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STUDIES ON THE RELATIONSHIP BETWEEN *BESNOITIA* OF BLUE WILDEBEEST AND IMPALA, AND *BESNOITIA BESNOITI* OF CATTLE

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Until fairly recently there was little incentive to speculate on the evolutionary history of *Besnoitia besnoiti* (Marotel, 1912) because the parasite had not been encountered occurring naturally in animals other than cattle. The recent discovery of *Besnoitia* cysts occurring mainly in the cardiovascular system of blue wildebeest [*Connochaetes taurinus* (Burchell, 1823)], impala [*Aepyceros melampus* (Lichtenstein, 1812)], and a kudu [*Tragelaphus strepsiceros* (Pallas, 1766)] in the Kruger National Park, provided vistas of new concepts on *inter alia* the evolution, epizootiology and pathogenesis of the disease (Basson, Van Niekerk, McCully & Bigalke, 1965; McCully, Basson, Van Niekerk & Bigalke, 1966). In these antelopes the majority of cysts were found in the peripheral veins of the limbs and neck, in which they were attached somewhat loosely to the intima.

Impala and kudu, and to a considerably lesser extent, blue wildebeest, are quite numerous in the districts of Transvaal where bovine besnoitiosis is enzootic. As these antelopes are fairly closely related to cattle taxonomically, a systematic relationship might well exist between their parasites. It is evident that these antelopes could be of considerable significance in the epizootiology of the disease should the *Besnoitia* they harbour prove to be identical to *B. besnoiti* in all respects. This investigation on the identity of the *Besnoitia* harboured by these antelopes and their relationship to *B. besnoiti* of cattle was therefore undertaken.

In the vicinity of Skukuza in the Kruger National Park the incidence of besnoitiosis is high in impala and very high in blue wildebeest, but only one infected kudu has been found to date (McCully *et al.*, 1966). For this reason only blue wildebeest and impala were used to provide the *Besnoitia* cysts required for this investigation. Rabbits are very susceptible to infection with proliferative (Pols, 1954, 1960) and cyst forms (Bigalke, 1960, 1965) of *B. besnoiti* of cattle. They develop skin lesions which are very similar to those seen in cattle and hence serve as good experimental models for studies on bovine *B. besnoiti* infections. Both rabbits and cattle were therefore inoculated with organisms from crushed cysts. Cattle and sheep were subsequently infected with strains isolated in rabbits. The reactions were compared with those observed in similar experiments with *B. besnoiti* (Bigalke, 1965). Extensive cross-immunity tests were done in these hosts between the antelope and bovine strains. Because the parasites from antelopes and cattle were so similar in their morphology, comparative studies were heavily biased towards the biological side.

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In this article experimental evidence is presented which indicates that the organisms found in blue wildebeest, impala and cattle represent distinct strains or biological races of *B. besnoiti*. A preliminary report on this work has been submitted for publication (Bigalke, Van Niekerk, McCully & Basson, 1966).

Materials and Methods

In this report *Besnoitia* cysts are referred to as cysts; organisms expressed from cysts are called cyst organisms, whereas those lying free in tissues or intracellularly are designated as proliferative organisms.

Collection of cysts and preparation of suspensions of cyst organisms

Blue wildebeest and impala shot in the vicinity of Skukuza for various purposes (McCully *et al.*, 1966) were immediately examined for cysts in the jugular veins; blood smears were then made, citrated blood collected from some of the positive cases and the carcasses transported to the Veterinary Investigation Centre at Skukuza. On arrival they were skinned, fully necropsied and cyst-bearing veins dissected from surrounding tissues and placed in Hanks' solution containing lactalbumin hydrolysate and 2,000 units penicillin, 2,000 micrograms streptomycin, 1,500 micrograms neomycin and 40 micrograms fungizone per ml, henceforth referred to as " cleaning fluid " in this report. Cysts were excised from the opened veins by means of a curved scissors and transferred to a tube surrounded by crushed ice and containing " cleaning fluid ". As soon as a sufficient number of cysts had been collected from a particular host-species they were crushed in a mortar, after removal of " cleaning fluid ", and the freed organisms suspended in Hanks' solution containing 0·5 per cent lactalbumin hydrolysate and 200 units penicillin, 200 micrograms streptomycin and 2 micrograms fungizone per ml. The approximate number of cyst forms was determined with the aid of a haemocytometer, simultaneously checking for motility, and the appropriate dose inoculated into rabbits or cattle. Other cysts were kept at 4° C overnight for *in vitro* cultivation purposes (*vide infra*—Cell culture studies).

Injection of rabbits

The majority of the rabbits used were young males bred at the Veterinary Research Institute, Onderstepoort. They were housed in wire cages, fed on concentrates and water and their temperatures recorded daily.

Of the initial group of eight rabbits, three were injected with cyst organisms from blue wildebeest and three from impala by the subcutaneous route as indicated in Table 1; two were injected subcutaneously with a very large dose of organisms (175×10^6) from blue wildebeest to study their toxicity. Blood smears were prepared daily and examined for signs of infection.

Citrated blood collected from the ear veins of reacting rabbits was sent, suitably cooled, by rail to the Veterinary Research Institute, Onderstepoort. This was injected into further rabbits by the subcutaneous or intraperitoneal routes and the process of subinoculation repeated in an attempt to perpetuate the strains. The volume of blood used for subinoculation varied from 5 to 20 ml.

To exclude the possibility of contamination of the strain with *Toxoplasma gondii* (Nicolle & Manceaux, 1908) 0·5 ml peritoneal exudate from rabbit No. 4741 of the 27th level of passage of a strain from blue wildebeest, was injected by the intraperitoneal route into each of eight six-weeks-old mice. Rabbit No. 4753 was injected

concurrently with the same volume of peritoneal exudate to control the infectivity of the inoculum, and challenged with *B. besnoiti* of cattle as indicated below. Six weeks later the brains of the mice were examined for *Toxoplasma* cysts by the method of Beverley & Watson (1961).

Rabbits No. 2 and No. 4 were killed for necropsy and histopathological examination 24 days after infection with cyst organisms. Rabbit No. 4360 which died 38 days after infection was examined similarly. The other rabbits injected with cyst organisms from antelopes were killed and examined approximately three months after infection, i.e. after their immunity had been challenged (Table I). Rabbits No. 4385, No. 4421, No. 4730 and No. 4739 of the 2nd, 3rd, 26th and 27th levels of passage respectively were destroyed and examined at various time intervals after infection. The same applied to rabbits No. 4629, No. 4708 and No. 4721 of the 14th, 24th and 25th levels of passage that died during passage of the blue wildebeest strain (Table 3). In addition smears were made from the peritoneal exudate, spleen, liver and testes of rabbits No. 4628, 4629, 4670, 4707, 4708, 4720 and 4721 of the 14th, 15th, 19th, 23rd, 24th and 25th passage levels respectively, all of which died during passage of the strain.

The immunity of the majority of the survivors was challenged form 18 to 68 days after infection. The Schoeman and, to a considerably lesser extent, the Fuls strains of *B. besnoiti* isolated in rabbits in 1961 (Bigalke & Schutte, 1960) and 1953 (Pols, 1954) from acute cases of bovine besnoitiosis were used for this purpose. Blood drawn by cardiac puncture from parasitaemic rabbits, was injected by the intraperitoneal or subcutaneous routes. Susceptible rabbits injected concurrently served as controls. Some of these and other rabbits infected with bovine *B. besnoiti* strains were also destroyed for comparative necropsy and histopathological studies.

Injection of cattle with cyst organisms

Four South Devon-Afrikaner crosses and two grade Friesians were obtained from an area where bovine besnoitiosis was not known to occur. They were approximately seven months old and, with the exception of one steer (No. 3920), all entire males. They were transported to Skukuza and housed in a stockade. Two were injected with organisms from each antelope species as indicated in Table 4, whilst the remaining two animals served as controls. Their temperatures were recorded and blood smears examined daily.

Ten weeks later their immunity was challenged by intravenous injection of 20 ml rabbit blood of the Schoeman strain of *B. besnoiti*. To prove that the ensuing febrile reactions seen in the controls were due to the challenging infection, 10 ml of blood was subinoculated into rabbits. The cattle were slaughtered 25 days after the challenge and examined for cysts; particular attention was paid to the peripheral veins, and tissues were collected for histopathological studies.

Injection of cattle and sheep with blood-passaged strains

Four grade Hereford oxen reared under tick-free conditions and varying in age from three to six years were injected with blood of the 1st, 6th, 6th and 3rd levels of passage respectively, from rabbits or sheep which reacted after infection with *Besnoitia* organisms originating from the blue wildebeest and impala (Table 5). Temperatures were recorded and blood smears examined daily. Their immunity was challenged with the Schoeman strain five to nine weeks later. Twenty ml blood

from every bovine which showed a febrile reaction within four weeks of the challenge was subinoculated into two or more rabbits in order to determine whether the reaction was due to *B. besnoiti*.

Of five Merino ewes and three wethers, varying in age from 18 months to three years, four were injected with the strain isolated in rabbits from blue wildebeest as indicated in Table 6. By subinoculation of blood at the height of the ensuing febrile reaction the strain was taken through four consecutive passages in sheep. Rabbits and cattle were also injected with blood from reacting sheep. Six of the sheep and all the rabbits were challenged with the Schoeman strain four to ten weeks after infection. Two susceptible three-year-old sheep were also infected with this strain for comparative purposes.

All the cattle and sheep were slaughtered two to five months after infection and examined for cysts. Tissues were collected for histopathological studies from four sheep.

Cell culture studies

A stable line of normal lamb kidney cells (developed by Dr. P. G. Howell) grown in Roux flasks and two ounce medical flat bottles was used for this purpose. The medium, which was renewed twice weekly, was Hanks' solution containing 0·5 per cent lactalbumin hydrolysate, 10 per cent bovine serum and antibiotics as indicated above.

The buffy coat obtained by differential centrifugation of blood, and a testicular homogenate of a rabbit from the seventh level of passage of the blue wildebeest strain were sown into two Roux flasks. Two weeks later organisms indistinguishable from *Besnoitia* were found in small numbers in the sediment of centrifuged medium. These isolates were multiplied in culture as described previously (Bigalke, 1962). Two rabbits were injected with culture material containing approximately 30×10^6 extracellular organisms. From one of these which developed a febrile response 20 ml of blood was injected into a third rabbit.

Organisms were grown at the Veterinary Investigation Centre, Skukuza, from cysts harvested from blue wildebeest and inpala, and kept in " cleaning fluid " at 4° C overnight before crushing the cysts to liberate the organisms.

Organisms from cysts found in veins of sheep No. 18661 and 18662 (Table 6) were multiplied in culture and a rabbit was injected with an inoculum containing approximately $1{\cdot}325 \times 10^6$ extracellular organisms. When the rabbit reacted 20 ml blood was subinoculated into a second rabbit.

The immunity of all the rabbits was challenged three to six weeks after infection as described previously.

Glycerinated suspensions of cultured organisms have been stored frozen at —76° C.

Morphological studies on the parasites

Maximum diameters of spherical and subspherical cysts in haematoxylin and eosin-stained sections of veins and other tissues from blue wildebeest, impala and cattle were compared with the aid of an ocular micrometer. The respective numbers of cysts measured were 13, 5 and 16.

Cyst organisms expressed into Hanks' solution from cysts obtained from blue wildebeest and cattle were dried on slides, stained with Giemsa and 100 were measured. The arithmetic mean and standard deviation of these measurements were calculated.

RESULTS

Criteria which were accepted as evidence of successful infection were somewhat different from those used in bovine *B. besnoiti* studies. Because of the somewhat fluctuating normal temperatures and the influence of ambient temperatures, only responses exceeding 104° F in rabbits and sheep and 103° F in cattle were regarded as significant in this report. Apart from a microscopically detectable parasitaemia, a characteristic feature of bovine *B. besnoiti* infections in rabbits is an increase in the number of large mononuclear leucocytes (monocytosis) as judged arbitrarily from blood smears (Pols, 1960). Although less pronounced the monocytosis was also found to be of value in this study. Demonstration of proliferative organisms and the typical cysts in particular, was naturally regarded as irrefutable evidence of infection. Once cross-immunity between parasites from antelopes and cattle had been established, the presence or absence of immunity to challenge with a bovine strain was regarded as reliable circumstantial evidence of successful infection, or failure thereof.

Infection of rabbits with cyst organisms

The two young males and one female injected with organisms from blue wildebeest (Table 1) developed sustained, if not very pronounced, febrile reactions after incubation periods varying from six to eight days (illustrated in Fig. 1). These reactions lasted for 11 to 18 days and were associated with a distinct monocytosis. In spite of a very thorough examination no parasites could be found in blood smears. No other symptoms were noticed. Twenty-four hours after infection rabbit No. 4 had a temperature of 105° F, which rapidly returned to normal. This may have been due to a toxin associated with the organisms, of which more convincing evidence was obtained when the two rabbits (not listed in Table 1) injected with the large dose of organisms were found dead 18 hours later.

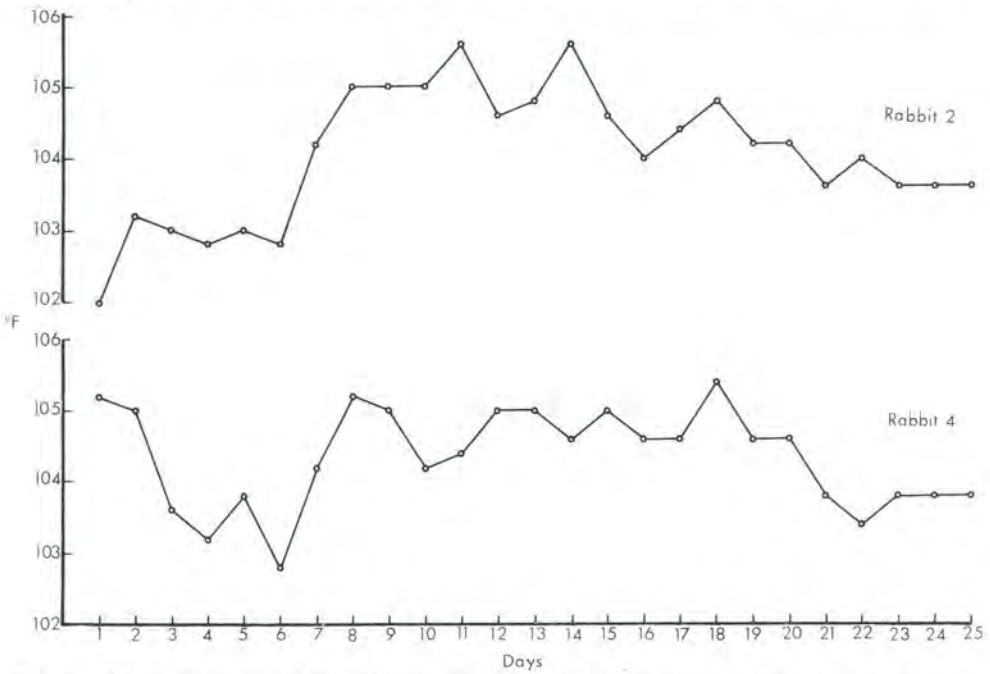

FIG. 1.—Febrile reactions seen in rabbits infected with cyst organisms of *Besnoitia* from blue wildebeest (Rabbit No. 4) and impala (Rabbit No. 2)

TABLE 1.—*Infection of rabbits with cyst organisms*

Source of cysts	Infection				Result					Immunity Test			
	Rabbit recipient	Inoculum	Dose	Route	Incubation period (days)	Duration fever (days)	Proliferative organisms	Cysts	Fate	Days after infection	Strain	Passage level	Result
Blue wildebeest	4363	cyst forms	$4{\cdot}01\times10^{6}$ ($\times3{\cdot}0$ ml)	s.c.	7	12	—	—	S	67	Schoeman	151	No reaction
	4366	cyst forms	$0{\cdot}74\times10^{6}$ ($\times3{\cdot}0$ ml)	s.c.	8	14	—	—	S	67	Schoeman	151	No reaction
	4	cyst forms	$5{\cdot}63\times10^{6}$ ($\times3{\cdot}0$ ml)	s.c.	6	14	—	—	K	—	—	—	—
Impala.....	4360	cyst forms	$7{\cdot}63\times10^{6}$ ($\times1{\cdot}5$ ml)	s.c.	8	17	—	—	D	—	—	—	—
	4364	cyst forms	$2{\cdot}81\times10^{6}$ ($\times1{\cdot}5$ ml)	s.c.	9	21	—	—	S	67	Schoeman	151	No reaction
	2	cyst forms	$5{\cdot}18\times10^{6}$ ($\times3{\cdot}0$ ml)	s.c.	6	15	—	—	K	—	—	—	—

Legend.—s.c. = subcutaneous
i.v. = intravenous
S = Survived
D = Died
K = Killed

The group of rabbits inoculated with organisms from impala developed similar reactions (Table 1). The incubation periods were the same and the febrile reactions (Fig. 1) lasted from 14 to 18 days. These animals also failed to develop the oedematous swellings so typical of bovine *B. besnoiti* infections in rabbits (Pols 1954, 1960) and again no parasites could be found in blood smears. One rabbit (No. 4360) that died 38 days after infection had bilateral pneumonia. No organisms could be found in sections and it can be safely assumed that besnoitiosis was not the cause of its death. The same rabbit had a temperature of 106° F twenty four hours after infection, presumably also due to a toxin of parasitic origin.

No proliferative forms or cysts could be found histologically in rabbits No. 2 and No. 4, killed 25 days after infection (Tables 1 and 2).

The three remaining rabbits were solidly immune to the challenging infection, thus providing good evidence of cross-immunity between the antelope and bovine strains. No cysts or proliferative organisms could be found in them histologically when they were killed four weeks later (Tables 1 and 2).

Serial passage of strains in rabbits

A strain from blue wildebeest has been maintained in rabbits by serial passage of blood. A total number of 66 rabbits have been used to date of which 59 became infected as determined by one or more of the following criteria: immunity to challenge, development of a distinct febrile reaction (only in later levels of passage), and demonstration of proliferative forms of *Besnoitia*.

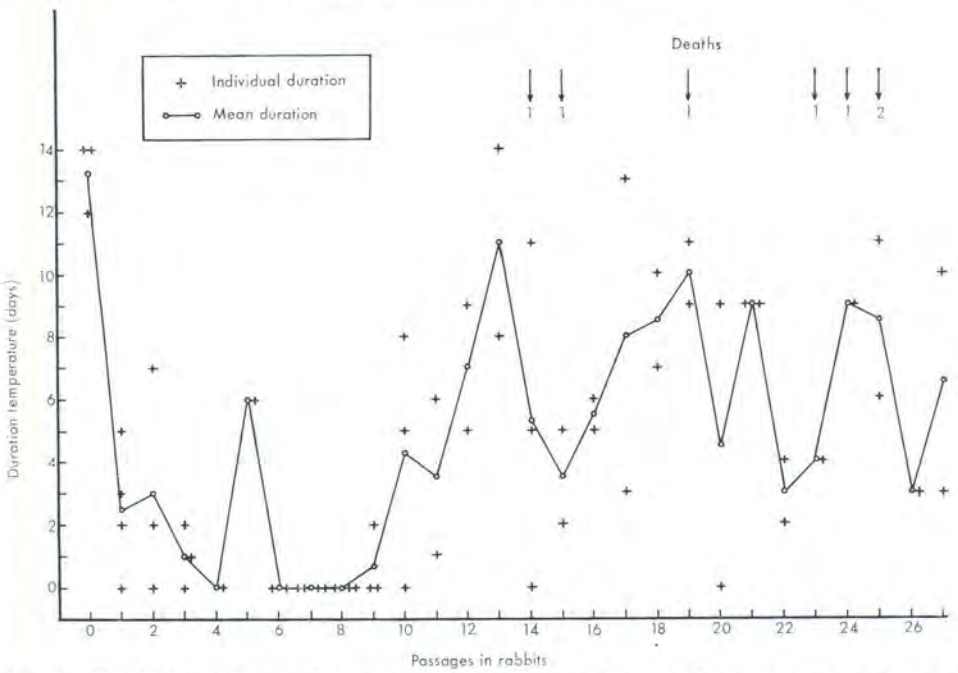

FIG. 2.—Graph showing the individual duration and mean duration of febrile reactions for each level of serial passage in rabbits of the blue wildebeest strain of *Besnoitia*. Passage level 0 on the abscissa represents the original infection with cyst organisms, passage level 1, the 1st level of passage, etc.

During the earlier levels of passage thermal reactions were either much less distinct than with the original isolation or quite absent (Fig. 2), and it became very difficult to know when to subinoculate blood in order to maintain the strain. The monocytic response was an aid in some cases, but was not always present. In many rabbits immunity to challenge was the only indication of successful infection. Clinically very little was seen in early passages apart from fever. During later passages there were definite signs that reactions were becoming much more severe. A transient unilateral scrotal oedema was noticed in two rabbits but the typical anasarca syndrome of the bovine strain did not appear, even in fatal cases. There was a striking increase in the duration and severity of the febrile reactions from the 13th passage onwards (Fig. 2) and monocytosis was marked. The presence of an abnormal number of immature erythrocytes in blood smears attested to the development of anaemia. Whereas no deaths due to besnoitiosis occurred before the 14th level of passage, seven rabbits died from the disease during the next 14 serial passages, i.e. 11·9 per cent of the total number and 26·9 per cent of those used for the latter 14 passages. The symptoms shown by the fatal cases were progressive emaciation, inappetence and weakness.

Direct demonstration of proliferative organisms in blood smears only succeeded at the 14th passage level in rabbit No. 4629 which died from the disease, and it remained consistently difficult to demonstrate organisms by this method, viz. in only four (all fatal cases) out of a total number of 59 animals. Intra- and extracellular proliferative organisms were, however, fairly plentiful, or plentiful, in smears made from the liver, spleen, testes and peritoneal exudate of the seven rabbits that died from besnoitiosis. As no serious attempts were made to find proliferative organisms in tissues other than the peripheral blood of rabbits which survived—testicular and peritoneal fluids were examined in isolated instances—their association with death was probably more apparent than real, viz. rabbit No. 4421 referred to below.

TABLE 2.—*Distribution of organisms in rabbits as detected by histopathological examination*

Origin of *Besnoitia* strain	Cyst organisms from antelopes	Rabbit-passaged wildebeest strain	Rabbit-passaged bovine strain
Total No. rabbits examined.....	6	7	9
Total No. rabbits positive.....	0	4	8
Skin.....	—	1*	5
Scrotum.....	—	—	4
Testes.....	—	—	5
Epididymis.....	—	—	4
Pampiniform plexus.....	—	—	5
Lymph nodes.....	—	4	6
Muscle.....	—	2	2
Myocard.....	—	3	—
Lung.....	—	2	1
Trachea.....	—	1	—
Kidney.....	—	2	—
Liver.....	—	2	—
Stomach.....	—	1†	—
Intestine.....	—	1	—
Urinary bladder.....	—	1	—
Adrenal.....	—	1	—

Legend.—* Localized lesion at injection site
† Possibly a localized lesion at injection site

TABLE 3.—*Lesions in rabbits as detected by histopathological examination*

Item	Antelope Strains													Bovine Strains								
	Cyst organisms					Rabbit-passaged strain								Cyst organisms	Rabbit-passaged strain							
Rabbit Number	4	2	4360	4364	4366	4363	4385	4421	4721	4708	4629	4739	4730	4333	4340	4329	4328	4276	4325	4330	4362	4429
Sex	F	F	M	M	M	M	F	M	M	M	M	M	M	M	F	M	M	F	M	M	M	M
Donor	W	I	I	I	W	W	W	W	W	W	W	W	W	B	B	B	B	B	B	B	B	B
Passage level in rabbits	0	0	0	0	0	0	2	3	25	24	14	27	26	0	147	147	147	139	147	147	151	151
Fate	K	K	D*	K	K	K	K	K	D	D	D	K	K	K	K	K	K	K	K	K	K	K
Days after infection	24	25	38	91	100	100	14	14	16	22	26	33	35	20	7	10	12	12	14	20	33	39
Lesions:																						
Skin	++†	+†		±	±	—	±	—	±	—	+†	—	—	++	—	++	++	++	++	++	—	—
Scrotum					±	—						—	—	++		++	++		++	+		+
Testes Aspermatogenesis			++	++	—	—		±	++	—	+	+	—	++		++	+		++	++	±	±
Necrosis/Orchitis			—	±	—	—		—	+	—	+	—	—	++		++	++		++	++	±	±
Epididymis			±					±	—	—	+	±	—	++		++	++		++	±	—	±
Pampiniform plexus					±			±	++	±		—	—	++		++	++		++	+		
Lymph nodes Hyperplasia	—	—					—	±	—	—		±	—	+	±	±	±	—	±	+		—
Necrosis/Lymphadenitis	—	+					—	—	++	++		—	—	±	—	—	—	—	—	—		—
Large veins (extremities)									++	+			±									
Muscle Degeneration/Necrosis	—	—		—	—	—	±	—	—	+		—	—		±	—	—	++	—	—		—
Interstitial myositis	—	—		—	—	—	—	+	+	+		—	—		—	—	—	++	—	—		—
Myocard Degeneration/Necrosis	—	—		±	—	—	±	++	+	±	++	—	—	—	—	—	±	—	±	—	—	±
Interstitial myocarditis	±	—		—	—	—	—	+	—	±	+	—	—	+	—	±	—	—	±	—	—	—
Spleen	±	+		++	±	++	—	+	±	+	+	±	±	++	±	++	++	+	++	+	+	±
Lung	+	±		—	±	±	±	±	++	+	++	—	±	+	±	+	—	±	+	±	—	—
Trachea	—	—					—		+		±				—		—		—	—		
Kidney Nephrosis	—	—		±	—	—	±	±	±	+	±	—	—	+	—	—	—	+	+	±	—	—
Interstitial nephritis	—	—		—	—	—	—	—	±	—	±	—	—	—	—	—	—	—	—	—	—	—
Liver	—	—		—	—	±	—	+	++	++	+	—	—	+	—	±	±	+	±	—	—	—
Stomach								—	—	±	+++‡	—	—							—		
Intestine	—	—						—	—	+	—	—	—	—				—	—	—		
Urinary bladder		—						—			+					—		—	—	—		
Adrenal	—						—	—	—	++	+	—	—		—	—	±		—	—		
Peritoneum									+	+												

Legend.—F = Female; M = Male; W = Blue wildebeest; I = Impala; B = Bovine; K = Killed; D = Died; * = Died of pneumonia; † = Injection site only; ‡ = Possibly at injection site; — = No lesions; ±, +, ++, +++ = Lesions in increasing order of severity

Histopathological examination also revealed the presence of proliferative forms in moderate to fairly large numbers in various tissues of three fatal cases, and rabbit No. 4421 of the 3rd level of passage, which was killed. In Table 2 the distribution of these parasites in various organs is compared with that observed in a similar group of rabbits infected with bovine strains of *B. besnoiti*. A notable difference was the virtual absence of organisms from the skin, scrotum, testes, epididymis and pampiniform plexus of rabbits infected with the blue wildebeest strain, and their relative abundance in these sites in the latter infection. Conversely, parasites were fairly common in the visceral organs of rabbits infected with the former strain, but apparently rare in these locations in animals infected with bovine strains. Another noteworthy feature was the apparent absence of cysts in the skin and other tissues of rabbits infected with the blue wildebeest strain.

A more detailed description of the pathological changes will be given in a subsequent publication. Briefly, the most prominent lesions seen in rabbits infected with the blue wildebeest strain were peritonitis, splenomegaly, lymphoid hyperplasia, focal disseminated hepatitis, testicular atrophy, vasculitis and thrombosis. Apart from splenomegaly and lymphoid hyperplasia, rabbits infected with bovine strains usually developed testicular and skin lesions not seen with the former, i.e. necrotic orchitis, periorchitis, subcutaneous oedematous swellings, dermatitis and dermal necrosis involving the ears, nose, lips, scrotum and limbs. A comprehensive list of the microscopic lesions observed in rabbits infected with antelope and bovine strains of *Besnoitia* is presented in Table 3. The rabbits are enumerated in sequence of increasing intervals of time after infection. Although the number of cases were limited, histopathological studies confirmed the clinical observations of low initial virulence and increasing pathogenicity of the passaged blue wildebeest strain. The most outstanding lesions seen were lymphadenitis, focal disseminated hepatitis, focal myocarditis, pneumonitis, vasculitis and thrombosis, i.e. the viscera were mainly affected. In contradistinction to this, bovine strains were highly pathogenic to rabbits from the outset; involvement of the internal organs was less pronounced, but severe testicular and skin lesions were seen as described above. These lesions appeared to be primarily associated with vascular involvement.

No *Toxoplasma* cysts could be found in the brains of mice injected with rabbit peritoneal exudate containing proliferative organisms of *Besnoitia*, which are morphologically very similar to *T. gondii* (Frenkel, 1953), and could therefore be confused with the latter. The control rabbit injected concurrently developed a pronounced febrile response associated with a monocytosis, i.e. the type of reaction seen with the blue wildebeest strain of *Besnoitia*, and was immune to challenge with a bovine strain.

The attempt to maintain a strain isolated from impala in the same way as the blue wildebeest strain was unsuccessful. The strain was lost at the fifth level of passage when the two recipients failed to give any indication of infection. Subsequent immunity tests showed that one of them had in fact contracted the disease.

Infection of cattle with cyst organisms

The only fairly consistent clinical sign shown by the cattle injected with these large doses of cyst organisms was a sustained, if not very pronounced, thermal reaction (Table 4 and Fig. 3). The febrile reactions lasted six and ten days in the two animals infected from impala. Two intracellular proliferative forms of *Besnoitia* were found in a monocyte of a blood smear made from bull No. 3895 on the third day of pyrexia. Bull No. 3893, infected from blue wildebeest, developed a distinct fever which lasted for nine days (Fig. 3). The temperature fluctuated around the 103° F level for an additional three weeks. The reaction was less distinct in bull No. 3867. There was a slight fever of 103·6° F three days after infection which lasted 24 hours, and a much more pronounced one of 105·6° F 11 days later lasting two days. Blood smears were negative for parasites.

TABLE 4.—*Infection of cattle with cyst organisms*

Source of cysts	Infection				Reaction					Immunity Test		
	Bovine recipient	Inoculum	Dose	Route	Incubation period (days)	Duration fever (days)	Max. temp. ° F	Proliferative organisms	Cysts	Strain	Passage level	Result
Blue wildebeest	3893	cyst forms	$40{\cdot}2\times10^{6}$ ($\times9{\cdot}0$ ml)	s.c. & i.v.	8	9	105·2	—	—	Schoeman	166	No reaction
	3867	cyst forms	$54{\cdot}2\times10^{6}$ ($\times10{\cdot}0$ ml)	s.c. & i.v.	14	3	105·6	—	—	Schoeman	166	No reaction
Impala.....	3920	cyst forms	$18{\cdot}4\times10^{6}$ ($\times9{\cdot}5$ ml)	s.c. & i.v.	8	6	104	—	—	Schoeman	166	No reaction
	3895	cyst forms	$34{\cdot}4\times10^{6}$ ($\times10{\cdot}5$ ml)	s.c. & i.v.	8	10	104·4	+	—	Schoeman	166	No reaction
Controls.....	3887	—	—	—	—	—	103·8	—	—	Schoeman	166	Fully susceptible
	3872	—	—	—	—	—	105·2	—	—	Schoeman	166	Fully susceptible

Legend.—i.v. = intravenous
also see Table 1.

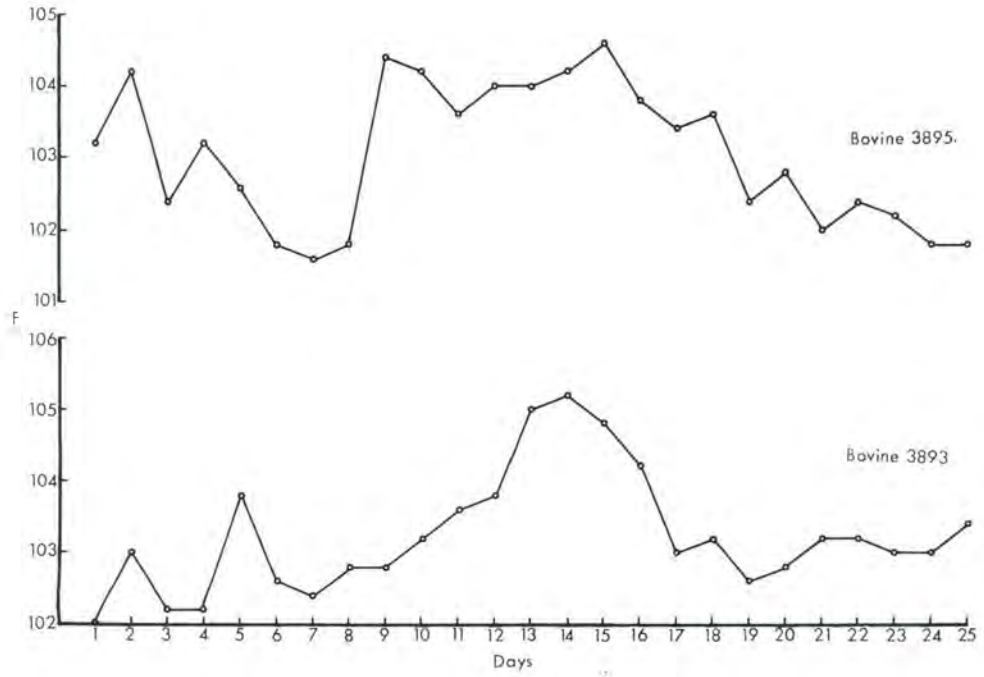

FIG. 3.—Febrile reactions seen in cattle infected with cyst organisms of *Besnoitia* from blue wildebeest (Bovine No. 3893) and impala (Bovine No. 3895)

Monocytosis was also observed, but as it also occurred in the controls it was not considered as reliable an indication of a besnoitiosis reaction as in rabbits. Other symptoms, which were so slight as to be of doubtful significance, included slight listlessness and a serous nasal discharge seen in bull No. 3895 during the early stages of the febrile reaction.

Prior to the challenge the controls developed unexplained fevers of very short duration, hence the somewhat confusing maximum temperatures listed in Table 4. Both animals reacted to the challenging infection after an incubation period of five days. They showed thermal reactions lasting six days, moderately enlarged lymph glands and slight oedematous swellings over the sternum. Rabbits injected with blood at the height of these reactions showed typical symptoms of the bovine *B. besnoiti* type and many proliferative organisms in testes smears. Nothing similar was seen in the four infected bulls, although isolated febrile peaks occurred from 24 to 96 hours after the challenge.

No cysts could be found in the usual sites (McCully *et al.*, 1966) in any of the cattle in spite of a very thorough examination at autopsy.

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Infection of cattle with blood-passaged strains

TABLE 5.—*Infection of cattle with antelope strains maintained in rabbits and sheep*

Source of strain		Infection			Reaction		Immunity Test			
Strain	Donor	Bovine recipient	Dose of blood (ml)	Route	Proliferative organisms	Cysts	Days after infection	Strain	Passage level	Result
Blue wildebeest.....	Rabbit 4380	450	50	i.v.	—	—	37	Schoeman	151	No reaction
	Sheep 18661	2065	40	i.v.	—	—	64	Schoeman	162	No reaction
	Sheep 18662	2231	80	i.v. & s.c.	—	—	50	Schoeman	162	No reaction
Impala.....	Rabbit 4438	2026	25	i.v.	—	—	41	Schoeman	156	No reaction

Legend.—See Table 1

TABLE 6.—*Infection of sheep with a blue wildebeest and a bovine strain*

Source of strain			Infection			Reaction					Immunity Test			
Strain	Passage level in rabbits	Donor	Sheep recipient	Dose of blood (ml)	Route	Incubation period (days)	Duration fever (days)	Max. temp. (° F)	Proliferative organisms	Cysts (No.)	Days after infection	Strain	Passage level	Result
Blue wildebeest..	6	Rabbit 4466	18661	24	i.v.	8	6	106	—	+ (8)	68	Schoeman	161	No reaction
		Sheep 18661	18662	40	i.v. & s.c.	9	6	105·4	—	+ (22)	57	Schoeman	161	No reaction
		Sheep 18662	18664	40	i.v. & s.c.	11	6	105·8	—	+ (21)	53	Schoeman	162	No reaction
		Sheep 18664	18187	40	i.v.	11	3	105·8	—	+ (22)	41	Schoeman	162	No reaction
		Sheep 18187	17071	60	i.v. & s.c.	10	5	105·4	—	+ (1)	29	Schoeman	162	No reaction
	6	Rabbit 4467	18663	20	i.v.	9	3	105·2	—	+ (2)	75	Schoeman	162	No reaction
	11	Rabbit 4594	17009	20	i.v.	6	2	107·8	—	—	—	—	—	—
		Rabbit 4594	16751	40	i.v.	6	9	106·2	—	+ (1)	—	—	—	—
Bovine.....	165	Rabbit 4585	14066	20	i.v.	3	11	107	—	—	—	—	—	—
		Rabbit 4585	17264	20	i.v.	5	7	106·4	—	—	—	—	—	—

Legend.—See Table 1

Not one of the four animals showed a sustained febrile response suggestive of successful infection (Table 5); their morning temperatures never exceeded 103° F. It was therefore surprising to find that they did not develop reactions attributable to bovine besnoitiosis either when challenged. Three days after challenge by the subcutaneous route, ox No. 450 showed a febrile reaction lasting two days, but his blood was not infective to two rabbits during this period. Ox No. 2065, challenged by the intravenous route, developed severe urticaria, hyperaemia and pruritus immediately after injection. Pyrexia which lasted four days also occurred. Again blood was not infective to rabbits. The other two oxen showed no indications of infection. No cysts were found in any of these animals.

Infection of sheep with the blue wildebeest strain

Distinct febrile reactions which lasted from three to eight days were seen in all the sheep after incubation periods varying from six to eleven days (Table 6). In addition there were general signs of illness such as inappetence, hyperpnoea and lassitude. They all recovered, however, and were immune to the challenge.

No proliferative organisms were seen in blood smears, but small numbers of cysts were demonstrated in seven of the eight sheep in one or more of the following sites:— peripheral veins (38 in all, including one in the subcutis), nasal mucosa (30), endocardium (8), tendon sheaths (1). The cysts in the veins were loosely attached to the intima, as seen in antelopes. One cyst was found in a sheep that had not been challenged with the bovine strain, and was therefore undoubtedly of blue wildebeest origin. Organisms from cysts obtained from the other animals, when multiplied in tissue culture, produced the antelope type of reaction in rabbits, i.e. fever and monocytosis, but no oedematous swellings or microscopically demonstrable parasitaemia. This provided further evidence that the cysts had originated from the antelope strain. Conversely, no cysts were found in the two sheep infected with the bovine strain only.

Only one of the four rabbits injected with blood from reacting sheep became infected as indicated by its immunity to challenge.

The symptoms shown by the two sheep infected with the bovine strain were similar to those described above. One pregnant ewe aborted a somewhat decomposed foetus 10 weeks after the height of the reaction.

Cell culture studies

The first direct evidence that organisms indistinguishable from *Besnoitia* had been isolated from antelopes and were being successfully passaged in rabbits, was provided by finding these organisms in cell cultures sown with testes and buffy coat from a rabbit, which showed no sign of infection other than a slight monocytosis. Two rabbits injected with organisms from the second passage in culture developed relatively mild reactions, similar to that of the donor above. This was not altered by short term passage in rabbits and they were all immune to the challenging infection.

Besnoitia was also cultivated from cysts harvested from blue wildebeest and impala. Parasitized cells enlarged considerably and could be recognized under the microscope.

With the exception of the organisms cultivated from sheep, all isolates have been transferred serially in culture. From time to time glycerinated suspensions of infected cells and parasites have been stored frozen at —76° C, both as safeguard against loss of strains, and for reference purposes. The parasites have survived in excess of nine months to date.

Morphological studies on the parasites

The maximum diameter of cysts from blue wildebeest, impala and cattle was 567, 472 and 394 microns respectively; from this it would appear that cysts in blue wildebeest are slightly larger than those in impala which, in turn, are larger than those in cattle. These measurements were, however, obtained from such a small sample that it is pointless to try and prove that they are statistically significant. Furthermore since these measurements were obtained from histopathologic sections, they represent transverse and tangential views at different levels through spherical objects. Hence only maximum measurements were considered. All the cysts seen in blue wildebeest and impala were non-compartmental.

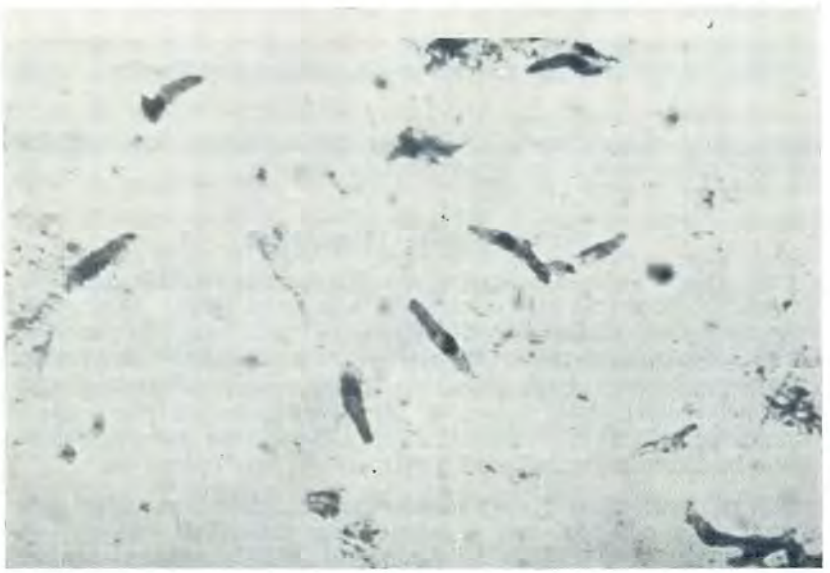

PLATE 1.—Giemsa-stained organisms expressed from a *Besnoitia* cyst from a blue wildebeest. ×1200

Cyst organisms from blue wildebeest and cattle were identical in appearance in both wet and stained preparations (Plates 1 & 2). Those from blue wildebeest varied in length from 6·0 to 10·4 microns (mean 8·6 ± S.D. 1·3) and in width from 1·5 to 3·7 microns (mean 2·5 ± S.D. 0·2). Those from cattle varied from 6·7 to 10·4 microns (mean 8·4 ± S.D. 0·2) and in width from 1·5 to 3·7 microns (mean 1·9 ± S.D. 0·1).

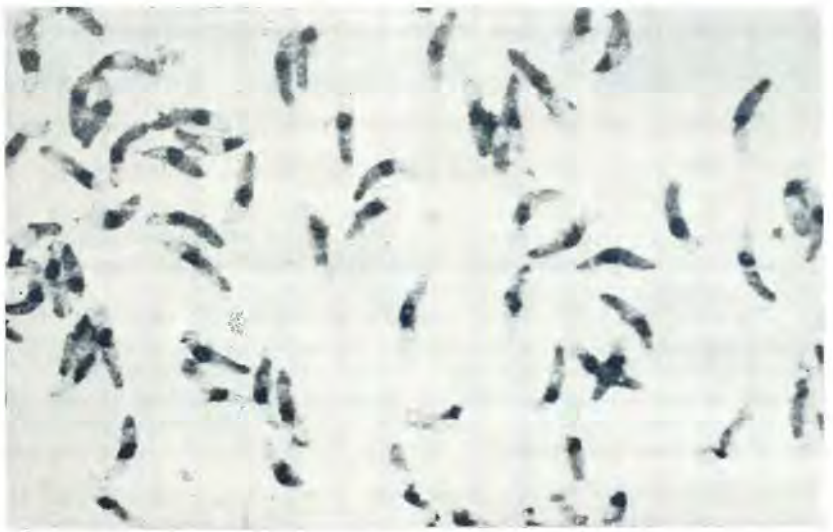

PLATE 2.—Giemsa-stained organisms expressed from a *Besnoitia* cyst from a bovine. × 1200

Specimen slides of cysts from both antelope species and cyst organisms from blue wildebeest have been stored in the departmental collection.

DISCUSSION AND CONCLUSIONS

The main object of this investigation was to determine whether *Besnoitia* found in blue wildebeest and impala was related to *B. besnoiti* of cattle. Organisms of the genus *Besnoitia* are very similar morphologically and these were no exception. Cyst organisms were indistinguishable. Although cysts were essentially identical in structure, there were indications of size differences, the sequence in descending order being those in blue wildebeest, impala and cattle. Since measurements from single sections are not considered suitable for statistical analysis, some other method such as either serial sections or direct measurement of fresh cysts will have to be used.

Contrary to expectations it soon became apparent that *Besnoitia* of these antelopes behaved somewhat differently in rabbits from that which had been observed with bovine isolates. In spite of careful examination of blood smears no proliferative organisms could be found microscopically in any of the six rabbits infected with cyst organisms. Only the transmission of the infection with blood indicated their presence. The only clinical signs shown by these rabbits was a thermal reaction and a monocytosis; none died as result of the infection and when they were killed no cysts were found in skin or other tissues. In contradistinction to this, Bigalke (1965) demonstrated proliferative forms in blood smears of 18 out of 19 rabbits infected with cyst organisms of *B. besnoiti* obtained from six chronically infected cattle; most of these rabbits developed the typical symptoms described by Pols (1960) which included oedematous swellings of the scrotum, prepuce, vulva, legs, ears and face, and necrosis of the scrotum, and cysts were present in skin sections of seven of eight survivors examined.

The strain isolated from blue wildebeest produced mild reactions during the early stages of serial transfer in rabbits, but the reactions became more severe later on. The mortality rate which was nil from the 1st to the 13th passage levels, increased to 26·9 per cent from the 14th to the 27th. Another indication of increased pathogenicity was that the febrile reactions, which were not detectable during some of the earlier passages became more pronounced and lasted longer than before. Proliferative organisms have been found in small numbers in blood smears of only four of the 59 infected rabbits, all of which were fatal cases, but they were plentiful in a wide range of internal organs. Despite this abundance of parasites, skin pathology, so characteristic of infections with bovine strains, has essentially remained in abeyance; this also applies to the development of cysts in the skin. It certainly seems as if the parasites from blue wildebeest and impala apparently giving preference to the internal organs, have a much lower affinity for the integumentum of rabbits than the bovine parasite. The finding of cysts *inter alia* in the renal cortex and mesenterium of impala by McCully *et al.* (1966) also comes to mind in this respect. Comparative histopathological studies in experimentally infected rabbits (and sheep—*vide infra*) might cast much light on the evolutionary history of the pathogenesis of the besnoitioses in general. The question arises whether the enhanced pathogenicity of the blue wildebeest strain was due to an increase in virulence as result of adaptation of the parasite to its new host, or whether it arose because there were slightly more organisms present in the blood. In our opinion both factors were probably involved, and possibly interrelated, but quantitative studies would be necessary to prove this hypothesis.

The impala strain could not be maintained in rabbits by blood-passage for more than four generations due to the increasing mildness of the reactions. If the organism from impala also has a preference for the visceral organs over circulating blood, as seems very likely, better results may be obtained if spleen and liver suspensions are used for subinoculation instead of blood. This is another striking example of a biological difference between the bovine parasite and that of impala, the former being very easy to maintain by passage of blood.

Cattle were susceptible to infection with cyst organisms of both antelope strains. The only symptoms seen were relatively mild febrile reactions. Two proliferative organisms were found in blood smears of one animal injected from impala, but none in the others. Similar reactions have been seen in cattle infected with cyst forms of bovine origin (Bigalke, 1965), a difference being, however, that cysts were found in some of them, whereas there were none evident here.

The cattle injected with infective rabbit or sheep blood of both antelope strains did not develop detectable reactions. Neither could proliferative organisms and cysts be found in any of them. Immunity to challenge was the only evidence that they were successfully infected. These reactions, if one is justified in calling them such, were considerably milder than anything seen hitherto in comparable infections with bovine strains.

On the whole the biological differences between the antelope and bovine strains, so clearly illustrated in rabbits, were less pronounced in cattle. It must be stressed, however, that the factors governing the pathogenicity of bovine strains of *B. besnoiti* in cattle are as yet poorly understood, as it is difficult to produce typical clinical cases of bovine besnoitiosis experimentally. Hence it would be unwise to try to give a detailed comparison of the pathogenicity of antelope and bovine strains in cattle at this stage.

Sheep were apparently much more susceptible to infection with the rabbit-passaged blue wildebeest strain than cattle. They developed pronounced febrile reactions accompanied by general symptoms of illness, but no anasarca was seen. Small numbers of cysts were found in the peripheral veins, nasal mucous membranes and endocardium of most of the animals. In this respect the picture was qualitatively virtually identical to that seen in antelopes (McCully *et al.*, 1966). Sheep would therefore possibly prove to be acceptable models in which to study the pathogenesis of the disease of antelopes. No difficulty was experienced in passaging the strain in sheep by intravenous subinoculation of fairly large doses of blood, but proliferative organisms could not be found in blood smears. Although two sheep injected with a bovine strain showed similar symptoms, no cysts could be found in them.

The results discussed hitherto include a considerable amount of evidence in favour of the existence of differences in biological behaviour between the antelope and bovine strains of *Besnoitia*, particularly as expressed in rabbits. Extensive investigations were also made on the immunogenic relationship between these strains by subjecting cattle, sheep and rabbits which had been infected with the antelope strains to a challenging infection with bovine strains of *B. besnoiti*.

There is good evidence that *B. besnoiti* of cattle is immunogenically stable. Complete cross-immunity has been shown to exist in cattle and rabbits between all the isolates tested to date (Bigalke, 1960, 1965). Although the isolates from antelopes studied here may represent only a fraction of their whole *Besnoitia* population, it seems reasonable to assume that they are also immunogenically stable. The validity of the use of the cross-immunity test as a taxonomic tool in this investigation therefore appears to have been established beyond doubt.

Unequivocal evidence of cross-immunity between *Besnoitia* of blue wildebeest and impala on the one hand, and *Besnoitia* of cattle on the other was obtained in rabbits, cattle and sheep. This means that the organisms from antelopes had immunogenic antigens in common with those of cattle, a phenomenon usually regarded in microbiological systematics as good evidence of very close taxonomic relationship.

On the strength of their immunogenic indivisibility and their biological distinctiveness we suggest that the organisms of blue wildebeest, impala and cattle be regarded as strains or biological races of *B. besnoiti*. It is quite conceivable that the impala strain may eventually be found to be identical to the blue wildebeest strain in all respects, but comparative studies have been too limited to warrant discussion at this stage.

The Fuls and Schoeman strains of bovine *B. besnoiti* (*vide supra*—Materials and Methods) have remained remarkably consistent in their biological behaviour through 500 and 170 serial rabbit passages. Although the blue wildebeest strain has been through only 27 such passages and has become very virulent for rabbits, it has remained consistently different from the two bovine strains in other respects and there is reason to believe that it will retain its character. On this assumption it can be argued that these distinct biological differences are expressions of genetically endowed metabolic differences between the antelope and bovine strains of *B. besnoiti*. This would mean that some degree of evolutionary divergence has taken place over a longer or shorter period of time between these closely related parasites, after they had presumably, although not necessarily, originated from a common ancestor in a wild animal host. Apparently this divergence is not so great as to have led to the development of separate species. The fact that they share immunogenic antigens shows that they also have genetic material in common with each other. Hence their separation must have occurred fairly recently in an evolutionary sense.

The practical importance of the evolutionary divergence of these parasites lies in the bearing it may have on the epizootiology of the disease. From the available evidence it seems unlikely that the strains isolated from blue wildebeest and impala will produce the typical disease picture of bovine *B. besnoiti* infection in rabbits. Although experimental proof is still scanty, because only a small number of cattle have been infected with antelope strains, this reasoning can probably be extended to cattle. From this follows that it would be unwise at this stage to regard antelopes infected with the two strains studied in this investigation as reservoirs of bovine besnoitiosis as we know it today. If the above reasoning that the biological differences have a genetic basis is correct, the situation is unlikely to change in the immediate future. This however, does not exclude the possibility that the antelope strains could become adapted to cattle by an evolutionary process.

The susceptibility of these antelopes to the bovine strain of *B. besnoiti* still needs to be determined. It is not entirely inconceivable that antelopes may be carriers of bovine strains in areas where bovine besnoitiosis is rife, and possibly play an important part in the epizootiology of the disease. Obviously investigations on the possible incidence (and nature) of besnoitiosis in antelopes occurring on farms where the disease is prevalent in cattle are necessary.

A most promising consequence of this study that warrants further investigation is the possible use of an antelope strain as a living vaccine for cattle in the enzootic regions. Much more information is needed on the pathogenicity of these strains, particularly when passaged in cattle, and the degree and duration of the immunity they induce, especially in the face of a natural challenge. A somewhat puzzling feature seen in a number of the cattle subjected to immunity tests was a mild febrile reaction that usually developed 24 to 96 hours after the challenge. Since it was not associated with parasitaemia detectable by subinoculation of blood, it was thought to be due to a hypersensitivity reaction. This, however, is merely a supposition and needs to be confirmed.

There were indications that cyst organisms were toxic to rabbits, rapidly causing death if injected in large doses. Similar observations have been made with *B. besnoiti* of cattle (Besnoit & Robin, 1912; Bigalke, 1965).

Summary

The relationship of *Besnoitia* encountered in blue wildebeest and impala to *B. besnoiti* of cattle was investigated by studying the susceptibility of rabbits, cattle and sheep to infection with antelope strains, and their subsequent immunity to a challenge with bovine strains.

Rabbits were susceptible to infection and a strain from blue wildebeest has been maintained through 27 serial passages by subinoculation of blood during the reactions. Reactions were considerably different from those that have been experienced with bovine strains. The typical skin lesions did not develop; it was much more difficult to find proliferative forms microscopically in blood smears, even in fatal cases but they were plentiful in internal organs of the latter. No cysts were found in the skin or other tissues. Initially the reactions were very mild, but the strain appeared to adapt itself to the new host and eventually became highly pathogenic.

Cattle were also susceptible to infection with antelope strains, but developed mild reactions. Some showed a febrile response, whereas in others immunity to challenge was the only indication of infection. Proliferative organisms were seen in blood smears of only one of the eight animals infected and no cysts could be found in any of them.

Sheep were infected with a strain from blue wildebeest. They developed fairly severe reactions from which all recovered. No proliferative organisms were seen in blood smears, but small numbers of cysts were found in the peripheral veins and nasal mucosa.

In spite of the abovementioned distinctiveness in biological behaviour, infection with these strains from antelopes conveyed an immunity to challenge with bovine strains in the three host-species used. This indicates a close immunogenic, and hence taxonomic relationship between the three strains. It is therefore suggested that the parasites of blue wildebeest, impala and cattle be regarded as distinct strains or biological races of *B. besnoiti*.

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