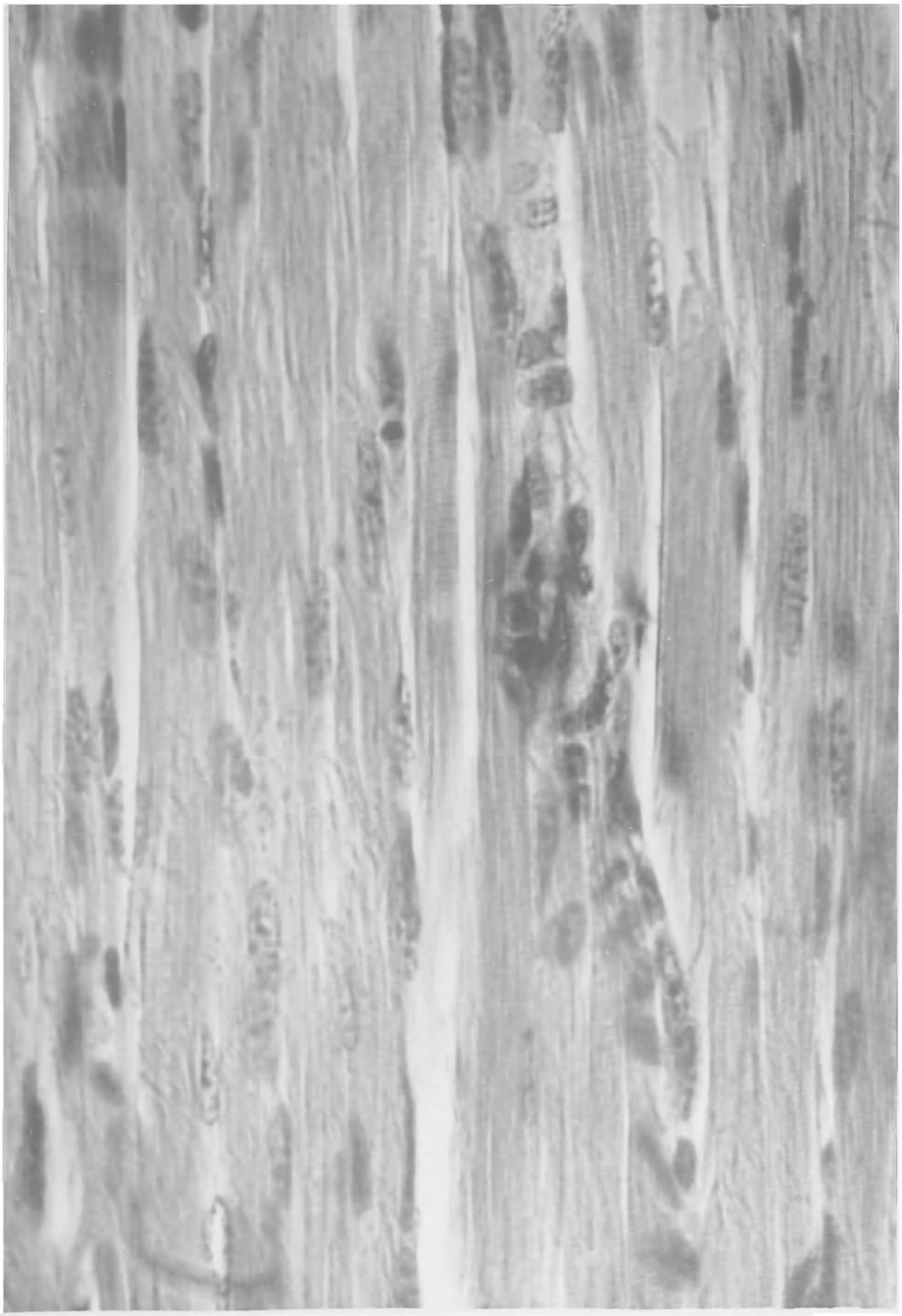

Fig. 29. Lamb No. 43.

More mature muscle fibers showing cross striation. Note the nuclei at the tip of the young muscle fibers. The nuclei are centrally located.

Hemztoxylin and Eosin stain x 750

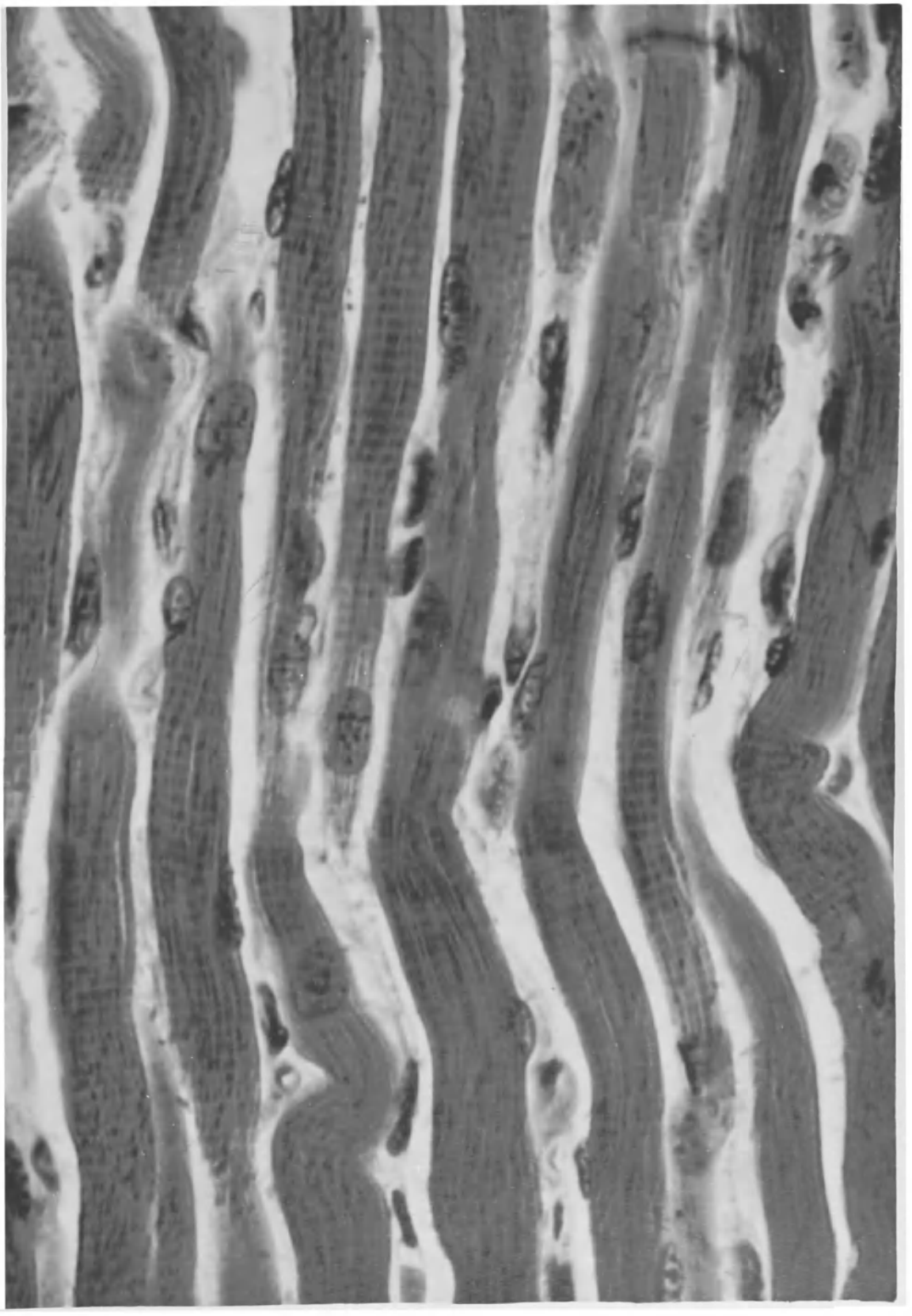

Fig. 30. Lamb No. 50.

Fully regenerated muscle fibers are characterized by pronounced cross striation.

Hematoxylin and Eosin stain x 750

Fig. 31. Lamb No. 42.

Regenerating muscle with numerous nuclei arranged in a row in the center of the muscle fiber.

Hematoxylin and Eosin stain x 1150

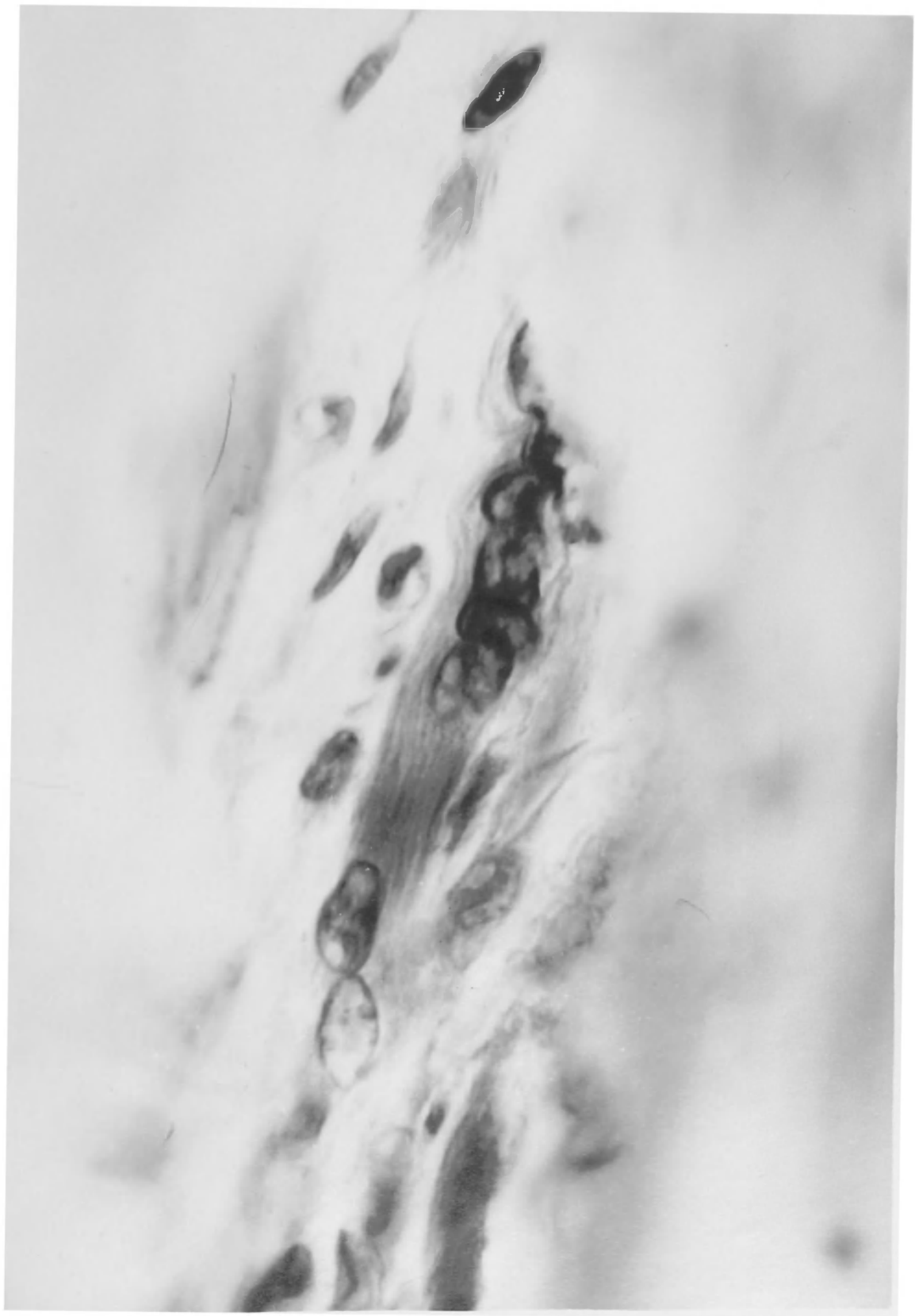

Fig. 32. Lamb No. 42.

Almost fully regenerated muscle from a lamb treated with alpha-tocopherol for 40 days. Note the increased number of muscle nuclei.

Hematoxylin and Eosin stain x 600

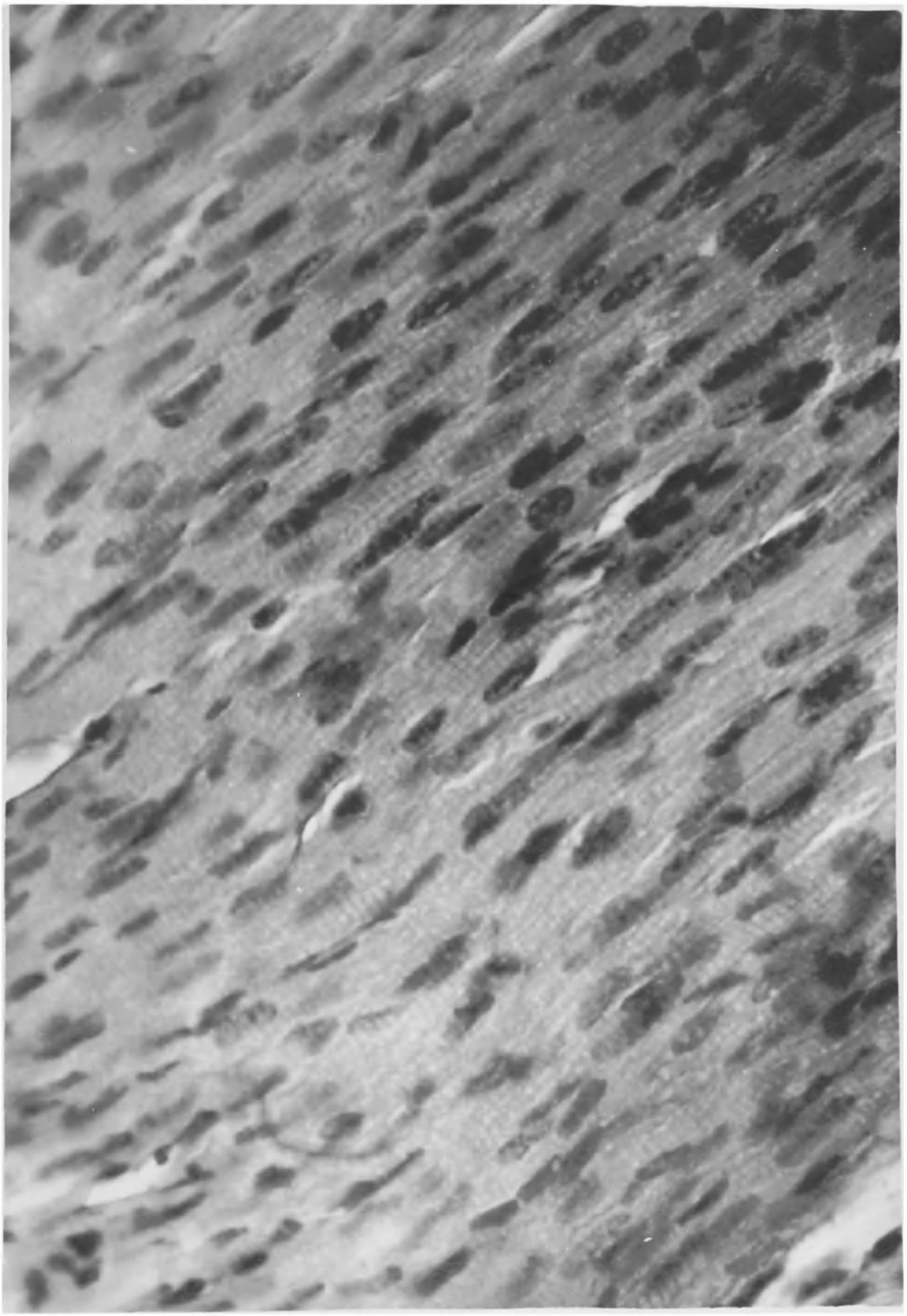

Fig. 33. Lamb No. 13.

Transversal section of the skeletal muscle from a positive control lamb.

Hematoxylin and Eosin stain x 170

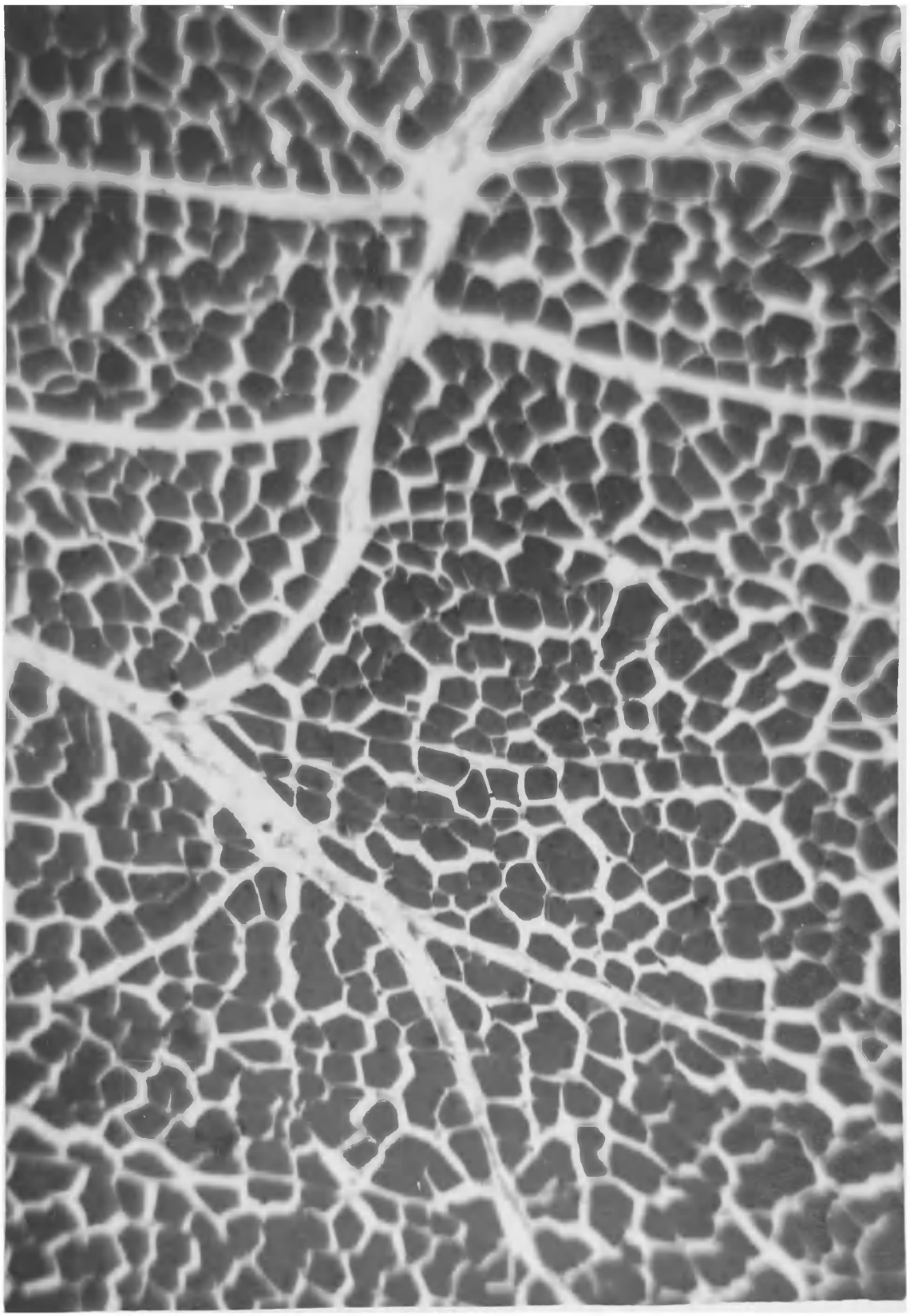

Fig. 34. Lamb No. 40.

Longitudinal section of the skeletal muscle from a positive control lamb.

Hematoxylin and Eosin stain x 170

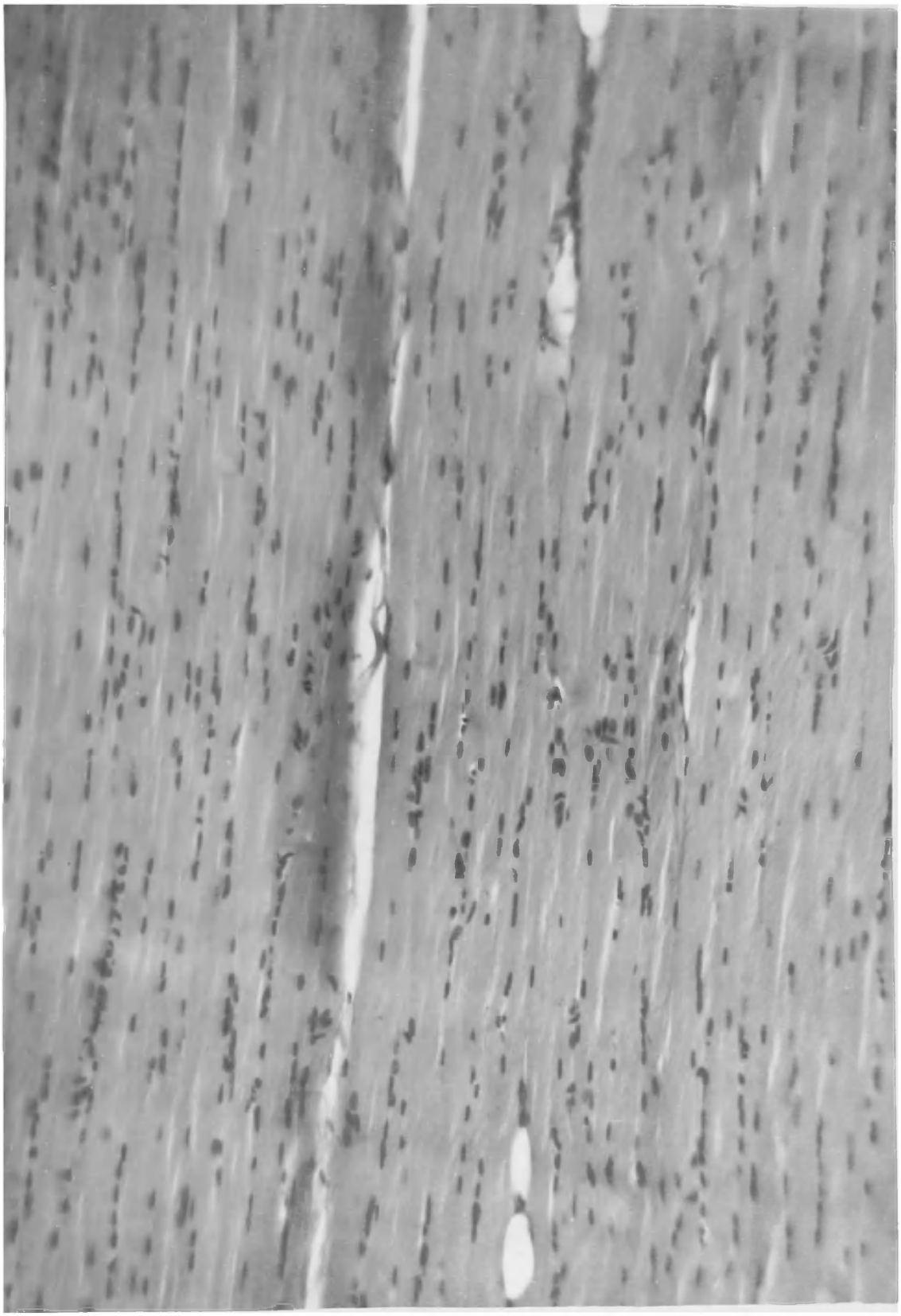

Fig. 35. Field Case.

Zenker's degeneration of the skeletal muscle. The area is infiltrated with phagocytic cells.

Hematoxylin and Eosin stain x 170

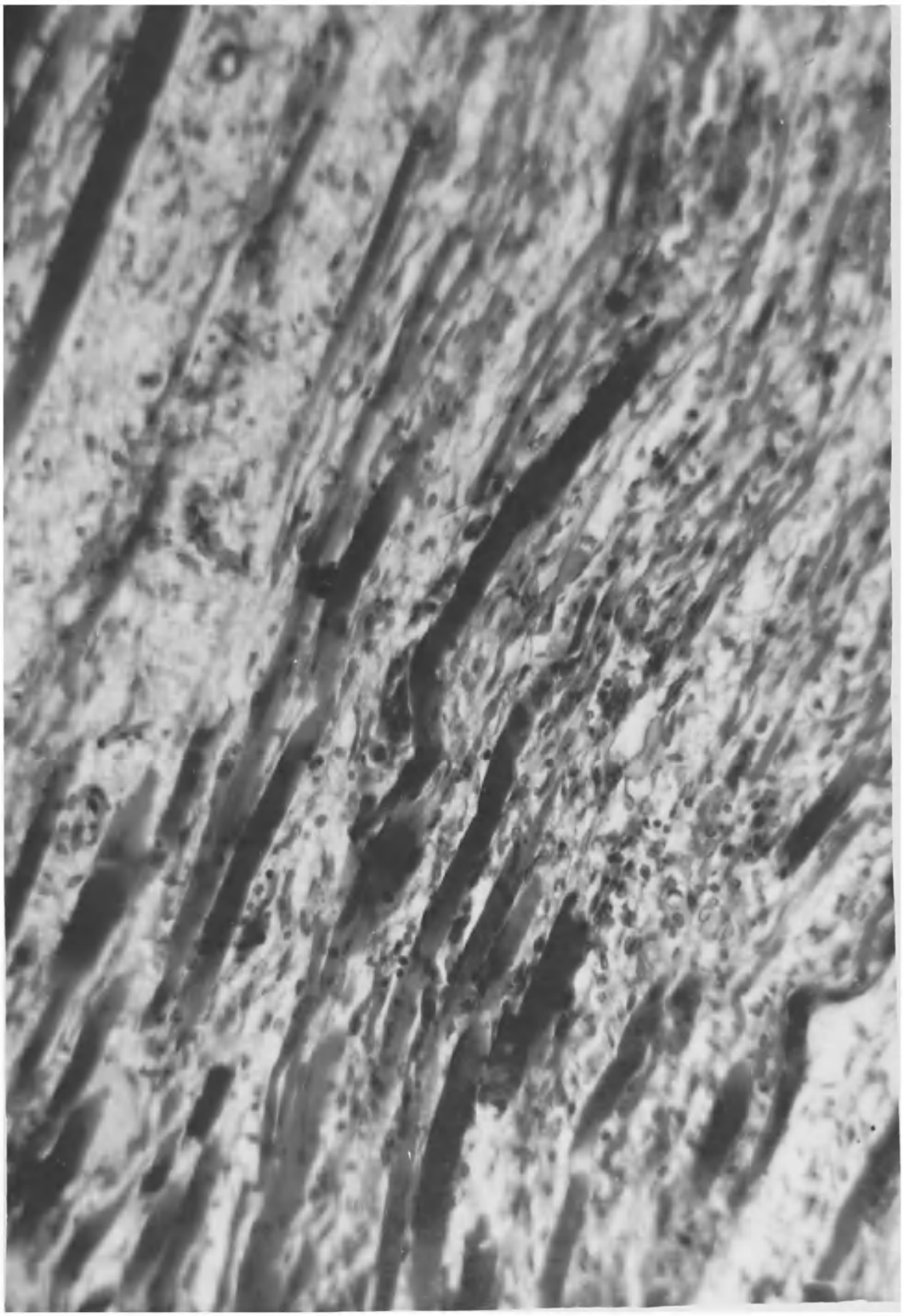

Fig. 36. Field Case.

Loss of striation, fragmentation and cellular infiltration of the muscle fibers. Some muscle fibers in the area are normal.

Hematoxylin and Eosin stain x 1150

Fig. 37. Field Case.

Regeneration of muscle fiber in the area of degeneration.

Hematoxylin and Eosin stain x 1150

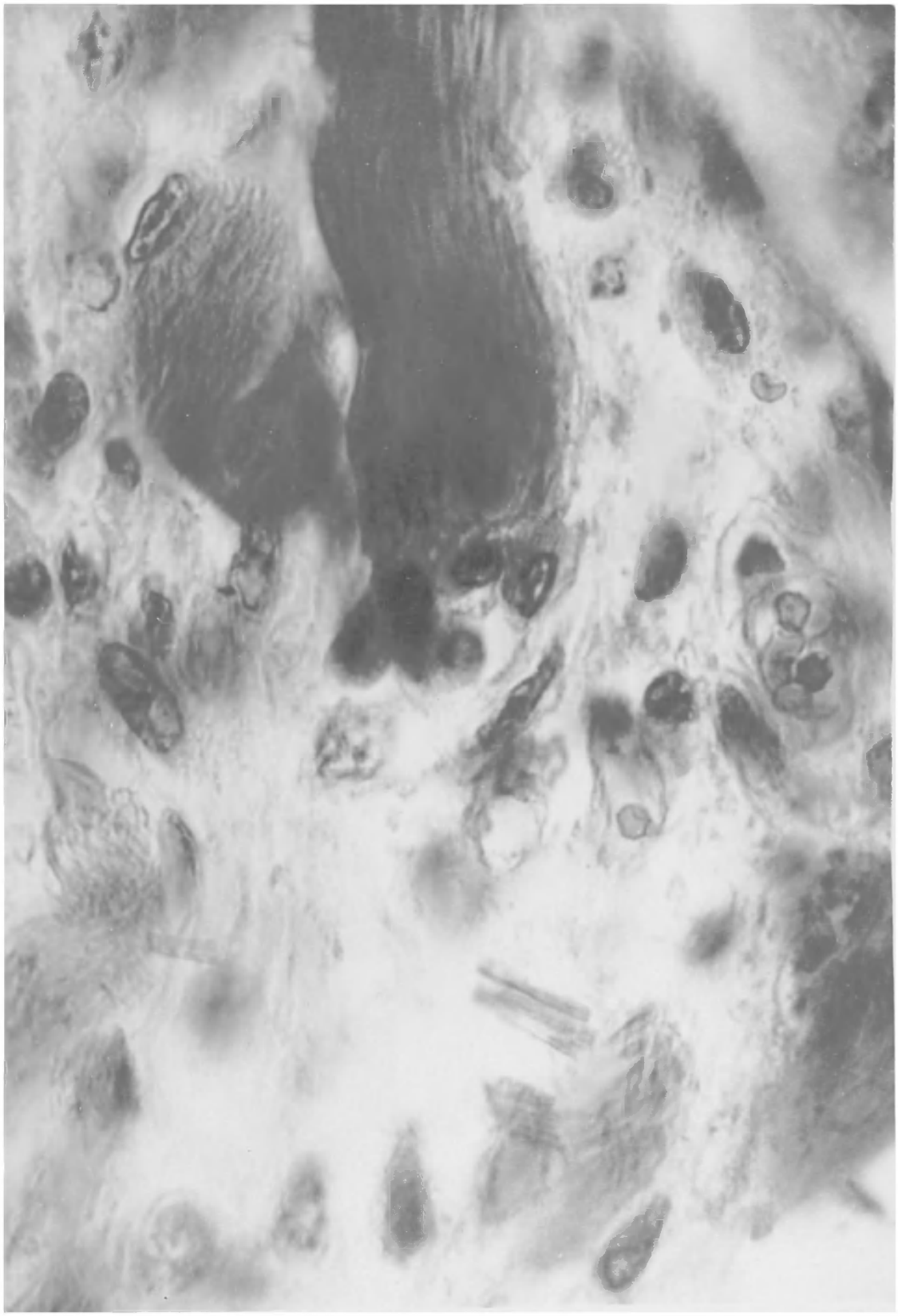

Fig. 38. Field Case.

Heart muscle. Degeneration of the muscle fibers. Note increased cellular activity and normally appearing Purkinje fibers.

Hematoxylin and Eosin stain x 170

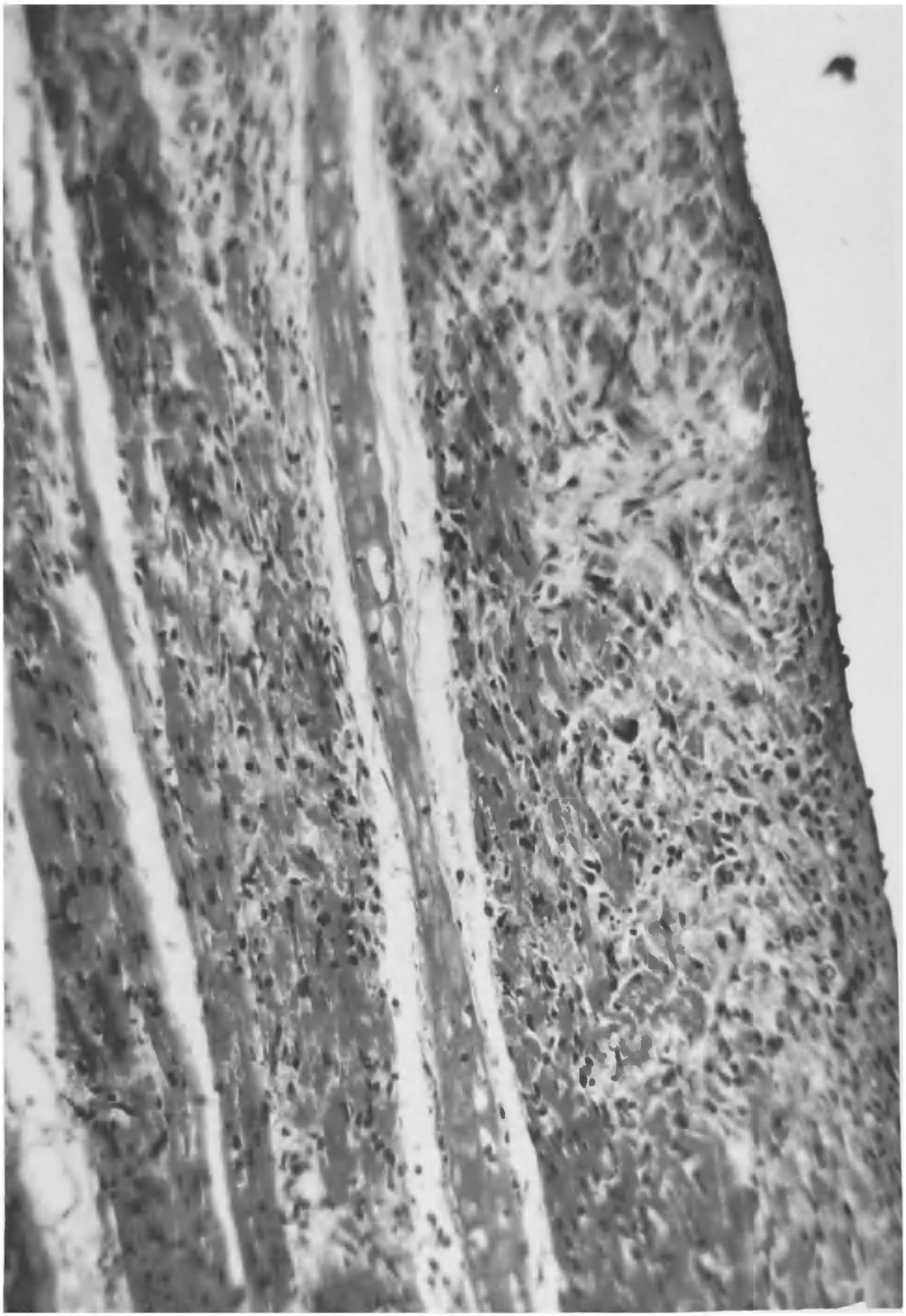

Fig. 39. Field Case.

Cardiac muscle regeneration. New muscle fibers are growing in the area of degeneration.

Hematoxylin and Eosin stain x 750

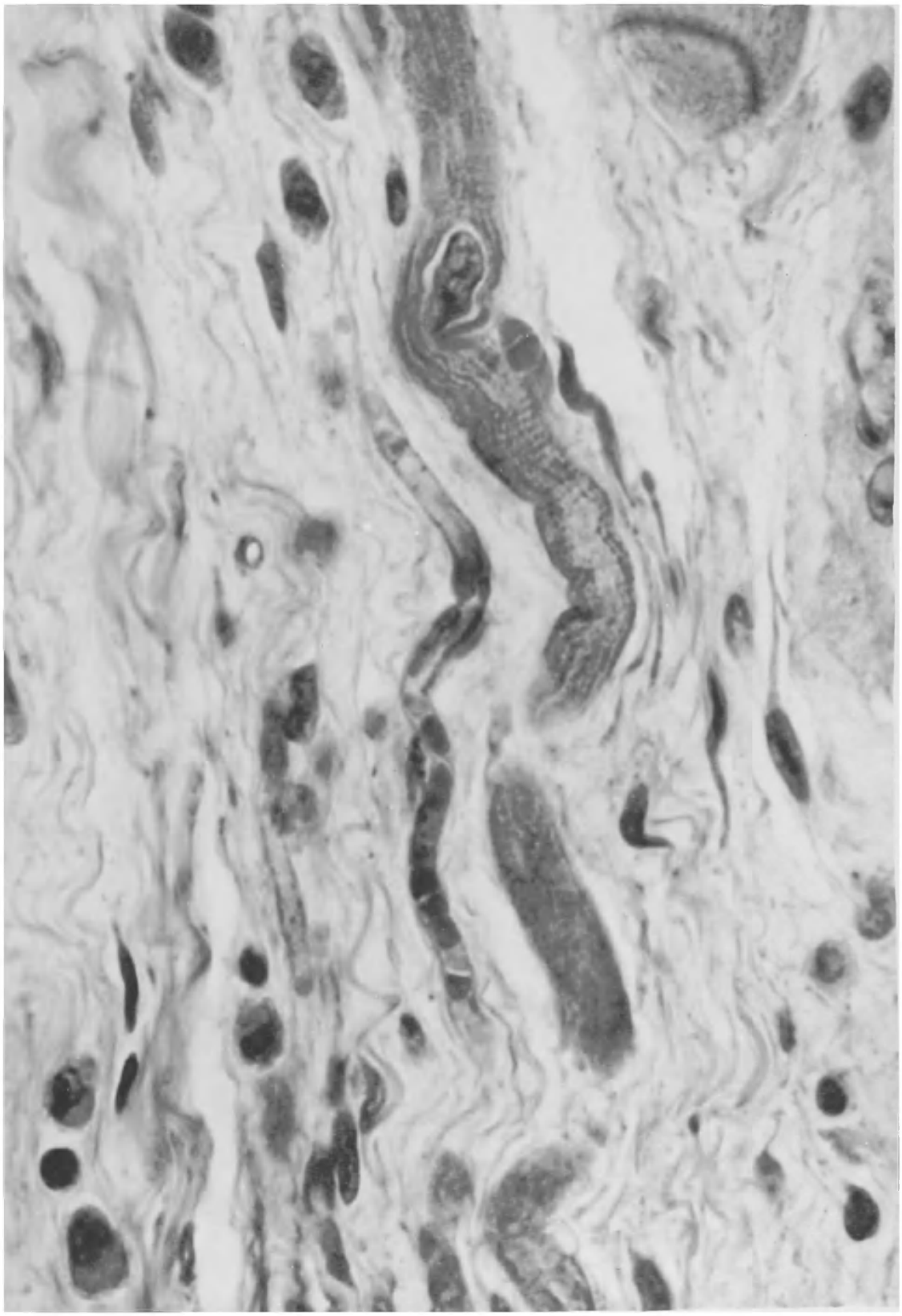

Fig. 40. Field Case.

Extensive lesions in the endocardium and myocardium from a lamb affected with "Stiff-Lamb" disease.


713 X
METRIC 1
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6

Fig. 41. Field Case.

Whitish lesions affecting most of the right ventricle.

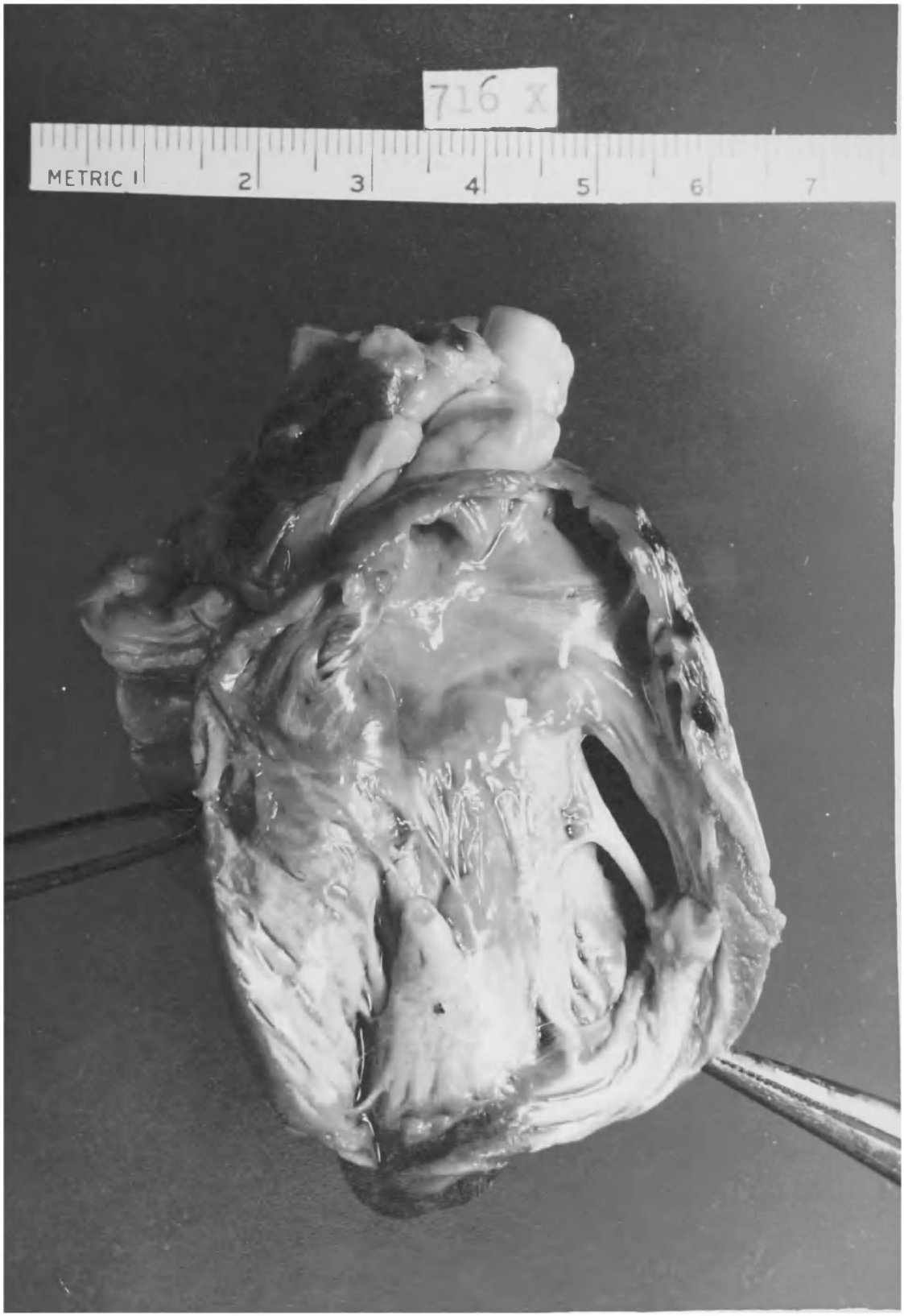
716 X
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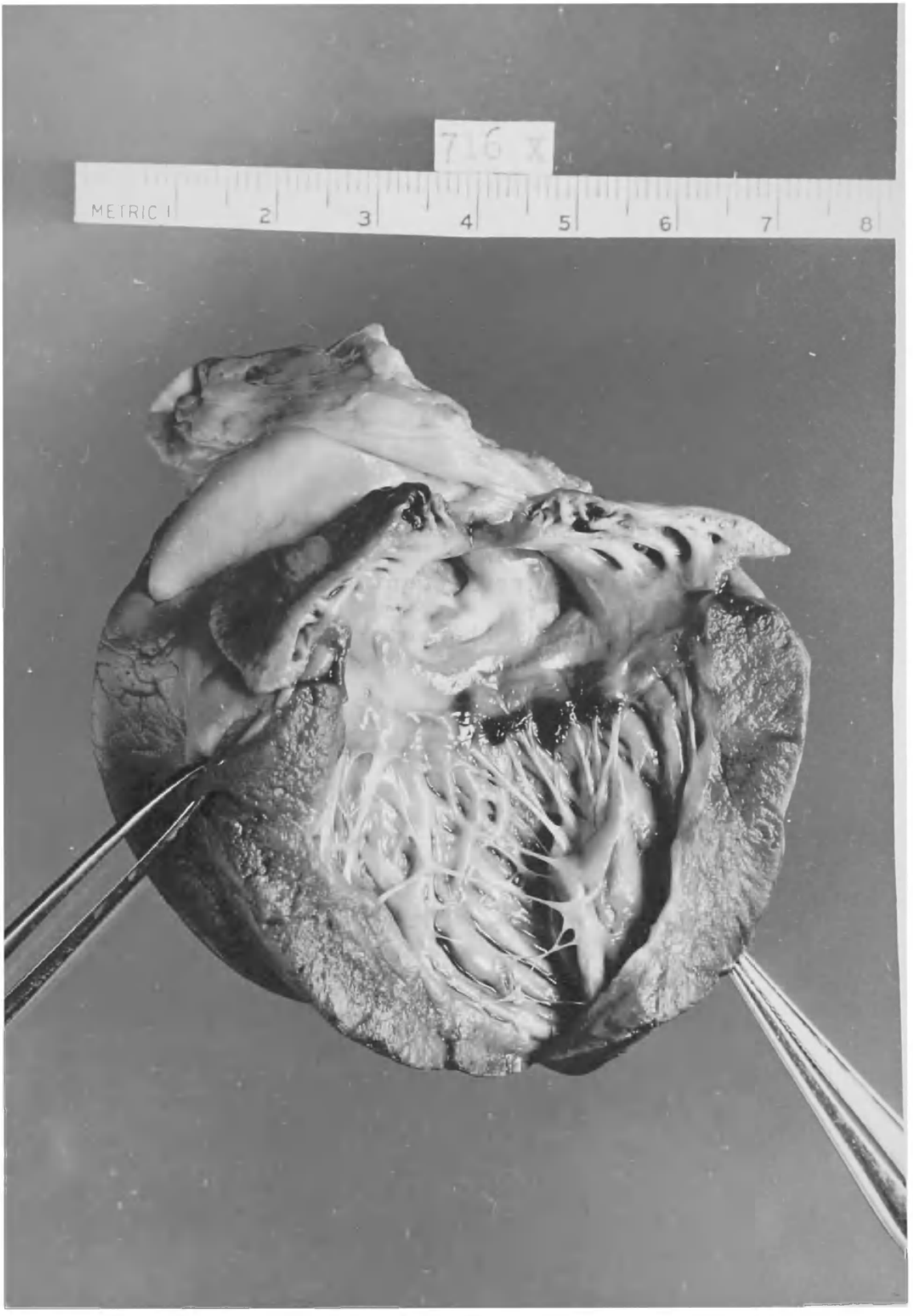
Fig. 42. Field Case, (same as Fig. 41).
Diffuse involvement of the left ventricle and atrium characterized by whitish areas. Note the lesions throughout the cardiac wall.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