

OBSERVATIONS ON THE PATHOGENESIS OF BOVINE AND ANTELOPE STRAINS OF *BESNOITIA BESNOITI* (MAROTEL, 1912) INFECTION IN CATTLE AND RABBITS

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ABSTRACT

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Histopathological studies were made on cattle and rabbits, artificially infected with either proliferative organisms of low and high passage level bovine strains of *Besnoitia besnoiti* (Marotel, 1912) grown in tissue culture and in rabbits, or cyst organisms from a natural bovine case of besnoitiosis. Rabbits similarly infected with either proliferative organisms of a low level rabbit-passaged blue wildebeest [*Connochaetes taurinus* (Burchell, 1823)] strain or cyst organisms from naturally infected blue wildebeest and impala [*Aepyceros melampus* (Lichtenstein, 1812)] were likewise used for comparative studies. Degenerative and necrotic vascular lesions, vasculitis and thrombosis, mainly of the medium and smaller veins and some arteries, were the most outstanding changes in the acute stages of the disease. These changes coincided with the parasitization of certain cells in the vessels such as the endothelium, where the organisms proliferate before the onset of the cystic stage. These basic lesions were responsible for oedema, degenerative changes and even infarction, particularly in the testes and skin. A histiocytic reaction and mild eosinophil infiltration were some of the other characteristic features.

The cystic stage in cattle apparently developed in enlarged histiocytes, which were recognizable 11 days after infection. These host cells became multinuclear and seemed to be responsible for the production of the cyst wall. The cysts reached maturity 71 days after infection. Reactions to cysts apparently commenced before any degenerative changes in the organisms were detectable and could have been preceded by such changes in the cyst wall. The antelope strains of *B. besnoiti* were only very mildly pathogenic to rabbits, but passage during the acute stage of the disease in this host increased the pathogenicity considerably. Rabbits which were infected with bovine strains developed severe testicular and skin lesions, but these lesions were either absent or relatively mild in rabbits infected with antelope strains. In the latter, lesions were usually confined to internal tissues and organs such as the myocardium, gut and lungs and, more rarely, even in the adrenal, liver, kidneys and urinary bladder. However, cysts were rarely encountered in both these groups.

INTRODUCTION

Since the original report on bovine besnoitiosis by Besnoit & Robin (1912) several investigators, notably Besnoit & Robin (1914), Hofmeyr (1945), Smith & Jones (1957), Pols (1960), Schulz (1960), Vsevolodov (1961) and McCully, Basson, van Niekerk & Bigalke (1966), have contributed to our present knowledge of the pathology of the disease. Due to the difficulty of producing typical clinical bovine cases experimentally (Pols, 1954a, 1960; Bigalke, 1967, 1968) and the fact that early symptoms may pass undetected in the naturally acquired disease, studies have been mainly confined to subacute or chronic natural cases. The pathogenesis of the early phase of besnoitiosis has therefore remained obscure.

New opportunities to study the histopathology of the earlier stages of the bovine disease in detail, and thus obtain a better understanding of the pathogenesis were provided by: (1) The discovery that rabbits are susceptible to artificial infection with proliferative forms (Pols, 1954a) and cyst forms (Bigalke, 1960, 1967; Neuman, 1962) of *Besnoitia besnoiti* (Marotel, 1912), and that they develop lesions very similar to those seen in natural bovine cases. (2) The development of a successful method of producing typical clinical cases in cattle by inoculation of large numbers of organisms grown in cell culture, or with rabbit tissues heavily parasitized with proliferative forms (Bigalke, 1968).

The discovery of *Besnoitia* in certain antelopes such as the blue wildebeest [*Connochaetes taurinus* (Burchell, 1823)] and the impala [*Aepyceros melampus* (Lichtenstein, 1812)] (Basson, McCully, van Niekerk & Bigalke, 1965; McCully *et al.*, 1966) and subsequent studies on its re-

lationship to *B. besnoiti* of cattle (Bigalke, van Niekerk, Basson & McCully, 1967) called for a comparison between the pathogenic effects of strains from antelopes and cattle.

A short résumé of the most prominent lesions seen in rabbits infected artificially with bovine and antelope strains of *B. besnoiti* and a detailed account of the finding in cattle infected with antelope strains were given by Bigalke *et al.* (1967). The purpose of this article is to give a comprehensive account of studies on the pathogenesis of besnoitiosis caused by bovine strains of *B. besnoiti* in cattle, and of bovine as well as antelope strains in rabbits. This in particular will be in relation to the developmental cycle of the parasite and the genesis of the cysts from the earliest stage to full maturity.

MATERIALS AND METHODS

Cattle

With the exception of Ox 4828, which was a South Devon-Afrikaner cross and Friesian Heifers 7602 and 7607, the cattle used in this experiment were all grade Herefords. Their ages varied from 8 months to almost 8 years. They were housed and fed as previously described (Bigalke, 1968). They were infected with bovine strains of *B. besnoiti* by various methods which are summarized in Table 1.

Rabbit tissues containing proliferative forms

Ox 2923 was inoculated intravenously with a heparinized testes suspension prepared from four acutely diseased rabbits harbouring the 542nd passage-level of the Fuls strain. As outlined previously (Bigalke, 1968) and in Table 1, Heifer 3088 received blood and testes

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suspension from a rabbit harbouring a strain recently isolated from feral *Stomoxys calcitrans* Linnaeus, 1758. Control 7602 was inoculated intravenously with a testes suspension prepared from five healthy rabbits and was slaughtered 11 days later.

Organisms of a recently isolated strain grown in vitro

Oxen 3894, 3515, 4828 and Cow 781 were inoculated intravenously with heparinized suspensions of organisms grown in lamb kidney monolayer cultures for three days (Ox 3894) or two days (remaining animals) before they were harvested (Table 1; Bigalke, 1968). They received the 2nd, 8th, 8th and 13th *in vitro* passage levels respectively of a strain which originated from chronically infected Bull 3329. The strain had been isolated in culture from the testes of acutely diseased rabbits inoculated previously with a suspension of cyst-bearing skin from this bull. On 26 June 1967, at the 4th passage level in culture, the strain was stored frozen as a stabulate and used to prepare inocula 2 to 11 months later. Control 7607 received the same number of normal lamb kidney cells intravenously as Cow 781 and was slaughtered 8 days later.

Large volumes of blood from experimentally produced acute bovine cases

Oxen 2431 and 3565 received transfusions from cattle infected with recently isolated strains, and Ox 3588 a high passage level strain (Bigalke, 1968, Chapter 12).

After infection, the temperatures of the cattle were recorded, blood smears from the tip of the tail were examined and clinical signs noted daily. Skin biopsies of approximately equal size were taken regularly, mainly from the neck region of the more severe cases. The animals were also examined for cysts in the scleral conjunctiva as described by Bigalke (1968). Autopsies were performed on the two animals that died and the others which were slaughtered after different intervals. The vessels of the extremities were routinely opened and examined for the presence of lesions and cysts. Specimens from various tissues, including skin from several sites, were collected in 10 per cent buffered formalin for a survey on the distribution of cysts and lesions.

Specimens from a few natural cases of bovine besnoitiosis, including two bulls, which were available in the files at Onderstepoort were included in the studies.

Rabbits

Most of the rabbits used were young males bred at Onderstepoort. Their management has been described (Bigalke *et al.*, 1967). They were infected with *B. besnoiti* as follows (Table 2):

Cyst organisms of bovine B. besnoiti: A suspension was prepared in Hanks' solution from a piece of cyst-bearing skin from Ox 3865, as described previously (Bigalke, 1967), and 8 ml injected subcutaneously into Rabbit (R) 4333.

Proliferative organisms of rabbit-passaged bovine B. besnoiti: Blood was drawn from parasitaemic rabbits infected with the Schoeman strain of *B. besnoiti* (Bigalke *et al.*, 1967; Bigalke, 1968) and 5 ml injected intraperitoneally into each of 8 rabbits.

Cyst organisms of B. besnoiti from blue wildebeest and impala: Three of the six rabbits used were inoculated with cyst organisms from blue wildebeest and three with cyst organisms from impala. The subcutaneous route, as described previously, was used (Bigalke *et al.*, 1967). R4363, R4364 and R4366 were challenged with the rabbit-passaged bovine strain approximately two weeks before they were killed.

Proliferative organisms of rabbit-passaged B. besnoiti from the blue wildebeest: Eight rabbits that were used during the course of serial passage of this strain (Bigalke *et al.*, 1967) were also included in this study.

The rabbits were examined for signs of besnoitiosis as described previously (Bigalke *et al.*, 1967; Bigalke, 1968). Those that did not die from the disease were destroyed at various stages for comparative necropsy and histopathological studies. Specimens from all the various organs and tissues, including the pampiniform plexus, epididymis, testes and adrenal, were collected in 10 per cent buffered formalin. These specimens and those collected from the bovines were embedded in paraffin wax and cut at 3 micron thickness with a sliding microtome. Haematoxylin and eosin (HE) stain was generally used. Special stains employed included Masson's trichrome (Anon., 1960), Alcian blue (Anon., 1960), Gridley's stain for fungi (Anon., 1960), Periodic acid-Schiff (PAS) (Pearse, 1961), Gomori's methenamine silver impregnation (GMS) (Anon., 1960), Feulgen-PAS-naphthol yellow (FSNY) (Himes & Moriber, 1956), oil red O (ORO) (Anon., 1960) and Van Gieson's picrofuchsin (VG) (Lillie, 1954).

Parasitized hypertrophic cells, developing cysts and mature cysts (*vide infra*) in the skin were measured at various intervals after infection. The largest diameter of a cell with its group or colony of organisms and cyst wall was measured. Septate cysts (*vide infra*) and compressed or very elongated cysts were ignored. In determining the age of the infection Day 1 was taken one day after infection and not one day after the onset of fever. This was done because of the very short incubation period of the experimental cases and the diphasic fever reaction seen in some of them.

RESULTS

All the clinical observations and some of the pathology of the experimental animals have been reported previously (Bigalke *et al.*, 1967; Bigalke, 1968). Only the relevant macro- and microscopical lesions and the development of the cysts will therefore be dealt with here.

Cattle

Macroscopic lesions

Acute cases

Cow 781 which received organisms grown in tissue culture and Ox 2923, a recipient of a rabbit testes suspension, died acutely 8 and 11 days respectively after infection. Generalized pronounced anasarca with accompanying subcutaneous congestion, many small disseminated haemorrhagic foci and marked subcutaneous thrombosis were present in Ox 2923 in comparison with similar but milder localized lesions mainly in the extremities, including the head, of Cow 781. In both cases congestion and disseminated per diapedetic petechiae, ecchymoses and/or effusions in the skin were prominent, especially in the extremities. On dissection the walls of many of the veins in the subcutis, head and limbs contained small haemorrhagic foci, the intima was uneven and covered by pinkish grey to reddish material or the lumina were entirely obliterated by thrombi. The jugular veins were only mildly affected, but the other larger veins and arteries appeared normal. Fairly large and prominent but localized irregular areas of pseudo-membranous rhinitis with disseminated petechiae were present.

Congestion, oedema and haemorrhagic foci in the haemal nodes and peripheral lymph nodes as well as localized areas of muscular degeneration and necrosis

TABLE 1 Data concerning artificial cases of besnoitiosis in cattle

Bovine No. . . .	2923	3088	3894	3515	4828	781	2431	3565	3588
Age in years . . .	3½	3	2½	3	1½	7½	3¼	2¾	3¼
Splenectomized	No	No	No	Yes	No	Yes	No	No	No
Date	5.5.67	6.2.67	8.6.67	6.9.67	19.10.67	7.5.68	8.3.66	15.6.67	9.5.67
Source infective inoculum . . .	Rabbit testes	Rabbit testes and blood	Cell culture	Cell culture	Cell culture	Cell culture	Bovine blood	Bovine blood	Bovine blood
Total No. of parasites	141.75 × 10 ⁶	Not counted	5.985 × 10 ⁶	1068 × 10 ⁶	984.9 × 10 ⁶	104 × 10 ⁶	Not counted	Not counted	Not counted
Dose in ml	54	12 + 100	19	42	42	21	4000	3500	4000
Route	i.v.	i.v.	i.v.	i.v.	i.v.	i.v.	i.v.	i.v.	i.v.
Febrile reaction* (days post-infection)	2	5 (7)	4	1	1	2	4 (8)	5	2
Termination (days post-infection)	11 (N)	77	71	108	96	8 (N)	385	77	94

i.v. = intravenous
(N) = died naturally
* = second febrile reaction written in parenthesis
OX 3588 proved positive only on gross examination

were found in both animals. The muscles of Ox 2923 were more severely and extensively affected. The longissimus dorsi, subscapularis, supraspinatus, triceps brachii, coraco-brachialis, biceps femoris, quadriceps femoris group, semimembranosus, pectorals and muscles of the neck were involved. The affected areas were either pale pink or beige in colour and usually well-demarcated. Prominent serofibrinous arthritis with petechial haemorrhages of all the fetlock joints, adjoining serofibrinous tendovaginitis and many petechiae or ecchymoses in the nerves of the fore limbs were present in the more advanced case (Ox 2923). A mild splenomegaly was also noticeable in this animal; Cow 781, however, had been splenectomized two years before infection.

The livers of both animals were mottled and degenerative with evidence of bile stasis and small petechiae in the gall bladder. The lungs and trachea were congested, either mildly or markedly oedematous and speckled with a few petechiae. Similar haemorrhages occurred in both larynx and pharynx and mild foci of necrotic laryngitis were also seen in Ox 2923. Areas of mild fibrinous peritonitis and pleuritis were noticed in the same animal. Cow 781 had a few petechial haemorrhages subendocardially in the left ventricle, kidneys, pituitary gland, abomasum and uterine mucosa. A few erosions occurred in the abomasum of the same animal.

Chronic cases

The lesions found in the chronic cases were similar to those reported earlier (McCully *et al.*, 1966). Most of the cases, except Heifer 3088 and Ox 3515, were either mild or fairly mild and did not show any prominent skin lesions when they were slaughtered. In the mildest cases a small number of cysts could only be found in the veins of the extremities, the most frequent site being in the facial vessels, followed by the veins of the limbs. Cysts were usually more numerous on the valves of the affected veins and at sites where tributaries entered. With increasing severity of the disease, cysts became more widespread in their distribution and were observed in the eyes (conjunctiva, limbus and iris), nasal mucosa (septum, turbinates and ethmoid), subcutis, paranasal mucosa and jugular veins. The pharynx, larynx, trachea, tendon sheaths, bursas, intermuscular fascia and some of the muscles such as the semitendinosus, semimembranosus, quadriceps femoris group and those of the tail and face were parasitized in severely affected chronic cases which showed skin lesions. In these cases the skin of the face around the mouth, eyes and ears, side of the neck, pasterns, gaskins, breech, tail, hips, thirls and point of the hock was thickened and revealed partial alopecia.

Controls

Heifers 7602 and 7607 failed to reveal any lesions or parasites.

Microscopic lesions

Pathogenesis of skin lesions and development of cysts

The main features of the progressive development of the lesions associated with proliferative stages of *B. besnoiti* and the development of cysts found in the skin biopsies and specimens taken at autopsy are listed in Table 3. The following is a summarized account of this development on consecutive days after infection. Only additional changes are mentioned on consecutive days.

Day 1 and 2: No material studied.

Day 3: One case examined. Congestion, few very small haemorrhages, leukostasis and slight perivascularitis noticed, mainly in the stratum papillare (s.pap.).

Small numbers of both eosinophils and mononuclear cells present.

Day 4 and 5: Six cases. Eosinophil reaction more advanced in one case. Perivascular proliferation and mild vasculitis present [Plate 5 (34)].

Day 6: Four cases. Abovementioned lesions somewhat more advanced and present in both s.pap. and stratum reticulare (s.ret.). Few *Besnoitia* organisms present in the endothelium of a vein in one case. Monocytes recognized.

Day 7: Three cases. Organisms in two cases also found intracellularly outside vessels. Groups of 3 to 5 organisms present in some endothelial cells and each group found within a vacuole. Slight endothelial hyperplasia, early thrombosis of some small vessels [Plate 5 (36)] and prominent oedema noticed.

Day 8: Five cases. Three cases had groups of proliferative organisms, which numbered up to six, in the endothelium [Plate 3 (20 and 21)]. Some organisms seen outside blood vessels. Few small haemorrhages present. Proliferated perivascular cells, possibly histiocytes, became hypertrophic [Plate 1 (1)]. Apparent increase of mast cells and more advanced thrombosis of smaller vessels noticed in s.pap. of two cases [Plate 5 (35)].

Day 9: One case. Numerous intra- and extracellular organisms and prominent thrombosis of small veins in both s.pap. and s. ret.

Day 10: One case. *Besnoitia* also seen extravascularly in mononuclear cells. Many thrombi present, some of which caused infarction of the skin with subsequent necrosis; s.pap. very hypercellular, especially around vessels. Endothelial hypertrophy and proliferation more prominent.

Day 11: Four cases. Two animals had small numbers of both intra- and extravascular hypertrophic cells (22 μ) which contained one or more organisms [Plate 1 (2)]. These represented very young developing cysts. Some intraneural hypertrophic cells without organisms were also present. The cytoplasm of these cells was reddish-blue and mucoid in appearance. Thrombosis occurred in both small and large veins [Plate 3 (16, 17 and 18)] and the walls of some revealed partial fibrinoid necrosis [Plate 3 (19)]. Localized swelling and coagulative necrosis of the epidermis, as a result of thrombosis, with secondary bacterial invasion and an increase in mononuclear cells, were apparent.

Day 12 (one case) and 13 (two cases): As for day 11.

Day 14 (two cases) and 15 (one case): Cell reaction in and around vessels was more pronounced and consisted mainly of monocytes, but also of some lymphocytes and eosinophils. Histiocytic proliferation encountered both outside and within vessel walls; proliferation of endothelial cells very prominent.

Day 16: Two cases. Hypertrophic cells [Plate 1 (3)] with small colonies of organisms became multinucleated (2 to 4 nuclei) and increased in size (45 μ). A peripheral collar of purplish blue material in these developing young cysts evidently represented the developing cyst wall. It was lined by an inner light blue zone, a subequivalent vacuolated area and a somewhat pinkish central part [Plate 1 (4 to 7)]. Thrombi were absent.

Day 18: One case. Some suspected extracellular organisms still noticeable.

Day 19 and 20: One case each. Many young cysts present (48 to 67 μ) with an inner eosinophilic cytoplasm containing increased numbers of nuclei (4 to 5) and organisms. S.pap. more affected than s.ret.

Day 22 (two cases) and Day 23 (one case): Largest developing cysts had 2 to 10 nuclei and measured 64 to 88 μ [Plate 1 (8)].

Day 24: One case. Developing cysts measured up to 70 μ . Pronounced vasculitis and perivasculitis, mainly of mononuclear type.

Day 25 and 26: One case each. Largest developing cysts 93 to 112 μ ; absence of oedema.

Day 27: One case. Developing cysts up to 105 μ frequently lined up in the course of vessels some even in valves [Plate 2 (13)]. A few contained as many as 14 nuclei each.

Day 28 and 29: One case each. Immature cysts of 108 to 135 μ . Mitotic figures present in host cell nuclei of young cysts [Plate 2 (15)].

Day 30 and 33: One case each. Largest immature cysts measured 165 to 170 μ . The growing colony of organisms in the host cell evidently enveloped by a thin membrane [Plate 2 (10)]. The nuclei were pushed outwards toward the inner lighter blue zone of condensed peripheral cytoplasm. The distinct outer peripheral zone represented the developing and maturing cyst wall. Some septate cysts with two to four compartments were present. These apparently developed from single host cells which had been parasitized by more than one organism, each proliferating to form separate colonies [Plate 1 (9)] as suggested by Pols (1960). Because of their large size, septate cysts were not measured and their measurements are therefore not included in this report. A small number of disintegrating cysts with pycnotic nuclei and more intense eosinophilia of the organisms and host cell cytoplasm was noticed on Day 30.

Day 35 and 36: One case each. Largest immature cysts measured from 165 to 228 μ .

Day 38 (one case) and 40 (two cases): Immature cysts measured 220 to 252 μ . *Besnoitia* organisms occupied most of the central area of the multinucleated giant cell and were surrounded by the nuclei, a narrow eosinophilic zone of cytoplasm and the well-demarcated peripheral, blue cyst wall [Plate 2 (11)]. The mononuclear reaction was more pronounced in the s.pap.

Day 42 (one case) and 43 (two cases): Immature cysts up to 240 μ . One necrotic cyst was present and conglomerations of cysts were noticed [Plate 2 (14)].

Day 44 and 45: One case each. Cysts with a maximum size of 236 to 300 μ . Some evidently approaching maturity, almost the entire host cell cytoplasm being occupied by organisms.

Day 50 and 51: One case each. Largest cysts measured 240 to 275 μ . Conglomerations of cysts appeared to result from fusion of the walls of enlarging, adjoining cysts. Perivasculitis was still evident.

Day 71: One case. Some of the cysts were up to 330 μ in diameter and probably either mature or approaching maturity. Granulomatous reactions including mild to moderate degrees of accompanying fibroplasia appeared around both apparently live and degenerative or necrotic cysts [Plate 5 (40)]. The walls of some viable appearing cysts became thinner, irregular in outline and increasingly eosinophilic as the surrounding reactions developed [Plate 5 (37)].

Day 75: One case. Some of the cysts reached a size of 360 μ in diameter but most were only about 300 μ . The cyst walls were still bluish in colour.

Day 77: One case. The largest cyst had a diameter of 390 μ . Most of the cysts were present in the s.pap.

Eosinophils were numerous in the granulomatous reactions. The cyst wall had an uneven and somewhat fibrous outer surface which seemed to indicate that an outer limiting membrane was absent.

Day 96: One case examined and the largest cyst measured 360 μ .

Day 108: Only one case with the maximum diameter of a single cyst being 390 μ .

Day 385: One case. A few cysts reached 375 μ in diameter [Plate 2 (12)]. Granulomatous reactions were even noticed around cysts with apparently live organisms. An increasing number of cyst walls became reddish, especially those with surrounding reactions. The walls appeared to be digested in an irregular fashion by the giant cells [Plate 5 (37)].

The rate of growth of the parasitized hypertrophic host cell, the potential cyst, to the fully mature cyst from Day 8 to Day 385 is presented graphically in Fig. 1; this shows that maturity could be reached approximately 71 days after infection.

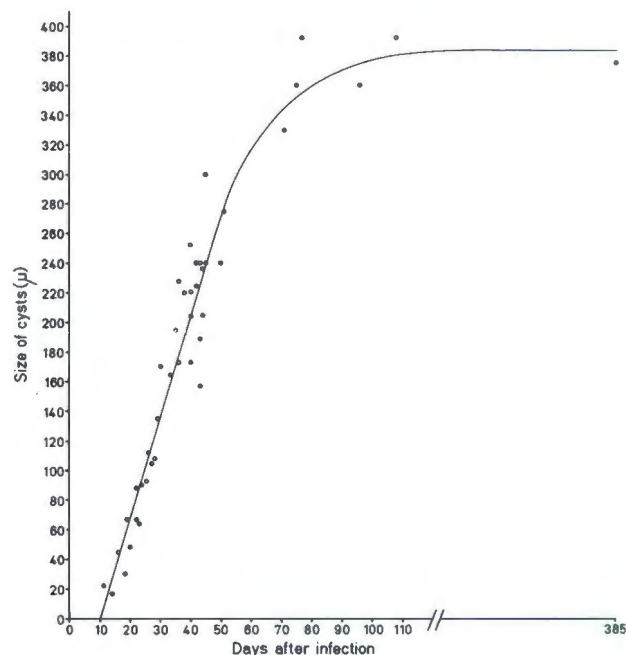


FIG. 1 Rate of cyst growth in cattle

Histopathology of acute cases

For the purpose of this report the acute phase of the disease refers to the febrile and anasarctous stages of Besnoit & Robin (1914). It terminates at approximately Day 25 when most of the oedematous changes have subsided and cysts of up to 100 μ are present (Table 3). Only two bovines died during this stage and in both cases the primary lesion was evidently of vascular nature. Many of the vessels, from the smallest venules in the skin [Plate 5 (35)] and *subcutis* to certain larger veins such as the facials [Plate 3 (16 and 17)] with an approximate diameter of 4 mm, were necrotic and contained thrombi. The intima and media were primarily affected for varying depths with a fibrinoid type of necrosis, but their involvement was not necessarily complete or symmetrical. Small haemorrhages within the vessel walls were also frequently seen. A few small arteries with distinct elastica internas were also affected. Varying numbers of endothelial cells were parasitized and contained one or more *Besnoitia* organisms [Plate 3 (20 and 21)].

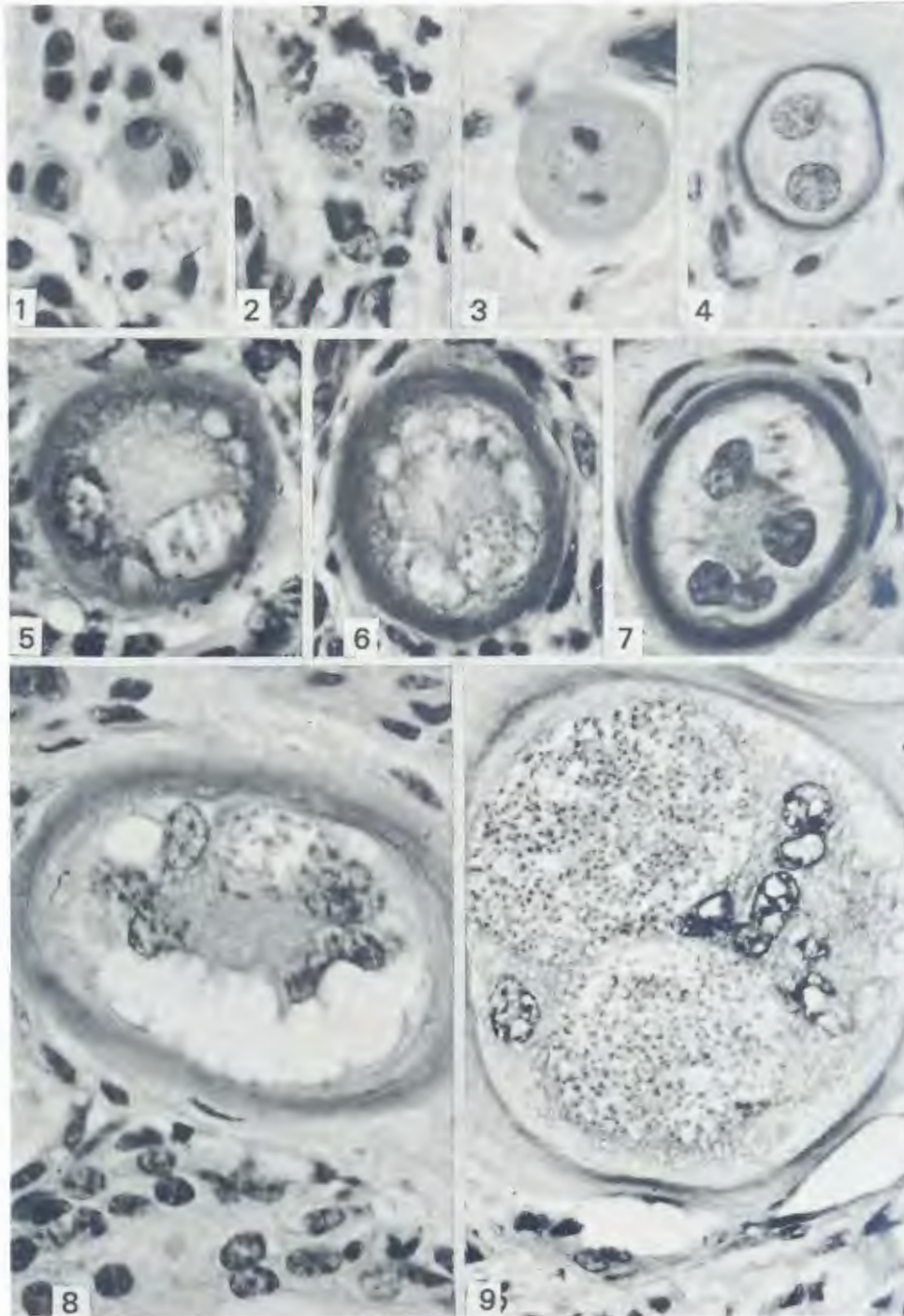


PLATE 1 Photomicrographs of HE stained sections of bovine skin depicting cyst formation. 1. Enlarged cells in the stratum papillare. $\times 750$. 2. Enlarged cell containing one parasite which is situated in a vacuole. $\times 750$. 3. Enlarged mitotic host cell containing two parasites. An exceptionally thick wall is already present. $\times 750$. 4. Binucleated host cell in the wall of a small vessel (lower left). The developing cyst wall is distinctly noticeable. $\times 750$. 5. Multiplication of organisms in the host cell and vacuolar appearance of outer zone of cytoplasm. The developing cyst wall is not distinctly demarcated. $\times 750$. 6. A developing colony of organisms (lower right) in a host cell which has well vacuolated cytoplasm. The colony is clearly demarcated from the surrounding host cytoplasm by a thin membrane. $\times 750$. 7. Almost complete occlusion of a small vessel by an enlarged host cell with four nuclei and developing colony (top right). $\times 750$. 8. Considerably enlarged host cell with a thickened cyst wall which is still indistinctly demarcated. The number of nuclei has increased, the vacuoles are very enlarged and a colony of organisms is noticeable at the top. Day 22. $\times 750$. 9. Developing septate cyst evidently due to two separate colonies developing in one host cell which has eight nuclei and a fairly well delineated cyst wall. Day 30 $\times 500$.

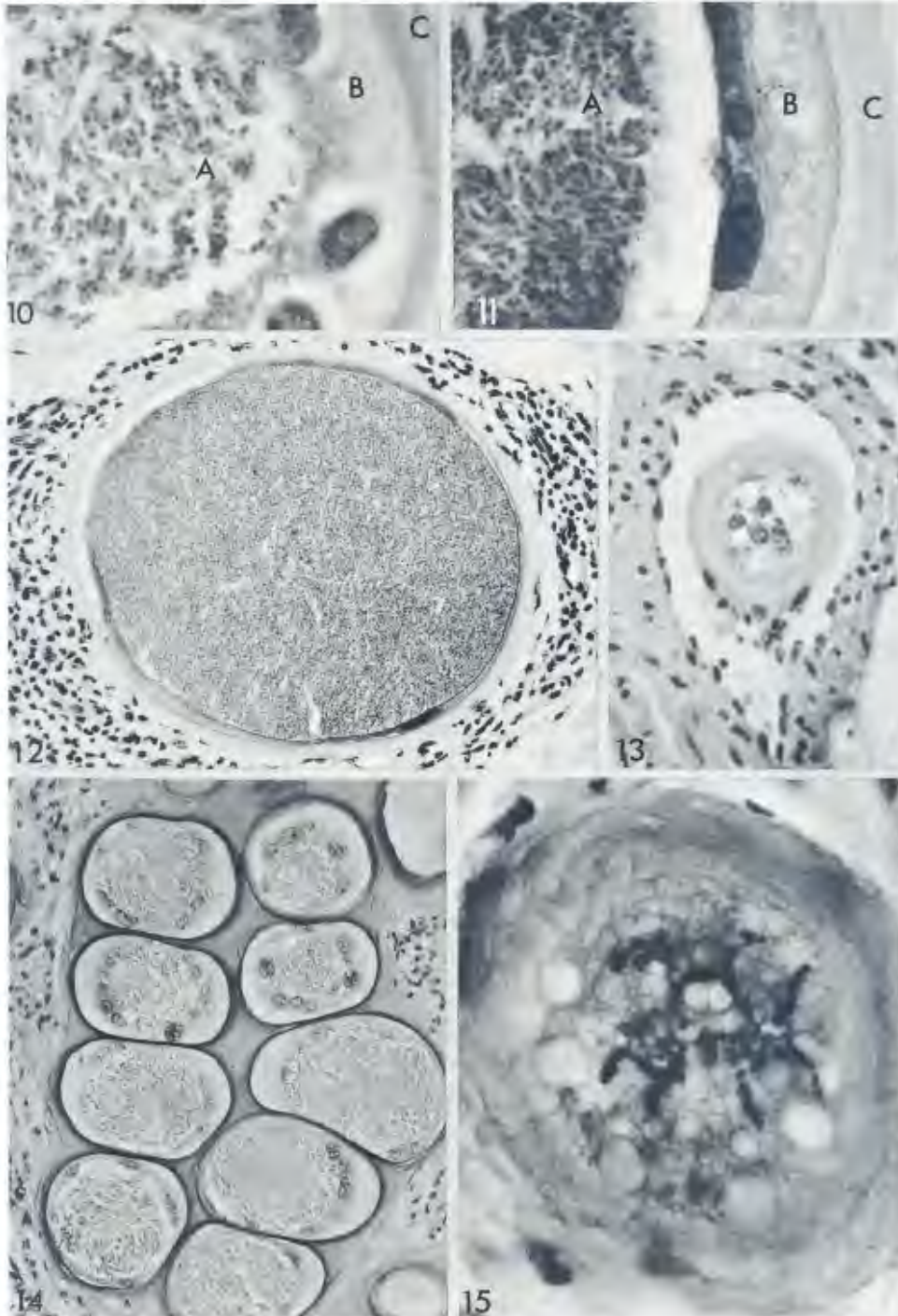


PLATE 2 Photomicrographs of HE stained sections of bovine skin depicting cyst formation. 10. Part of a developing cyst with a colony of organisms (A), which is enveloped in a distinct membrane, compressed peripheral cytoplasm and nuclei (B) and part of the developing cyst wall (C). Day 33. $\times 750$. 11. Part of another cyst in a more advanced state of development. The colony of cyst organisms (A), compressed cytoplasm with nuclei (B) and cyst wall (C) are easily distinguishable. A distinct line of demarcation can be seen between B and C. Day 38. $\times 750$. 12. A mature cyst surrounded by a mononuclear reaction. Cyst wall, compressed cytoplasm and colony of organisms can be differentiated. Day 385. $\times 200$. 13. A developing cyst within a valve of a vein. Day 27. $\times 335$. 14. A conglomerate of developing cysts in the skin with confluence of cyst walls and a surrounding mild host response. Day 43. $\times 150$. 15. Mitosis of host cell nuclei in a developing cyst. Day 28. $\times 750$.

TABLE 3 *Development of skin lesions in cattle*

Stage	Type of lesion	Onset (days after infection)
Acute: (Inc. period 1-5 days)	Perivasculitis, perivascular proliferation and vasculitis	3-4
	<i>Besnoitia</i> noticed	6
	Proliferation of <i>Besnoitia</i> in endothelium	7-10
	Oedema	7
	Thrombosis	7
	Hypertrophic histiocytes	8
	Very early cystic stage (15-25 μ)	11
	Multinucleated hypertrophic cells (cystic stage)	16
	Developing cysts (30-100 μ)	16-25
Termination of stage of oedema	25	
Subacute:	Young cysts (> 100 μ)	26
	Septate cysts	30
	Necrotic cysts	30-77
	Fairly mature cysts (300 μ)	70
Chronic:	Mature cysts (> 300 μ)	71
	Reddish cyst wall with HE	385

Inc. = incubation

The parasites were invariably found within vacuoles. Primitive mesenchymal cells of the intima were apparently also parasitized. Organisms were also found elsewhere in the walls of the vessels, within the endothelium of the vasa vasorum of larger vessels and in the fibrinoid thrombi, or extravascularly. A small number of hypertrophic cells, some containing organisms and representing very young cysts, were present in the intima, media, within nerves or adjacent to vessels. Vasculitis and perivasculitis of the type described above were prominent features. Mild but prominent per diapedetic cutaneous and subcutaneous haemorrhages were present. Oedematous changes were severe, particularly in Ox 2923. Focal disseminated areas of the epidermis and some of the superficial dermal papillae and large hair follicles were either degenerative or necrotic. In certain areas such as the ear some of the epithelial cells became enlarged and swollen. Superficial secondary bacterial infection occurred in some of the necrotic foci.

The same primary vascular lesions, constantly associated with *Besnoitia* organisms, and the accompanying secondary lesions such as haemorrhages, oedema, necrosis, pseudomembranes and erosions were found in the nasal mucosa, trachea and larynx. Parasitization of the vessels and extensive vascular lesions were present in the muscles [Plate 4 (30 to 33)] and in one nerve. Waxy changes, Zenker's necrosis with fragmentation, microcavitation, lysis and even mineralization were pronounced features in the muscles [Plate 4 (29)]. Thrombosis was also encountered in veins of some of the joint capsules, tendons and hypophyses. The iris of one animal contained a few small haemorrhagic foci. The changes in the liver consisted mainly of cloudy swelling, vacuolar changes and large hyaline droplet degeneration [Plate 3 (22)]. A few very small foci of necrosis were also seen. The sinusoids sometimes contained an increased number

of eosinophils and mononuclears. Only small numbers of suspected parasites were detectable [Plate 3 (23 and 24)]. The kidneys were only mildly affected with vacuolar degeneration and a few foci of very mild interstitial round cell reaction. In the lungs small numbers of proliferative organisms occurred in the alveolar walls and within the endothelium of the branches of the pulmonary artery. They were associated with a mild pneumonitis, congestion, very mild oedema and some emphysema. Very mild suspected cloudy swelling of the myocardium was present in one case only. A few tiny mononuclear cell aggregates were also noticeable. Some of the lymph nodes were hyperplastic, congested and haemorrhagic and contained a few small foci of fibrinoid necrosis in the sinuses. Activation and proliferation of the reticulo-endothelial cells were prominent and both hypertrophic and multinuclear giant cells were present in the sinuses and medullary cords of both cases [Plate 4 (27)]. Small numbers of organisms were noticed [Plate 4 (26)]. The intracellular organisms were usually single, seldom in groups. A haemal node of one animal revealed extensive necrosis and haemorrhages in the presence of suspected organisms [Plate 4 (28)].

Histopathology of subacute and chronic cases

The subacute phase of the disease follows the acute phase. For the purpose of this report it is limited to the period during which the cysts grow from about 100 μ to approximately 300 μ in diameter. The chronic phase is regarded as the stage after which the cysts have reached full maturity. Most of the histopathology of the subacute stage has been dealt with above (Days 26 to 71).

Sections from one of the subacute cases available in the pathology files at Onderstepoort revealed severe testicular lesions. Extensive coagulative necrosis of the seminiferous tubules with dystrophic calcification was present [Plate 5 (38)]. The adjacent tubules revealed

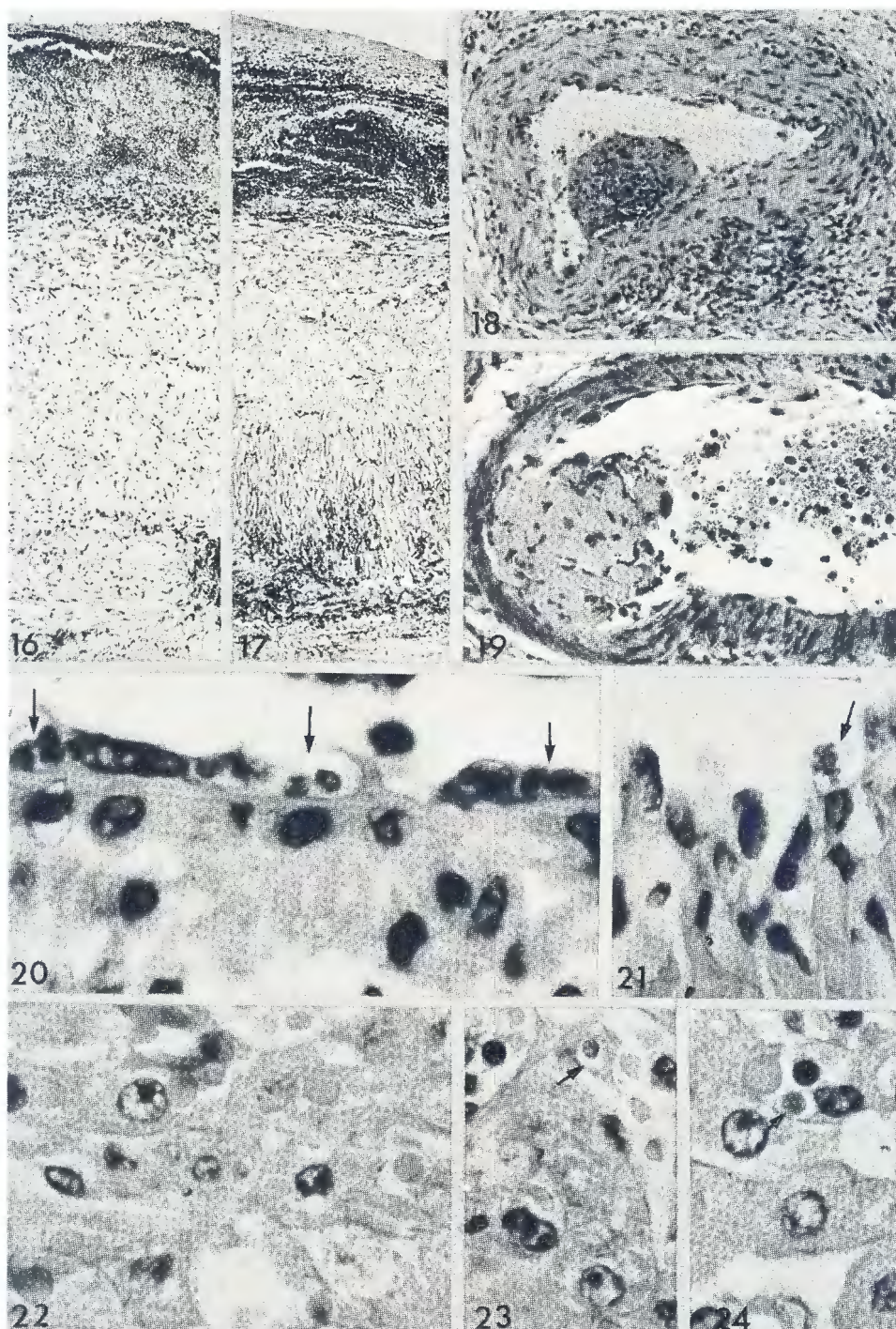


PLATE 3 Photomicrographs of HE stained tissue sections from acute cases of bovine besnoitiosis. 16 and 17. Facial veins of Ox 2923 showing an intimal reaction and mural thrombosis. Day 11. $\times 75$. 18. Muscular vein in the teat of Cow 781 showing thrombosis adjacent to a segment of the wall which is degenerated and inflamed. Day 8. $\times 75$. 19. A vein in the subcutis with partial fibrinoid necrosis and adjacent thrombosis. Ox 2923 at Day 11. $\times 200$. 20. *Besnoitia* organisms (arrows) in the endothelial cells of a vein in the teat of Cow 781. Day 8. $\times 1200$. 21. Cow 781 showing a colony of proliferative organisms (arrow) in an endothelial cell of a vein, Day 8. $\times 750$. 22. Liver of Cow 781 with hyaline droplet degeneration. Day 8. $\times 750$. 23 and 24. Liver of the same case with suspected organisms in Kupffer cells (arrows). Day 8. $\times 750$.

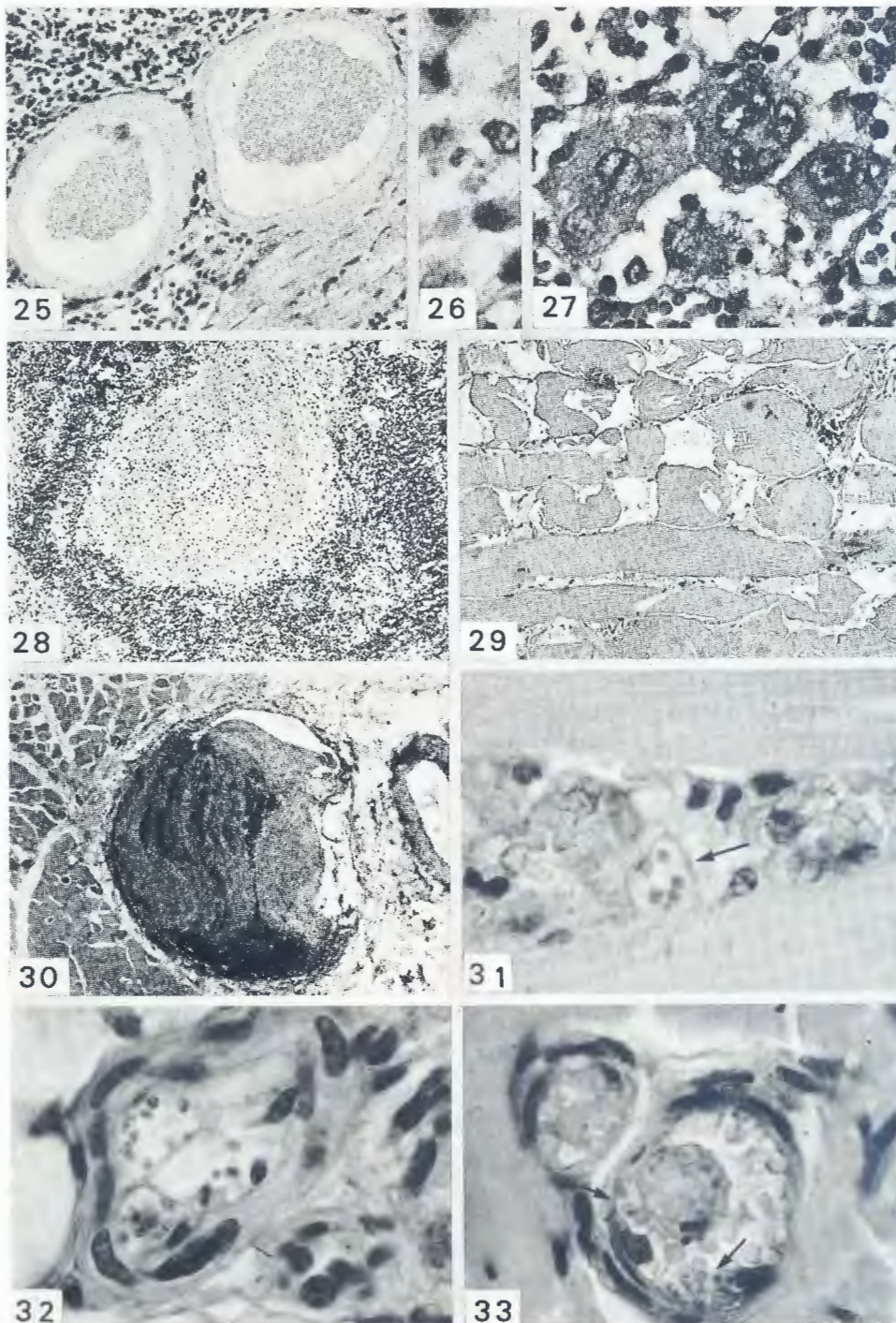


PLATE 4 Photomicrographs of HE stained tissue sections mainly from acute cases of bovine besnoitiosis. 25. Cysts within a lymph node of a natural case. $\times 200$. 26. Organisms (centre) within a lymph node of Cow 781. Day 8. $\times 750$. 27. Multinucleated giant cells in the medullary cords of a lymph node of the same animal. Day 8. $\times 500$. 28. Necrosis and haemorrhages in a haemal node of the same animal. Day 8. $\times 75$. 29. Muscular degeneration and necrosis in the same case. Day 8. $\times 75$. 30. Intermuscular thrombophlebitis in Ox 2923. Day 11. $\times 30$. 31. A distinct colony of proliferative organisms (arrow) in an affected intermuscular vein. Day 8. $\times 750$. 32. Several colonies of proliferative organisms in the endothelial cells of an intermuscular vein. Day 8. $\times 750$. 33. An intermuscular venule with a small thrombus and proliferative organisms in the endothelial cells (arrow) Day 8. $\times 750$.

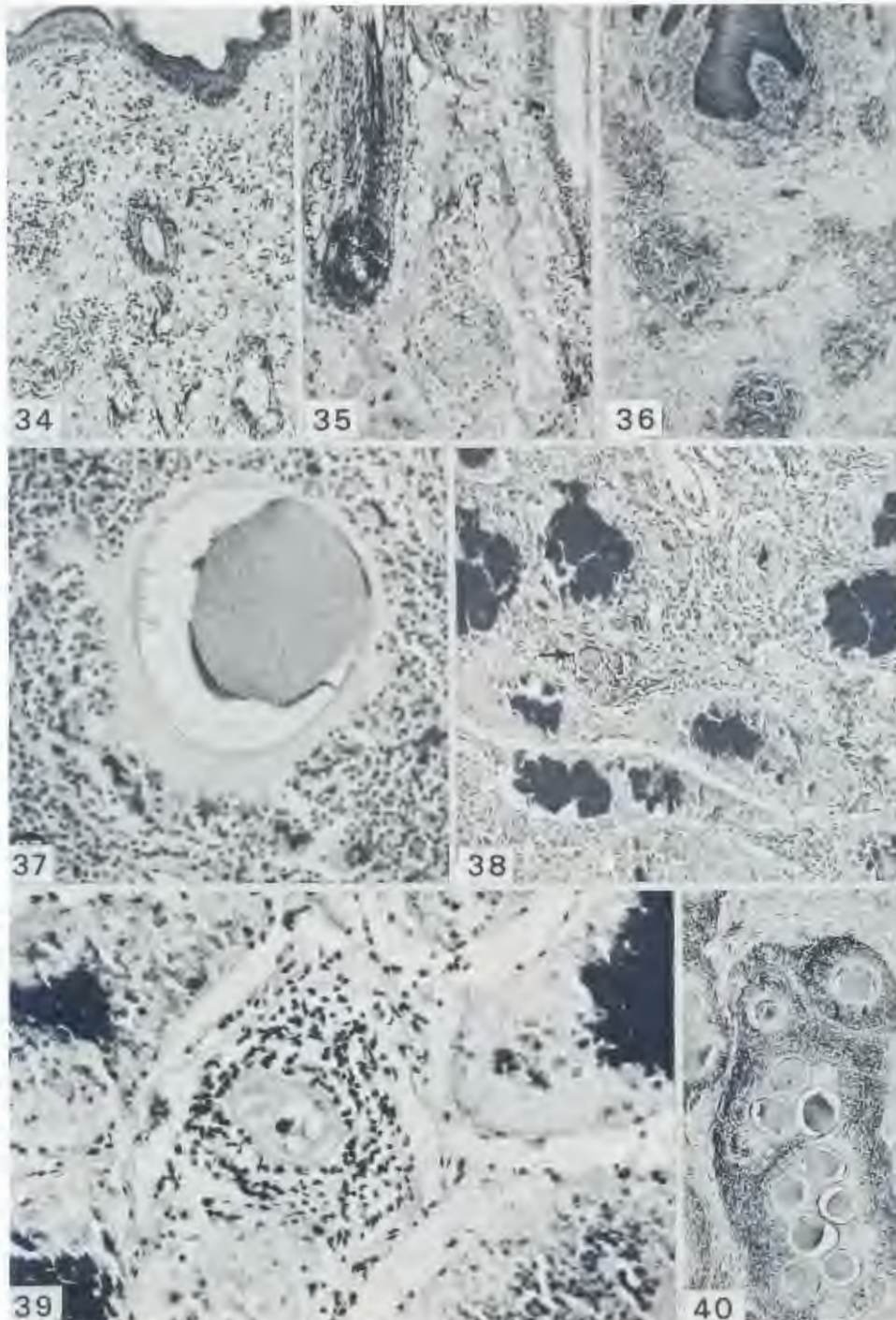


PLATE 5 Photomicrographs of HE stained tissue sections from cases of bovine besnoitiosis. 34. Mononuclear reaction in the skin. $\times 75$. 35. Thrombosis in the skin. $\times 75$. 36. Perivascular mononuclear reaction and small thrombi in the teat. $\times 30$. 37. Granulomatous reaction around a mature cyst resulting in an irregular removal of the cyst wall. $\times 150$. 38. Necrotic orchitis with mineralization in the testis of a natural case. Giant cells are also noticeable (arrow). $\times 75$. 39. Testis of the same case illustrated in 38. Two developing cysts surrounded by a mononuclear reaction are present in the centre between necrotic and mineralized seminiferous tubuli. $\times 200$. 40. A conglomerate of cysts in the skin surrounded by a marked mononuclear reaction. $\times 30$.

complete aspermatogenesis and the presence of small numbers of multinucleated spermatidic giant cells. A mild interstitial lymphocytic reaction was present in both the necrotic and adjacent areas, but was more pronounced around the small number of cysts which had a maximum diameter of 105 μ [Plate 5 (39)]. Vasculitis was also seen. Sections, of the lymph nodes of another case, which were studied contained a fair number of small cysts in the cortex [Plate 4 (25)], particularly in the subcapsular sinus and capsule but some were even present in the reaction centres. The few necrotic cysts were not accompanied by any granulomatous reaction.

The histopathology of chronic besnoitiosis has previously been reported in considerable detail (McCully *et al.*, 1966). It suffices to state here that these observations have all been confirmed and that most of these changes have again been dealt with (*vide supra* Days 71 to 385). Contrary to the previous statement that the reaction around cysts appeared to be most severe in the muscles, it has now become evident that the skin reactions can be just as marked.

Although it has been reported by Bigalke (1968) that the skin and subcutis of such areas as the tip of the tail, face, upper eyelid and breech are more heavily parasitized than those of the gluteal region, behind the scapula and neck, it has subsequently been established that the posterior part of the chin is one of the most severely affected sites. The host response to the cysts in the skin was also most severe in the area of the coronet, breech, chin, nostrils and shoulder.

Controls. No comparable lesions or parasites were encountered.

Histochemical findings

These results are outlined in Table 4. The cyst organisms contained PAS-positive material which seemed to decrease with maturation of the cyst and advancing age. The cyst wall consisted of an inner strongly PAS-positive and outer mildly PAS-positive zone, both of which stained blue with alcian blue and pale blue with Masson's trichrome stain. It was usually negative for collagen fibres with VG, except for a thin peripheral adjoining layer of collagen in the mature cysts. In some of these cysts the wall stained light pinkish red and could possibly be regarded as very mildly positive for collagen. The cytoplasm of the multinucleated host cell lying adjacent to the inner side of the cyst wall in young cysts was strongly PAS-positive.

TABLE 4 *Histochemical studies on Besnoitia cysts*

Stain	Cyst wall	Organisms
Haematoxylin-eosin	Purplish red, eosinophilic	Eosinophilic
Periodic acid-Schiff	+, \pm	+, -
Alcian blue	+	-
Feulgen-PAS-naphthol yellow	PAS+, \pm	PAS +, -
Gomoris' methenamine silver	-, -	+, -
Gridley's stain for fungi.	\pm , -	+, -
Masson's trichrome	Eosinophilic	Pale blue
Oil red O	-	-
Van Gieson's picrofuchsin	- \pm	-

+ = positive reaction
 \pm = mildly positive reaction
 - = negative

The first and second symbols respectively indicate reactions in immature and mature cysts

Rabbits

Macroscopic findings

Recipient of cyst organisms of bovine B. besnoiti

The most striking features were the skin lesions over the nose and of the supraorbital areas, base of the ears [Plate 6 (41)] and lower legs and scrotum, and the subcutaneous oedema of the ears, throat, limbs and axillary region. The skin was purplish-red in colour and hardened in some parts. Prominent fibrinous periorchitis, mild splenomegaly, mild pulmonary oedema, hyperplasia, oedema and haemorrhages of the lymph nodes draining the extremities and foci of hepatic degeneration were the other most significant lesions.

Recipients of proliferative organisms of rabbit-passaged bovine B. besnoiti

In general the lesions were similar to those of the previous group. The skin, lymph nodes, lungs and left ventricle were lightly sprinkled with petechial haemorrhages. Oedema of the subcutis was occasionally more advanced in one leg or ear and, in some of the cases, also present around the mouth, face and croup. Fibrinous or sero-fibrinous periorchitis was a constant feature and either haemorrhagic or necrotic orchitis were manifested in three out of four cases. Thrombosis was observed in the pampiniform plexus of one rabbit. Pulmonary oedema and some foci of necrosis or mild fatty metamorphosis of the liver occurred in 33 per cent of the cases. Muco-catarrhal rhinitis was seen in one animal.

Recipients of cyst organisms of B. besnoiti from blue wildebeest and impala

Pneumonia, evidently unrelated to besnoitiosis, was found in one case and suspected splenomegaly in the others. Typical lesions of besnoitiosis did not develop. The three rabbits which were challenged with the bovine strain did not reveal any of the typical skin or testicular lesions found in the previous groups and were evidently immune to this strain as reported by Bigalke *et al.* (1967).

Recipients of proliferative organisms of rabbit-passaged B. besnoiti from the blue wildebeest

The lesions in this group varied somewhat from those caused by the bovine strain. Cachexia was a prominent feature in 38 per cent of the animals. Furthermore, in spite of a transient clinical scrotal oedema in two rabbits, the typical scrotal and testicular lesions were absent at autopsy. The testes of some, however, appeared mildly

atrophic and flabby. The typical subcutaneous oedema found in other parts as well as the prominent skin lesions seen in the first two groups were never observed. Mild splenomegaly and lesions in the lymph nodes (*vide supra*) were noticed in several cases, but suspected foci of hepatic necrosis in only one animal. Pulmonary oedema occurred in 25 per cent of the rabbits and localized unilateral pneumonia associated with *Besnoitia* organisms in one case. Hydrothorax and serofibrinous peritonitis were seen in 25 per cent and mild hydropericardium in 12 per cent of the animals. Phlebitis of the veins of the neck and some of those from the limbs was apparent in 25 per cent of the cases. Other striking unusual features were the presence of acute or ulcerative gastritis and mild pseudomembranous rhinitis. Each of these lesions was found in one case only.

Microscopic findings

Bigalke *et al.* (1967) have tabulated and discussed the most significant findings which are again summarized in Table 2. Some of the very mild inflammatory foci encountered in the brain and kidneys of a few rabbits were suspected to be due to nosematosis. Mild coccidial lesions in the liver were observed in a small number of rabbits.

Recipient of cyst organisms of bovine B. besnoiti

Marked congestion, haemorrhages and oedema apparently preceded pronounced localized necrosis of the skin [Plate 6 (42)]. These primary lesions were accompanied by fibrinoid and kariorrhctic necrosis of the venules. A few vessels were partially thrombosed. The subsequent cell reaction consisted mainly of mononuclear cells, histiocytes and eosinophils and involved both veins and arteries. The hypertrophic and hyperplastic endothelial cells and other intimal cells frequently contained *Besnoitia* organisms, but these were also encountered in the tunica media, adventitia and extravascularly, either free or within histiocytes. Necrotic vasculitis and thrombosis of some of the scrotal and testicular vessels, especially the spermatic arteries and veins, with infarction of both *testes* and *scrotum* were prominent features. Severe necrotic changes, congestion, oedema and haemorrhage were noticeable within the infarcted areas. Many of the seminiferous tubules were necrotic. Aspermatogenesis was pronounced, Sertoli cells being the only remaining cellular elements in most of the tubuli. The tunica vaginalis was very heavily parasitized, partially necrotic and the visceral and parietal layers were connected by fibrinous material. The cell reaction consisted mainly of round cells, eosinophils and neutrophils with the former predominating in the interstitium of the testes. Interstitial *epididymitis* of the same nature with partial necrosis was also present. Organisms, either singly or in small groups, were always found in association with the lesions, mainly within or around the veins, but unequivocal evidence of their presence in the tubuli was absent.

The *lymph nodes* were mildly hyperplastic, oedematous and some contained small numbers of eosinophils and suspected *Besnoitia* organisms. An increased number of polymorphonuclear cells, especially eosinophils, was also present in the *spleen*. This organ was congested and contained numerous large macrophages with phagocytized polymorphonuclears, polymorphonuclear debris, structures resembling dead or disintegrating organisms and haemosiderin.

The *liver* was sprinkled with a few degenerative and necrotic midzonal foci. The Kupffer cells in these areas were activated and the sinusoids contained granulocytes.

A small artery in the *lung* had a thrombus and foci of pneumonitis and some foam cells were apparent. A few foci of round cell reaction were noticeable in the *endocardium*.

Recipients of proliferative organisms of rabbit-passaged bovine B. besnoiti

The lesions were very similar to those found in the first group, but the larger number of recipients in the second group supplied some additional information on the histopathology. More severe vascular degeneration, fibrinoid necrosis and thrombosis of the vessels in the *skin*, *spermatic cord*, *epididymis* and *testes* were observed. Both small and medium-sized veins and arteries were involved [Plate 7 (50 to 53)]. The spermatic vessels were usually most severely affected and thrombosis gave rise to infarcts [Plate 7 (50)]. The host response was similar to that of the first group [Plate 6 (47)]. Polymorphonuclears were prominent in the necrotic areas of the skin and scrotum. The entire wall of the scrotum was usually affected. Single organisms or proliferative intracellular groups of 6 to 8 were present in the skin, testes, pampiniform plexus, epididymis, spermatic cord, lymph nodes, lungs and muscles. The necrotic areas in the testes were well-demarcated, of a coagulative type and usually associated with venous thrombosis [Plate 7 (50 and 53)]. Prominent leukocytic reactions [Plate 6 (48) and 7 (54)] and haemorrhages were frequently seen in the necrotic mass [Plate 7 (55)] and some of the tubules were sometimes mineralized. Most of the cellular elements in the tubules as well as the Sertoli cells were destroyed in these areas. Degenerative changes, including a hyaline droplet type with partial or almost complete aspermatogenesis, were frequent findings. Spermatidic, phagocytic, multinuclear giant cells with 4 to 27 nuclei and mononuclear reactions were encountered fairly regularly in these areas [Plate 6 (48 and 49)]. The ductus deferens of one rabbit was surrounded by mononuclear cells and organisms. Two cysts were located in the scrotum of a case 39 days after infection [Plate 6 (46)]. Localized *muscular* degeneration, Zenker's necrosis and lysis, interstitial myositis with vascular lesions and histiocytic proliferation were prominent features in a few cases. The *myocardium* of one animal contained a few small foci of Zenker's necrosis and one small round cell focus. Reticulo-endothelial hyperplasia and numerous macrophages containing phagocytized debris (*vide supra*) were evident in the *spleen*. The *lymph nodes* were somewhat enlarged, sometimes mildly oedematous and those draining the extremities were populated to varying degrees with macrophages similar to those encountered in the spleen. Proliferative organisms were seen in small numbers in some of these nodes. Foci of mixed cell pneumonitis associated with parasites and mild oedema were again present in the *lungs* of some animals. The *liver* and *kidney* lesions were usually mild and the changes were by no means regular or uniform. Foci of very mild disseminated hepatitis and nephritis and foci of hepatic necrosis were occasionally found, but a mild granular type of nephrosis was present in the majority of cases. Single cases with mild mononuclear *rhinitis*, *adrenitis* of the adrenal medulla and focal disseminated *kerato-scleritis* were encountered. Two hypertrophic cells and suspected proliferative organisms were noticed in the adrenal medulla.

Recipients of cyst organisms of B. besnoiti from blue wildebeest and impala

The blue wildebeest strain provoked mild foci of *pneumonitis* and mild typical splenic lesions. The latter, however, were more prominent in those rabbits which

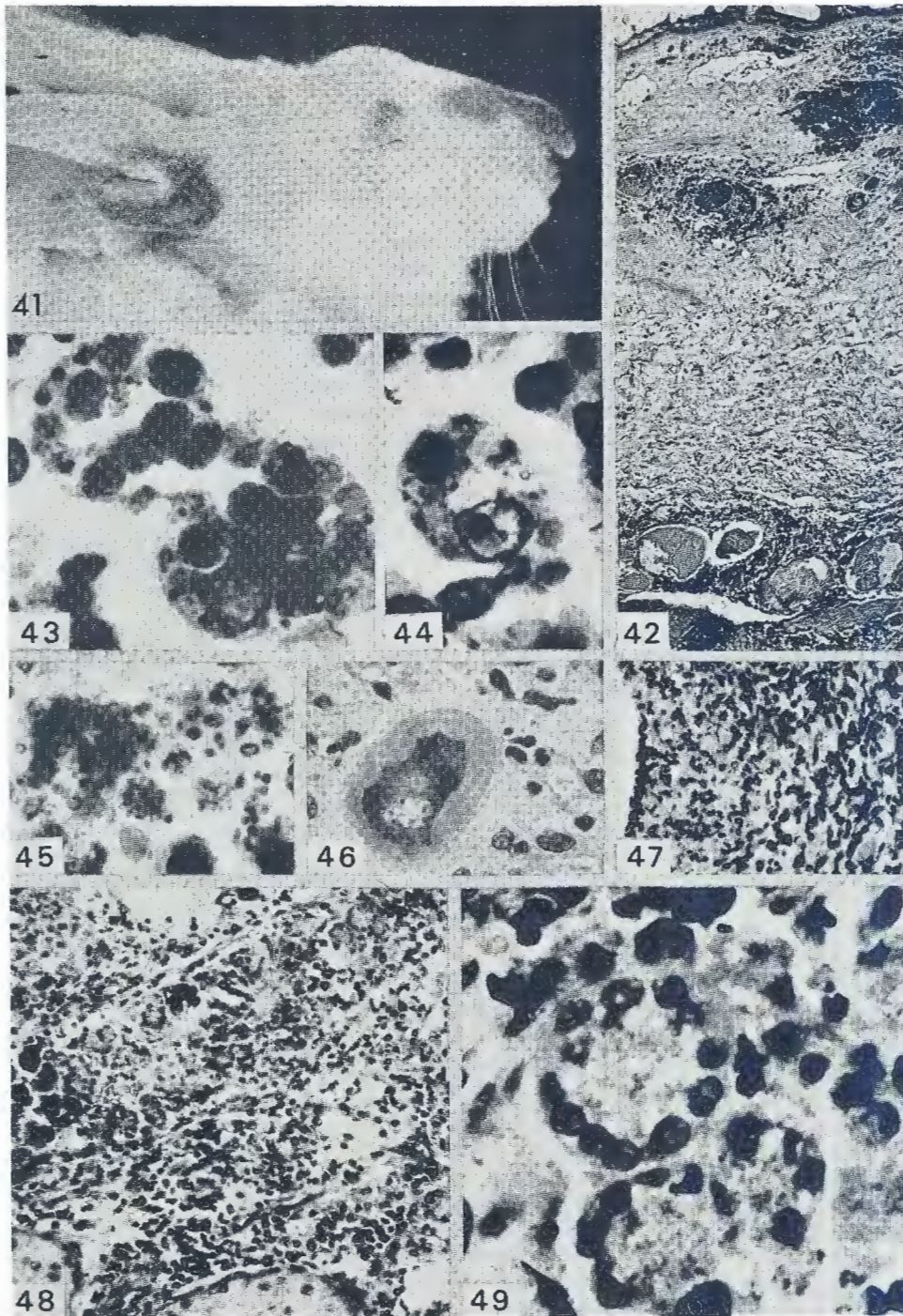


PLATE 6 Photograph and photomicrographs of HE stained rabbit tissues illustrating lesions of besnoitiosis. 41. R4333 given bovine cyst organisms: Haemorrhagic skin lesions on the ears, nose and supraorbital area (shorn for illustration). Day 20. 42. R 4333 showing severe cutaneous congestion, haemorrhages, oedema and thrombosis, and subcutaneous muscular necrosis. Day 20. $\times 75$. 43. R 4364 given antelope cyst organisms: Phagocytized necrotic eosinophils and *Besnoitia* debris in the spleen. $\times 1200$. 44. R4364, with suspected *Besnoitia* debris in a macrophage of the spleen. $\times 1200$. 45. R 4363 given antelope cyst organisms: *Besnoitia* debris and necrotic eosinophils in the spleen. $\times 1200$. 46. R 4429 given rabbit-passaged bovine organisms: A small developing cyst in the scrotum (rare finding). Day 39. $\times 500$. 47. R 4329 given rabbit-passaged bovine organisms: Necrosis and mononuclear reaction in the inner zone of the scrotum. Day 10. $\times 200$. 48. R 4325 given rabbit-passaged bovine organisms: Testis with marked interstitial mononuclear reaction, tubular necrosis, formation of spermatidic giant cells and aspermatogenesis. Day 14. $\times 200$. 49. Testis of R 4325 with spermatidic giant cells containing some calcific granules. Day 14. $\times 200$.

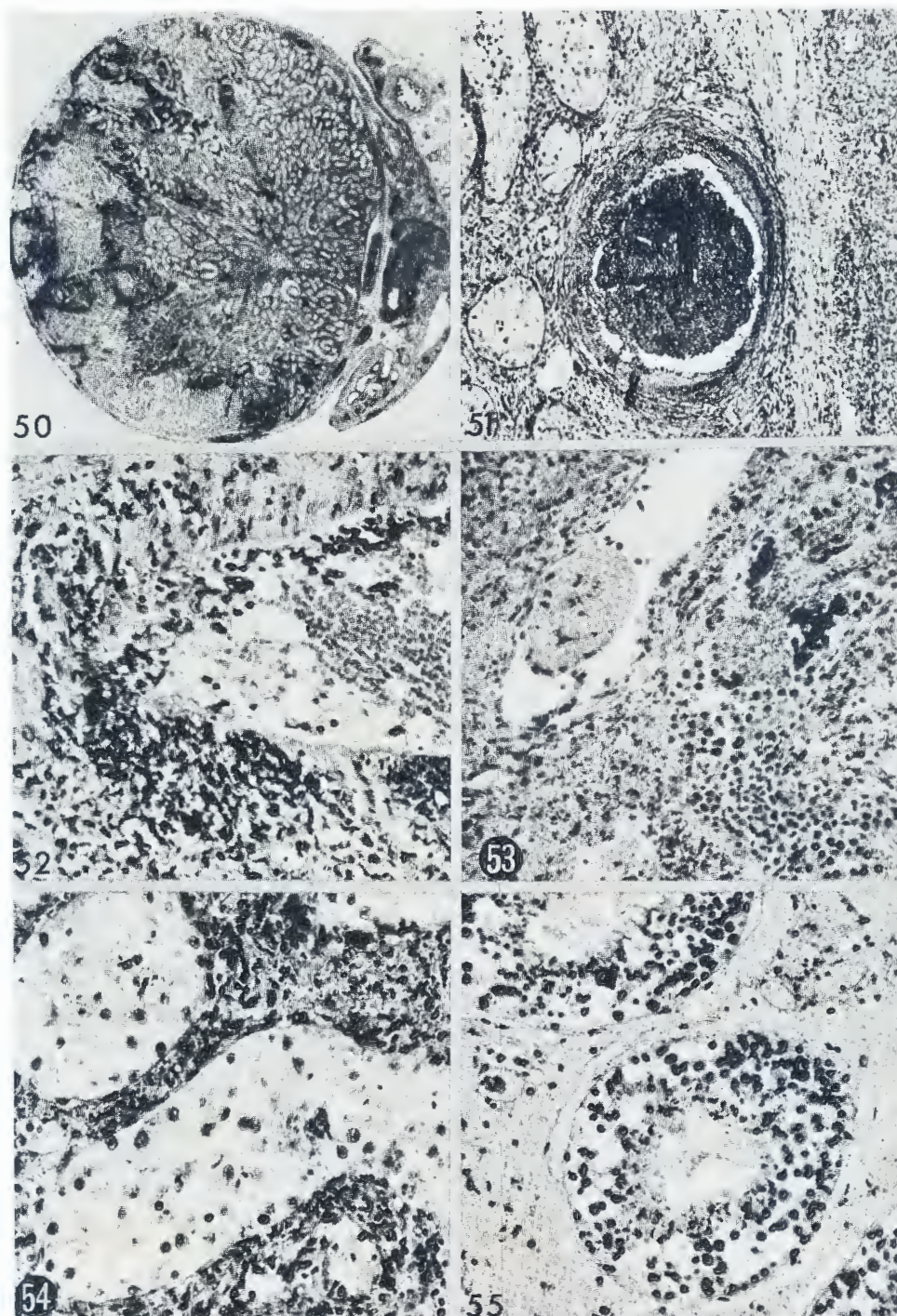


PLATE 7 Photomicrographs of HE stained sections from rabbits which received the rabbit-passaged bovine strain of *B. besnoiti*. 50. Cross section of the testis showing infarction and involvement of the adjacent epididymis and spermatic artery. R 4328 on Day 12. $\times 4$. 51. Aspermatogenesis and mononuclear infiltration in the interstitium and tunica albuginea. R 4325 on Day 14. $\times 75$. 52. Eosinophilic arteritis of the spermatic artery of R 4328. Day 12. $\times 200$. 53. An area in the testis of R 4325 showing marked necrotic orchitis, some dystrophic calcification and thrombo-phlebitis. Day 14. $\times 200$. 54. Interstitial reaction, necrosis and complete aspermatogenesis with only Sertoli cells remaining. Testis of R 4325. Day 14. $\times 200$. 55. Haemorrhage and necrosis in an infarcted area of the testis of R 4328. Day 12. $\times 200$.

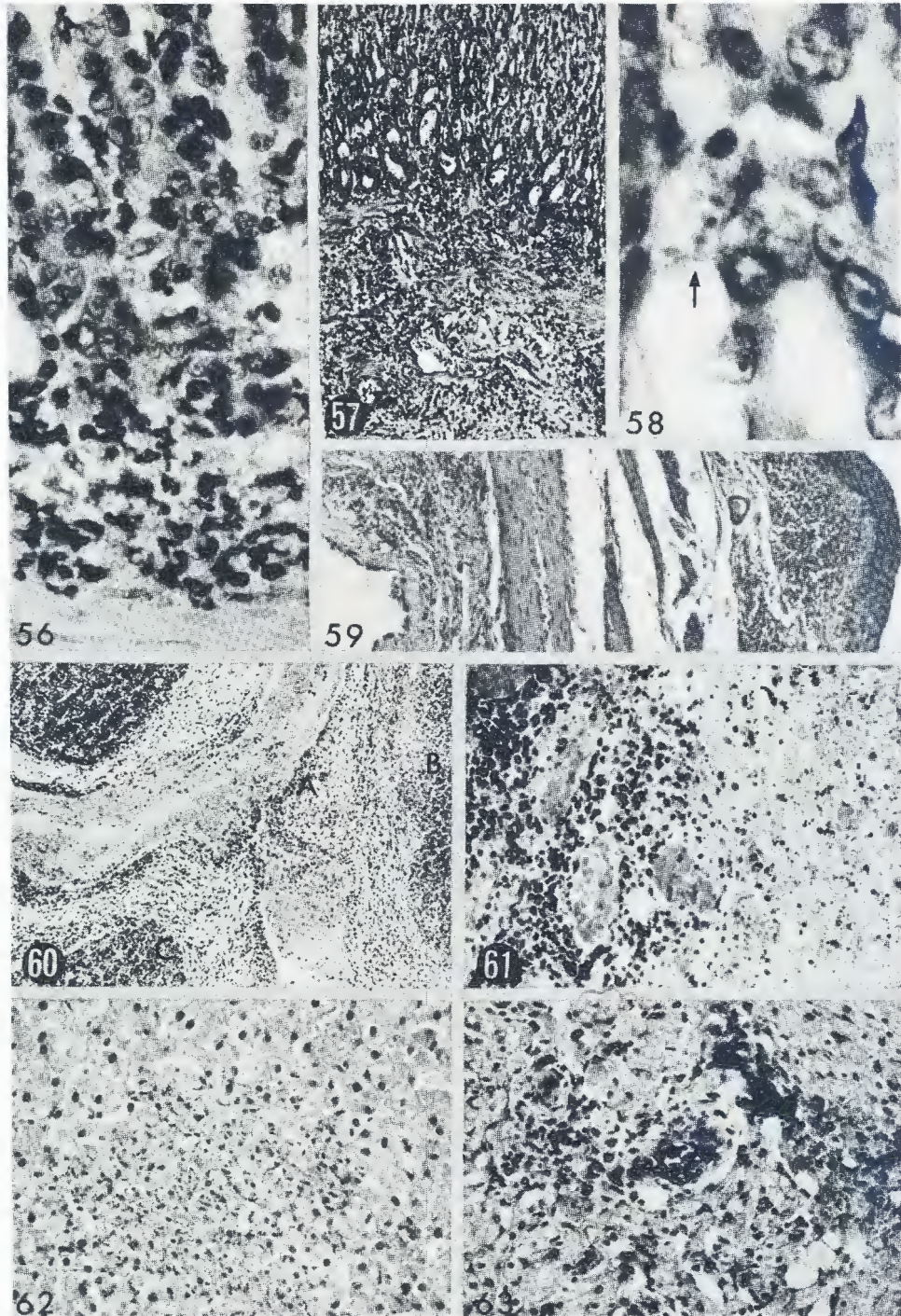


PLATE 8 Photomicrographs of HE stained sections from rabbits which received the rabbit-passaged antelope strain. 56. A portion of the stomach of R 4629. A polymorphonuclear infiltrate and organisms can be recognized. Day 26. $\times 500$. 57. Lower magnification of the stomach of the same rabbit showing the cell reaction in the submucosa and mucosa. Day 26. $\times 75$. 58. High magnification of the stomach showing a small colony of organisms (arrow). Day 26. $\times 1200$. 59. Urinary bladder of the same case. A mononuclear reaction is present in the propria and serosa. Day 26. $\times 75$. 60. Vasculitis, and mononuclear reaction in a trabecula (A) of a lymph node with necrosis of some of the adjacent lymph follicles (B & C). R 4721 on Day 16. $\times 75$. 61. Marked necrosis and congestion in a lymph node of R 4708. Day 22. $\times 200$. 62. Focal necrotic adrenalitis in the adrenal cortex of R 4708. Day 22. $\times 200$. 63. Mononuclear reaction and haemorrhage in the myocardium of R 4629. Day 26. $\times 200$.

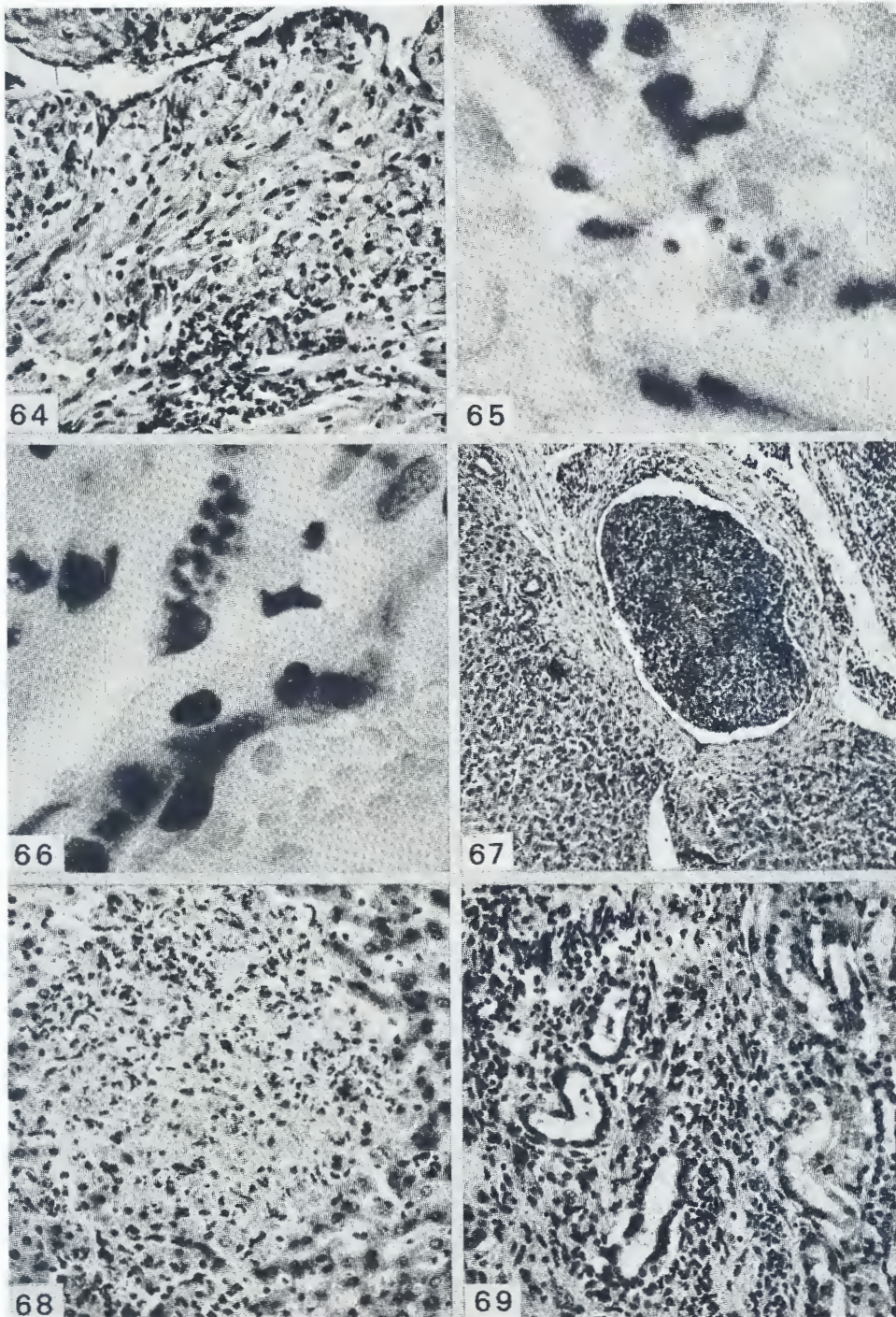


PLATE 9 Photomicrographs of HE stained sections from rabbits which received the rabbit-passaged antelope strain. 64. Mononuclear reaction, mild haemorrhage below the endocardium and within the myocardium of R 4629. Day 26. $\times 200$. 65. A group of *Besnoitia* organisms in the myocardium of R 4629. Day 26. $\times 200$. 66. A large colony of proliferative organisms in the wall of a vein in R 4721. Day 16. $\times 1200$. 67. Thrombosis in the liver of R 4421. Day 14. $\times 75$. 68. Focal necrotic hepatitis in R 4721. Day 16. $\times 200$. 69. Interstitial mononuclear reaction in a localized area of the renal cortex of R 4721. Day 16. $\times 200$.

were challenged with the bovine strain and contained many macrophages with phagocytized polymorphonuclear cells and suspected parasitic debris [Plate 6 (43, 44 and 45)]. No other lesions or organisms were detected. Of those rabbits which received the impala strain the one with the pneumonic lesions harboured no *Besnoitia* organisms. The lesions in the *spleen* were identical to those of the recipients of the blue wildebeest strain and more severe in the rabbits that were challenged. Mild foci of *interstitial orchitis* and *aspermato-genesis* in the presence of a small number of spermatidic giant cells were observed in one rabbit. Areas of Zenker's necrosis and mild foci of granulocytic endocarditis were present in the *myocardium*.

Recipients of proliferative organisms of rabbit-passaged B. besnoiti from the blue wildebeest

The primary lesions of vasculitis, vascular degeneration or necrosis involving either the intima or entire wall of the veins and some arteries in association with vascular parasitization [Plate 9 (66)] and thrombosis, described above were also present in this group. These lesions were, however, usually infrequent and mild or absent in the commonest localities such as the skin and testes. Moreover, the parasite evidently often invaded unusual sites and internal organs such as the stomach, myocardium and kidneys.

The lesions in the *spleen* were again similar to those of the first group, but usually not equally severe, some being very mild. The changes in the *lymph nodes* included prominent lymphadenitis, necrosis and vasculitis [Plate 8 (60 and 61)]. The cell reaction appeared to be mainly of mononuclear type but eosinophils were sometimes also plentiful.

Organisms were very numerous in the necrotic areas and both congestion and oedema were very marked in some cases. Perilymphadenitis was evident in a few nodes. The *skin* was only very mildly affected in two cases in localized small areas such as the ventral side of the abdomen where mild purulent dermatitis in the presence or absence of necrosis, oedema and haemorrhages were found, apparently at the inoculation site. R4935, which received the highest passage level of the organisms, had areas of more marked dermatitis which contained a few hypertrophic histiocytes. Mild interstitial *orchitis* was observed in 43 per cent of the cases, but partial or complete aspermato-genesis following degeneration of the tubuli was more frequent. Spermatidic giant cells appeared in only one case and a granulomatous *periorchitis* was present in another rabbit. Evidence of infarction was never obtained. *Epididymitis* or *periepididymitis* and inflammation of the spermatic cord were either very mild or mild, inconstant and present in 57 per cent of the males. Thrombosis was encountered in one vessel of the *pampiniform plexus*. The *myocardium* was affected in 75 per cent of the cases and although usually mild and localized, various types of changes were noticeable in one or more animals. These included epicarditis, focal disseminated myocarditis [Plate 8 (63) and Plate 9 (64)], proliferative endocarditis, valvular endocarditis of the atrio-ventricular valves, fibrinoid necrosis and thrombosis of some of the small vessels, small haemorrhages, cloudy swelling and foci of Zenker's necrosis. These lesions were usually associated with the primary type of vascular lesions and the presence of either proliferative organisms [Plate 9 (65)] or, exceptionally, cysts (R4935). Mononuclear cells were mainly involved in the host response. Mild fibrosis was seen in two rabbits. Two cases of mild localized interstitial or necrotic *myositis* were found. Round cell vasculitis and the

presence of *Besnoitia* organisms were characteristic features. Zenker's necrosis, myolysis and both mild endothelial and sarcolemmal proliferation were present in these cases.

Apart from some pulmonary oedema and areas of eosinophilic *pneumonitis*, one case of localized eosinophilic *bronchopneumonia* in association with organisms was encountered. Mild endarteritis in a branch of the pulmonary artery, was seen once. Foamy macrophages occurred occasionally in the alveolar septa. The *liver* lesions, particularly the coagulative necrotic foci [Plate 9 (68)] were generally more severe and advanced than in the previous groups. The necrotic foci usually had a midzonal distribution and were present in 37 per cent of the cases. Small numbers of eosinophils often infiltrated these lesions. Mild round cell reactions and some vasculitis were present in the portal areas of most of the rabbits. Thrombosis of only one portal vein was noticed [Plate 9 (67)]. The blood vessels and sinusoids contained increased numbers of monocytes. The Kupffer cells were often mildly activated and probably proliferative. Single cases of mild localized foci of round cell *pyelitis* and *nephritis* in association with *Besnoitia* were encountered [Plate 9 (69)]. Organisms within areas of vasculitis, round cell reactions and some eosinophils were found in the *duodenum* of one animal and in the *stomachs* of two cases [Plate 8 (56, 57 and 58)]. Both the mucosa and serosa were affected. Necrosis of all the mucosal elements and haemorrhages containing many eosinophils and organisms were apparent in the gastric fundus of one animal. The proliferative organisms were either in groups of 7 to 8 or single and sometimes lined up in a row as if in capillaries and lymphatics. Endothelial cells were evidently parasitized, but the possible parasitization of gastric epithelium could not be verified. Similar lesions were encountered in one *urinary bladder* [Plate 8 (59)] and one *gall bladder*. As in both stomach and duodenum, evidence was obtained that the serosa of these organs was prominently involved in the reaction. The *adrenals* of two rabbits revealed foci of disseminated round cell reactions and necrosis in both cortex and medulla [Plate 8 (62)]. The necrotic foci in one case contained many eosinophils and surrounding vacuolar degeneration as well as generalized cortical hypertrophy were noticed. Organisms were present in fair numbers and a few enlarged and binucleated histiocytic cells were observed. One case revealed foci of necrotic *tracheitis* accompanied by vasculitis and proliferative organisms. In one animal the examination of the *bone marrow* proved to be positive for organisms. Mild *rhinitis* in association with polymorphonuclear and hypertrophic cells was seen in R4935.

DISCUSSION

The regular parasitization mainly of small and medium blood vessels by proliferative organisms in the acute cases of bovine besnoitiosis which were produced in the present investigation, as well as the abundance of *Besnoitia* cysts in these vessels of various antelopes and in cases of chronic bovine besnoitiosis (McCully *et al.*, 1966), indicate that vascular lesions are probably of primary significance in the pathogenesis of besnoitiosis. In the acute stages vasculitis, fibrinoid vascular necrosis and thrombosis evidently caused congestion, haemorrhages, oedema, degeneration, necrosis and infarcts, particularly in the skin and testes. The acute skin lesions in rabbits, however, were frequently predominantly haemorrhagic and necrotic in nature and did not necessarily always represent infarcts. Hypertrophy and hyperplasia of various perivascular and vascular cells such

as the endothelium were also present in the acute stages of the disease. Vasculitis and perivasculitis were constant findings in all the affected tissues. Initially, up to about 10 to 12 days after infection, the organisms frequently seemed to parasitize and proliferate in certain specific cells of the intima, such as the endothelial cells, as well as in the tunica media and adventitia. The identity of the other parasitized vascular and extravascular cells, however, could not be established with any certainty. During this early stage most of the adverse effects such as the acute vascular and subsequent secondary lesions were either initiated or fully manifested. The effect of the parasite on these tissues did not seem to be solely traumatic in nature. The severity of the primary vascular lesions indicates that the effect is probably mainly due to a toxin which increases the vascular permeability and has a degenerative and necrotizing effect, particularly on the vessels.

The host reaction was mainly confined to the walls of the blood vessels and the adjacent tissues where proliferation of histiocytes and a mononuclear reaction were most prominent. Small and variable, but nevertheless significant, numbers of eosinophils and some neutrophils were usually present, especially during the initial stage, and their numbers increased within or near necrotic areas and around dead cysts. This increase was also reflected in the spleen of the rabbit, where fairly large numbers of phagocytized necrotic polymorphonuclears and *Besnoitia* organisms were particularly striking features. A slight increase in number of most of these cells in the skin of acute cases was suspected.

The process of cyst formation of *B. besnoiti* has been dealt with by Pols (1954 a, b; 1960) and Schulz (1960), but a detailed day to day account of the progressive development has not been given. The recently improved methods of artificial infection of cattle (Bigalke, 1968) made it possible to produce a fair number of typical clinical cases which provided suitable specimens for histopathological studies. From these specimens it became evident that cyst formation follows the proliferative stage and that the hypertrophic host cells which are subsequently parasitized are not endothelial cells but probably histiocytes, as suggested by Pols (1954 b, 1960). These cells are usually found in the intima, or anywhere within the vessel walls or in a juxta-vascular position. The first evidence of parasitized hypertrophic cells was obtained 11 days after infection. These cells became progressively enlarged and multinucleated. The peripheral layer of cytoplasm became basophilic with HE stain and also strongly PAS-positive. These changes evidently indicated an increased production of muco-polysaccharides in a very active and reactive host cell. An outer hyaline layer or wall, which has a strongly PAS-positive inner and mildly PAS-positive outer zone, is apparently produced by the parasitized cell. This PAS-positive reaction decreased with advancing age and a mild or very mild positive reaction for collagen was obtained with VG in some of the mature cysts, especially on the periphery of the wall. Unequivocal evidence of an extra-cellular fibrotic process contributing largely towards or solely forming the cyst wall, as suggested by Pols (1960) and Schulz (1960), except in cysts surrounded by granulomatous reactions, was not obtained. In our opinion the parasitized host cell is responsible for the secretion and formation of the wall and some collagen fibres are probably incorporated in the process. The host cell is evidently physiologically hyper-activated and even functionally altered from its normal type of reaction by the biological interaction

between host cell and parasite. This type of interaction has been reported in coccidia (Pellérdy, 1965). It is admitted, however, that the entire enigma of the specific identity of the host cell and the production of the cyst wall will have to await electron microscopical studies.

The organism within the host cell is apparently contained in a vacuole. It multiplies and the enlarging colony of organisms gradually compresses the multiple host cell nuclei and cytoplasm towards the periphery of the developing cyst and ultimately against the cyst wall that has been produced. A distinct membrane enveloping the colony of organisms has been noticed. This could be derived from the invaginated host cell plasma membrane, which would signify that the host cell has phagocytic properties and could be classified as a type of histiocytic cell. As stated by Pols (1960), septate cysts apparently develop within host cells which have either phagocytized or were parasitized by more than one organism, two or more colonies in each cell being the ultimate result. Cysts with compressed peripheral nuclei, large almost spherical colonies of organisms and sizes varying from 300 to 390 μ were regarded as mature. This stage was reached 71 days after infection when some of the cysts had attained a diameter of 330 μ . There seemed to be very little morphological difference between these cysts and older and somewhat larger ones that were encountered in other animals up to Day 385. The data on cyst development in Fig. 1 and Table 3 could therefore be used for age determination of cysts, differentiation between acute, subacute and chronic stages of the disease and estimation of the approximate age of an infection of natural bovine cases. Bigalke (1968), however, has presented evidence which indicates that the whole complement of cysts is probably formed within a relatively short period of time closely associated with maximum proliferation, i.e. in the acute stage of the disease, rather than with the time of infection. It is therefore clear that the length of the incubation period may exert an appreciable influence on any calculation of the time of infection. Bigalke (1968) found that the incubation period varied from 1 to 13 days, depending on the method of infection. In cattle infected by methods which could occur in nature the average was 13 days. The animals used in this study, however, had an average incubation period (taken from the time of infection to the first rise in temperature) of only 2.7 days. If it is assumed that the average is 13 days in all cattle infected naturally it would mean that approximately 10 days should be added to any calculation of the day of infection based on the data given here. Other unstudied factors which might influence the growth-rate of cysts are parasitic strain or bovine host differences, the number of developing cysts in an animal, and its nutritional state. It also stands to reason that the smaller the number of cysts available for measurement the less accurate calculations of the age of an infection will be. The reliability of evaluations based on the graph in Fig. 2 therefore still needs to be determined in practice.

The development of the cyst has been studied in the closely related parasite *Besnoitia jellisoni* Frenkel, 1953 by Frenkel (1953, 1965). From his description it is evident that the development of the *B. besnoiti* cyst matches that of *B. jellisoni* in most respects. The only important discrepancy concerns the cyst wall nuclei, which Frenkel believes to be of parasite rather than of host origin. Our conclusion that these nuclei are those of the parasitized, hypertrophic host cell is, however, in agreement with that of Pols (1960) who also studied the development of the *B. besnoiti* cyst.

Limited cystic disintegration was first noticed 30 and 42 days after infection respectively in two animals. Contrary to the observations by Schulz (1960) that only disintegrating cysts provoked a host response, it was evident that both mononuclear and granulomatous reactions were present around many cysts in which no degenerative or necrotic changes could be detected in the colonies of organisms by light microscopy. This was a constant finding in all the chronic cases and is in accordance with the observations of Besnoit & Robin (1914), who suggested that the leukocytic reaction could bring about premature death of the organisms. This could be preceded by necrotic changes in the cyst wall or host cell. The presence or absence of degenerative changes in either cyst wall, host cell or organisms in such cysts, however, should be verified by electron microscopy. On the other hand, the presence of live cysts and even disintegrating ones in the absence of an accompanying host response was not uncommon.

The typical clinical manifestation of scleroderma in chronic cases can be explained mainly by the development and growth of large numbers of cysts in the skin, especially in the dermal papillae and other parts of the stratum papillare, and by the granulomatous reaction and accompanying fibrosis around these cysts, hyperkeratosis and acanthosis. Infarction and the severe reaction in the stratum papillare would interfere with circulation and account for the formation of sitfests and alopecia.

The general pattern of the disease seen in rabbits inoculated with the rabbit-passaged bovine strain differed from the disease in cattle infected with recently isolated strains on four main points. Proliferative organisms were more frequently encountered in tissues other than the endothelium, arteries were frequently severely affected, cysts developed rarely and active macrophages with abundant parasitic debris and eosinophils and some neutrophils were commonly present in the spleen. A few cysts were encountered in one rabbit only. Chronic besnoitiosis, characterized by the presence of numerous cysts, can evidently be produced in the rabbit by using either proliferative organisms (Pols, 1960) and cyst organisms (Bigalke, 1967, 1968) from natural bovine cases, or by employing recently isolated strains (Pols, 1954 b, 1960), but not with the high passage-level rabbit-passaged strain used in these experiments. There appears to be a negative correlation between the passage level and the number of cysts formed and Bigalke (1968) has suggested that the strain concerned is losing its cyst-producing ability, much as *B. jellisoni* is known to do after about 30 acute passages in mice (Frenkel, 1965).

The severe testicular lesions in both cattle and rabbits indicate that the effect is not always transient or aspermatogenic, but that the large necrotic foci could affect the fertility of these animals permanently. Both Pols (1960) and Schulz (1960) have presented evidence to this effect.

The bovine strain of *B. besnoiti* was more pathogenic to rabbits than the antelope strains. However, after passage, the pathogenicity of the latter increased considerably. Furthermore, the distribution of the lesions differed markedly from that of the bovine strain as reported previously (Bigalke *et al.*, 1967). Skin lesions were either absent or very rare, invariably very mild and apparently confined to the inoculation site. Those in the male genital organs, if present, were also mild and included mainly aspermatogenesis. Infarction was never observed in the testes. The rabbit-passaged blue wildebeest strain had a strong tendency to parasitize internal

organs and tissues more frequently than the bovine strain. Localized areas of mononuclear gastritis, enteritis, myocarditis, pneumonia, hepatitis, nephritis, cystitis and adrenalitis, usually in association with necrotic changes, were noticed in some of the rabbits that were infected with the blue wildebeest strain. The size, distribution and severity of the lesions varied considerably. The changes in the lymph nodes were also more severe than those in rabbits which received the bovine strain, and cysts developed only in the myocardium of R4935 which received the highest passage level. The severe reaction in the spleens of Rabbits 4363, 4364 and 4366 was evidently caused by the challenge with the rabbit-passaged bovine strain which was administered two weeks prior to death. The absence of typical skin and testicular lesions, however, evidently indicated that the antelope strain had produced an immunity against the bovine strain (Bigalke *et al.*, 1967).

The absence of cysts and typical lesions of besnoitiosis in bovines that received the blue wildebeest strain, has been reported previously (Bigalke *et al.*, 1967).

SUMMARY

Nine cattle and nine rabbits that were artificially infected with either proliferative organisms of low or high *in vitro* and/or rabbit-passaged level bovine strains of *B. besnoiti*, or cyst organisms from a natural bovine case were used for histopathological studies. Fourteen rabbits similarly infected with either proliferative organisms of a low level rabbit-passaged blue wildebeest strain or cyst organisms from naturally infected antelopes were likewise used for comparative studies. The most significant findings were the following:

1. Degenerative and necrotic vascular lesions, vasculitis and thrombosis, mainly of the medium and smaller veins and some arteries, were the most outstanding changes in the acute stages of the disease. These changes coincided with the parasitization of certain cells in the vessels such as the endothelium, where the organisms proliferate before the onset of the cystic stage. These basic lesions were responsible for oedema, dystrophic changes and even infarction, particularly in the testes and skin. A histiocytic reaction and mild eosinophil infiltration were some of the other characteristic features. Differences between the disease in cattle and rabbits are reported.

2. The cystic stage was initiated by the parasitization of non-epithelial cells which appeared to be histiocytes. These enlarging parasitized cells were first noticeable 11 days after infection, whereas non-parasitized hypertrophic histiocytes were already present after 8 days. They became multinuclear and seemed to be responsible for the production of an outer bluish PAS-positive wall enveloping the host cell and colony of organisms. Cysts reached maturity about 71 days after infection and a graph is included to illustrate the rate of cyst growth. Reactions to the cysts apparently commenced before any degenerative changes in the organisms were detectable by light microscopy.

3. The antelope strains of *B. besnoiti* were only very mildly pathogenic to rabbits but passage during the acute stage of the disease in this host increased the pathogenicity considerably. A striking difference between the rabbits which were injected with bovine and antelope strains was the absence, or relative mildness, of testicular and skin lesions in the latter as compared to their severity in the former. In the case of the antelope strains, lesions were usually confined to internal tissues and organs such as the myocardium, gut and lungs and

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more rarely even in the adrenal, liver, kidneys and urinary bladder. Cysts rarely developed in rabbits which received the rabbit-passaged bovine strain, and were found only in one animal which was infected with a high passage level of the antelope strain.

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