A STUDY OF THE PATHOLOGY OF EMBRYONATING CHICKEN EGGS INOCULATED WITH VIBRIO FETUS

By

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INTRODUCTION

Vibrio fetus infection of the genital tract of cattle has been known for 38 years. The disease received little attention until recently due to the difficulty encountered in the isolation and cultivation of the micro-organism. *V. fetus* has been classified as second only to *Brucella abortus* as the cause of abortion in untested herds; and as the primary etiological factor in abortions from brucellosis-free herds. Brucellosis has been and still is the greatest single cause of abortion in the cattle population as a whole.

Improved methods of diagnosis and the increased number of brucellosis-free herds have accounted for the present increased interest in vibriosis. Emphasis has been placed on the clinical and diagnostic aspects of the disease.

Gross and microscopic lesions of the aborted bovine fetus and fetal membranes were described in early reports on the condition. Contemporary workers usually slight these aspects of the disease or refer to the literature. The extensive degenerative changes usually occurring in the conceptus prior to its premature expulsion generally make the tissues unfit for intensive study. This deterioration and the prohibitive costs involved in using cattle as experimental animals enhanced the desirability of utilizing a suitable laboratory animal in preliminary studies on the pathology of

vibrionic abortion. The common laboratory animals have been generally regarded as refractory to infection with V. fetus. Embryonating chicken eggs were selected for this study because they are a good experimental medium and had been shown by Plastridge and Williams to be susceptible to V. fetus.

HISTORICAL REVIEW

The first authentic record of the recovery of a vibrio from cases of abortion was contained in the Report of the Departmental Committee Appointed by the Board of Agriculture and Fisheries to Inquire into Epizotic Abortion (1909). This vibrio had been repeatedly isolated from outbreaks of abortion in ewes. Committee members were able to experimentally infect other ewes, pregnant for the first time, with this organism. Unsuccessful attempts were made to infect pregnant cows.

A further report by the same committee (McFadyean (1913)), however, stated that infective material obtained from ewes was administered to seven pregnant bovines intravenously, through natural orifices, or by both channels and that one cow became infected.

Unsuccessful attempts were made to infect other species. One goat aborted four days after being inoculated with virulent material, but no vibrios were found in the cotyledons, discharges, or uterus. They speculated that the period between inoculation and abortion was too short to permit much increase in the number of organisms. These investigators also found that guinea pigs and rabbits did not suffer any illness when inoculated with vibrios; but in one of the few cases where pregnant guinea pigs were used abortion occurred

12 days post injection.. These workers reported that two natural outbreaks of vibrionic abortion in cows, one in Ireland and one in Wales, had been investigated.

McFadyean and Stockman (1913) in an appendix to this report stated that the cow that became infected was given two intravenous injections of virulent ovine material 39 days apart. A dead hairless fetus was aborted 18 days after the last injection.. Vibrios were observed in stained smears of stomach contents and cotyledons.. A contaminated culture was obtained from fetal stomach contents.

Smith (1918) observed that the lesions associated with a spirillum infection of cattle were largely, if not exclusively, restricted to the fetal membranes; and that the fetus suffered secondarily from a gradually increasing interference with the placental circulation in much the same manner as with Brucella abortus. It was found impossible to foretell whether a given fetus would yield cultures of brucella, the vibrio or none at all..

The main fetal lesions were edema of the subcutaneous tissues and effusions into the peritoneal and pleural cavities.. These fluids, as a general rule, were more or less heavily tinged with blood, and frequently associated with delicate, loose, shreddy deposits of fibrin or more rarely with heavy, whitish pseudomembranes in the abdomen and much less abundantly on the pleura and pericardium.. The visceral lesions were chiefly autolytic changes following death.. Focal lesions were not present.

The stomachs usually contained quantities of meconium apparently swallowed or possibly driven into the abomasum by antiperistaltic movements. The stomachs of normal fetuses contained a colorless, translucent, very thick, viscid fluid which in older fetuses might contain a few pellets of meconium and perhaps some hairs. The diseased fetus, however, almost invariably had in the stomachs a very turbid, thick, yellowish, flaky fluid. Not infrequently there were also found small, whitish, soft, disc-like masses which could be traced back to epithelial excrescences of the amnion which had sloughed off and been swallowed by the fetus.

The younger fetuses almost invariably showed congenital pulmonary atelectasis, however the lungs of the older aborted fetuses sometimes contained air. In some, the air tubes contained fluid identical with that found in the stomachs. This fluid often filled the trachea and bronchi completely.

A thick, bloody fluid usually collected in a thin layer under the renal capsule. The tissue surrounding the kidneys was also frequently distended with bloody fluid.

Histological sections of fetal tissue did not reveal anything characteristic of the infection. The epithelial coverings of the digestive tract were partially or wholly desquamated which was interpreted as a post-mortem change.

Smith and Taylor (1919) named the spirillum associated with disease of the fetal membranes of cattle Vibrio fetus n. sp., and speculated that it possibly was identical with

the organism associated with ovine abortion in England (McFadyean (1913)).

Smith, Little, and Taylor (1920) described the lesions found in the placenta of one case of vibrionic abortion. The chorion varied from a smooth, translucent, slightly congested membrane to one opaque, thickened and leathery. The opacity was due to an infiltration which resulted in the formation of slightly elevated plaques not removable by gentle scraping. In other places the infiltration was discrete in the form of whitish opacities, one-half to one mm in diameter. There were also scattered minute tufts of adventitious villi, which were completely cheesy. The subjacent tissue was edematous and varied in thickness. Part of the cotyledons were normal and part were diseased. Some of the latter were yellowish and pultaceous throughout. The still normal cotyledons contained, usually on the margin, necrotic, yellowish villi. More rarely such villi were scattered throughout the cotyledon.

Fixed sections from various regions of the placenta showed necrosis of villi, loss of surface epithelium with leucocytic infiltration of the underlying tissue in certain areas. Where the epithelium was present, no bacteria were found in the cells as with Br. abortus. The endothelium of the capillaries had proliferated in places and it partially or nearly filled the lumina. Bacteria resembling vibrios were detected within these cells and in groups among necrotic villi.

Smith (1919) reported on additional cases of vibrionic abortion and observed that in most cases the placenta was retained. Four pregnant cows were given intravenous injections of V. fetus. The cultures were grown on sealed agar slants with or without added bits of guinea pig spleen and calf serum water for four to six days. Although live calves were born, lesions were found in the placenta of two of the animals, and the organism was observed in stained smears of placental tissue.

Smith (1923) injected six cows intravenously with V. fetus and two of these animals received in addition a subcutaneous injection. Two cows aborted and four were killed at varying periods after exposure. Five of the six showed lesions due to V. fetus in the fetal membranes, and the organism was observed in stained smears from various areas of the fetal membranes, fetuses, and exudate in the uterochorionic space. Pure cultures of the organisms were obtained from three cases and a mixed culture of V. fetus and a streptococcus in a fourth case. The cultures used in these experiments had been isolated within a four month period prior to the inoculations.

Rhoades and Hardenbrook (1947) were unsuccessful in attempts to prove the pathogenicity of a culture of V. fetus. Three cows between four and six months pregnant were used. One cow received two intravenous injections and subsequently delivered a live calf at term. Another cow given an intrauterine injection aborted four days after the inoculation.

A streptococcus was isolated. All injections were made of 24-hour tryptose broth cultures of varying densities when compared to the McFarland nephelometric scale. A suspension of V. fetus was mixed with the feed of a third cow at three-day intervals. A total of 1,290 ml of 24-hour tryptose broth cultures were administered over a period of 25 days. One month after the last exposure the cow was slaughtered. Cultures from various organs and tissues were negative for V. fetus. The authors speculated that attenuation of the culture had occurred by growth on artificial media.

Sjollema, Stegenga, and Terpstra (1949) concluded that V. fetus infection in cattle was a venereal disease transmitted at service. The infection caused, primarily, a catarrhal inflammation of the cervix and uterus. Infected cows failed to conceive, or the fertilized ovum soon died. These animals sometimes continued to have regular heat periods, but in some the periods were considerably extended. Sometimes abortion occurred at a later stage of established pregnancy.

These investigators found that usually an immunity was established within a three months period, and that the infertility was thus of a temporary nature. It was found that infected bulls could harbor the organisms for long periods without showing any abnormality of the genital organs, and transmit the infection at the time of service. The focus of infection in the bull was not determined.

Roberts, Gilman, and Larsen (1950) inoculated V. fetus into the conjunctival sac of three pregnant cows and the vagina of a fourth at the time of estrus without producing any evidence of disease.

Plastridge (1951) isolated V. fetus from scrapings of the uterine mucosa of two of three cows slaughtered because of failure to conceive after repeated services.

Beveridge and Burnet (1946) reviewed the literature dealing with the experimental utilization of embryonating chicken eggs. The method had been used in the study of chemotherapy, immunological reactions with antisera, and selective localization of the infective agent similar to that occurring in the natural disease. Embryonating chicken eggs had also been used for growing bacteria otherwise not cultivable, for the production of antigen for compliment fixation tests, and for viral and rickettsial investigations. These workers credited the cultivation of spirochaetes in the chick embryo by Levaditi in 1906 as being the first occasion on which the fertile egg was used to propagate an infective agent.

Rous and Murphy (1911) reported that a cell-free extract of a transmissible avian sarcoma caused tumor growth on the inner surface of the chorioallantoic membrane of developing hens' eggs. These workers were credited with being the first to use the chorioallantoic membrane for the study

of problems in experimental pathology and, more specifically, virology.

Woodruff and Goodpasture (1931) found that ectodermal and entodermal cells of the chorioallantoic membrane of the chick, as well as embryonic chick skin, were susceptible to infection with the virus of fowl-pox at an early stage in the development of the embryo. Goodpasture and his associates have been credited with the recognition of the potentialities of this method for virus research.

Goodpasture and Anderson (1937) observed that Streptococcus viridans, Aerobacter aerogenes, Eberthella typhi, Brucella abortus and Mycobacterium tuberculosis avium were able to multiply within the protoplasm of mesodermal or epithelial cells, or both, of the chorioallantoic membrane of embryonating chicken eggs. These, together with Staphylococcus aureus, Streptococcus haemolyticus and Corynebacterium diphtheriae, would grow extracellularly in the presence of necrosis, but apparently did not invade living tissues except by the mechanism of phagocytosis. The organisms were dropped on the exposed chorioallantoic membranes and the invasiveness studied. A polymorphonuclear and a mononuclear leucocytic response was generally noted.

Plastridge and Williams (1943) inoculated five embryonating chicken eggs with cultures of V. fetus on the twelfth day of incubation. All died within a period of five days and growth of the organism occurred in the allantoic fluids.

MATERIALS AND METHODS

The culture of V. fetus used in this study was isolated from the abomasal contents of a bovine fetus aborted at an estimated three to three and one-half months after conception. Histopathological changes typical of vibriosis occurred in the fetal membranes (Smith, Little, and Taylor (1920)). Organisms having typical morphological characteristics were observed in stained smears of the abomasal contents. The tube agglutination test was positive in the 1-200 dilution (Plastridge, Williams, and Petrie (1947)).

The organism was maintained in semisolid thiol medium for one month (Huddleson (1948)). A tube of this medium was seeded from the original culture and incubated for 72 hours at 37 C. Seven tubes of the same medium were each inoculated with 0.1 ml of the growth obtained from the first transfer. These tubes, plus one uninoculated tube were incubated for five days at 37 C. The culture was aspirated from the tubes using a sterile Pasteur pipette and diluted four times with a diluent consisting of 0.1 per cent tryptose and 0.5 per cent sodium chloride in distilled water (Wilson (1949)). The mixture was vigorously shaken and the resulting suspension centrifuged for 35 minutes in an angle centrifuge* at

*Ivan Sorvall, Serv-all Laboratory Aids, New York.

approximately 350 revolutions per minute. The cloudy supernatant was removed with a Pasteur pipette and centrifuged at approximately 2450 r.p.m. for 30 minutes. The clear supernatant was decanted and the sediment resuspended in one to two ml of the diluent. The bacterial suspension was then compared to the number six tube of the McFarland nephelometer, using a photelometer* containing a green filter. The number six tube gave a reading of 50, while the suspension of V. fetus gave a reading of 60 on the photelometric scale. This suspension constituted the infective inoculum for the original passage.

Inoculum for control eggs of the original passage was prepared by treating a volume of sterile incubated thiol medium, equal to that containing the organisms. Diluent was added to the sediment obtained by the final centrifugation in a volume equal to that used in the standardization of the suspension of V. fetus.

The eggs used in this investigation were purchased from the Poultry Department, Michigan State College.

An electric type incubator** was used for incubation of the eggs. The temperature varied between 99 and 100 F, while the wet-bulb thermometer ranged from 78 to 84 F.

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*Cenco-Sheard-Sanford Photelometer, Central Scientific Co., Chicago.

**Reliable Incubator and Brooder Co., Quincy, Illinois.

For the original passage, 17 eggs were candled on the seventh day of incubation. During candling the air sac of each egg was delineated on the shell by a penciled line.. A mark was made approximately one-quarter of an inch above the penciled line, thus being over the air sac, and directly over the embryo.

The area around the mark was painted with a red tincture of phemerol (1-500 dilution), before a hole approximately 1 x 3 mm was made in the shell over the mark. Care was taken not to puncture the shell membrane. The area was again painted with phemerol before inoculation and before the hole was sealed with melted paraffin.

One ml glass tuberculin syringes fitted with three-quarter inch 26 or 27 guage needles were used in making the inoculations. Each inoculation was made with the egg racked so that the long axis was horizontal and the drilled side uppermost. The needle was inserted horizontally through the drilled hole the full length of the shaft, and 0.1 ml of the inoculum was slowly injected..

Twelve of the eggs were inoculated with the suspension of V. fetus and five with the control fluid.

The eggs were candled, on an average of twice a day, to detect dead embryos. Each time the eggs were candled they were turned to prevent adhesions.

After 48 hours incubation, eight of the infected and one of the control eggs were dead. The allantoic and

amniotic fluids were harvested from the eggs containing live embryos by the following method. The shell over the air sac was painted with tincture of phemerol (1-500) and allowed to dry. Sterile pinceps were used to prepare a hole in the shell approximately one cm in diameter over the center of the air sac. A sterile one and one-half inch 18 guage needle was fitted to a two ml short barreled glass syringe and used first to expose the embryo, and then to withdraw one to two ml of the amniotic and allantoic fluids. A drop of fluid was checked for active motility, using a microscope equipped with a 10x ocular and a 3 mm objective. The remainder was placed in a sterile rubber capped vial. The fluids from the four live embryos injected with the control fluid were pooled and used as the control inoculum for the second passage.

Inocula for subsequent passages were prepared in a manner similar to that used for the second passage, with the exception that the infective material was harvested from dead embryos in all but one instance.

The presence of viable V. fetus and the absence of contaminants in the inocula for the passages was demonstrated by seeding tubes of the thiol medium with 0.1 ml of the inoculum immediately after completing the egg inoculations for each passage.

Eggs were inoculated on the sixth, seventh, eighth, ninth, eleventh, twelfth, and fourteenth day of incubation.

The variability inherent in the method of inoculation combined with the use of eggs at different stages of incubation resulted in the inoculum possibly being injected into one of several sites. This may have accounted for a part of the variability in mortality obtained in the 23 serial passages.

All inoculated eggs were examined for gross pathological changes. Embryos were picked at random from the second, third, fourth, seventh, ninth, twentieth, twenty-first, twenty-second, and twenty-third serial passages and fixed in Zenker's solution (Mallory, 1938). The embryos were usually dead and the chorioallantoic membranes showed post-mortem changes. Each embryo was removed from the shell, and the abdominal cavity opened before placing it in the fixative. When the chorioallantoic membrane was in good condition, i.e., when the embryo was still alive or had apparently just died, it was fixed in situ. The chorioallantoic membranes were fixed for two to four hours, while the embryos were left in the fixative for eight to 16 hours. Both were washed in running tap water for 24 hours, and preserved in 80 per cent alcohol.

The chorioallantoic membranes were trimmed to squares of one to one and one-half cm on a side. Three blocks were cut from the body and one from the head of each embryo. The blocks were trimmed to a thickness of five to seven mm.

The chorioallantoic membranes were dehydrated for two hours in 95 per cent and one hour in absolute alcohol. The

embryos were placed in 95 per cent for six hours and absolute alcohol for two hours. The embryos and membranes were then treated in an identical manner for the remainder of the embedding process. The tissues were cleared in cedarwood oil for 16 hours, and infiltrated with 48-52 C melting point paraffin for 24 hours. The paraffin was changed at the end of nine hours. The tissues were embedded in 56-58 C melting point paraffin.

All sections were cut at the six micron setting of the microtome* and stained with Harris' Hematoxylin and Eosin (Mallory (1938)).

*Spencer Lens Co., Buffalo.

RESULTS

Mortality

During the 23 serial passages there were 184 deaths (84.01 per cent mortality) in the 219 embryonating chicken eggs inoculated with virulent material (Fig. 1). The peak mortality occurred during the third 24 hour period following inoculation (Table I).

TABLE I

PER CENT OF TOTAL MORTALITY OCCURRING AT INTERVALS FOLLOWING INOCULATION

24 hour Periods	Per cent of Total Mortality
1	17.94
2	25.00
3	33.70
4	13.59
5	4.89
6	1.63
over 6	3.25
	100.00

Only eight of the surviving eggs hatched, while 27 were killed at various times. There were six deaths (15.79 per cent) in the 38 inoculated control eggs. The deaths in the inoculated controls occurred within a 48 hour period following injection and two-thirds of these occurred within the first 24 hours. The deaths in the controls were attributed to trauma.

The mortality figures were influenced by sacrificing inoculated embryos at varying periods of time. However, the mortality in the eggs inoculated with virulent material tended to be highest and more uniform during the first 13 passages (Table II). Although insufficient numbers of embryos were used in some age groups, the results showed a tendency toward a higher mortality in the eggs inoculated before the eleventh day of incubation (Table III).

A characteristic zone of multiplication of V. fetus was usually evident within 24 hours after seeding tubes of thiol medium with a portion of the virulent inoculum for each passage. In some instances, the multiplication was not evident for as much as four days after inoculation. The visible zone produced by the organisms was grayish-white in color and at first very thin, and from three to six mm below the surface of the medium. Later, this zone increased in thickness (one and one-half to three mm thick) and occurred on the surface of the medium.

The presence of contaminants in the inoculum for the control eggs was not demonstrated.

TABLE II

INFLUENCE ON MORTALITY OF SERIAL PASSAGE AND AGE OF EMBRYO AT INOCULATION

Passage	Age in days of embryo at inoculation	Number of eggs		Per cent mortality	
		Experimental	Control	Experimental	Control
1	7	12	5	66.6	20.0
2	9	12	4	75.0	0.0
3	12	7	2	71.4	0.0
4	7	12	4	100.0	50.0
5	12	9	4	100.0	50.0
6	8	12	3	100.0	0.0
7	11	8	2	100.0	0.0
8	7	8	4	100.0	0.0
9	11	8	3	100.0	0.0
10	7	8	4	100.0	0.0
11	9	6	2	100.0	0.0
12	12	3	1	66.6	100.0
13	8	9		100.0	
14	12	11		54.5	
15	6	4		100.0	
	14	3		0.0	
16	8	8		100.0	
17	11	8		87.5	
18	8	8		100.0	
19	11	7		28.5	
20	11	8		87.5	
21	6	8		100.0	
	14	12		100.0	
22	11	12		27.2	
23	8	16		93.8	

TABLE III

SUMMARY OF INFLUENCE ON MORTALITY
OF AGE OF EMBRYOS AT INOCULATION

Age in days	Experimental eggs			Control eggs		
	Number inoculated	Number of deaths	Per cent mortality	Number inoculated	Number of deaths	Per cent mortality
6	12	12	100.0			
7	40	36	90.0	17	3	17.6
8	53	52	98.1	3	0	0.0
9	18	15	83.3	6	0	0.0
11	51	35	68.6	5	0	0.0
12	30	20	66.6	7	3	42.9
14	15	12	80.0			

Macroscopic Lesions

In general, the lesions were more numerous and more severe in the eggs examined during the third and fourth 24 hour period after injection of virulent material (Table IV).

The chorioallantoic membrane was usually thickened and frequently contained petechial and ecchymotic hemmorrhages. Some degree of a generalized congestion of the embryo was almost invariably observed (Fig. 2). Petechiae and ecchymoses were found in the cutaneous tissues, especially of the extremities (Fig. 3).

The liver was usually enlarged, often congested and showed varying degrees of mottling (Fig. 3). The mottled

effect was due to the presence of circumscribed, sometimes slightly depressed, areas up to 5 mm in diameter that were lighter in color.

The kidneys showed varying degrees of enlargement and congestion.

Splenomegaly was sometimes the only lesion observed in embryos that did not succumb to the infection. Gradations in size of the spleen up to four times that of the controls were seen (Fig. 3).

Other lesions often observed were hemorrhages in the wall of the gizzard, ascites, and pulmonary congestion (Fig. 3). Hemorrhages in the lungs, brain, and epicardium were noted in a few instances.

TABLE IV

SUMMARY OF GROSS PATHOLOGY

Passage	Age in days of embryo at inoculation	24 Hour periods after inoculation				
		1	2	3	4	5
1	7	0	0	1,2,6	6	
2	9		0	1,2,7		
3	12	0	0	0	1,5,8,11,12	
4	7	0	0	1,6,8,10,14,15		0
5	12		1,6		1,5,10	
6	8			1,5,6,10,14		
7	11	0		5,8,10	5,8,9,10,15	
8	7	0	5	1,5,10		
9	11	0	1,2,5,8,10,13,17	4		
10	7	5,8	1,5,8,10	5,8,10		
11	9	0	5,8,10	3,8,10		
12	12		1,10	5,6,8,10		
13	8	0	5,10	5,8,12,13	5,8,10,14	
14	12		0			5,8,10
15	6 14	0	5	1,5		
16	8		5,10	1,10,11,13,18,19	5,8,10,11,13,14	5,8,10,13,14
17	11		1,10,15	1,5,10		
18	8	5		5,10,14		
19	11			1,5,6,8,10,13,15		
20	11	5,11,15	5,6,8,10	5	5,8,10	
21	6 14	5,9,12 9,12,17	5	5,8,10,11,12,13	5,8,12,13,16	
22	11				1,5,8,10,12,13,14	5,10,11,12,16
23	8	5	5,6,8,9,10,12,15		1,5,6	

24 Hour periods after inoculation		Key
6	Over 6	
		0-No significant lesion
		1-Hemorrhages in chorio-allantoic membrane
4,7		2-Thickening of chorio-allantoic membrane
		3-Hemorrhages in yolk sac
		4-Bloody amniotic fluid
		5-General congestion of embryo
		6-Cutaneous hemorrhages
		7-Smaller embryo
		8-Enlarged liver
		9-Congested liver
		10-Mottled liver
	5,8,10	11-Renal enlargement
	8,10	12-Renal congestion
		13-Splenomegaly
	1,8,10,13	14-Hemorrhages in serosa of gizzard
		15-Ascites
	8,9,10,11,13,16	16-Pulmonary congestion
	10,11,12,13,16	17-Pulmonary hemorrhages
		18-Hemorrhages in brain
	9,10,13,15,16	19-Epicardial hemorrhages
	2,13	
1,2,5,9,10,15		

Microscopic Findings

The chorioallantoic membranes of only a few embryonating chicken eggs, inoculated with *V. fetus*, were available for histopathological study. Proliferation and degenerative changes ranging from vacuolization to necrosis occurred in the ectoderm. In some areas the cytoplasm was swollen, somewhat granular in appearance and stained a pink color. The nuclei showed pyknosis and karyolysis. Slight proliferation of the entodermal cells occurred. Some infiltration of heterophils occurred in both the ectoderm and entoderm.

Congestion, hemorrhage and edema of varying intensity occurred in the mesoderm. Leucocytic infiltration, usually perivascular in nature, was marked in some instances (Fig. 7). Heterophils and/or macrophages were the predominating cells. Mesenchymal cell proliferation was noticeable in some instances (Fig. 8). Islands of epithelial cells occurred in the mesodermal layer. There was a tendency for macrophages to adhere to the endothelial lining and infiltrate the walls of arterioles. In some instances histiocytes and the proliferation of endothelial cells completely blocked the lumen of the arterioles (Fig. 9, 10). Pyknosis and karyorrhexis were noted in the nuclei of some endothelial cells (Fig. 9).

The chorioallantoic membranes of inoculated control eggs showed proliferation of the ectodermal and entodermal layers, and sometimes degenerative changes in the ectoderm.

Edema, heterophils and islands of epithelial cells were observed in the mesoderm (Fig. 4, 5, 6).

The livers of the infected embryos were commonly congested. In some instances the congestion was so marked that the parenchymal cells were compressed and the nuclei pyknotic (Fig. 13, 14, 15, 16).. There was some mobilization of macrophages and the Kupffer cells often appeared swollen. In one, a large area of necrosis and hemorrhage was somewhat separated from the more normal tissue by a zone containing macrophages and some proliferating fibroblasts (Fig. 17). Focal areas of necrosis without a leucocytic response occurred in other instances..

Marked congestion of the kidneys was usually present (Fig. 18, 20). There was an increased number of macrophages in the interstitial tissue. Macrophages, heterophils, erythrocytes and cellular debris were observed in the lumina of the convoluted tubules.. Some of the glomeruli were as much as two or three times larger than those of the controls (Fig. 19, 21, 22). The major part of this enlargement apparently was due to engorgement with blood. Pyknotic nuclei, apparently of vascular endothelial cells, and fibrin were observed in the glomeruli.. Some proliferation of vascular endothelial cells was thought to have occurred. Macrophages had infiltrated the capillary tuft; also macrophages, desquamated glomerular epithelial cells, heterophils, multinucleated cells, and erythrocytes were observed in the subcapsular space.

The spleen was usually congested. Some necrotic foci were present. Germinal cell proliferation was apparent. Some macrophages and a few heterophils as well as lymphocytes were present. The reticular elements were not as prominent as in the controls..

Foci of necrosis and hemorrhage were observed in the brain (Fig. 23, 24, 25). These areas occurred in the cerebrum and the cerebellum.

The lungs showed congestion, hemorrhage and fibrinous exudation (Fig. 26). Fibrin was sometimes observed in the peritoneal cavity. The generalized congestion of the tissues of the embryo observed at autopsy was verified by microscopic examination. The occurrence of hemorrhages in the outer muscular wall of the gizzard and the subcutaneous tissues were likewise confirmed by histological examination (Fig. 27, 28, 29).

DISCUSSION

The pathogenicity of a culture of *V. fetus* for embryonating chicken eggs was indicated by the 84.01 per cent mortality and the 3.6 per cent hatchability obtained in the 219 eggs used in the 23 serial passages. Deaths due solely to trauma undoubtedly influenced these figures. However, 12.3 per cent of the eggs were sacrificed prior to hatching. These factors tended to counterbalance each other. This high mortality corroborated the finding of Plastridge and Williams (1943) that the organism was pathogenic for embryonating chicken eggs.

Although the volume of inoculum per egg was standardized at 0.1 ml, the number of organisms per ml was not controlled. However, an attempt was made to select for passage the amniotic and allantoic fluids that contained the largest number of actively motile organisms per microscopic field.

When India ink was inoculated into two 12 day old embryonating eggs using the technique employed in this study, it was found that the inoculum was deposited in the allantoic cavity. However, the use of eggs at different stages of incubation during serial passage probably resulted in the inoculum being injected at times into the amniotic fluid or the yolk sac.

Jawetz and Meyer (1944) found that the number of virulent or avirulent Pasteurella pestis used to inoculate the chorioallantoic membranes of fertilized eggs on the twelfth day of incubation influenced the mortality rate and the lesions obtained. These workers also found that the number of avirulent organisms in the infecting dose which permitted the hatching of a significant proportion of chick embryos varied greatly with the route of administration and with the age of the embryo. Embryos less than 11 days old were quite highly susceptible and permitted the extensive multiplication of avirulent organisms leading to toxemia and death.

In this experiment, the injection of eggs incubated less than 11 days resulted in a 93.5 per cent mortality, while the injection of 11 and 12 day embryos resulted in a 67.9 per cent mortality. This was a difference of 25.6 per cent. Usually, however, the younger embryos were so susceptible that death ensued prior to the production of significant lesions, except for hemorrhage and generalized congestion.

The use of eggs at different stages of incubation, the variable number of organisms per injection, and the method of inoculation, together with the sacrificing of eggs prior to hatching would affect the total mortality and the per cent mortality obtained in each passage. However, the tendency for higher and more uniform mortality rates in the first 13 passages and the apparent increase in the number

and severity of lesions in the last ten passages indicated that possibly adaptation of the organism had occurred. Another possibility was that the number of organisms injected was decreased in the last ten passages. This factor was discounted somewhat by the production of visible multiplication of the organism in thiol medium within 24 hours after inoculation except in three passages where 48 hours were required. The observation had been made previously that there appeared to be a correlation between the number of viable organisms seeded and the time required for the production of visible zones of growth.

Plastridge (1951) reported that vaccination of calves under one year of age with live suspensions of V. fétus was started in two state owned herds during 1949, and that several years would be required to evaluate the results. Possibly, if adaptation of V. fetus occurred during serial passage in embryonating chicken eggs, such an adapted strain could conceivably be used in the vaccination of mature cattle and sheep. Especially, if in this process of adaptation, the pathogenicity for these valuable food producing animals was lost, and the immunizing properties were retained.

The organism apparently showed an affinity for the blood vascular system. The lesions produced in the arterioles of the chorioallantoic membranes of infected eggs were strikingly similar to those described in the bovine placenta by Smith, Little and Taylor (1920) (Fig. 9, 10). Similar

lesions were also observed in the placenta of the bovine fetus from which the culture used in this study was isolated (Fig. 11, 12). The proliferation of vascular endothelial cells was thought to have occurred in affected glomeruli (Fig. 21, 22). This together with the presence of pyknosis and the infiltration of macrophages was interpreted to mean that localization of V. fetus in the capillary tufts had probably occurred.

Smith (1918) observed bacteria resembling vibrios in the vascular endothelial cells of blood vessels in the bovine fetal placenta. Although the tissues of the embryonating eggs used in this study were not stained for the detection of bacteria, evidence was obtained which suggested that injury to the endothelial cells had occurred. The generalized congestion, edema, and hemorrhage of the tissues of embryonating eggs could be explained by the increased permeability of damaged endothelial cells (Fig. 2, 3). Further evidence of injury to these cells was obtained in a few of the eggs that survived the infection for at least three days. This evidence consisted of the arteriolar lesions in the chorioallantoic membranes and glomerulitis (Fig. 9, 10, 21, 22). Apparently the pathogenicity of V. fetus was confined primarily to the vascular endothelial cells of embryonic tissue.

Although focal areas of necrosis in the liver have not been described as occurring in aborted bovine fetuses, V.

fetus sometimes causes such lesions in premature lambs (Baker and Stone (1939)). Possibly species differences and the occurrence of ovine abortions nearer term would account for this difference in the manifestations of the disease. Focal necrotic areas were produced in the livers of chicken embryos infected with *V. fetus* isolated from a bovine fetus.

SUMMARY AND CONCLUSIONS

1. Vibrio fetus was propagated through 23 serial passages in embryonating chicken eggs, and its pathogenicity for this host was confirmed.

2. The production of arteriolar lesions in the chorioallantoic membranes of infected embryos similar to those in the bovine fetal placenta demonstrated the suitability of embryonating chicken eggs for preliminary studies on the pathology of vibrionic abortion.

3. The arteriolar lesions in the chorioallantoic membranes and the glomerulitis illustrated the pathogenicity of V. fetus for embryonic vascular endothelial cells.

4. The tissues and organs most generally affected by the organism were: chorioallantoic membrane, cutaneous tissues, liver, kidney, spleen, gizzard and brain. The generalized congestion, edema and hemorrhage of the tissues of embryonating eggs were explained by the occurrence of increased permeability of damaged endothelial cells.

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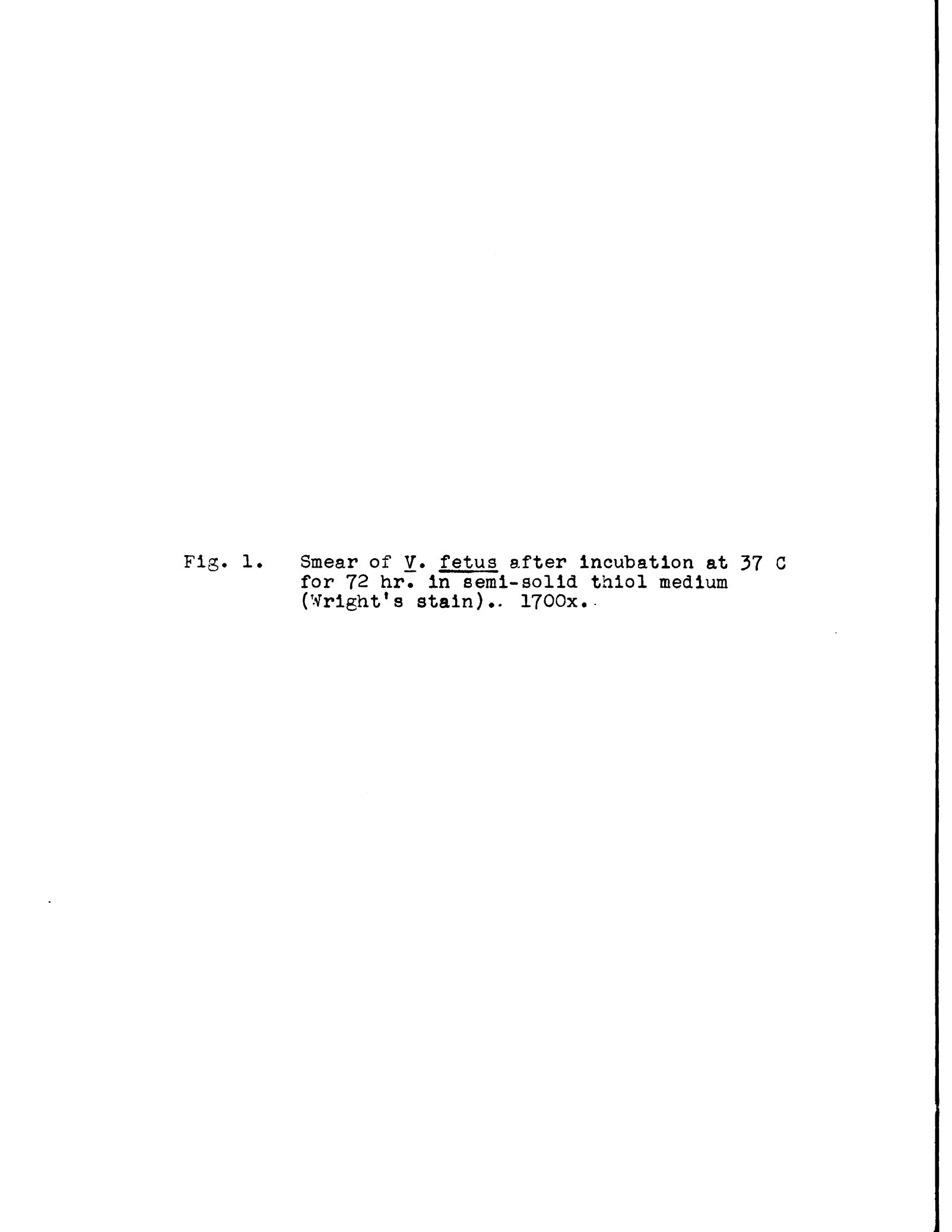

Fig. 1. Smear of *V. fetus* after incubation at 37 C for 72 hr. in semi-solid thiol medium (Wright's stain).. 1700x.

Fig. 1

Fig. 2. Infected and control embryos on the eleventh day of incubation. The infected embryo (left) shows generalized edema, marked congestion and hemorrhage of the cutaneous tissues. Both embryos were injected on the seventh day of incubation (eighth passage). Kodachrome print.

Fig. 3. Infected and normal embryos on the fifteenth day of incubation.. The infected embryo (left) was inoculated on the eleventh day of incubation.. Note the enlarged, congested and mottled liver, enlarged spleen (2x normal size), enlarged kidneys, ecchymosis on gizzard, and congested extremities (seventeenth passage). Kodachrome print.

Fig. 2

Fig. 3

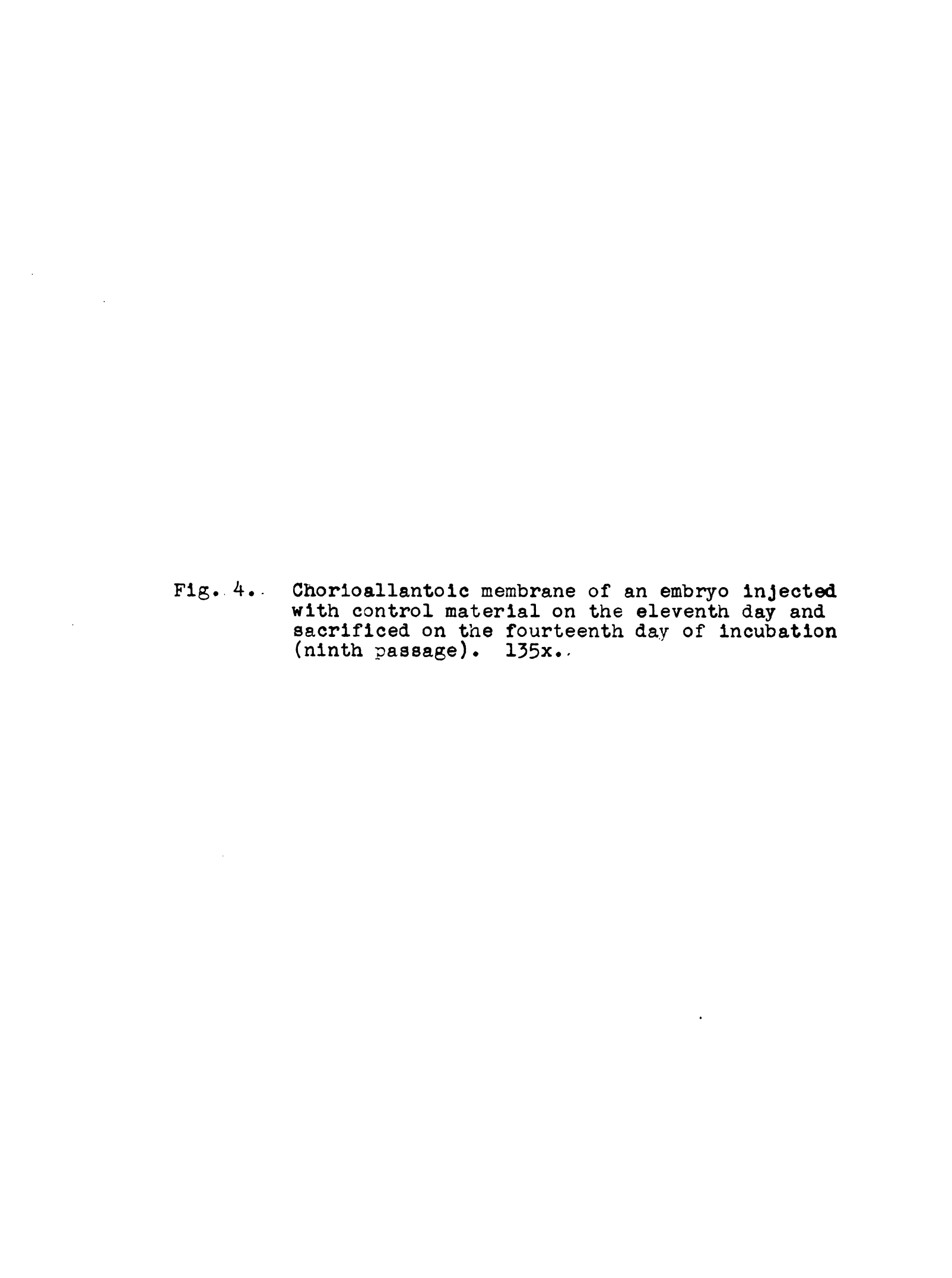

Fig. 4. Chorioallantoic membrane of an embryo injected with control material on the eleventh day and sacrificed on the fourteenth day of incubation (ninth passage). 135x.

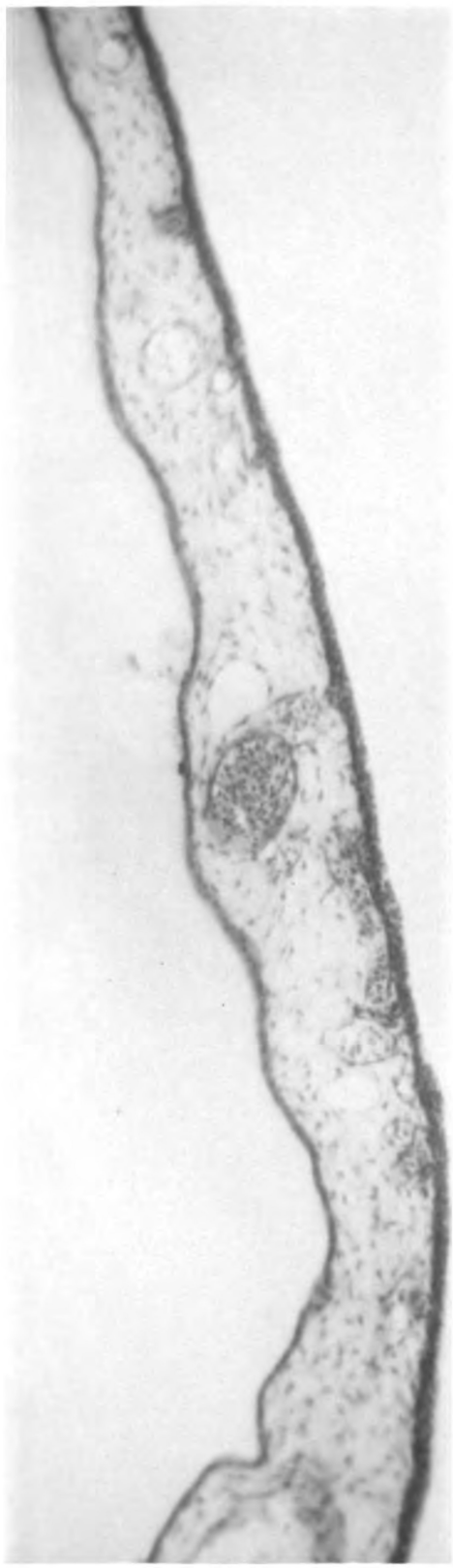

Fig. 4

Fig. 5. Traumatic lesion in the chorioallantoic membrane of an embryo inoculated with control fluid on the ninth day and sacrificed on the twelfth day of incubation (second passage). 135x.

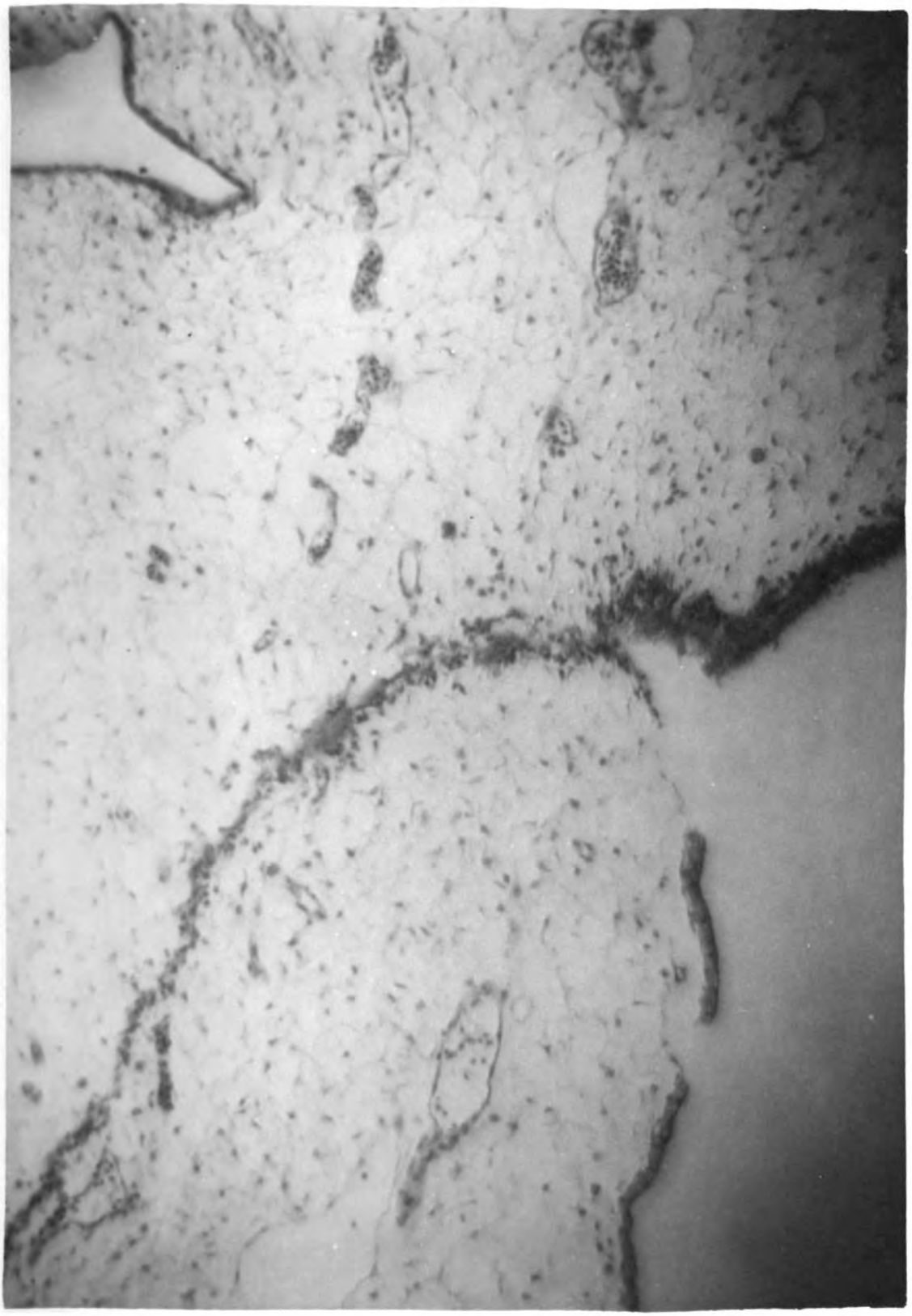

Fig. 5

Fig. 6. Higher magnification of Fig. 5 showing edema and islands of epithelial cells associated with capillaries along the apparent site of inoculation. 550x.

Fig. 6

Fig. 7. Chorioallantoic membrane of a 14 day embryo inoculated on the eighth day of incubation.. Note the edema and increased cellularity (twenty-third passage).. 135x.

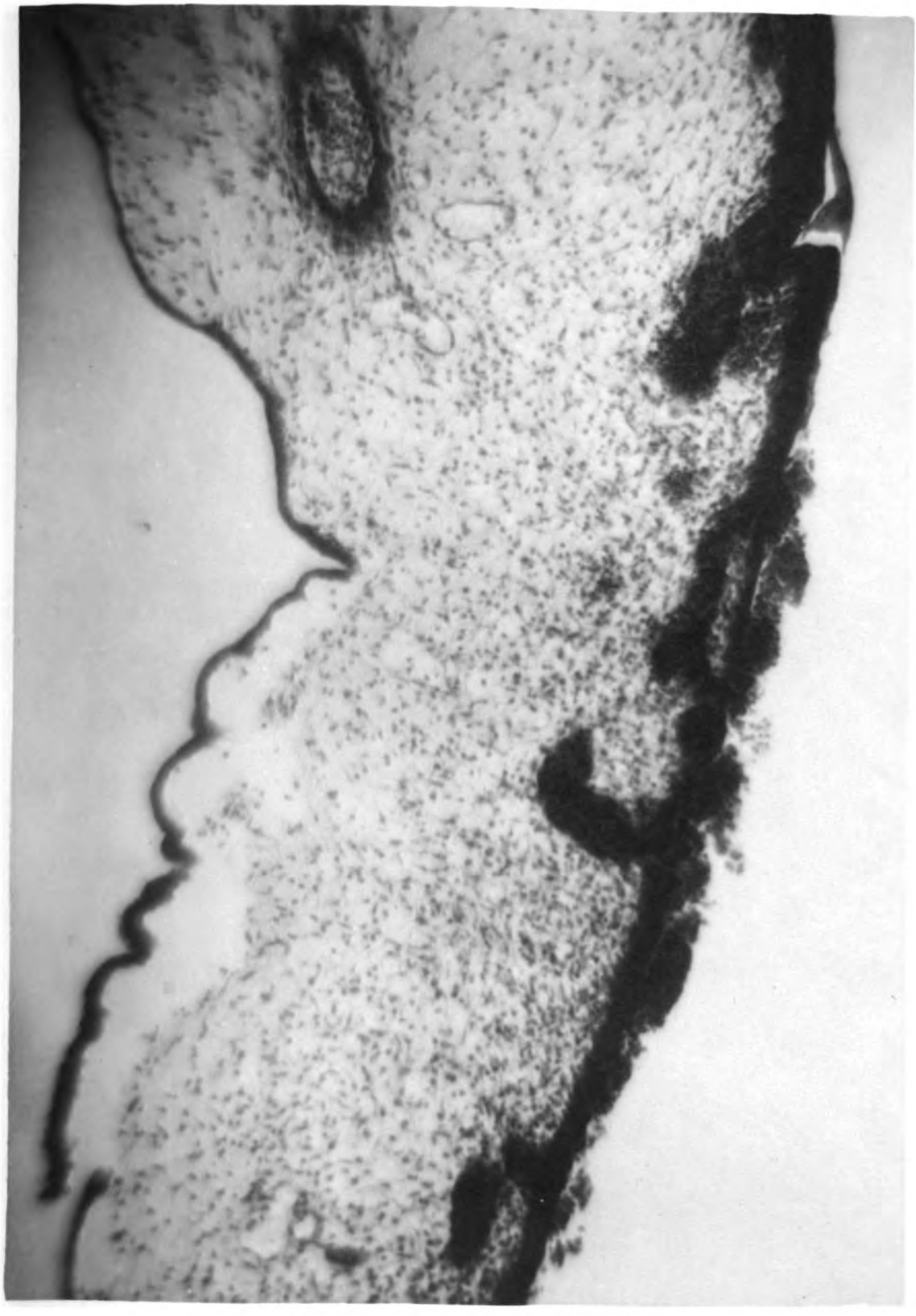

Fig. 7

Fig. 8. Higher magnification of Fig..7 showing edema,. infiltration of heterophils and proliferation of mesenchymal cells. 550x.
1-Heterophil.
2-Mesenchymal cell.

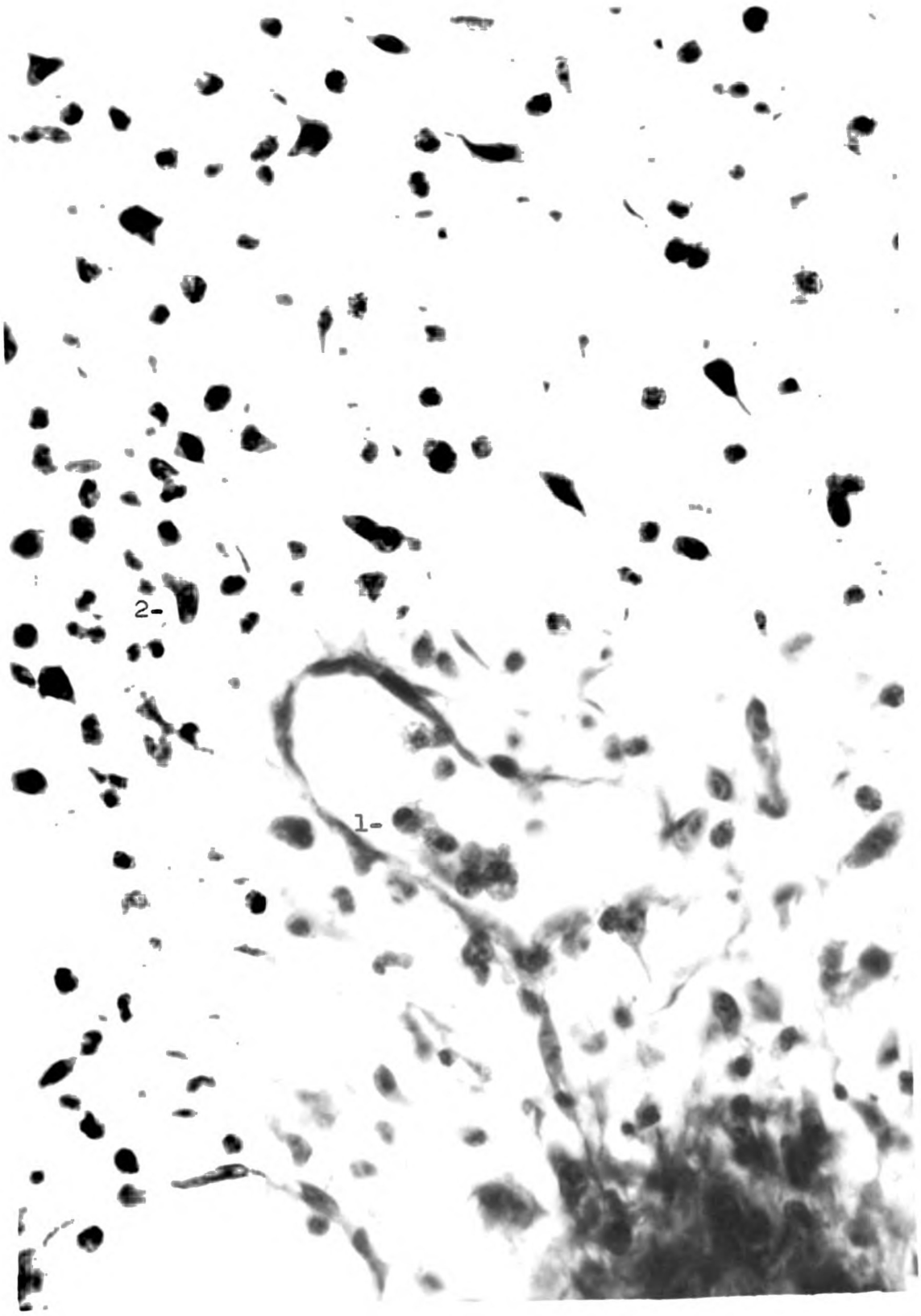

Fig. 8

Fig. 9. Arterial lesion in the chorioallantoic membrane of a 14 day embryo inoculated on the eleventh day of incubation (ninth passage). 550x.

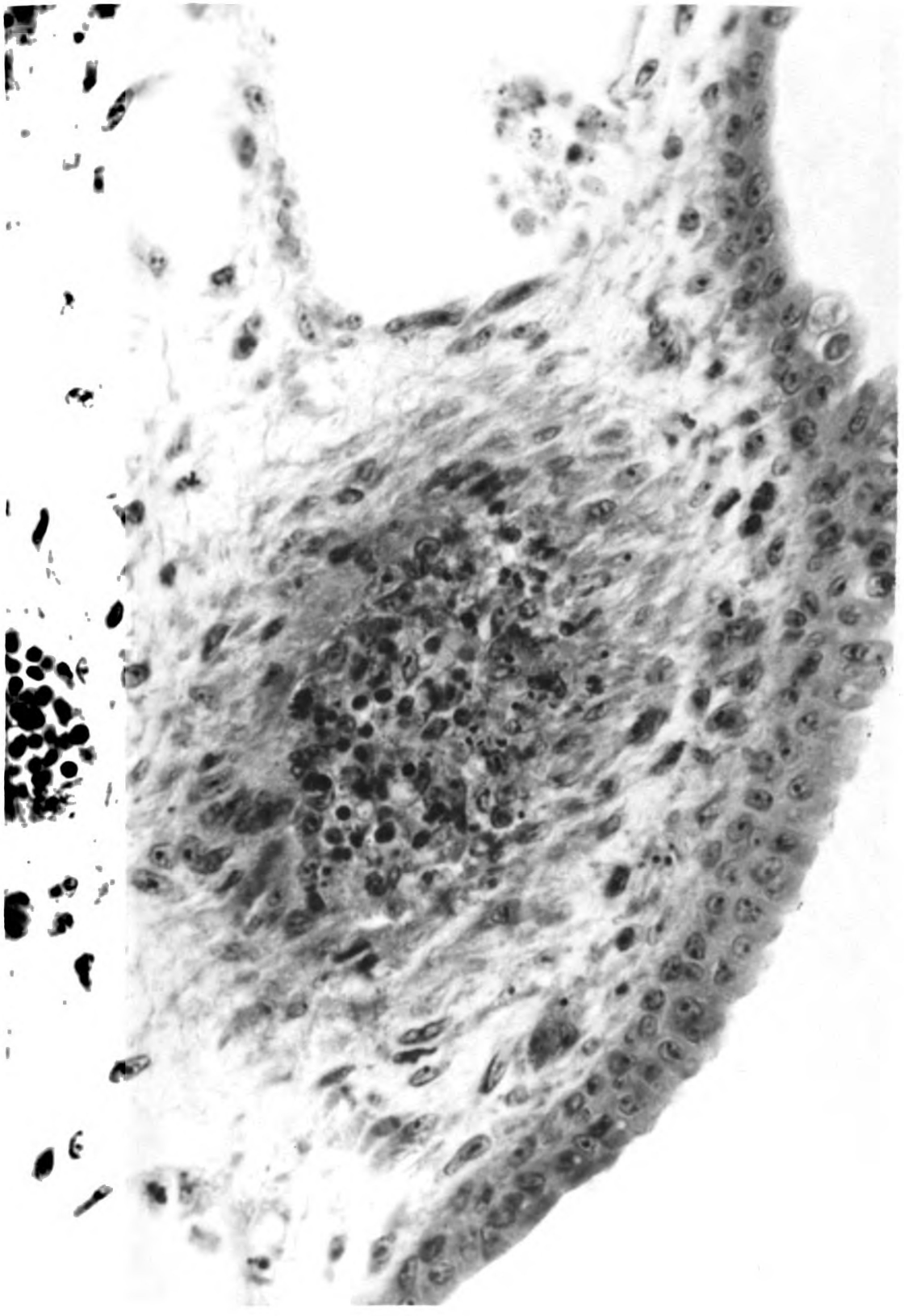

Fig. 9

Fig. 10. Arteriolar lesions in the chorioallantoic membrane of a 15 day embryo (fifth passage). 550x.

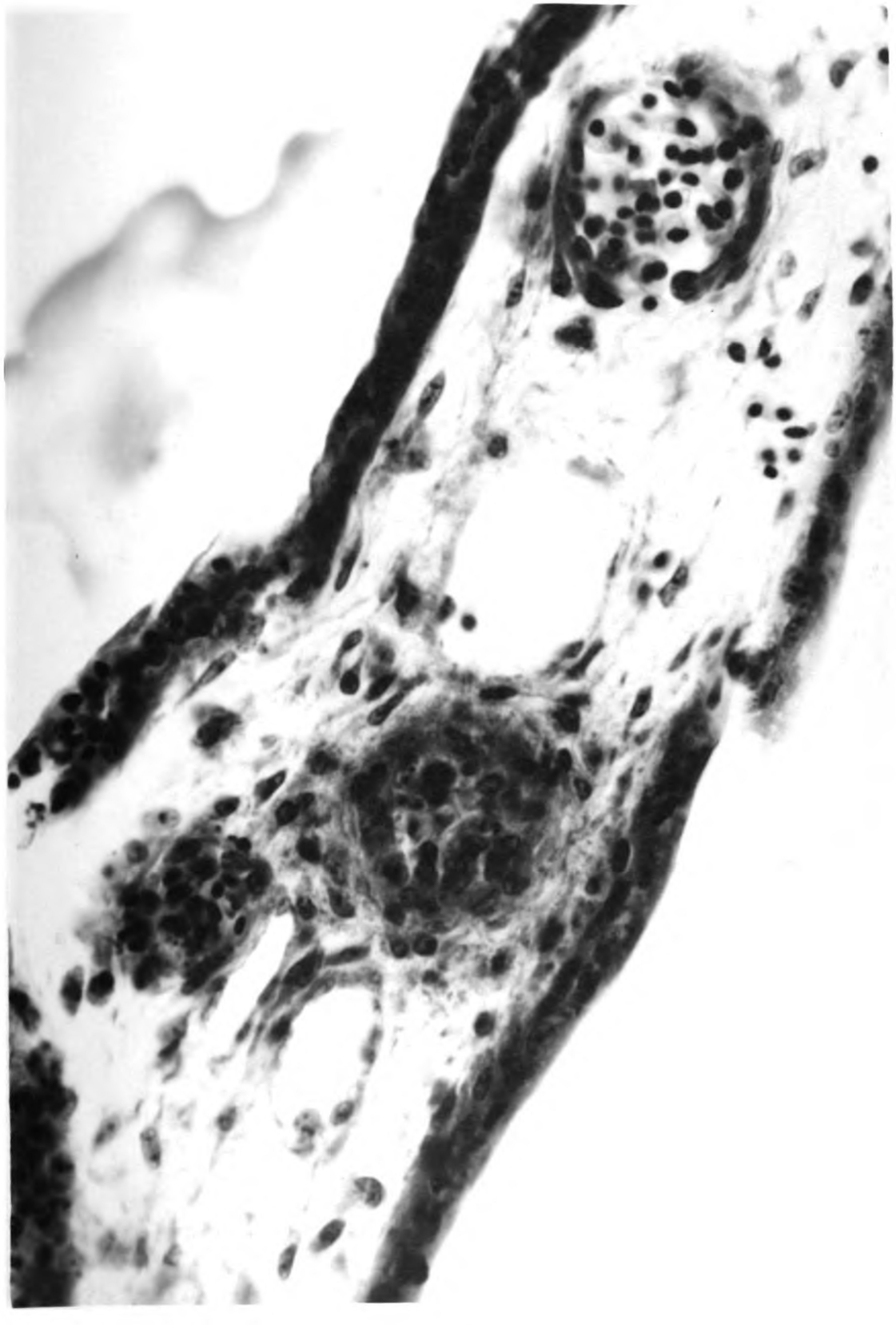

Fig. 10

Fig. 11.. Arterial lesions due to V.. fetus in the bovine fetal placenta.. 135x..

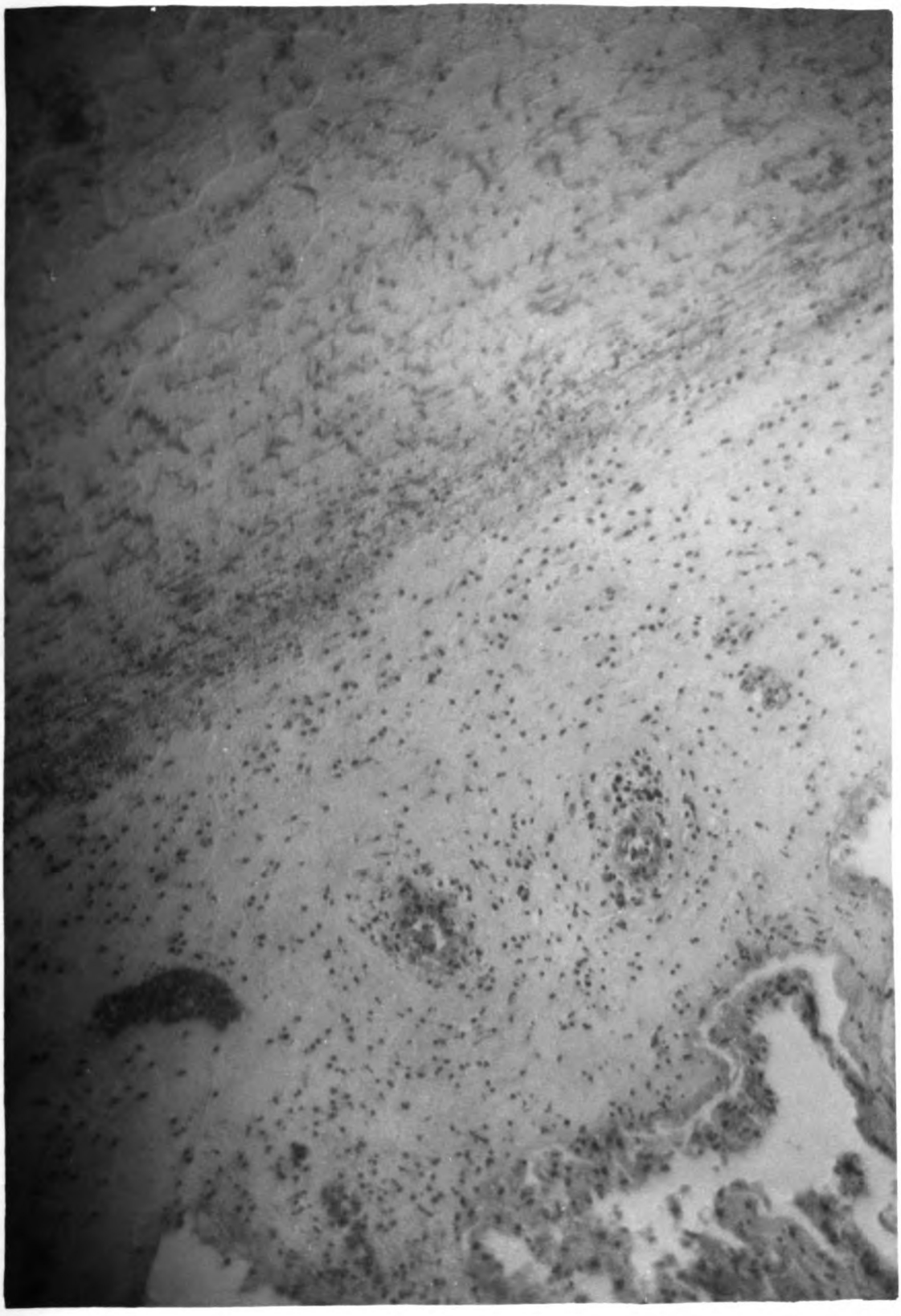

Fig. 11

Fig. 12. Arterial lesion in the bovine fetal placenta. Note the pyknotic nuclei of endothelial cells and leucocytes. Macrophages have infiltrated the area. 550x.

Fig. 12

Fig. 13. Liver of a 12 day embryo injected with control fluid on the ninth day of incubation (second passage). 135x.

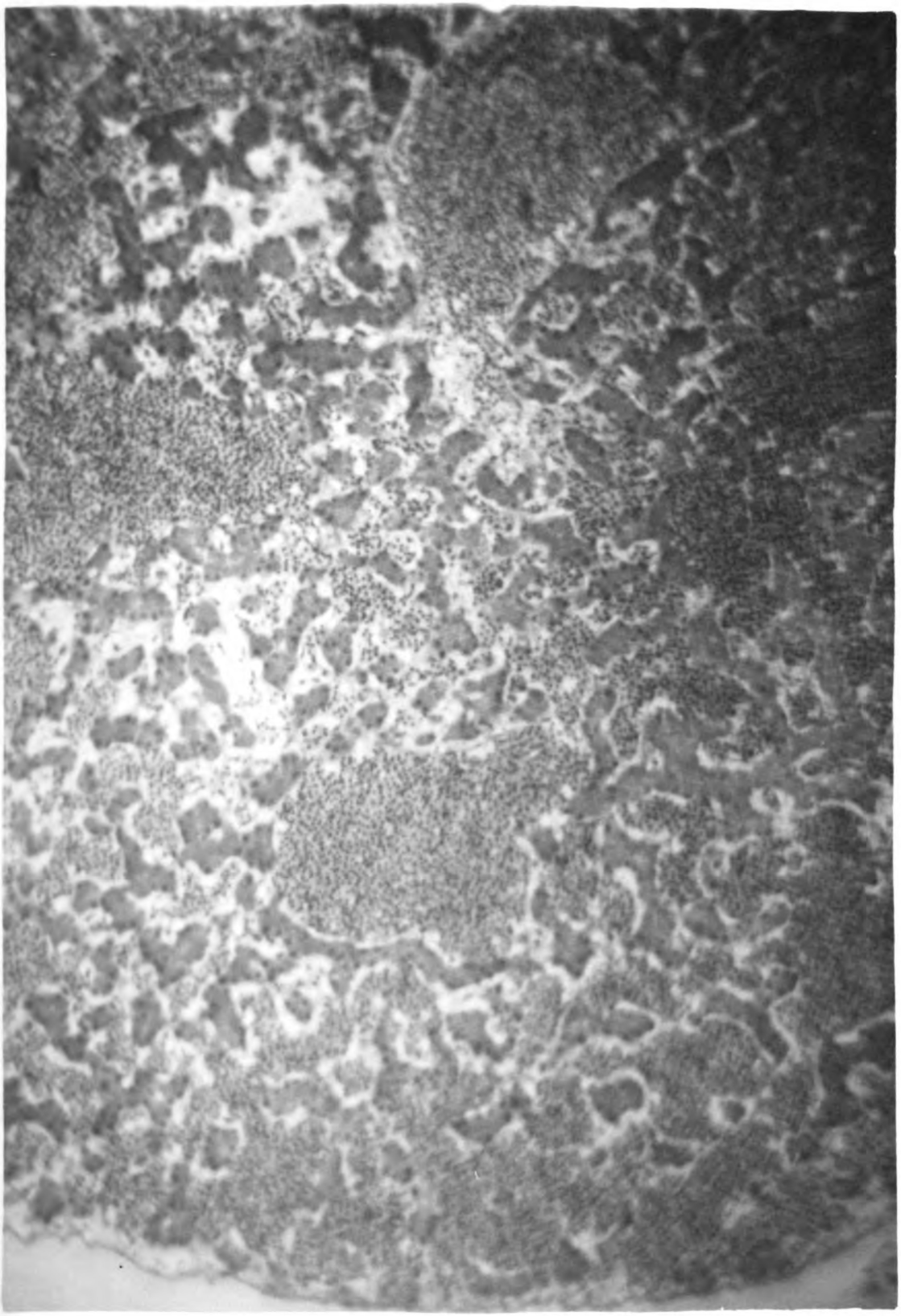

Fig. 13

Fig. 14. Liver of a 12 day embryo injected on the ninth day of incubation showing marked congestion, paucity of parenchymal cells and pyknotic nuclei of the remaining cells (second passage). 135x.

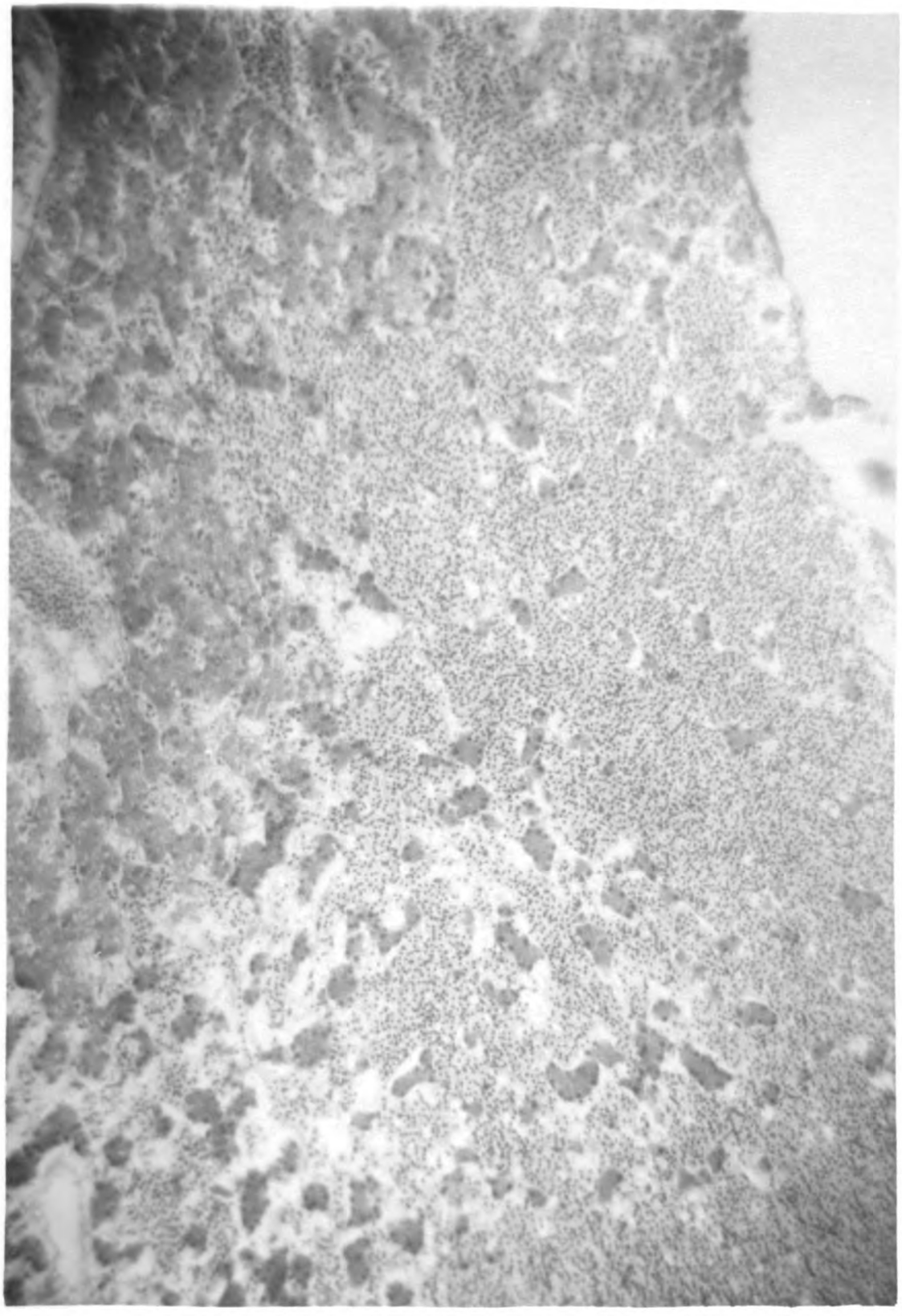

Fig. 14

Fig. 15. Liver of a control embryo sacrificed on the fourteenth day of incubation. 135x.

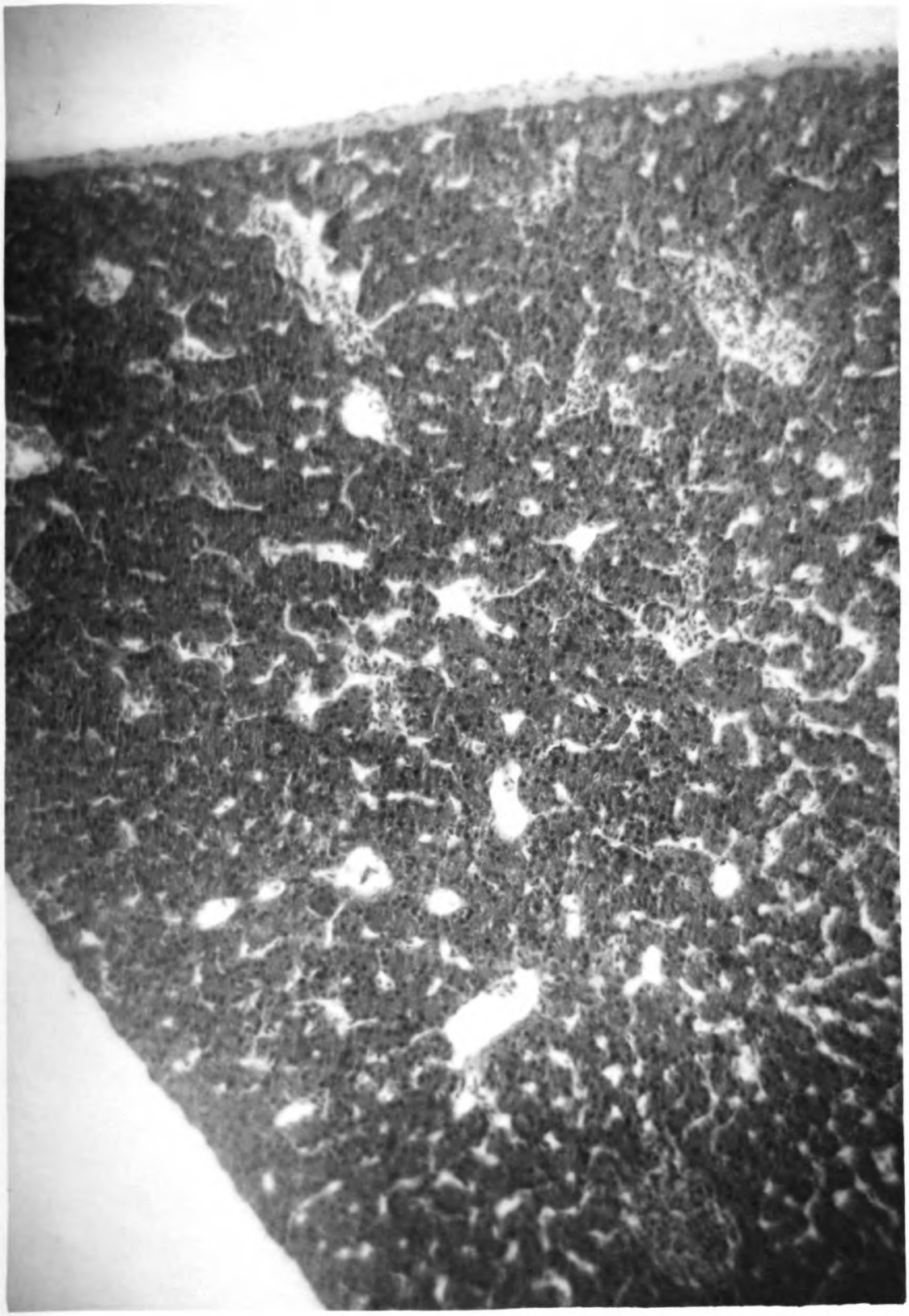

Fig. 15

Fig. 16. Marked congestion of the liver together with fibrin in the peritoneal cavity of a 14 day embryo injected on the eighth day of incubation (twenty-third passage). 135x.

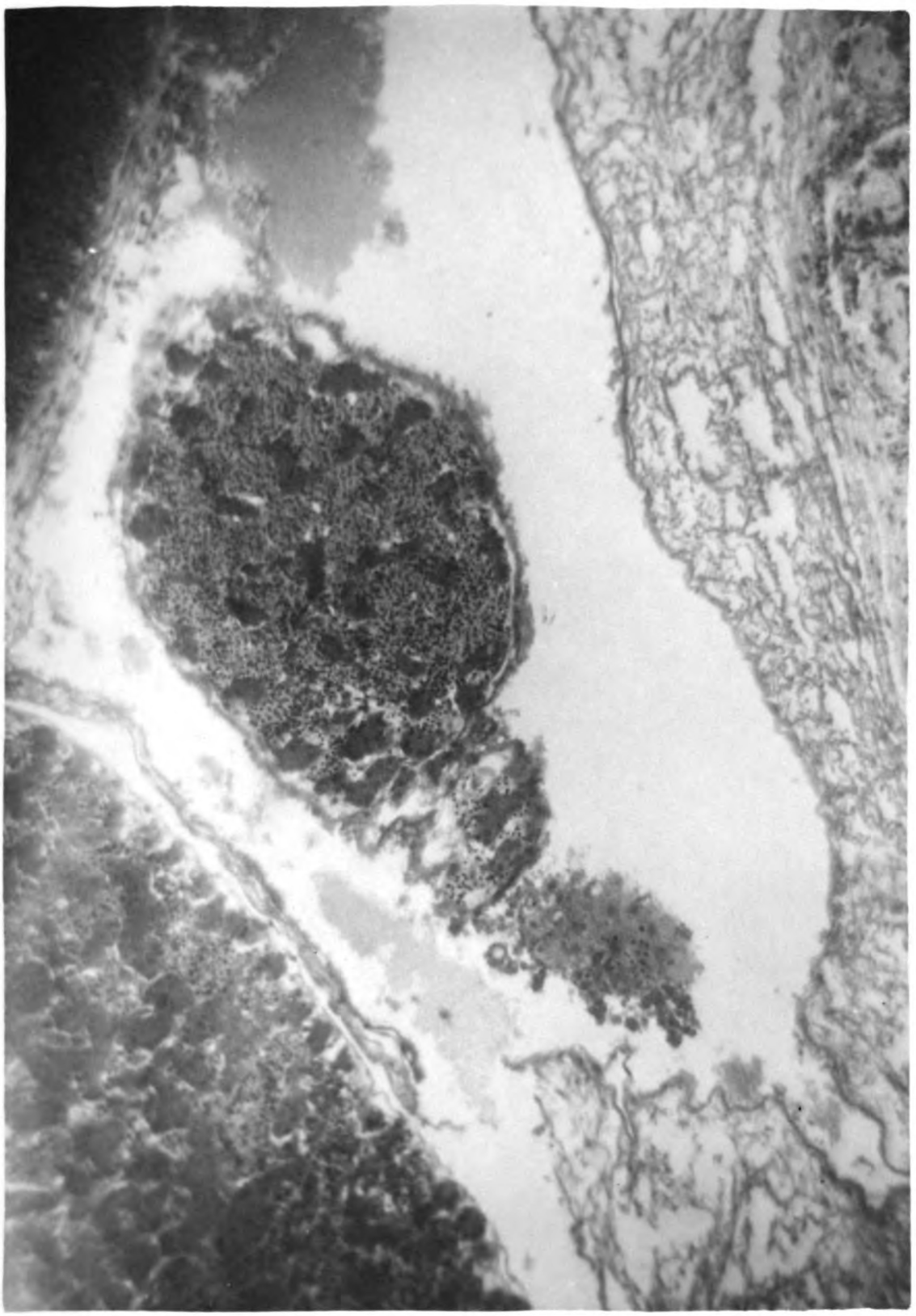

Fig. 16

Fig. 17. Necrotic liver tip of a 15 day embryo inoculated on the eleventh day of incubation (twenty-second passage). 135x.

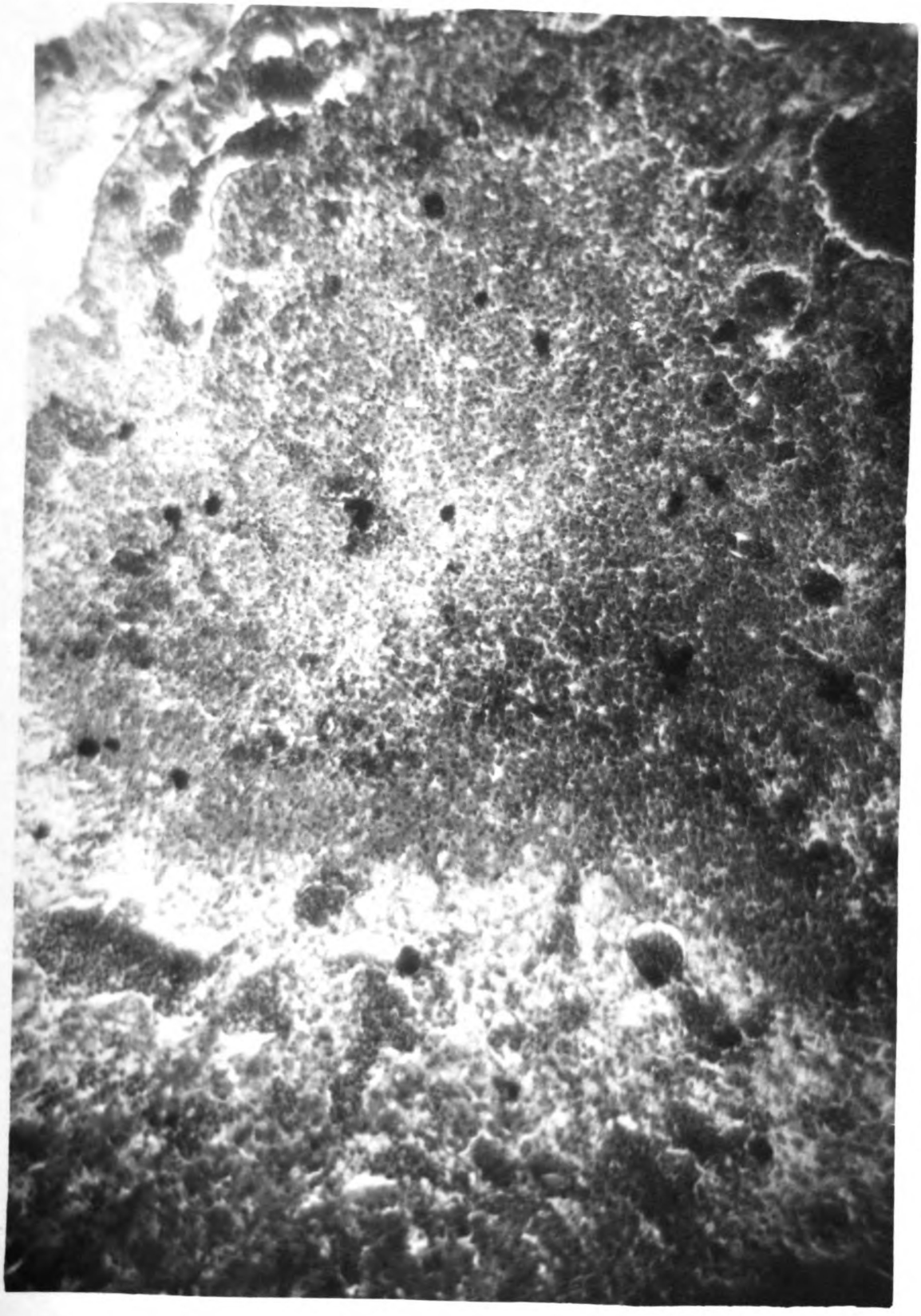

Fig. 17

Fig. 18. Kidney of a normal 14 day embryo. 135x.

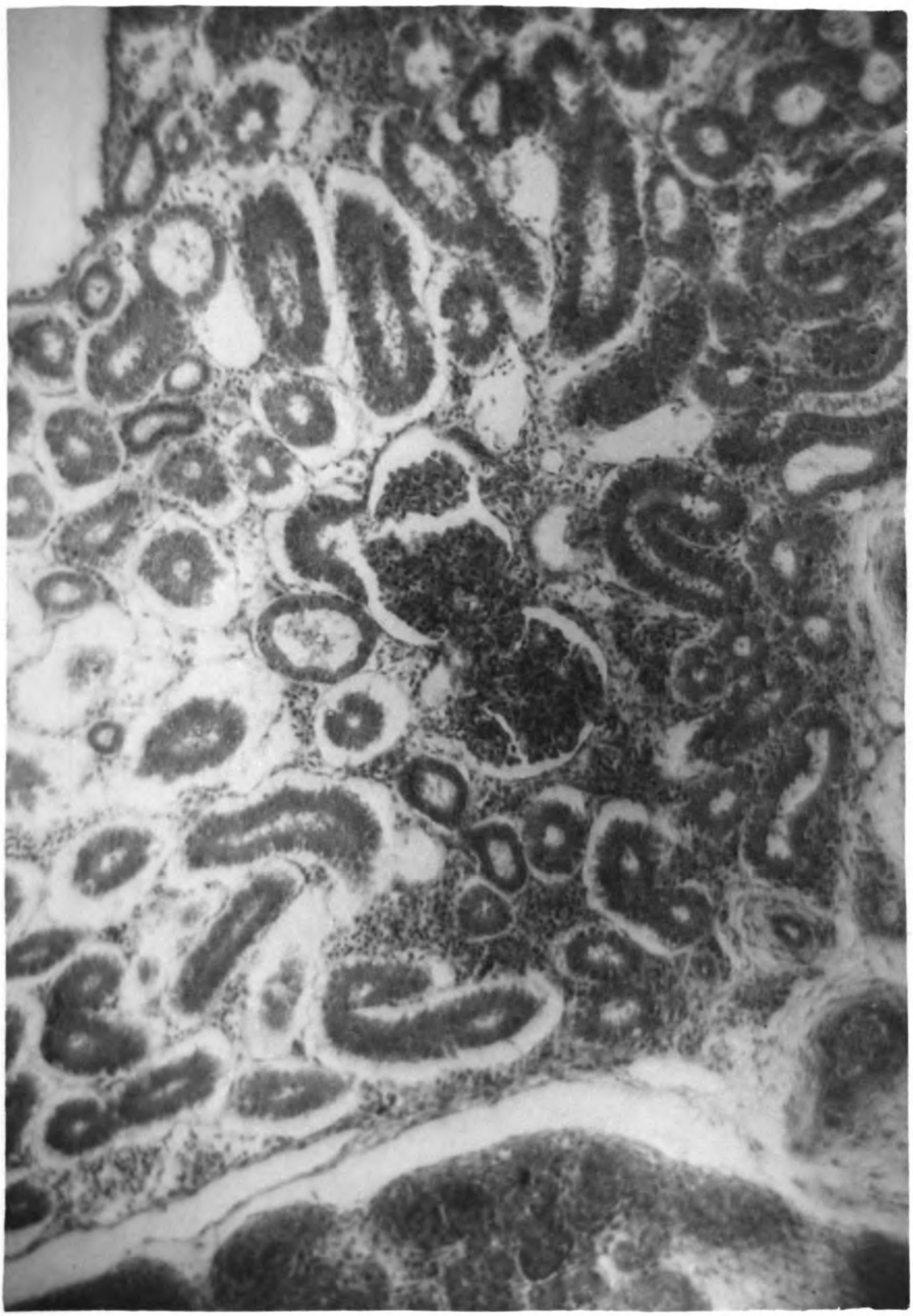

Fig. 18

Fig. 19. Higher magnification of normal glomeruli shown in Fig. 18. 550x.

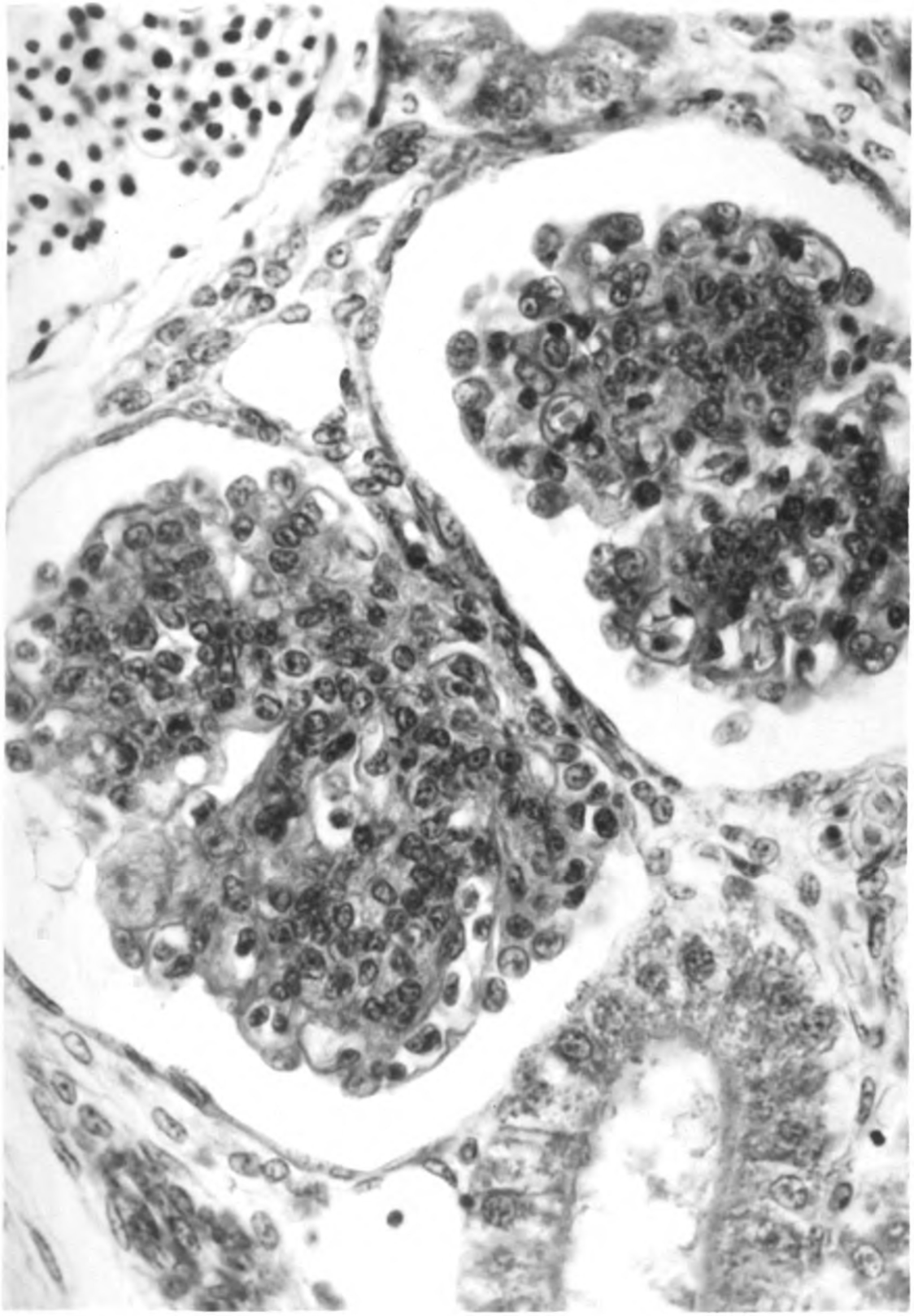

Fig. 19

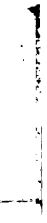

Fig. 20. Kidney of a 15 day embryo injected on the eleventh day of incubation showing marked congestion and an enlarged glomerulus (twenty-second passage). 135x.

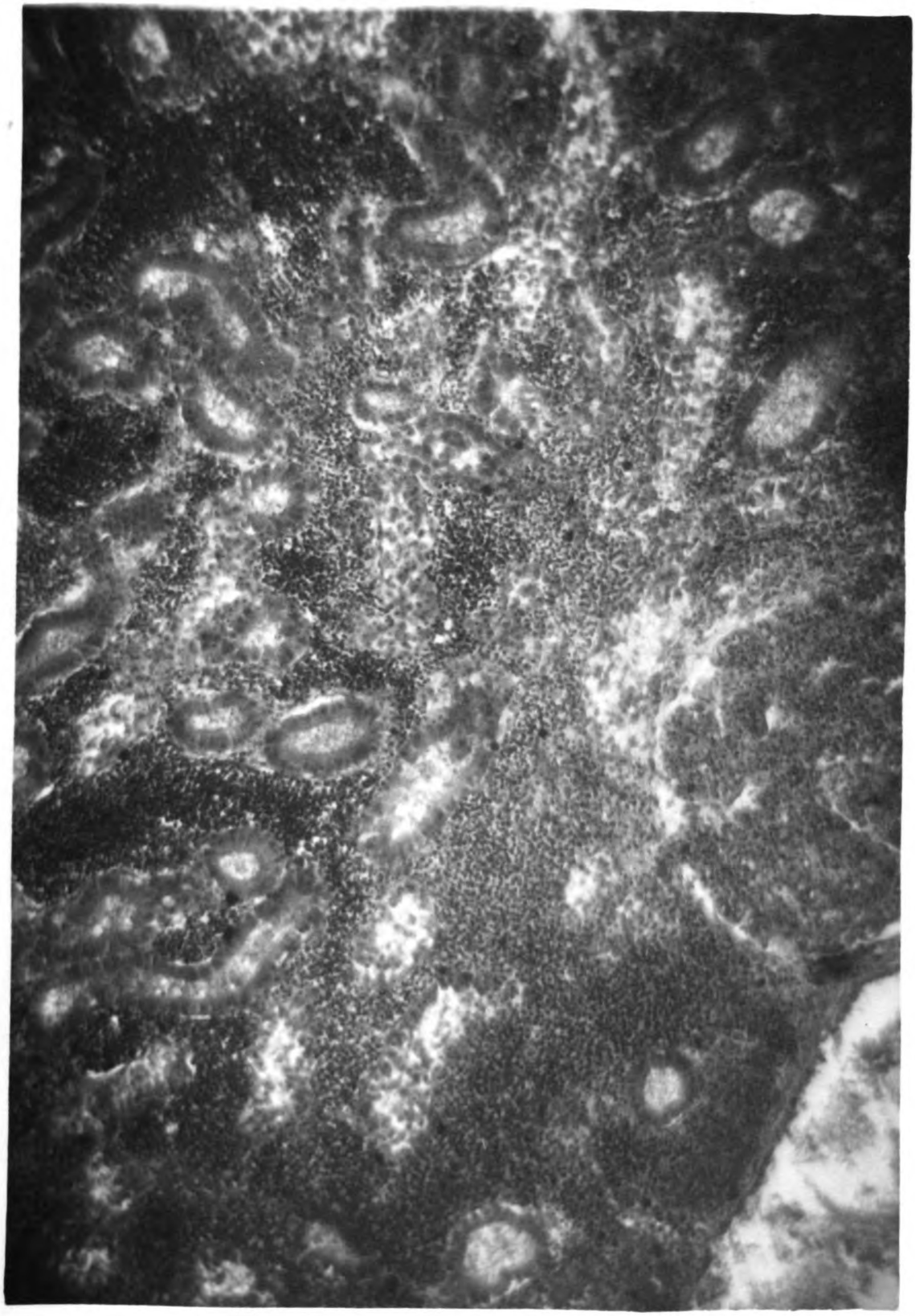

Fig. 20

Fig. 21.. Higher magnification of enlarged glomerulus shown in Fig. 20. Note the engorgement with blood and presence of pyknotic nuclei of glomerular endothelial cells.. 550x.

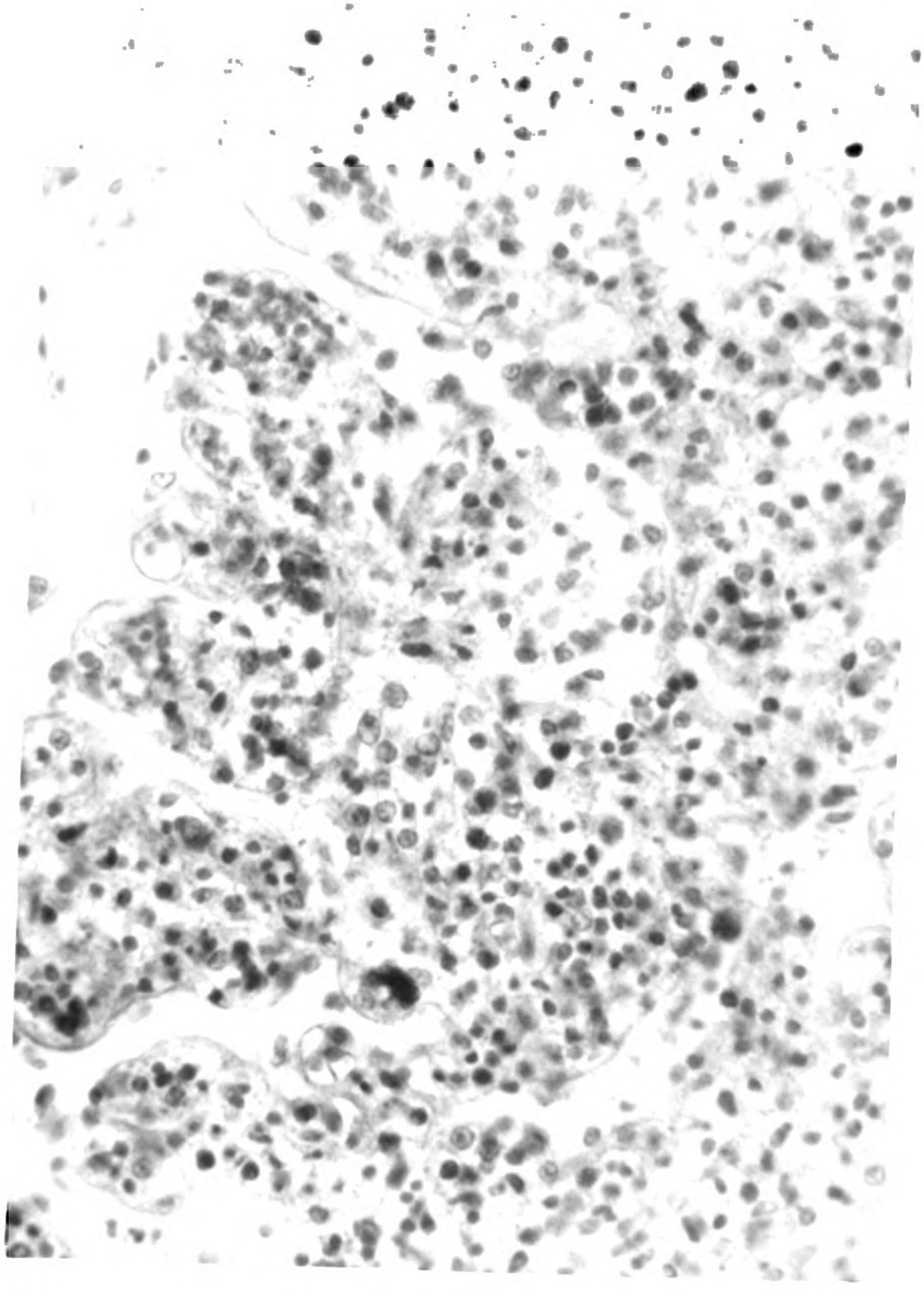

Fig. 21

Fig. 22.. Glomerulus of a 14 day embryo inoculated on the eighth day of incubation (twenty-third passage).. 550x.

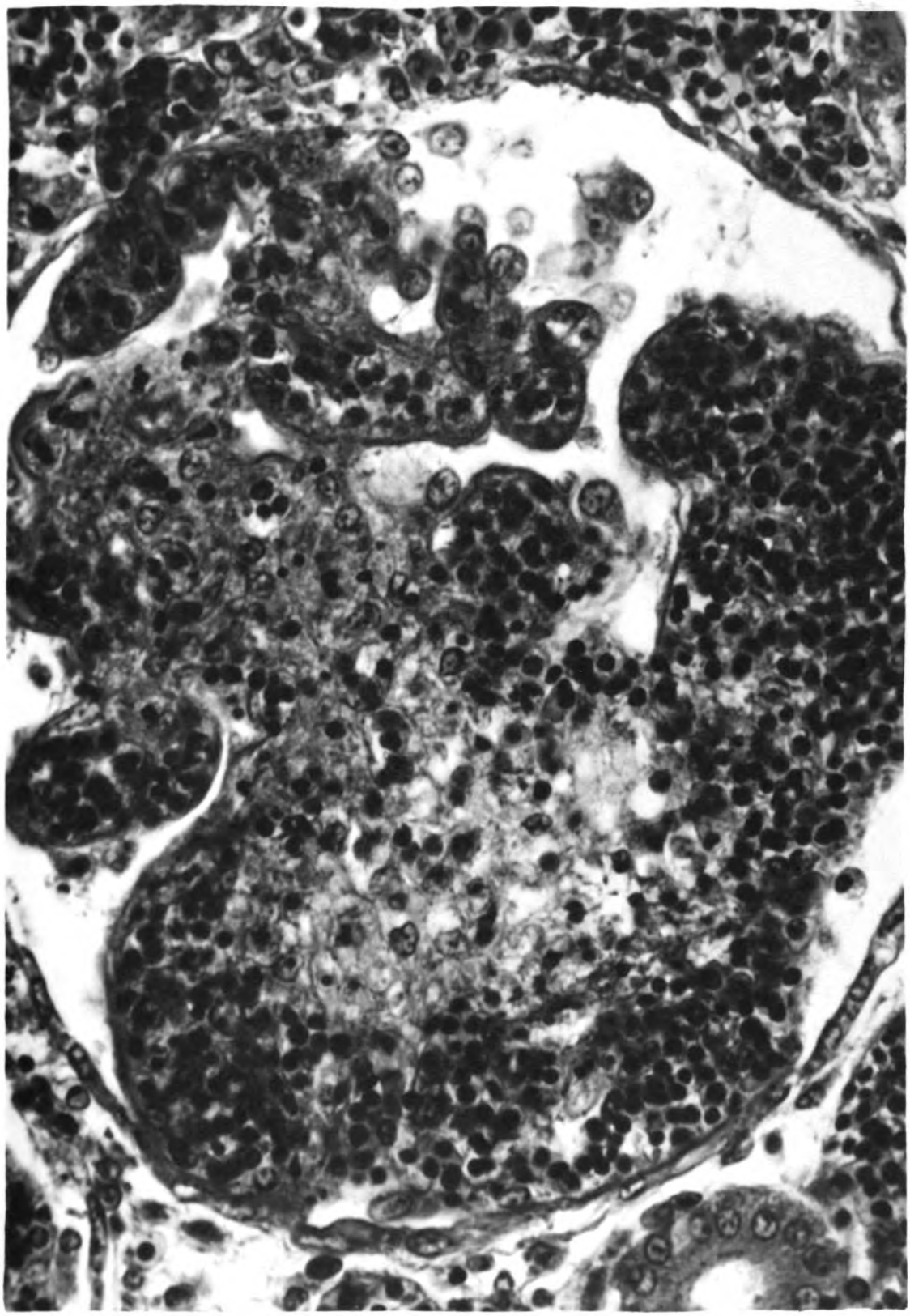

Fig. 22

Fig. 23. Normal cerebrum of an embryo on the fourteenth day of incubation. 135x.

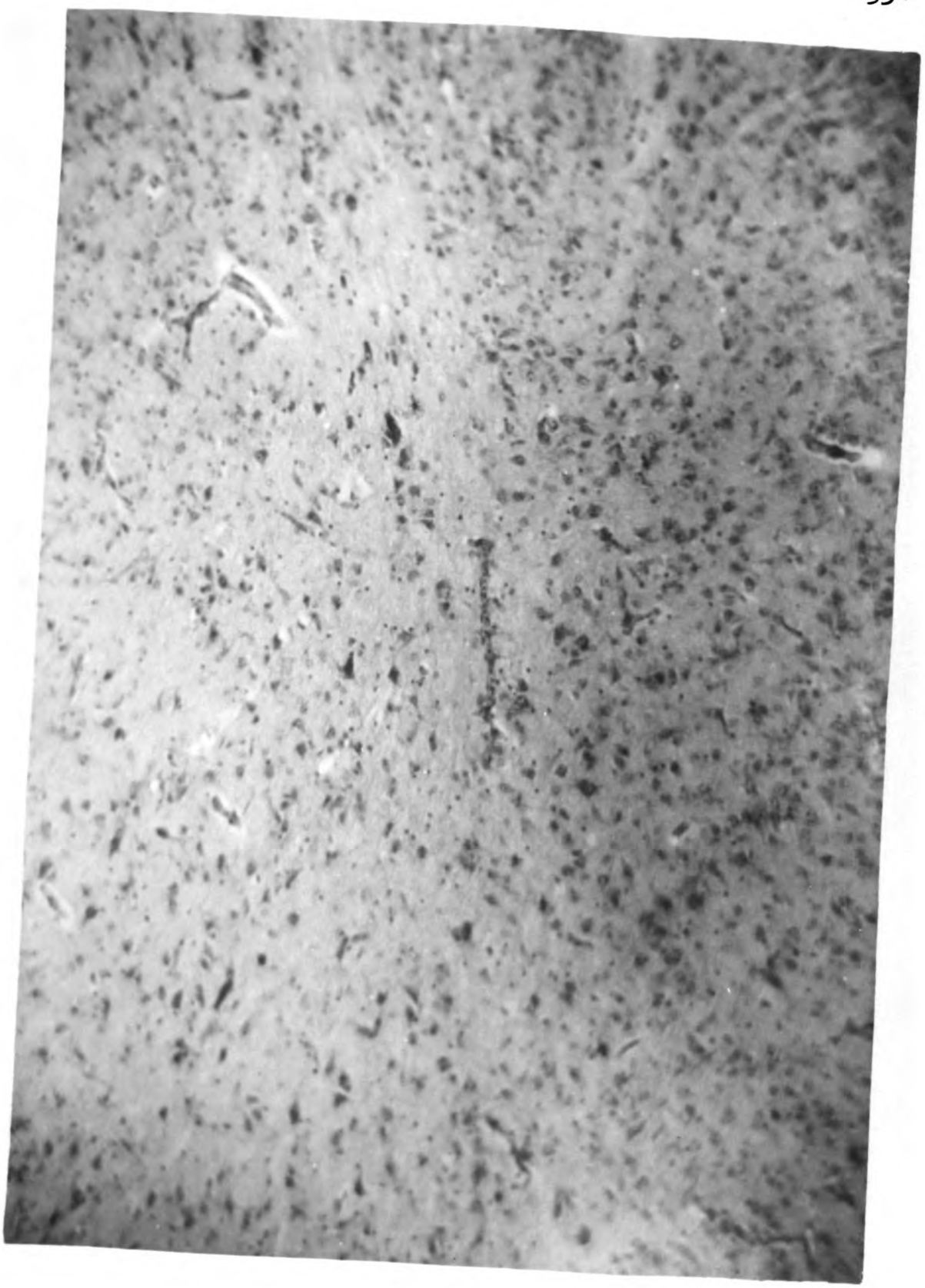

Fig. 23

Fig. 24. Small area of necrosis and hemorrhage in the cerebrum of a 15 day embryo inoculated on the eleventh day of incubation. Note the congestion (twenty-second passage). 135x.

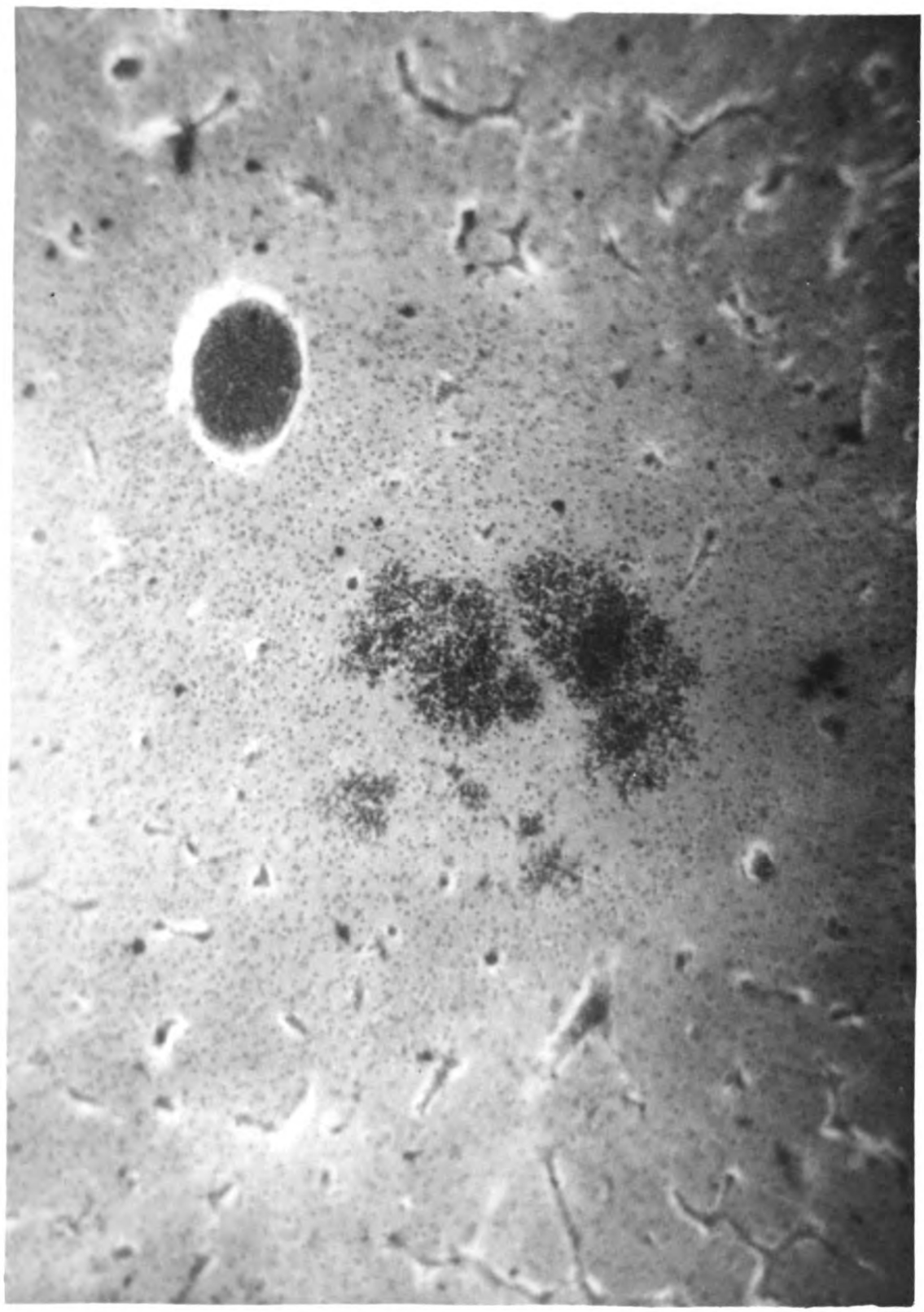

Fig. 24

Fig. 25.. Portion of a large area of necrosis and hemorrhage in the cerebrum of a 14 day embryo inoculated on the eighth day of incubation (twenty-third passage). 135x.

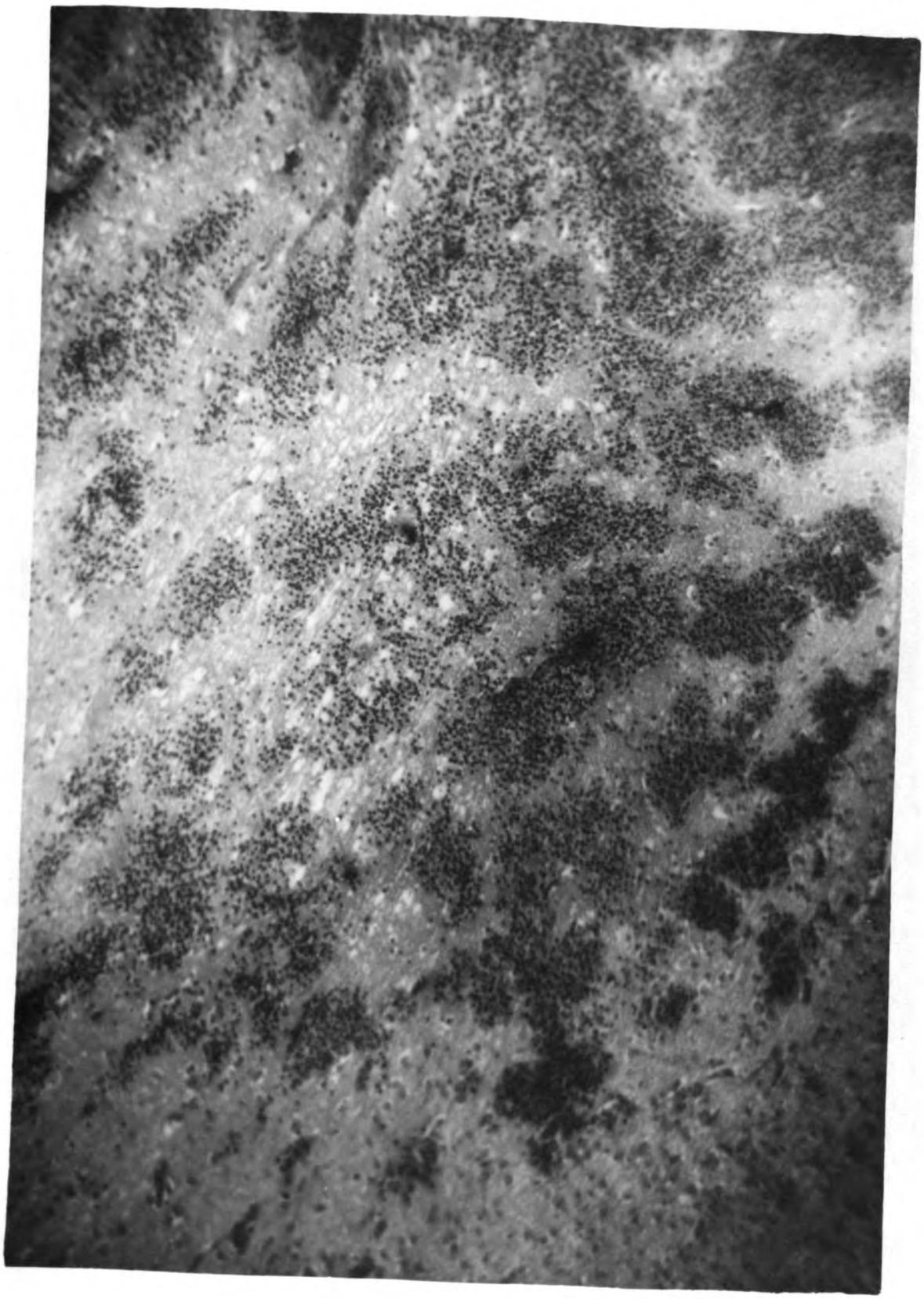

Fig. 25

Fig. 26. Congestion, hemorrhage and fibrinous exudation in the lung of a 15 day embryo injected on the eleventh day of incubation (twenty-second passage). 135x.

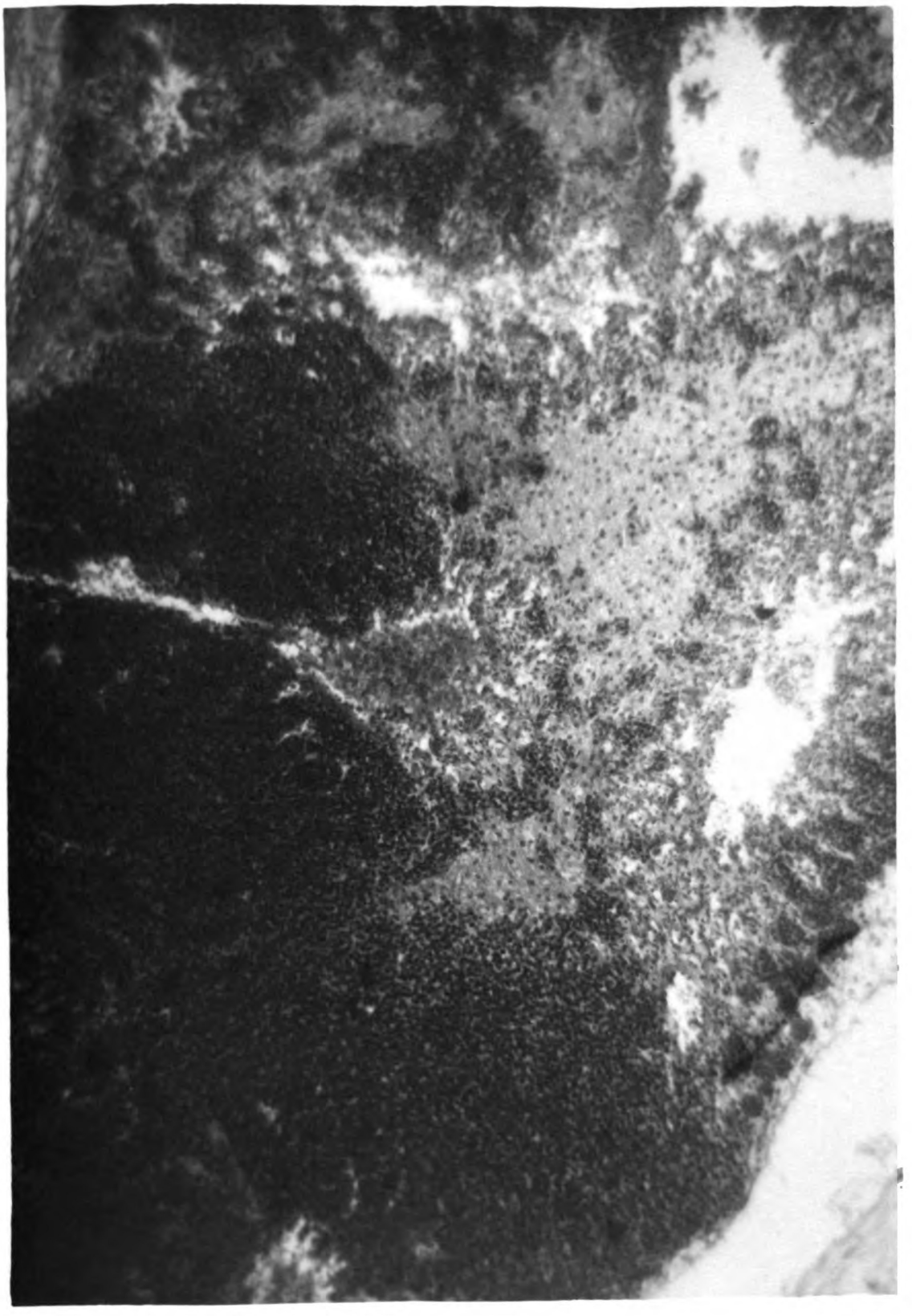

Fig. 26

Fig. 27. Hemorrhage in the muscular wall of the gizzard of a 14 day embryo inoculated on the eleventh day of incubation (seventh passage). 135x.

Fig. 27

Fig. 28. Skin and subcutaneous tissues of a normal embryo after 14 days incubation. 135x.

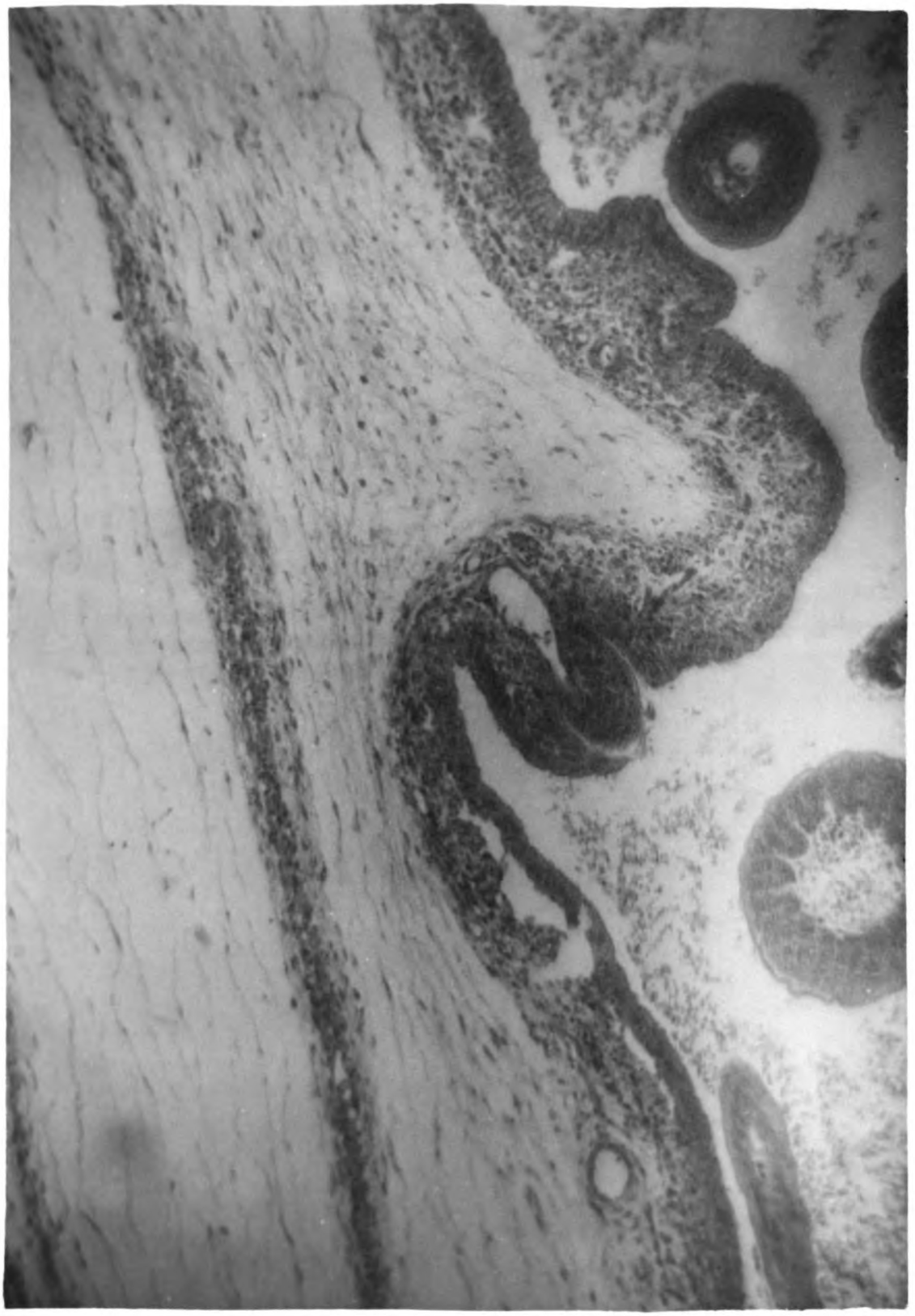

Fig. 28

Fig. 29. Congestion in the skin, subcutaneous tissue and muscle of a 15 day embryo inoculated on the twelfth day of incubation (fifth passage). 135x.

Fig..29