Onderstepoort J. vet. Res. (1968), 35 (1), 3–138
Printed in the Repub. of S. Afr. by the Government Printer, Pretoria

NEW CONCEPTS ON THE EPIDEMIOLOGICAL FEATURES OF BOVINE BESNOITIOSIS AS DETERMINED BY LABORATORY AND FIELD INVESTIGATIONS*

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CONTENTS

* Submitted in partial fulfilment of the requirements for the degree of Doctor of Veterinary Science in the Faculty of Veterinary Science, University of Pretoria, Pretoria, 1 March 1968.
Received for publication on 1 March 1968. Editor.

NEW CONCEPTS ON THE EPIDEMIOLOGICAL FEATURES OF BOVINE BESNOITIOSIS

1. General Introduction

Besnoitia besnoiti (Marotel, 1912), the aetiological agent of bovine besnoitiosis, has been known since 1912 when the thick-walled cysts harbouring the parasites were discovered by Besnoit & Robin (1912) in a chronically infected cow. Although there has been much speculation on the natural mode of transmission, reviewed by Pols (1960), all attempts to transmit the disease were singularly fruitless until fairly recently.

Besnoit & Robin (1912), Franco & Borges (1916, cited by Pols, 1960), Cuillé & Chelle (1937a) and Pols (1960) failed to infect cattle, rabbits, rats and mice by administration of suspensions prepared from cyst-bearing tissues obtained from chronically infected cattle by various parenteral routes and natural openings. Cuillé, Chelle & Berlureau (1936), however, succeeded in transmitting bovine besnoitiosis from natural cases to susceptible cattle by subinoculation of blood during the initial febrile stage of the disease. This observation was subsequently confirmed by Pols (1954a), who showed that proliferative forms of *B. besnoiti* present in the blood were responsible. Blood ceased to be infective as soon as the short-lived febrile reaction subsided (Cuillé & Chelle, 1937a; Pols, 1960), a situation which continues to prevail during the life-long period of cyst-involvement (Bigalke, 1967).

To Pols (1960) these findings suggested that acute cases might serve as a source of infection for susceptible animals via blood-sucking arthropod vectors. However, even at the height of the besnoitiosis season fresh clinical cases are rare and the abovementioned hypothesis would fail to explain how the parasite survived the interepizootic season, unless this occurred in the presumed vector. A suitable carrier was obviously indicated and in spite of the abovementioned experimental evidence to the contrary, the chronically infected beast seemed the most likely reservoir. Since the mortality rate is very low there is no spate of such animals in the enzootic regions.

The infectivity of inoculated cyst organisms was therefore reinvestigated. Instead of using cattle, rabbits shown by Pols (1954a) to be highly susceptible to artificial infection with proliferative forms, were inoculated with suspensions of cyst-bearing tissues by the writer (1958, cited by Bigalke, 1960, 1967). When it was observed that they developed typical besnoitiosis reactions the experiment was extended to cattle. These also became infected, but usually developed a mild form of the disease not easily seen unless specifically looked for. Neuman (1962b), working in Israel, confirmed that cyst forms were infective to rabbits and also found gerbils (*Meriones shawi tristrami* Thomas, 1892) to be susceptible. Thus the chronically infected, so-called " recovered " natural case harbouring what must amount to many millions of cysts in the dermis and elsewhere in the body was brought back into the epidemiological picture as an extremely likely potential, life-long reservoir.

This finding led to a series of investigations on one of the most harassing problems facing the research worker on besnoitiosis, i.e. the natural transmission of the disease. The problem was attacked from various direct and indirect angles with the object of clarifying the epidemiological picture and obtaining guiding principles on methods of control. The results of these studies form the subject matter of this dissertation.

Although Cuillé & Chelle (1937b) and Pols (1960) recorded negative results, the question whether cohabitation between chronically infected and susceptible cattle would result in transmission of besnoitiosis was reinvestigated on the premises of the Veterinary Research Institute, Onderstepoort, where natural cases had not been known to occur.

A preliminary investigation had indicated that tsetse flies were capable of transmitting cyst organisms mechanically from chronically infected cattle to rabbits (Bigalke, 1960). These experiments were extended to include cattle, and then repeated to a greater or lesser extent with horse flies (tabanids), stable flies and mosquitoes, depending on the availability of the insects and the facility with which they could be handled. The possibility of cyclical development of *B. besnoiti* in these vectors was also considered. Since transmission had occurred in the above-mentioned experiment under conditions of cohabitation it was also decided to reinvestigate the feasibility of transmission of cyst and proliferative forms of *B. besnoiti* by the oral, conjunctival and nasal routes.

Surveys on the incidence of bovine besnoitiosis were conducted on seven farms in the enzootic region by using the method of examination of the scleral conjunctiva for cysts described by Bigalke & Naudé (1962) as diagnostic procedure. All the infected animals on one of these farms were subsequently either isolated or disposed of, and the procedure repeated annually over a seven-year period to determine whether quarantine measures would influence the incidence of besnoitiosis, which was to be expected if chronically infected cattle served as reservoirs of the disease.

Specific attempts were made to produce typical clinical cases of bovine besnoitiosis by various experimental methods with the object of discovering the trigger mechanism(s) responsible for their production. Chronological data obtained during these studies on the time taken for cysts to become visible in the scleral conjunctiva were analyzed for use in some of the chapters dealing with naturally acquired infections.

Finally the epidemiology of the disease is discussed as it emerges from these and some other investigations conducted by the writer.

2. Definitions of terms and abbreviations

For the purposes of this dissertation, unless otherwise stated:—

The parasite is *B. besnoiti.*

B. besnoiti refers to various bovine strains.

The Fuls strain of *B. besnoiti* was isolated from a natural acute case in the Rustenburg district of Transvaal (Pols, 1954a). It has subsequently been passaged 555 times in rabbits by subinoculation of blood during the acute phase.

The Schoeman strain of *B. besnoiti* was isolated in rabbits from a natural acute case in the Waterberg district of Transvaal in 1960 (Bigalke & Schutte, 1960) and subsequently passaged 94 times as above.

The Lamprechts A strain of *B. besnoiti* was isolated in rabbits from a natural acute case in the Thabazimbi district of Transvaal and subsequently passaged 10 times as above.

The Lamprechts B strain of *B. besnoiti* was isolated in a rabbit from a natural chronic case in the Thabazimbi district of Transvaal in 1959.

Proliferative forms (or organisms) are parasites that occur intra- or extracellularly in the blood and some other tissues during the acute stage of the disease only.

Cyst forms (or organisms) are parasites that occur within cysts in the skin and other tissues during the later stages of the disease, or have been expressed mechanically from cysts.

The incubation period is the time-interval between injection, or the last feeding session of potentially infective flies, and the first rise in temperature above the normal level of 102 to 103°F in rabbits and 102°F in cattle.

Clinical cases of besnoitiosis are cattle that are showing, or have shown, the classical symptoms of anasarca and scleroderma.

Inapparent, subclinical or mild cases of besnoitiosis are cattle that are not showing, or have not shown, the classical symptoms although parasites may be demonstrable.

Acute cases of besnoitiosis are cattle that are in the early stage of the disease when the main symptoms are anasarca and fever.

Chronic cases of besnoitiosis are cattle that are in the later stage of the disease when the main symptom is scleroderma.

Typical symptoms of besnoitiosis in rabbits are anasarca and necrosis on one or more of the sites described by Pols (1954a, 1960) and Bigalke (1960, 1967).

Hanks' solution is a balanced salt solution containing 0·5 per cent lactalbumin hydrolysate, 10 per cent bovine serum, and 100 units penicillin, 100 micrograms streptomycin and 1 microgram fungizone per ml.

" Cleaning fluid " is Hanks' solution containing 0·5 per cent lactalbumin hydrolysate and 2000 units penicillin, 2000 micrograms streptomycin, 1500 micrograms neomycin and 40 micrograms fungizone per ml, but no serum.

Blood containing 1 per cent sodium citrate was regularly used for subinoculation.

Viscera, in this article, refer to spleen, liver, kidney and lungs only.

SC is the scleral conjunctiva of cattle.

Tollies are oxen under two years of age.

Mara is the Mara Agricultural Research Station, an experimental farm of the Department of Agricultural Technical Services at which most of the field observations were done.

S.Afr. is the Republic of South Africa.

3. General Aspects concerning Materials and Experimental Methods used

Unless otherwise stated the following applies:—

Rabbits: Sexually mature rabbits, of which the majority were males, were used. They had been bred at this Institute. They were housed singly in cages and fed on green lucerne in experiments conducted before 1965, and on a balanced concentrate ration and water thereafter. After injection or exposure to *B. besnoiti* infection their temperatures were recorded, and blood smears prepared from the ears examined daily for signs of besnoitiosis, viz. parasitaemia and an increase in the number of large mononuclear cells (monocytosis). Rabbits were also inspected for typical symptoms. Four to eight weeks after infection skin specimens collected from the nose and lip by biopsy, or after spinal dislocation, were examined for cysts in sections prepared and stained with hematoxylin and eosin by standard histological methods.

Cattle: Grade Herefords, of which the majority were oxen, that had been bred at this Institute under tick-free conditions and that had been used for babesiosis or anaplasmosis vaccine production previously, were used. They were housed singly in tick-free stables and fed on a balanced ration and lucerne hay. After exposure or injection their temperatures were recorded, blood smears prepared from the tip of the tail were examined and clinical signs noted daily. They were examined for cysts in the SC (see below) and in skin specimens of roughly equal size collected by biopsy or at autopsy from the neck and prepared for histological examination as above. Depending on the state of knowledge at the time, they were also examined for cysts in the SC and elsewhere as described in the relevant chapters below. They were invariably negative for SC cysts prior to the experiments.

Demonstration of cysts in the SC of cattle: The dorsal portion of the SC of both eyes of each animal was examined very carefully from a distance of about 15 to 25 cm for cysts and their approximate number estimated. With the head tilted towards good light, preferably direct sunlight, they resembled minute white grains of sand bulging out of the SC. They became more clearly visible if the SC was stretched by firmly depresssing the posterior portion of the upper eyelid against the eye-ball causing it to bulge outwards.

If necessary cysts or cyst-like structures were removed by biopsy to verify the diagnosis microscopically. Novesine (Wander), a surface-acting anaesthetic, was instilled into the eye concerned. After about 5 to 10 minutes, when the drug had taken effect, a small portion of the SC was lifted with a fine artery forceps and one or more cysts excised with a small pair of scissors. The specimens were placed in normal saline containing antibiotics in the same concentrations as in " cleaning fluid " in appropriately labelled specimen bottles and stored in a refrigerator as soon as possible if not examined immediately. Examination consisted of careful scrutiny of each specimen under a stereoscopic microscope at magnifications of 8 to 16 $\times$ for cysts. If present, one or more were dissected free from surrounding tissues and crushed between two slides. The slides were allowed to dry, fixed and stained as described below and examined for cyst organisms.

Hamsters: Three to six months old Syrian hamsters of both sexes bred at this Institute were used. They were fed on a balanced concentrate ration and some green lucerne. Each experimental group (Chapter 9) was housed in a cage. They were inspected for signs of ill health. Smears were prepared from the viscera and peritoneum of all hamsters that died or were killed within 15 days of infection and examined for parasites. Skin specimens were collected from the lips and noses of some of the survivors three to four weeks after infection and examined for cysts.

Challenge of immunity: Even a mild infection with *B. besnoiti* produces a solid immunity in rabbits and cattle (Pols, 1960; Bigalke, 1967). In this investigation immunity or susceptibility to challenge were therefore regarded as a reliable indication of a previous successful infection or failure thereof. The Fuls and Schoeman strains were used for this purpose. Blood was drawn from parasitaemic rabbits by cardiac puncture. In the case of cattle 20 to 40 ml were injected by the intravenous or subcutaneous route; in rabbits 5 to 20 ml were injected by the latter or intraperitoneal route. With few exceptions, when cattle served as recipients, susceptible rabbits inoculated concurrently, which invariably reacted, served as controls. If a febrile response ensued in a challenged bovine, a rabbit inoculation test (see below) was conducted.

Rabbit inoculation test: All cattle that developed febrile reactions after exposure, injection or challenge, and a few rabbits that showed doubtful reactions, were bled. Susceptible rabbits were immediately inoculated with 15 to 40 ml bovine, or 5 to 20 ml rabbit blood by the intraperitoneal or subcutaneous route to determine whether or not the reaction was due to *B. besnoiti*. The rabbits were examined for signs of infection as described above and challenged if they failed to show an unequivocal reaction, viz. proliferative forms and/or cysts.

Smears from insect bite wounds: Smears were made from the droplets of blood that exuded spontaneously or under pressure from bite wounds inflicted by various insects used in the transmission experiments. They were stained as described below and examined for organisms.

Staining of slides: Blood smears and other slides were allowed to dry, fixed in May-Grünwald solution for 2 to 3 minutes, and stained in a 5 per cent solution of Giemsa in buffered distilled water for 35 minutes.

Counts of organisms: The numbers of extracellular organisms in suspensions of tissues or cell cultures were estimated with the aid of a haemocytometer. In cases where excessive numbers of organisms or cells and debris made counting inaccurate the suspensions were diluted 1 in 10 with normal saline.

4. Natural Transmission of Bovine Besnoitiosis under Conditions of Cohabitation

Introduction

From their perceptive field observations in southern France, Cuillé & Chelle (1937b) deduced that acute and chronic cases of besnoitiosis served as sources of infection for susceptible cattle. They regarded chronic cases as being of greater importance in this respect, particularly those that developed only small localized areas of scleroderma and depilation after having shown typical anasarca. Such animals were not disposed of because farmers regarded them as cured even though they harboured large numbers of viable cysts.

Cuillé & Chelle noticed that in certain regions the introduction of such cases into clean herds led to outbreaks of the disease. Cohabitation was, however, not the only requirement for transmission, for when four healthy bovines were kept in close contact with two experimentally infected animals at the Veterinary School of Toulouse they failed to contract the disease. A similar observation was made on a farm outside the known enzootic region. Furthermore, fresh cases only occurred during the summer and autumn. From the regional distribution and seasonal incidence the writers inferred that transmission must take place indirectly via an intermediate host confined to certain parts of the country, and because they were unable to transmit the disease with cyst-bearing tissues they concluded that organisms ingested by this hypothetical host must first undergo cyclical development before they became infective.

As far as S.Afr. is concerned, Pols (1960) conducted a cohabitation experiment between chronically infected and susceptible cattle in which no transmission occurred over a 10 year period at this Institute. A similar statement was made by Schulz (1960). Hofmeyr (1945), however, believed that transmission followed on contact between infected and susceptible animals.

It became imperative to reconsider the part played by chronically infected cattle in the epidemiology when it was discovered that, despite claims to the contrary by Besnoit & Robin (1912), Franco & Borges (1916, cited by Pols, 1960), Cuillé & Chelle (1937a) and Pols (1960), cyst-bearing tissues were in fact infective to cattle and rabbits when administered by parenteral routes (Bigalke, 1960, 1967). The cattle all developed a mild form of the disease in which neither anasarca nor scleroderma were evident. Subsequently Bigalke & Naudé (1962) observed that by far the majority of natural cases on farms in the enzootic regions had also contracted a clinically inapparent form of the disease. The contention of Cuillé & Chelle (1937b) that cohabitation in the absence of a specific intermediate host would not result in transmission may therefore have been incorrect for two reasons: Firstly because cyst-bearing tissues, and hence cyst organisms, are directly infective; and secondly, because the possibility that the exposed cattle may have contracted a subclinical infection had not been considered. The latter argument also applies to the cohabitation experiment conducted by Pols (1960).

It was therefore decided to reinvestigate the question of whether transmission would occur if susceptible cattle were allowed to cohabit with severe chronic cases on the premises of this Institute. Shortly after the experiment was initiated a few cysts of *B. besnoiti* were noticed quite by chance in the SC of an ox leaning over a stable door. Upon tracing the history of this animal it was found that it had been exposed unwittingly to close contact with chronic cases in the belief that there was no danger of infection. A number of other animals that fell into the same category were detected and the observations made on them are included. Brief reference has been made to the results obtained in this experiment (Bigalke 1966a, 1966b).

Materials and Methods

Cattle exposed deliberately

Twelve black Friesian-Afrikaner cross heifers and two grade Friesian bulls (Table 2) were obtained from areas where bovine besnoitiosis is not known to occur. They were negative for cysts in the SC and skin. They were immunized against anthrax, quarter evil, anaplasmosis, babesiosis and heartwater and divided into two groups.

Group 1 consisted of six heifers and two bulls. On 22 November 1960 four heifers and the bulls were transferred to an open paddock with a surface area of about 840m^2 which harboured five bulls and two cows that were chronically infected with naturally acquired besnoitiosis. The remaining two heifers were added on 28 August 1961. Details on the breed, sex, origin and degree of infection of the infected in-contact animals are given in Table 1. The number of SC cysts is an estimate, whereas those in the skin represent the number counted in a single section. The cattle were fed and watered at communal troughs and hay racks. The temperatures of the eight exposed animals were recorded daily and blood smears examined thrice weekly during the first (1960 to 1961), and once weekly during the second year (1961 to 1962) of exposure. A rabbit inoculation test was conducted if an unexplained febrile reaction occurred. They were also examined for cysts in the SC and skin at approximately three-monthly intervals in the first, and at six-monthly intervals during the second year.

Group 2 consisted of the remaining six heifers. They were placed in an open paddock roughly two acres in size which contained four chronically infected cows (Table 1) and the four animals in Group 4 (Table 3). With the exception of the water supply, which consisted of a small dam fed by an irrigation furrow, the management was similar to that described above. The temperatures of the exposed animals were recorded thrice weekly and blood smears examined at weekly intervals for the duration of the experiment which lasted about two years. After 11 months four of the heifers were transferred to Group 1 (Table 2). For the rest the experimental procedures were the same as for this group.

Cattle exposed unwittingly

Four groups of cattle, representing a total of 18 head, of various breeds were housed in paddocks with mild and severe chronic cases. Since this had not been done with the specific purpose of determining whether transmission would occur no temperatures or blood smears were taken, and the animals were not examined for cysts prior to exposure, with the exception of those in Group 6. The latter had originated from Mara where besnoitiosis is enzootic (Chapter 11) and had been examined in connection with studies on the incidence of the disease. The rest came from farms where besnoitiosis is not known to occur and hence were presumably susceptible at the time of exposure. With the exception of Groups 4 and 5 which were treated like Group 2, the management was similar to that described for Group 1.

Group 3 consisted of nine oxen and one heifer that had been kept in an open paddock about 480 m^2 in size with four chronic cases (Table 1) for periods varying from 2 to 23 months. They were examined for cysts in the SC and skin as indicated in Table 3.

Group 4 comprised four oxen housed with Group 2. They were exposed to the chronic cases six weeks before the Group 2 animals were introduced, and examined for cysts concurrently.

Group 5 consisted of four oxen. They were kept in an identical paddock adjacent to Group 4 with a standard 1·37 m barbed wire fence as the only barrier for the first year after exposure, which coincided with that of Group 4. During the second year they were mixed with Groups 2 and 4. Examinations for cysts were conducted at the same time as for the latter.

Group 6 consisted of eight of a group of 16 bulls of various breeds which had no SC cysts when examined two months after arrival at this Institute from Mara. The rest had relatively small numbers of SC cysts but no skin lesions, i.e. they were mild chronic cases (Table 1). They were all housed in an open paddock with a surface area of about 300 m^2 for 9 months when the previously negative cases were re-examined for cysts (Table 3).

Challenge of immunity

All cattle in which no cysts could be found were either challenged at the end of the period of exposure (Table 2, Groups 1 and 2; Table 3, Groups 4 and 5), or shortly after examination for cysts had been completed (Table 3, Groups 3 and 6).

Random examination of cattle at the Institute

To exclude the possibility that bovine besnoitiosis was being transmitted randomly on the premises of the Institute, 92 head of cattle of mixed sexes and ages that had never been in direct contact with chronic cases of besnoitiosis were picked out at random and examined for SC cysts.

TABLE 1.—*History of chronically infected in-contact cattle*

Group No.	Bovine No.	Breed	Sex	District of origin	Date of purchase	Degree of infection		
						SC cysts	Skin cysts	Degree of scleroderma
1	130	Afrikaner.....	bull.....	Vryburg.....	18.6.59	3+	3+	S
	688	Afrikaner.....	bull.....	Groot Marico.....	20.5.60	2+	3+	S
	895	Afrikaner.....	bull.....	Mara.....	19.10.60	3+	+	FS
	894	Hereford.....	bull.....	Mara.....	19.10.60	2+	+	?
	896	Bonsmara.....	bull.....	Mara.....	19.10.60	+	+	?
	898	Bonsmara.....	cow.....	Mara.....	19.10.60	3+	+	FS
	902	Hereford.....	cow.....	Mara.....	19.10.60	2+	+	?
2, 4, 5	899	Bonsmara.....	cow.....	Mara.....	19.10.60	3+	+	FS
	900	Afrikaner.....	cow.....	Mara.....	19.10.60	3+	+	FS
	897	Hereford.....	cow.....	Mara.....	19.10.60	3+	2+	S
	901	Hereford.....	cow.....	Mara.....	19.10.60	3+	2+	S
3	4093	Friesian cross..	cow.....	Potgietersrus.....	9.9.49	3+	+	FS
	78	Afrikaner.....	ox.....	Potgietersrus.....	5.5.58	3+	2+	S
	130	Afrikaner.....	bull.....	Vryburg.....	18.6.59	3+	3+	S
	688	Afrikaner.....	bull.....	Groot Marico.....	20.5.60	2+	3+	S
6	799	Bonsmara.....	bull.....	Mara.....	20.8.60	+	+	O
	800	Red Poll.....	bull.....	Mara.....	20.8.60	+	+	O
	802	Red Poll.....	bull.....	Mara.....	20.8.60	+	+	?
	803	Red Poll.....	bull.....	Mara.....	20.8.60	+	0	O
	808	Afrikaner.....	bull.....	Mara.....	20.8.60	+	0	O
	810	Afrikaner.....	bull.....	Mara.....	20.8.60	+	0	O
	815	Hereford.....	bull.....	Mara.....	20.8.60	+	0	O
	818	Hereford.....	bull.....	Mara.....	20.8.60	+	+	O

Legend: SC = scleral conjunctiva
0 = no cysts
\+ = 1 to 19 cysts
2+ = 20 to 49 cysts
3+ = 50+ cysts
O = no scleroderma
? = doubtful scleroderma
FS = fairly severe scleroderma
S = severe scleroderma

Results

Cattle exposed deliberately

The results are summarized in Table 2.

Group 1

Four of the six heifers developed small numbers of SC cysts during the course of the experiment. These were detected in Heifers 9850 and 9691 at 3½ months, Heifer 9775 at 6, and Heifer 9588 at 9 months after exposure. The identity of the cysts was verified microscopically. The remaining heifers and the two bulls failed to show SC cysts during the period of exposure.

Since cysts take about eight weeks to become visible in the SC (Chapter 13) it can be deduced that Heifers 9850 and 9691 probably contracted besnoitiosis between the time of exposure at the end of November 1960 and early January 1961. Heifer 9850 showed two cysts in the left and one in the right eye, whereas Heifer 9691 had three in the left eye only. No new cysts appeared subsequently. Heifer 9775, in which five cysts were seen in the right and two in the left eye, probably contracted the infection between the end of August when she was exposed and mid-December 1961; and Heifer 9588, in which only one cyst was seen in the left eye, between February and the end of May 1961. A few cysts were found in skin sections of Heifer 9850 but the other animals were all negative.

No symptoms that could be ascribed to besnoitiosis were seen in any of the animals. Heifer 9839 developed an unexplained febrile peak lasting 24 hours about 11 months after exposure. A rabbit inoculation test was, however, negative. A fair number of cyst organisms were seen in blood smears of Heifer 9850 on two occasions shortly after SC cysts had first become visible. They were all situated extracellularly and were cigar-shaped or slightly crescentic with the one end more pointed than the other. The compact nucleus usually lay slightly off centre in the attenuated end. The finely vacuolated cytoplasm stained rather poorly and was light greyish-blue in colour. These organisms had obviously originated from cysts punctured accidentally, a rather common feature in more severe besnoitiosis cases, especially when smears are prepared from the tip of the tail where cysts may be quite common (Chapter 12).

Heifers 9600 and 9839 and bulls 660 and 667 in which no cysts could be demonstrated were, however, solidly immune to the challenge indicating that they had contracted completely inapparent infections at some time during the course of the experiment. The eight animals had therefore all contracted clinically inapparent forms of besnoitiosis with varying degrees of parasitosis during the two years of exposure within the confines of the small paddock. The infections had no apparent detrimental effect on the reproduction of the heifers because they all calved down normally within a year of exposure. They were served by the chronically infected bulls, of which only two had hard atrophic testicles and were obviously sterile, and by Bulls 660 and 667.

Small numbers of *Babesia bigemina* (Smith & Kilborne, 1893), *Babesia bovis* (Babes, 1888), *Anaplasma marginale* Theiler, 1910, *Eperythrozoon wenyoni* Adler & Ellenbogen, 1934, and erythrocytic stages of a *Theileria* sp., presumably *Theileria mutans* (Theiler, 1906), were observed in blood smears from time to time.

The cattle in this open paddock were exposed to mild infestation with *Rhipicephalus evertsi* (Neum.), to massive *Stomoxys calcitrans* (L.) attacks during December, January and February in particular, and probably to nocturnal worry by culicine mosquitoes and *Culicoides* spp. that are extremely plentiful at this Institute. Tabanids and hippoboscids were noticed from time to time but they were never very plentiful.

TABLE 2.—*Deliberate exposure of cattle to* B. besnoiti *infection*

Group No.	Bovine No.	Sex	Age at exposure (months)	Date of exposure	SC cysts		Skin cysts	Immunity test			
					Time after exposure (months)	Degree of infection	Degree of infection	Date	Strain	Passage level	Result
1	9850	heifer...	23	22.11.60	3·5	+	+	—	—	—	—
	9691	heifer....	26	22.11.60	3·5	+	0	—	—	—	—
	9588	heifer....	27	22.11.60	9	+	0	—	—	—	—
	9600	heifer....	27	22.11.60	—	0	0	16.10.62	Schoeman..	66	No reaction
	660	bull.....	21	18.1.61	—	0	0	24.8.62	Fuls.....	276	No reaction
	667	bull.....	21	18.1.61	—	0	0	24.8.62	Fuls.....	276	No reaction
	9839	heifer....	32	28.8.61	—	0	0	6.12.62	Schoeman..	69	No reaction
	9775	heifer....	33	28.8.61	6	+	0	—	—	—	—
2	9549	heifer....	29	8.12.60	5·5	+	0	—	—	—	—
	9679	heifer....	27	8.12.60	5·5	+	0	—	—	—	—
	9725*	heifer....	26	8.12.60	20	+	0	—	—	—	—
	9599*	heifer....	28	8.12.60	—	0	0	—	—	—	—
	9533*	heifer....	29	8.12.60	—	0	0	6.12.62	Schoeman..	69	No reaction
	9744*	heifer....	26	8.12.60	—	0	0	16.10.62	Schoeman..	66	No reaction

Legend: *Transferred to Group 1 on 7 Nov. 1961 Also see Table 1

Group 2

Small numbers of microscopically confirmed cysts were seen in the SC of both eyes of Heifers 9549 and 9679 5½ months after exposure. Since they were negative when examined two months previously it was calculated that they must have contracted the infection between late January and the middle of March 1961. The other four heifers remained negative for the rest of the year, and only eight months after they were transferred to Group 1, did one of them (Heifer 9725) develop two cysts in the SC of the right eye. The animal had been negative when examined five months previously and infection may have occurred at any time between early January and the middle of May 1962, viz. after the transfer. No cysts were found in skin sections of any of the six heifers.

Once again no symptoms which could be associated with besnoitiosis were noticed in any of the animals. Febrile reactions which reached 105°F and lasted 3, 7 and 15 days were seen in Heifers 9744, 9549 and 9725 at 3½, 1 and 2½ months after exposure respectively. However, rabbit inoculation tests were negative for besnoitiosis and although two of them (Heifers 9549 and 9725) showed SC cysts 5 and 18 months later, they were both negative when examined 2½ and 6 months after their respective fevers when sufficient time had elapsed for cysts to become visible. These febrile attacks were accompanied by mild stiffness and were probably due to three-day-stiffsickness.

No proliferative or cyst forms of *B. besnoiti* were seen in blood smears of any of the heifers. The blood parasites enumerated for Group 1 were, however, encountered from time to time.

Heifers 9533 and 9744 were immune to the challenging inoculum proving that they had contracted inapparent infections. It is a moot point whether it occurred before or after transfer to Group 1. This brought the total number of infected animals in the group to five. The sixth (Heifer 9599) was not challenged due to an oversight.

The ecological conditions differed somewhat from those applying to Group 1. The environment was much more natural in that there was more space, some grazing, and a dam served as a source of drinking water. Mosquitoes, tabanids and midges were more plentiful since breeding sites abounded, and there were no bulls in the paddock.

Cattle exposed unwittingly

The results are summarized in Table 3.

Group 3

Seven of the ten cattle in this group had SC cysts when examined. Their number varied from more than 20 in both eyes (Oxen 9805 and 9936) to only one in the right eye (Ox 9771). In all cases their identity was confirmed microscopically. A few cysts were found in skin sections of Ox 9805 but the others were all negative. No trace of scleroderma was seen in any of the animals. Two of the three in which no cysts could be found (Oxen 9803 and 9809) were immune to challenge. The third (Heifer 9811) developed slight fever lasting 24 hours on the eighth and fourteenth days post-inoculation, but since rabbit inoculation tests were negative it was concluded that they were not due to the challenge, and she was, therefore, also immune. Hence the animals had all contracted clinically inapparent forms of besnoitiosis during periods of exposure that varied from two months to two years under conditions that were basically the same as those pertaining to Group 1.

TABLE 3.—*Cattle exposed unwittingly to* B. besnoiti *infection*

Group No.	Bovine No.	Sex	Date examined	Age (months)	Cysts		Immunity test			
					In SC	In skin	Date	Strain	Passage level	Result
3	9805	ox.....	7.3.61	28	2+	+	—	—	—	—
	9936	ox.....	7.3.61	28	2+	0	—	—	—	—
	9806	ox.....	7.3.61	28	+	0	—	—	—	—
	9955	ox.....	7.3.61	25	+	0	—	—	—	—
	9935	ox.....	7.3.61	28	+	0	—	—	—	—
	9771	ox.....	7.3.61	28	+	0	—	—	—	—
	9160	ox.....	22.8.61	47	+	0	—	—	—	—
	9803	ox.....	7.3.61	28	0	0	19.6.61	Schoeman..	30	No reaction
	9809	ox.....	7.3.61	28	0	0	19.6.61	Schoeman..	30	No reaction
	9811	heifer....	7.3.61	28	0	0	22.6.61	Fuls.....	239	No reaction
4	9638	ox.....	10.3.61	32	2+	+	—	—	—	—
	9651	ox.....	10.3.61	31	+	n.e.	—	—	—	—
	9641	ox.....	10.3.61	31	+	n.e.	—	—	—	—
	9652	ox.....	10.3.61	31	+	n.e.	—	—	—	—
5	9330	bull.....	10.3.61	38	0	0	4.10.62	Fuls.....	280	No reaction
	9640	ox.....	10.3.61	38	0	0	4.10.62	Fuls.....	280	No reaction
	9624	ox.....	10.3.61	32	0	0	5.10.62	Schoeman..	65	No reaction
	9508	ox.....	10.3.61	33	0	0	5.10.62	Schoeman..	65	No reaction
6	813	bull.....	20.6.61	aged	2+	0	—	—	—	—
	807	bull.....	20.6.61	aged	+	0	—	—	—	—
	816	bull.....	20.6.61	aged	+	0	—	—	—	—
	798	bull.....	20.6.61	aged	0	0	22.6.61	Fuls.....	239	No reaction
	801	bull.....	20.6.61	aged	0	0	22.6.61	Fuls.....	239	No reaction
	811	bull.....	20.6.61	aged	0	0	22.6.61	Fuls.....	239	No reaction
	812	bull.....	20.6.61	aged	0	0	23.6.61	Fuls.....	240	No reaction
	814	bull.....	20.6.61	aged	0	0	23.6.61	Fuls.....	240	No reaction

Legend: n.e. = not examined Also see Table 1

Group 4

The four oxen all had SC cysts when examined for the first time approximately 4½ months after exposure. Their numbers varied from 1 in the left eye of Ox 9641 to close on 50 in both eyes of Ox 9638. Their identity was confirmed in the usual way. With the exception of one animal in which a slight decrease occurred, their numbers appeared to remain at a constant level over the two years of observation. Skin sections of Ox 9638 revealed small numbers of cysts. The others were not examined. At no stage was scleroderma noticed in any of the animals, not even in the latter in which the SC cyst estimate was comparable to what has been observed in some clinical cases (Chapters 9, 10 and 12). These animals had therefore also all developed a clinically inapparent form of the disease under ecological conditions that were identical to those applying to Group 2.

Group 5

No cysts could be found in the SC or skin of any of the four oxen. Yet they were all immune when challenged two years after exposure, proving that they had all contracted a completely inapparent infection.

Group 6

Three (Bulls 813, 807 and 816) out of the eight bulls that were negative at the first examination had small numbers of SC cysts when re-examined nine months after their arrival at this Institute. The identity of the cysts was confirmed microscopically. Skin sections were negative for cysts and none of the animals showed scleroderma. Four of the five with no demonstrable cysts were solidly immune when challenged. The fifth developed a mild febrile reaction 13 days after the challenge which lasted three days and reached a maximum level of 103·6°F; a rabbit inoculation test was, however, negative and it was concluded that he was also immune. Hence all the bulls had apparently contracted a subclinical form of the disease. Since they were negative for SC cysts when examined two months after arrival at this Institute, it can be assumed that the three bulls which developed cysts subsequently must have contracted the infection on the premises. The ecological conditions were very similar to those pertaining to Groups 1 and 3, except for the fact that the in-contact animals were not severe clinical, but clinically inapparent cases. The other five animals may either have contracted the disease at Mara or at the Institute. It will be assumed that the latter occurred.

It is evident from Table 4 that at least 13 out of 14 young head of cattle exposed intentionally to close or fairly close cohabitation with chronically infected clinical cases of besnoitiosis over the two-year period contracted the disease. Furthermore, that all 26 animals that had previously been exposed unintentionally in the belief that transmission would not take place, also contracted besnoitiosis. At least 39 (97·5 per cent) of the 40 head therefore became infected. Twenty-one (53·8 per cent) were mild cases with SC cysts and 18 (46·2 per cent) completely inapparent cases in which the only indication of infection was immunity to challenge. Cysts were found in skin sections of only four (11·1 per cent) of the 36 infected animals examined and in no instance were cysts seen in sections when they were not also present in the SC.

TABLE 4.—*Summary of numerical data in Tables* 1, 2 *and* 3

Group No.	Number of cattle per group	Number of in-contact cattle per group	Number of cattle that became infected			
			With SC cysts	With skin cysts	Immune to challenge	Total number infected
1.....	8	7	4	1	4	8
2.....	6	4	3	0	2	5*
3.....	10	4	7	1	3	10
4.....	4	4	4	1†	—	4
5.....	4	4	0	0	4	4
6.....	8	8	3	1	5	8
TOTALS..	40	31	21	4	18	39

Legend: * = One animal not challenged † = Sections from only one animal examined

Random examination of cattle at the Institute

Only one of the 92 head examined at random on the premises had SC cysts, i.e. a five-year old Friesian cross, Cow 2164. She had spent the first 14 days of her life on the grounds of the Institute, was then transferred to the Institute's farm close by where she stayed for roughly three years before being returned. As far as is known she had never been in close contact with besnoitiosis cases.

Forty of the abovementioned animals had been in close proximity to the paddock which housed Group 1 for periods varying from a couple of months to five years. Since no immunity tests were done, the possibility that a few of them had contracted completely inapparent infections cannot be ruled out entirely. If transmission was, however, occurring on a grand scale on the premises of this Institute a fair proportion of the 92 head would have shown SC cysts.

Apart from having no SC cysts prior to being drafted into the experiments, the 28 head of cattle bred at this Institute under tick-free but not insect-free conditions were all fully susceptible when infected with besnoitiosis during the course of this investigation. Thus additional proof was provided that transmission was not occurring at random on the grounds of the Institute.

Discussion

From the results obtained it is evident that the prevailing conditions of cohabitation between chronic cases of besnoitiosis and susceptible cattle resulted in an almost 100 per cent transmission rate, i.e. 39 out of 40 exposed animals showed evidence of infection, and it is tempting to assume that the one in which an immunity test was omitted also contracted the disease. The fact that it happened on the same premises, where failure had previously been recorded in a similar experiment (Pols, 1960), raises some pertinent questions.

Firstly it is necessary to consider whether the exposed cattle were not already infected when drafted into the experiments. It has already been stated that 18 of the animals, that were exposed unwittingly, were not examined for cysts beforehand. Since they originated from areas where besnoitiosis is not known to occur, the assumption that they were susceptible is reasonable. This is substantiated by the fact that some of them originated from the same farm as the 14 cattle which had no SC cysts prior to deliberate exposure and that seven of the latter developed cysts at this site subsequently, in five of which the time-interval between exposure and the first appearance of cysts was long enough to exclude the possibility of infection prior to exposure. Hence the conclusion that the cattle were all fully susceptible before they were exposed is justified on factual as well as good circumstantial evidence.

The second question that arises is whether besnoitiosis was not perhaps being transmitted on a grand scale at the Institute, and that the chronically infected cattle were not involved. This possibility is ruled out entirely by the very low incidence (1·09 per cent) in the cattle examined at random on the premises as compared to the very high incidence in the cattle exposed to this experiment (97·5 per cent). It also refutes the legitimate criticism that the experimental animals in the transmission experiments (Chapters 5, 6, 7, 8 and 9) were not being kept under insect-free conditions and may therefore have acquired the disease naturally.

Typical symptoms of besnoitiosis were not seen in any of the 39 animals that became infected. Not even a febrile response which could be ascribed to a reaction was noticed. The infections were all clinically inapparent and if the cattle had not been examined specifically for signs such as cysts in the SC and skin, and immunity, their diseased status would have escaped detection. Non-recognition of subclinical infection is therefore the only plausible explanation that can be offered for the discrepancy referred to above between the results obtained in this investigation and those recorded by Pols (1960), and by Cuillé & Chelle (1937b) at Toulouse.

A close correlation exists between the severity of the disease and the number of SC cysts (Bigalke & Naudé, 1962; Chapters 10, 11 and 12). It can therefore be deduced that at least three of the oxen exposed unwittingly must have contracted clinically inapparent infections associated with pronounced parasitosis, since they had more than 20 cysts in each eye. Although there was no evidence of scleroderma, it seems likely that they must have shown at least some signs of ill health such as fever accompanied by general symptoms of illness in the acute stage of the disease. It is possible that the observations, made in this experiment, are a reflection of what might happen on a farm when besnoitiosis starts to establish itself, viz. that a population of inapparent cases with varying degrees of parasitosis is built up before clinical cases start appearing (see also Chapters 10, 11 and 14). However, the reason why so many cases are clinically inapparent still remains to be determined (Chapters 12 and 14).

Of particular interest was the observation that subclinical cases with (53·8 per cent) and without (46·2 per cent) SC cysts occurred in almost equal numbers. This means that surveys based on examination of the SC might underestimate the overall incidence by as much as 50 per cent (Chapters 10 and 11). This study has also confirmed previous observations (Bigalke & Naudé, 1962) that inspection of the SC, which detected 53·8 per cent of cases, was a much more reliable and quicker diagnostic procedure than histological examination of skin sections that revealed only 11·1 per cent.

The most important question which arose from this experiment was how the cattle contracted the infection from the chronically infected carriers. Considerable speculation is justified because it forms the basis of most of the experimentation recorded in this study.

The carrier cattle were all in the chronic stage of the disease when their blood was no longer infective but artificial transmission could only be accomplished if organisms expressed from viable cysts were subinoculated (Bigalke, 1960, 1967). Any hypothetical natural mode of transmission must take cognizance of this fact and explain how organisms escape from the thick-walled cysts in which they are incarcerated. In severe chronic cases cysts are particularly plentiful in the dermis, fascia, mucous membranes of the upper respiratory tract, especially the nasal passages, larynx and the peripheral veins (Besnoit & Robin, 1912; Bennett, 1933; Hofmeyr, 1945; Schulz, 1960; McCully, Basson, Van Niekerk & Bigalke, 1966). One or more of these sites must in some way or other have furnished the organisms required for successful transmission in this experiment. A critical analysis of the epidemiological manifestations of the disease in the paddocks provided useful information in this respect.

Highly significant was the virtual absence of evidence of infection in cattle other than those that were in direct contact with the carriers. This indicated that contact had to be rather close before successful transmission occurred and suggested a contaminative type. There is, however, a rider to this: It was calculated that six of the seven cattle that developed SC cysts after exposure became infected at the height of the summer or in autumn, and the seventh in spring, which merely substantiates numerous previous observations (Cuillé & Chelle, 1937b; Barrairon, 1938, cited by Pols, 1960; Herin, 1952, cited by Pols, 1960; Pols, 1960; Bigalke & Schutte, 1960) that bovine besnoitiosis is essentially a disease of the warmer months and is hence probably arthropod-borne. It therefore seemed logical to seek firstly a type of transmission by an arthropod vector which would not result in a rapid dissemination of the disease on a wide scale or secondly, a contaminative one which for some obscure reason would be seasonal.

Mechanical transmission of bovine besnoitiosis by blood-sucking insects, for which some experimental evidence has already been forthcoming (Bigalke, 1960), is most likely to comply with the seemingly contradictory requirements of a seasonal incidence combined with a restricted range of dispersal. This form of transmission operates best at close quarters because of the relatively rapid loss of infectivity of the organisms which, in contrast to the position in cyclical transmission, do not multiply in the vector.

The severe chronic cases of bovine besnoitiosis in this experiment were an inexhaustible, omnipresent, potential source of infection for mechanical vectors by virtue of the many millions of cysts in the dermis. They must have been easily accessible to the probing mouthparts of a variety of blood-sucking insects in search of blood meals. And if the relatively thick walls of the cysts formed no obstacle to them, the mouthparts should have become contaminated with cyst organisms which may have been transmitted mechanically as has been shown to occur with tsetse flies (Bigalke, 1960). The possibility that some blood-sucking arthropod or other intermediate host might also become cyclically infected must not, however, be lost sight of entirely, even though it seems most unlikely from the evidence obtained in this experiment.

The ecological conditions that existed in the open paddocks concerned, were very favourable for transmission by blood-sucking arthropods. *S. calcitrans* was particularly active during the hot summer months in the small paddocks that housed Groups 1, 3 and 6 and on more than one occasion the cattle were observed huddled closely together, apparently seeking mutual protection from their persistent attacks. Large numbers of engorged flies were caught for trituration purposes in the paddock harbouring Group 1 (Chapter 7). Culicine mosquitoes and *Culicoides* spp. are fairly plentiful on the premises of the Institute during spring and very plentiful in summer and autumn (Neville, Veterinary Research Institute, Onderstepoort, personal communication, 1967). The close contact between the cattle in these paddocks was of course ideally suited to mechanical transmission.

The paddocks in which Groups 2, 4 and 5 were kept, were appreciably larger than the three camps mentioned previously with an environment in which one would like to imagine that besnoitiosis would occur naturally if it were arthropod-borne. The small dam in the camps, a slow-flowing furrow for stable effluent virtually adjacent to them, and a large irrigation dam (Bon Accord) fed by the Apies river close by, provided ideal breeding sites for tabanids, mosquitoes and midges. A Harris trap placed in one of the camps caught a sufficient number of tabanid flies for transmission studies (Chapter 6), indicating that they were not infrequent. Stable flies were very numerous in the trap at times, and a light trap not far off captured very large numbers of *Culicoides* spp. and some mosquitoes (Neville, *loc. cit.*). *R. evertsi* and *Boophilus decoloratus* (Koch) were also present but never very plentiful. Contact between the cattle was not as close as in the abovementioned paddocks and it is perhaps significant that at least one of the six exposed heifers contracted besnoitiosis only after having been transferred to one of them. However, all eight oxen became infected in the larger camps and some of them showed a surprisingly large number of SC cysts.

From the above reasoning it is clear that the possibility of mechanical transmission of bovine besnoitiosis by blood-sucking arthropods warrants very serious investigation.

It is not so easy to find arguments in favour of direct transmission from bovine to bovine, particularly if the proviso that it must be seasonal is added. Cysts in the dermis of the skin that are perhaps occasionally exposed accidentally by trauma, or rupture to the surface spontaneously, are most unlikely to form a sufficiently reliable source of infection to account for the very high infection-rate recorded in this experiment. Cysts are, however, also present in large numbers in the nasal passages of severe cases where they are much more superficial. It is not inconceivable that they might occasionally rupture onto the surface liberating cyst forms which may then be disseminated in droplets of nasal secretions and serve as a source of infection via the nasal passages, conjunctival sacs, oral route, or wounds and abrasions. An essential prerequisite would be that this must occur very rapidly after their release because the organisms are short-lived outside the body and the protective cyst wall.

In the relevant paddocks contact was close enough for direct transmission to take place if a mechanism that would provide a sufficient number of organisms at the right time of the year could be found. There is a remote possibility that complex unknown factors in the developmental cycle of the parasite might somehow be responsible for the seasonal incidence of the disease. Another possibility is that contaminative transmission occurs throughout the year, but climatic factors determine the severity; or that organisms liberated spontaneously are passively transferred by a non-biting arthropod like *Musca domestica* (L).

No evidence that besnoitiosis might be a venereal disease was obtained in this study. Heifers became infected irrespective of whether bulls were present (Group 1) or not (Group 2). Furthermore, there were no indications that the sexes differed in their susceptibility. Admittedly the number of exposed bulls was small.

Whatever the mode of transmission might be, these experiments have clearly indicated that clinical, and even clinically inapparent long-standing cases of besnoitiosis act as an excellent source of infection for susceptible animals. It therefore stands to reason that their elimination from farms where the disease is enzootic should lead to a marked reduction in its incidence.

5. Mechanical Transmission of *B. besnoiti* by Tsetse Flies

Introduction

The seasonal incidence of bovine besnoitiosis has led most people who have studied the epidemiology to believe that an arthropod vector is involved in its transmission. Cuillé & Chelle (1937b) specifically postulated a biological mode of transmission, whereas Barrairon (1938) and Herin (1952), both cited by Pols (1960), did not state explicitly whether they thought it would occur by biological or mechanical means. Pols (1960) recapitulated previous theories and speculated in a general way on both possibilities. Having successfully transmitted the infection from artificially infected to susceptible rabbits during the early, fleeting, parasitaemic stage of the disease by insertion of a syringe needle into an infected animal and immediate transfer to a susceptible one, he proposed the possibility of mechanical transmission from acute bovine cases by blood-sucking insects in nature. He conceded, however, that this method would not explain " how the first cases are produced at the beginning of the bovine besnoitiosis season ", and was faced with the lack of infectivity of cyst-bearing tissues containing cyst forms in his experiments, when he tried to implicate chronic cases in mechanical transmission. Pols also considered the possibility of biological transmission and suggested that a tick like *Hyalomma truncatum* Koch, with its long stout mouthparts might be involved. Schulz (1960) argued along similar lines. He favoured the biological type of transmission in which the organisms would complete part of their life-cycle in the vector so enhancing their virulence, enabling them to produce severe natural cases.

In the interim the writer, in 1958, had determined that cyst organisms were in fact transmissible to rabbits and cattle by parenteral routes. Hence the basic objection to a hypothesis that cyst-bearing cattle might serve as reservoirs for mechanical (or contaminative) transmission lapsed. In a subsequent pilot test it was shown that *Glossina brevipalpis* Newstead, was capable of transmitting cyst organisms mechanically to rabbits (Bigalke, 1960).

Cattle were therefore not only potentially infective during the short-lived period of parasitaemia but also during the life-long stage of cyst involvement of the skin and other tissues. The latter contention was proved to be true under natural conditions (Chapter 4), where transmission was distinctly seasonal and limited to in-contact cattle, which is highly suggestive of mechanical transmission. It was therefore decided to extend the preliminary studies on mechanical transmission of cyst and proliferative forms of *B. besnoiti* by tsetse flies considerably to include cattle and to determine the longevity of the organisms in the proboscis of these flies. The transmissibility of proliferative forms was also investigated.

Materials and Methods

Tsetse flies

Pupae of *G. brevipalpis* were obtained from Zululand, Natal, when available*. They were kept in fine sand in an insect cage. After they had emerged flies were placed singly either in 4 oz glass jars covered with mosquito or nylon gauze or in plastic cylinders covered with netting at both ends. They were kept in an insectary with a constant temperature of 26°C and relative humidity of 80 per cent. The flies were given the opportunity to engorge daily on the ears of rabbits when not being used for transmission purposes.

Mechanical transmission of cyst organisms

Donors: Bulls 130 and 688, and Cow 4093, all chronically infected with naturally acquired besnoitiosis (see Table 1 for details), were used. Cyst-bearing skin from these animals had previously been shown to be infective by subinoculation (Bigalke, 1960, 1967).

Transmission by syringe needle: A fold of thickened neck skin of Bull 130 was anaesthetized with novocaine (Winthrop-Stearns) and the hair carefully clipped with a pair of scissors so as not to injure the epidermis. A 20-gauge needle was inserted 3 to 4 mm into this and then immediately introduced into the skin of the neck of a rabbit; the process was repeated five times. Using a separate needle, the procedure was repeated with a second rabbit. The third needle was rinsed in a drop of saline on a slide after each prick. The slide was stained and examined for cyst organisms.

Transmission by tsetse flies to rabbits: The donors were restrained in a crush and the flies fed on various sites on which the hair had been carefully clipped. The tip of the tail was initially chosen because organisms originating from cysts pierced accidentally (see also Chapters 4 and 12) were frequently observed in blood smears prepared from this site. This, however, required epidural anaesthesia with 10 ml novocaine to prevent the animal continually flicking its tail and was soon abandoned in favour of more convenient sites showing severe thickening of the skin such as the neck and shoulders.

Flies were starved for at least 24 hours prior to use. Recipient rabbits were placed in a restraint cage leaving only the head and ears free. The gauze-covered end of a jar containing a fly was pressed tightly against the chosen site on the donor. When the fly had inserted its proboscis, stopped probing and appeared to be feeding, the jar was quickly transferred to the outside of the ears of the recipient rabbit where the process was repeated. In one instance transfer was made to the shaven abdomen of Rabbit 2668. The result was that each fly was continually being transferred from the donor to the recipient and back again to obtain as many immediate transfer feeds as possible before engorgement. The total number of transfers to the recipient was recorded in each case. Flies sometimes penetrated the skin at more than one site before being transferred; these additional bites were not recorded.

Rabbits were examined for signs of infection and a rabbit inoculation test conducted on one showing a doubtful reaction (Chapter 3). All rabbits that failed to react or developed doutbful reactions were challenged.

* Kindly supplied by Dr. E. B. Kluge, formerly of Nongoma, Natal, and Prof. R. M. du Toit of this Institute

Transmission by tsetse flies to cattle: The four recipient oxen varied from 1 to 3½ years in age. They were restrained in a crush, either in front or behind the donor. The flies were fed on the donors as described above and immediately transferred to the carefully clipped face, neck, chest or buttocks of the recipient. In the case of Oxen 9575 and 9546 the flies were fed in a single session as with the rabbits above, whereas feeding extended over several days with the others (Table 6). Ox 9546 was housed in an open paddock during the incubation and reaction periods, the others as described in Chapter 3. Rabbit inoculation tests were conducted in all cases. The cattle were examined for signs of infection and three were challenged.

Mechanical transmission of proliferative organisms to rabbits

Rabbits showing moderate to severe parasitaemia were used as donors. They had been infected either artificially with the serially passaged Fuls or Schoeman strain (Chapter 3) or by tsetse flies (Rabbit 2683—Table 5). In five instances the flies were fed on the ears and in two on the oedematous scrotal sacs of the donors, and then immediately transferred to the ears of the recipient, as described above. The rabbits were examined for signs of infection and a rabbit inoculation test conducted on Rabbit 2740. Five rabbits that failed to react were challenged.

Longevity of B. besnoiti *in tsetse flies*

The tsetse flies used in these experiments were fed, when possible to repletion, on seven susceptible rabbits at increasing intervals of time after they had been used for transmission purposes. The intervals varied from three to four weeks and longer. The rabbits were examined for signs of infection and challenged.

Results

1. *Mechanical transmission of cyst organisms*

Transmission by syringe needle

This preliminary experiment was done to simulate mechanical transmission as it might occur in nature. The recipient rabbits both developed typical besnoitiosis reactions after incubation periods of 27 and 34 days respectively. During the febrile reactions proliferative forms were demonstrable in their blood smears and one developed pronounced oedema of the vulva. Rabbit 2544 died from severe besnoitiosis four days after having shown the first symptoms, whereas Rabbit 2545 was bled to death for a rabbit inoculation test which was positive.

Transmission by tsetse flies to rabbits

Seven of the 11 rabbits used as recipients in this experiment became infected (Table 5).

Bull 130 was a good donor since all four recipients became infected. The tip of its tail was the feeding site throughout. Numerous cyst organisms were found in smears made from blood exuding from bite wounds, indicating that cysts were plentiful at this site and had been punctured by the probing haustellum. The smallest number of transfer feeds that resulted in transmission was 17, inflicted by four, and the largest number 20, inflicted by nine flies. The incubation period

TABLE 5.—*Mechanical transmission of cyst organisms from cattle to rabbits by tsetse flies*

Bovine donor No.	Rabbit recipient No.	Date of infection	Tsetse flies		Reaction					Immunity test			
			Number used	Total number transfers	Incubation period (days)	Proliferative organisms	Skin cysts	Symptoms	Fate	Time after last infection (days)	Strain	Passage level	Result
130	2668	31.3.60	4	17	8	+	n.e.	0	D	—	—	—	—
	2677	4.4.60	4	18	10	0*	0	0	S	28	Schoeman	11	No reaction
	2678	4.4.60	9	20	11	+	+	0	S	—	—	—	—
	2683	5.4.60 7.4.60	6	20	10	+	n.e.	+	D	—	—	—	—
4093	2723	2.6.60	8	27	—	0	n.e.	0	S	40	Fuls.....	210	Susceptible
	2733	2.6.60	10	46	—	0	n.e.	0	S	40	Fuls.....	210	Susceptible
	2734	3.6.60	7	29	—	0	n.e.	0	S	54	Fuls.....	212	Susceptible
	2735	3.6.60	3	22	—	0	n.e.	0	S	39	Fuls.....	210	Susceptible
688	2750	21.6.60	7	28	10	+	+	+	S	—	—	—	—
	2751	21.6.60	5	26	13	+	+†	+	S	—	—	—	—
	2808	7.9.60	3	15	?	0*	+	0	S	—	—	—	—

Legend: * = Rabbit inoculation test positive
† = Skin suspension infective to rabbit
+ = Present
0 = Absent
n.e. = not examined
S = Survived
D = Died

varied from eight to eleven days. During the febrile reactions, of which the maximum temperature varied from 103·2 to 104·8°F, proliferative forms were quite plentiful in three of the rabbits for one or more days (Table 5). Rabbits 2668 and 2683 died from a severe form of the disease two and four days after the start of their respective reactions. The latter showed pronounced oedema of the vulva before death. Rabbits 2677 and 2678 developed milder reactions manifested by fever and a typical monocytosis. Proliferative forms were found in blood smears of Rabbit 2678 on the fourth day of fever and cysts were present in small numbers in the dermis of the upper lip, base of ears and vulva when the animal was eventually killed. Although no parasites could be demonstrated in Rabbit 2677, a biological test was positive. Immunity to challenge provided further proof of successful infection.

Despite experiencing as many as 46 undelayed transfer feedings administered by ten tsetse flies, not one of the four rabbits for which Cow 4093 served as donor showed any sign of infection. They were all susceptible when challenged (Table 5). No cyst organisms were found in smears made from bite wounds of the flies, apparently because cysts were less plentiful in the tip of the tail and neck than in the other two donors (see Table 1 for comparisons on the degree of infection in neck skin).

Rabbits 2750 (male) and 2751 (female) infected from Bull 688 by 28 and 26 immediate transfer feeds developed typical besnoitiosis reactions after incubation periods of 10 and 13 days. Fever lasted for 14 and 11 days respectively and pronounced oedema of the scrotum, vulva, hind legs, face and ears was featured. Proliferative forms and cysts were much more plentiful in Rabbit 2751 in which cysts were also detected in the third eyelid with the aid of a stereoscopic microscope. A suspension prepared from skin of the upper lip and toe 128 days after infection was inoculated into Rabbit 2807, which became infected showing both proliferative forms and cysts. Rabbit 2808 infected by 15 transfer feeds inflicted by only three flies developed a much milder reaction, with a severe monocytosis as the only early sign. A concomitant rabbit inoculation test was, however, positive and cysts were present in small numbers in the dermis of the upper lip. Cyst organisms were numerous in blood smears made from bite wounds on the neck and tail of the donor, another indication that cysts were plentiful (Table 1).

The total number of undelayed transfer feeds in each attempt at transmission was determined by the willingness of the rather limited number of flies to feed. No attempts were made to establish the minimum number of feeds that would result in transmission.

Transmission by tsetse flies to cattle

The four recipients all became infected (Table 6). They developed rather mild reactions requiring various avenues of approach to prove that transmission had occurred.

Ox 8968 received 107 immediate transfer feeds from 29 flies via the tail of donor Bull 130 over three successive days. No early sign of besnoitiosis was seen. Its daily morning temperature never exceeded 102°F. Blood subinoculated into two rabbits, a day after the last feed, was not infective. Although no cysts could be found in skin sections, three were noticed in the SC of the right eye when the animal was slaughtered four months after infection. The diagnosis was verified microscopically. In addition he was immune when challenged.

TABLE 6.—*Mechanical transmission of cyst organisms from cattle to cattle by tsetse flies*

Bovine donor No.	Bovine recipient No.	Date of infection	Tsetse flies		Reaction						Immunity test			
			Number used	Total number transfers	Incubation period (days)	Duration fever (days)	Max. temp. °F	Proliferative organisms	Cysts	Rabbit inoculation	Time after last infection (days)	Strain	Passage level	Result
130	8968	22.5.60 3.5.60 4.5.60	29	107	?	0	102·4	0	+	0/2*	47	Schoeman	13	No reaction
688	9575	23.9.60	8	30	13	7	105·2	0	+	3/3	—	—	—	—
	9576	26.9.60 27.9.60 28.9.60 29.9.60 30.9.60	17	61	11	6	104·6	0	0	3/4	73	Schoeman	24	No reaction
	9546	1.11.60	8	32	13	7	107·2	0	0	0/4	57	Schoeman	25	No reaction

Legend: * 0/2 denotes no rabbits reacted out of 2 injected Also see Table 5

Ox 9575, infected from Bull 688 by means of only 30 transfer feeds on one day from different skin sites, developed a pronounced thermal response 13 days later which lasted seven days and reached a maximum of 105·2°F. No skin lesions were noticed but the animal showed anorexia, listlessness and polypnoea during the febrile reaction. Proliferative forms were not demonstrable in blood smears. However, three rabbits inoculated with blood during this period developed typical besnoitiosis reactions in which initially proliferative forms and later cysts were demonstrated. The first cyst was seen in the SC of the right eye of Ox 9575,53 days after infection. Three weeks later there were three in this and one in the left eye; the latter was removed to verify the diagnosis. A single cyst was also found in skin sections.

A slightly milder reaction developed in Ox 9576 infected by means of 61 transfer feeds delivered by 17 flies from the same donor over five successive days (Table 6). The first rise in temperature occurred 11 days after the last feed. Except for a single afternoon peak of 105·2°F on the fifth day, it never exceeded 103·4°F. Fever was not accompanied by any sign of ill health and proliferative forms were not demonstrable. Their presence in sub-microscopic concentrations was, however, again revealed by inoculation of blood into four rabbits, of which three developed typical besnoitiosis reactions. No cysts could be found in the SC or skin of Ox 9576, but he was immune when challenged 73 days after infection, providing further proof of successful transmission.

Ox 9546, housed in an open paddock where he was exposed to the elements after experiencing 32 undelayed transfer feeds by eight flies on one day, developed quite a severe febrile reaction 13 days later which lasted a week and reached a maximum of 107·2°F on one morning. During this period the animal was decidedly off colour. It fed poorly, drank excessively, showed polypnoea, photophobia and lassitude, and drooled from the mouth. It was therefore surprising that no proliferative forms were demonstrable, not even by rabbit inoculation tests conducted on four days of the reaction. No cysts could be found in the SC or skin sections either. In fact the only concrete evidence that the ox had contracted besnoitiosis was provided by its immunity (Table 6). It is not inconceivable that the severe symptoms shown were not due to besnoitiosis at all, but to some other condition, and that the actual reaction had been as mild as in Ox 8968.

Cyst organisms were once again plentiful in most smears made from blood exuding from the bite wounds of the flies on the two donors. The animals showed some discomfort when being bitten but it was not as pronounced as with tabanids and stable flies.

2. *Mechanical transmission of proliferative organisms to rabbits*

Only two of the seven rabbits used as recipients became infected (Table 7). In both instances the tsetse flies were fed on the oedematous scrotum of the donor rabbits instead of the ears as in the other five cases.

Eleven flies and 51 transfers—the largest number used—administered over three successive days transmitted besnoitiosis to Rabbit 2740. Eighteen feeds by six flies on two successive days infected Rabbit 2852. There was also a marked difference between their incubation periods (Table 7), but both developed febrile reactions that reached 104°F and during which proliferative forms were demonstrable in blood smears. Moderate anasarca of the scrotum, prepuce and ears was seen in Rabbit 2852 during this period. A few cysts were detected in the skin of the upper lip of Rabbit 2740 infected with the recently isolated Schoeman strain, but none was found in Rabbit 2852 infected with the frequently passaged Fuls strain.

TABLE 7.—*Mechanical transmission of proliferative organisms from rabbits to rabbits by tsetse flies*

Rabbit donor			Rabbit recipient No.	Date of infection	Tsetse flies		Reaction					Immunity Test			
No.	Strain	Passage level			Number used	Total number transfers	Incubation period (days)	Proliferative organisms	Skin cysts	Symptoms	Fate	Time after last infection (days)	Strain	Passage level	Result
2652	Fuls.....	200	2679	5. 4.60	3*	12	—	0	n.e.	0	S	39	Fuls.....	204	Susceptible
			2680	7. 4.60	3*	8	—	0	n.e.	0	S	53	Schoeman....	11	Susceptible
2699	Fuls.....	201	2689	12. 4.60	9*	28	—	0	n.e.	0	S	52	Fuls.....	206	Susceptible
2683	Bull 130....	0	2693	21. 4.60	9*	27	—	0	n.e.	0	S	43	Fuls.....	206	Susceptible
2722	Schoeman...	11	2740	8. 6.60	11*	51	17	+**	+	0	S	—	—	—	—
2730	Schoeman...	12		9. 6.60 10. 6.60											
2847	Fuls.....	220	2852	24.10.60 25.10.60	6†	18	6	+	0	+	S	—	—	—	—
			2853	24.10.60 25.10.60	5*	18	—	0	n.e.	0	S	35	Schoeman....	23	Susceptible

Legend: *Fed on ears of donor †Fed on oedematous scrotum of donor **Rabbit inoculation test positive Also see Table 5

It is interesting to note that Rabbit 2853 which received an equal number of transfer feeds from the ears instead of the scrotum of the same donor as Rabbit 2852 above, failed to become infected. The other four rabbits exposed similarly also failed to contract besnoitiosis, although their temperatures sometimes exceeded 103°F for unknown reasons. They were all susceptible when challenged.

3. *Longevity of* B. besnoiti *in tsetse flies*

The seven rabbits on which 63 tsetse flies, used previously for mechanical transmission, were fed to determine how long they remained infective, failed to contract besnoitiosis. It would appear, therefore, that the proboscis is cleansed of viable organisms within three hours of an infective feed. Non-infectivity persisted for four weeks and longer in 21 of the flies tested; some survived for just over three months. Hence there was no evidence of cyclical development in the fly and transmission by bite (" anterior station ") as occurs with the mammalian trypanosomes transmitted by *Glossina* spp. The possibility of biological development and transmission via the faeces was not investigated.

Discussion

Previous observations (Bigalke, 1960) on the mechanical transmission of *B. besnoiti* have been extended considerably. A second suitable, chronically infected bovine donor was found from which cyst organisms were transmissible from the tip of the tail and other skin sites. The third prospective host, a cow that had shown severe sclerodermatitis when purchased 12 years previously, and skin suspensions of which had been shown to be infective (Bigalke, 1967), was unsuitable for mechanical transmission purposes in this experiment. The absence of cyst forms in smears made from bite wounds on the cow, and their abundance in the two satisfactory hosts clearly indicate that cysts were much more plentiful in the feeding sites on the latter animals.

This illustrates an important feature of mechanical transmission from chronically infected cattle, i.e. that the concentration and distribution of cutaneous cysts determine the reliability of the reservoir.

From the results it is evident that rabbits developed typical besnoitiosis reactions as seen after artificial infection with proliferative (Pols, 1954a, 1960) and cyst organisms (Bigalke, 1967), as well as a solid immunity to well-established bovine strains.

Cattle were infected mechanically from the same two good donors shortly afterwards. They developed rather mild reactions with no sign of anasarca or scleroderma. No proliferative forms were seen in blood smears and their presence was only revealed indirectly by inoculation of rabbits. Cysts were, however, demonstrated in small numbers in two of them. In one animal the only evidence of infection was immunity to challenge. The overall picture was therefore rather similar to that observed when cattle were exposed to conditions of cohabitation (Chapter 4).

This experiment has provided proof that the relatively thick wall of the cyst presents no formidable obstacle to the probing haustellum of *G. brevipalpis*. Unless some undiscovered regurgitation of blood occurs from the gut or crop every time the fly inserts its proboscis, it must be assumed that one or more of the elements forming the biting fascicle become contaminated with organisms from cysts punctured accidentally during the feeding process, and that the parasites are deposited in the

tissues of the recipient. If the structure and mode of action of the haustellum are considered (Buxton, 1955; Gordon, Crewe & Willett, 1956), the labellar lobes with their prestomal teeth and rasps, by means of which the fly cuts its way through the tissues, seem the most likely site for the attachment of organisms. The food canal probably also provides many nooks and crannies but a mechanism for dislodging the organisms anteriorly cannot be envisaged. Metacyclic forms of trypanosomes are present in either the salivary glands or the hypopharynx which opens between the labellar lobes virtually at the anterior tip of the haustellum (Buxton, 1955). From these locations they are passively washed into the skin with the saliva (Wenyon, 1926; Buxton, 1955). But in this experiment the abrasive effects of the shearing action of the contaminated mouthparts against the tissues, and their rinsing with blood, tissue fluids and saliva must have been responsible for dislodgement of the organisms.

The infectivity of flies with contaminated mouthparts was of short duration. Only those that were interrupted during the feeding process and immediately transferred, deposited viable organisms in the skin of the recipients. This supports the contention that organisms were adherent to the exterior surface of the proboscis, rather than the food canal, where they were exposed to adverse environmental effects such as dehydration or mechanical cleaning of the fascicle by the fly.

Mechanical transmission also sometimes occurred if the donor (rabbit) was in the acute phase of the disease. In these cases the flies were fed on the oedematous scrotum of the donor which was apparently richer in parasites than the ears from which failure had been recorded (Bigalke, 1960). No attempt was made to transmit proliferative forms between cattle. It is, however, conceivable that cattle in the short-lived anasarca stage of the disease may occasionally also serve as donors for mechanical transmission, provided organisms are present in large numbers at the feeding sites.

The main objective of this experiment was to determine whether or not transmission would take place, and no attempt was made to establish the minimum number of immediate transfer feeds that would give a positive result. This happened to be 15 in rabbits and 30 in cattle. Hence a single fly should be capable of transmitting the disease if interrupted a sufficient number of times. Direct comparisons between the vecting abilities of different species of flies are, however, only justified if the differences are big, because there is a considerable variation in the numbers of cysts from site to site in the skin of the same animal (Chapter 12).

Tsetse flies have not been present for seven decades (Fuller, 1924) in the regions of Transvaal where besnoitiosis is enzootic. As the disease was only recognized in S.Afr. in the late thirties (Hofmeyr, 1945), the fly could not have been involved in its transmission since 1897 when *Glossina morsitans* Westwood, disappeared from the Transvaal. In Zululand, Natal, bovine besnoitiosis was only diagnosed recently (Bezuidenhout, State veterinarian, Nongoma, Natal, personal communication, 1967). *G. brevipalpis* and *Glossina austeni* Newstead, still occur in this area but they are rare and limited in their distribution to a small number of pockets of unadulterated riverine bush extending from St. Lucia Bay northwards as far as the Mocambique border (Du Toit, Veterinary Research Institute, Onderstepoort, personal communication, 1967). Hence there is no conclusive evidence that these flies have ever been involved in the transmission of the disease in this country. Other blood-sucking flies such as members of the families Culicidae, Ceratopogonidae, Tabanidae and Hippoboscidae and subfamilies Stomoxyinae and Muscinae are, however, plentiful in the enzootic regions. Of these the tabanids, all robust flies with formidable mouthparts capable of inflicting most painful bites, attract primary attention.

6. Mechanical Transmission of *B. besnoiti* by Tabanid Flies

Introduction

In the previous experiment, using *G. brevipalpis* as tool, mechanical transmission of besnoitiosis was shown to be feasible, particularly when chronically infected cattle harbouring numerous cysts of *B. besnoiti* served as donors. Since tsetse flies have not been present in the enzootic regions of Transvaal for seven decades, other vectors had to be sought. Tabanid flies were an obvious choice. Not only are they quite plentiful in these regions during the summer months (Bigalke & Schutte, 1960), but because of their extremely painful bites they are intermittent feeders *par excellence* and hence very well suited to mechanical transmission of various infectious diseases (see Zumpt, 1949 for a comprehensive review). If the haustellum of *G. brevipalpis* is capable of penetrating cysts it is perfectly reasonable to expect the same to apply to the array of robust mouthparts of tabanids.

As yet nobody has succeeded in breeding horse flies through more than one generation in the laboratory and the chances of establishing a thriving colony suitable for transmission purposes appear to be remote. One reason is that the larvae are very slow to mature (Gordon & Lavoipierre, 1962). Furthermore, the adults do not readily adapt themselves to captivity and are inclined to batter their wings very badly. Hence flies had to be caught wild, and in sufficiently large numbers to work with because only a relatively small proportion was prepared to take a blood meal under experimental conditions. Their reluctance to feed, combined with the fact that the numbers caught were not large, made it essential that co-operative flies be used for every conceivable purpose relative to transmission. The possibility of exposure to besnoitiosis prior to capture had to be considered and excluded by the use of appropriate controls.

The primary objective of this experiment was to investigate the feasibility of mechanical transmission of besnoitiosis from chronically infected bovine donors as obtained with a *Glossina* sp. However, appreciably more attention was paid to the survival-time and the possibility of multiplication of the parasite in these potential vectors. This was done by feeding, dissection, smear examination and subinoculation of triturated flies or portions thereof into rabbits. An attempt was also made to transmit proliferative forms mechanically.

Developmental stages of a parasitic flagellate were found in smears made from the intestinal tract of some of the flies. Since contact with cases of besnoitiosis could not be ruled out in the captured tabanids, the somewhat remote possibility that the flagellates constituted an unknown stage in the life-cycle of *B. besnoiti* had to be considered. The morphology of the different stages of the flagellates was therefore studied in the flies and in culture, and their infectivity to rabbits and cattle compared with that of *B. besnoiti*.

Materials and Methods

Tabanid flies

Female tabanids* were caught in a Harris trap (Harris, 1930, 1931) placed at various sites on the grounds of this Institute. Small numbers were captured on farms in the Rustenburg, Soutpansberg and Potgietersrus districts of Transvaal and forwarded in glass tubes with moistened cotton wool stoppers.

* Tabanidae used in these experiments were kindly identified by Pamela J. Usher, Natal Museum, Pietermaritzburg

As soon as possible after capture flies were transferred to a cage about 0·1 m^3 in size consisting of a wooden frame covered with mosquito gauze. It was kept in the basement of an insectary in which neither the temperature nor the humidity was controlled in spring, summer and autumn, but which was electrically heated in winter. The flies were routinely fed on cotton wool plugs soaked with brown sugar solution placed on the floor and suspended from the roof of the cage. Flies destined to be examined for flagellates within 24 hours of capture were kept singly in small (about 375 cm^3) gauze-covered cages.

The sugar solution was removed from the bigger cage about 24 hours before a proposed mechanical transmission experiment. The next step was to coax the flies to indulge in a blood meal by allowing them to run about on the writer's hand and arm placed inside the cage. As soon as a fly was felt to be penetrating the skin it was quickly transferred to one of the abovementioned small cages and coaxed to feed on the ears of a susceptible rabbit. This precaution was necessary to ensure that a fly was not already infective due to exposure prior to capture. When it had inserted its proboscis and was apparently attempting to feed it was rapidly removed and immediately used for the purpose required. Subsequently each fly was given a serial number and kept in the same insectary as the tsetse flies (Chapter 5) for further use or until it died.

Some flies were anaesthetized with ether or CO_2 and dissected. The abdomen was severed from the thorax and the intersegmental membranes between the sixth and seventh abdominal segments cut through on either side. By gentle traction, the gut from the midgut backwards and the genital tract were drawn into a drop of saline on a slide. The intestinal tract was used for preparation of smears, subinoculation and culture purposes. Some observations were also made on the degree of ovarian development and state of digestion of blood in the gut.

Mechanical transmission of cyst organisms by tabanids

Donors: Bulls 688, 130 and 1061 and Cow 934, all chronically infected with naturally acquired besnoitiosis, were used (see Table 1 for details on Bulls 688 and 130). Cow 934 and Bull 1061 came from Mara and were severe chronic cases of approximately $3\frac{1}{2}$ and $1\frac{1}{2}$ months standing (see Chapter 11 for details).

Transmission to rabbits: The donors were restrained in a crush and the flies fed on convenient sites showing scleroderma and some alopecia, such as the neck, over the scapula, lumbar region and flanks. When necessary the hair was carefully clipped.

Individual flies were prepared for transmission studies as described above. The cage containing a hungry fly was either pressed against the feeding site or against a fold of skin held between the fingers. As soon as a fly had penetrated the skin and was apparently feeding it was immediately transferred to the ears of the recipient rabbit, restrained as described in Chapter 5, where the process was repeated. In some instances flies were coaxed to bite the donor at more than one site before they were transferred to the recipient. If flies were still prepared to feed the process was repeated until they had engorged. With three exceptions, where it extended over two consecutive days, feeding was completed in a day (Table 8). The number of bites on the donor and immediate transfers to the recipient were recorded.

The 12 rabbits, thus exposed to infection, were examined for signs of besnoitiosis and seven were challenged.

Transmission to cattle: The five recipient oxen varied from 20 to 32 months in age. They were restrained as described in Chapter 5. Individual flies were fed on the donor as outlined above and immediately transferred to the clipped face, neck or buttocks of the recipient. The number of bites on the donor and immediate transfers were recorded. In all cases feeding extended over several days (Table 9). Oxen 9398, 188 and 9160 were maintained in an open paddock where they were exposed to the elements during the expected incubation and reaction periods; the others were kept in tick-free stables. Subinoculations into rabbits were conducted in all cases except Ox 9160. The cattle were examined for signs of infection and three were challenged.

Longevity of B. besnoiti *in tabanids as determined by feeding:* Single flies prepared for transmission as described above were fed on several sites on the donors. Depending on how soon they were going to be fed again, some were allowed to engorge whereas the majority were forcibly interrupted during the feeding process. They were subsequently coaxed to feed on susceptible rabbits at intervals of 30 minutes, 3 hours, 1, 2, 4, 5, 6, 7, 12 and 24 days after the potentially infective feeds (Table 10). Where the period exceeded 48 hours flies were allowed access to sugar water, with 24 hours starvation before attempted transmission. Most of the data on the longer intervals came from flies used previously for mechanical transmission and fed routinely on a susceptible rabbit before being used again. Many flies were subsequently triturated and subinoculated (see below) for comparative purposes. Rabbits were examined and challenged.

Longevity of B. besnoiti *in tabanids as determined by subinoculation:* Flies were killed with CO_2 or ether at increasing intervals after potentially infective feeds on the donors. Either the head, the abdominal organs, or the whole fly was triturated in normal saline containing 200 units penicillin and 200 micrograms streptomycin per ml and the resulting suspensions inoculated into rabbits (Table 11). Smears of the suspensions were examined as usual.

Mechanical transmission of proliferative forms by tabanids to rabbits

Rabbits showing parasitaemia after artificial infection with the Fuls strain, triturated tabanids and the Schoeman strain served as donors. Flies prepared as described above were fed on the oedematous scrotal sacs of one, and the slightly oedematous ears of three donors. They were then immediately transferred to the recipients and the process repeated as described above (Table 12).

Investigations on flagellates found in tabanids

Most of the flies screened for infection with flagellates had been captured recently. Some had already been used for mechanical transmission purposes. They were anaesthetized with a small dose of ether and by gently stroking the abdomen with an entomological needle it was usually possible to express a small drop of faeces onto a slide which was stained and examined. Infected flies were subsequently dissected as described above and below.

Morphology: Smears of crushed gut, gut suspensions and NNN cultures (Hoare, 1949) were stained and the various morphological types studied. The length and width through the widest end of 50 " barley corn " forms were determined with the aid of an ocular micrometer.

Cultivation: Recently captured, unused flies were killed with CO_2 or ether, placed in a sterile Petri dish, sprayed with 70 per cent alcohol to reduce surface contamination and allowed to dry. The gut was removed into broth or Hanks' solution. Initially no antibiotics were added to the medium; then a combination of penicillin, streptomycin, neomycin and fungizone in various concentrations was used; finally, after testing all the antibiotics individually against a strain of *Trypanosoma theileri* Laveran, 1902 (see below), a combination of penicillin (1000 units), streptomycin (1000 micrograms), and neomycin (1000 micrograms per ml) was utilized.

The guts were crushed in 5 ml medium in a mortar with a pestle. The suspensions were kept at 4°C overnight and aliquots sown into each of three or four tubes containing NNN medium. The tubes were incubated at 28°C, and smears prepared from the condensation fluid studied for signs of growth. After a second exposure to antibiotics, strains were passaged in culture by means of a bacteriological needle.

Infectivity studies on rabbits and cattle: Gut suspensions were prepared in normal saline from a number of recently captured flies that harboured flagellates. The viability of the parasites was checked by culture and examination of wet smears.

A suspension of one *Atylotus nigromaculatus* Ricardo, was inoculated into Rabbit 2889 by the intravenous and intraperitoneal routes, and one prepared from another fly into Rabbits 2952 and 2953 by the latter and subcutaneous routes respectively. Similarly Rabbit 2926 was inoculated with a suspension from two *Tabanocella denticornis* (Wiedemann) by the subcutaneous route. The rabbits were examined for signs of infection and challenged.

Samples of blood from all cattle were tested on NNN medium (see below) before attempted infection. The 5-year old Ox 9011 (Bigalke, 1967), immune to *B. besnoiti*, was inoculated subcutaneously with 4 ml of a gut suspension from one *A. nigromaculatus*. Broth washings of eight-day old cultures of the third and fourth subcultures of isolates from two *A. nigromaculatus* were pooled and 10 ml harbouring $24{\cdot}5 \times 10^6$ organisms per ml injected subcutaneously into the neck of $2\frac{1}{2}$-year old Ox 9575. Apart from routine examination of blood smears, the cattle were subsequently tested for the presence of flagellates by culture of defibrinated blood on NNN medium at least once weekly over a period of four weeks.

Comparative investigations on T. theileri

Bulls 130, 895, 2035, 2069 and Oxen 2068, 9011, 138, 738 and 739 were tested for natural infection with *T. theileri* as follows: Using aseptic precautions, 100 ml blood was drawn from their jugular veins, defibrinated and aliquots sown into five NNN medium tubes from each of the bottles. The tubes were incubated at 28°C, examined for flagellates and subcultured as described above.

Morphology: Smears made from positive cultures were used for comparing the morphology of bovine flagellates with those of tabanids.

Influence of antibiotics in vitro: Equal volumes of broth washings of the third subculture of a strain of *T. theileri*, isolated from Bull 130, were added to a series of tubes containing varying concentrations of the four antibiotics to be tested. The final concentrations per ml were: 1000, 500 and 250 units penicillin; 1000, 500 and 250 migrograms streptomycin; equal concentrations of neomycin; 6·25, 3·125 and 1·56 micrograms fungizone. The tubes were left at room temperature for two hours. Thereafter equal volumes of well-mixed broth from every tube were inoculated into each of three NNN culture medium tubes, and incubated and examined for growth as described above.

Infectivity studies on cattle: Two 28-month old Jersey oxen were inoculated with broth washings of the abovementioned strain. Ox 738 received 3 ml of a young culture of the eleventh subculture containing 1×10^6 flagellates per ml by the intravenous and 2 ml by the subcutaneous routes. Ox 739 received $2 \cdot 5$ ml of an old culture of the fourteenth subculture containing $2 \cdot 77 \times 10^6$ organisms per ml by the intravenous, 2 ml by the subcutaneous and $0 \cdot 5$ ml by the intradermal routes. The viability of the flagellates was checked and the cattle examined for successful infection over a period of four weeks as described above.

Results

1. *Some ecological and ethological observations on Tabanidae*

During the early stages of this investigation attempts were made to obtain tabanids from farms in the Rustenburg, Soutpansberg and Potgietersrus districts of Transvaal. Local enquiries had indicated that they were plentiful at the height of the summer and on two of the three farms some of their breeding sites had been identified by finding their larvae. These attempts were, however, never very satisfactory and were abandoned when it was found that vigorous, healthy flies to work with could be caught in sufficient numbers in a Harris trap installed on the premises of this Institute.

The total number of tabanids caught in this trap over a period of 15 months is illustrated in Fig. 1. Initially (September 1960) it was placed in a shady spot in amongst a clump of indigenous trees consisting mainly of *Acacia karroo* Hayne, next to an open muddy drainage furrow used for effluent from paddocks. Because very few flies were caught at this site the trap was transferred to a more open one close by in October, but the numbers remained small.

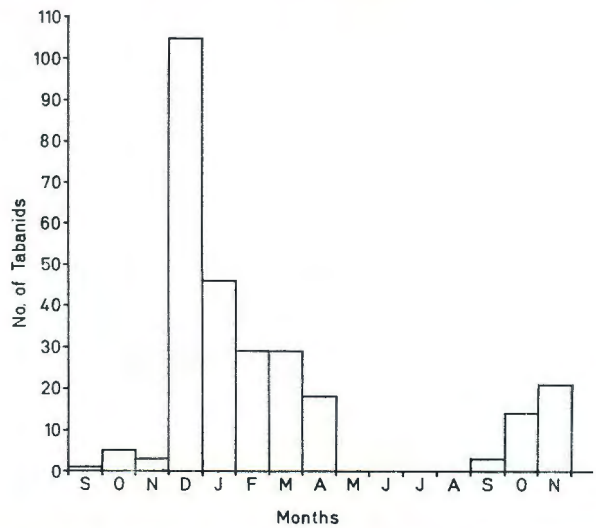


FIG. 1.—Monthly catch of tabanids in a Harris trap on premises of this Institute from September 1960 to November 1961

It was noticed that the majority of flies were caught during weekends when draught-mules and horses were turned out to graze in the vicinity of the trap. On the assumption that these animals served as bait the trap was transferred on 6 December to an open spot in a paddock close by in which the 14 cattle in Groups 2 and 4 (Chapter 4) were being kept. The immediate environment was regarded as suitable for the breeding of tabanids (see Discussion—Chapter 4). In the new position the trap was exposed to sunlight throughout the day. It was surrounded by feed troughs so as to attract the cattle, from which it was protected by barbed-wire fence.

Five flies had been caught in December prior to shifting the trap to its final site, an average of one a day. This increased to a mean of four a day for the rest of the month resulting in a peak total of 105. The catch was 45 in January 1961, and slowly declined reaching zero towards the end of April, where it remained until September, when it slowly started climbing again. The total number caught was 275 flies. Due to an enforced interruption in December 1961, counting was dispensed with and records only kept of those used for experimental purposes. Again the majority were caught in December and January. From the above-mentioned figures it would appear that the cattle did serve to attract flies to the trap. Kluge (cited by du Toit, 1954) made a similar observation with tsetse flies.

Inclusive of tabanids caught after November 1961, the following numbers and species were recorded: 279 *A. nigromaculatus*, 14 *T. denticornis*, 3 *Tabanus taeniola* Palisot de Beauvois, 2 *Haematopota albihirta* Karsch, and 2 unidentified members of each of the tribes Tabanini and Chrysospini, viz. a total number of 302 of which of each of the tribes Tabanini and Chrysopini, viz. a total number of 302 of which 92·4 per cent were *A. nigromaculatus*. Only 56 (18·5 per cent) were prepared to feed and could therefore be used for transmission studies. This proportion would have been considerably lower had all the caught flies been counted and included in the calculations.

The flies were very susceptible to dehydration in the exposed trap and the longer they remained in it the more battered their wings became. The trap was therefore emptied three times a day during December and January and twice daily during the rest of the year. When removed from the traps, flies refused to take a blood meal, but would probe with opened labella from time to time obviously looking for fluid which they sucked up avidly the moment sugar solution was offered. More flies were caught during hot humid spells that at any other time.

The above-mentioned figure of 18·5 per cent makes it clear that some difficulty was experienced in inducing flies to indulge in a blood meal. Thirst *per se* was apparently no incentive. On the other hand, it was necessary to remove the sugar solution from the cage at least 24 hours before a proposed transmission experiment. A fly full of sugar solution, as was sometimes literally the case, was never seen to take a blood meal. Species differed in their readiness to suck blood. Only 47 (16·8 per cent) of the 279 *A. nigromaculatus* fed, whereas 6 (42·9 per cent) out of 14 *T. denticornis* took a blood meal under the existing experimental conditions.

The attitude adopted by a fly taking a blood meal was very different from one drinking sugar water. It would deflect its maxillary palps laterally and retract the labium and labella dorsally thus uncovering the stylet-like mouth-parts which constitute the biting fascicle. It would then depress its head forcibly whilst piercing the skin with its mouthparts. Penetration occurred quite rapidly, although it was slow compared to *G. brevipalpis*, and much more painful. The pain was not as protracted

as with *S. calcitrans*. The act of penetration was accompanied by rocking of the head to and fro and vibration of the palps, but once the fly had inserted its proboscis and was apparently engorging this, as well as the pain, subsided. Sometimes the rocking and cutting were resumed when the fly was apparently not satisfied with the blood pool it was creating. Occasionally it withdrew its fascicle and inserted it at a new site. This did not happen as frequently as with *G. brevipalpis* and *S. calcitrans* and usually the fly had to be disturbed forcibly.

Small specimens of *A. nigromaculatus*, which has a rather short fascicle, sometimes appeared to have difficulty in reaching blood vessels in severe chronic cases with their thick, hard, scurfy skins. Compared to *G. brevipalpis*, that literally blew up with blood, engorgement was slow and the increase in abdominal volume almost imperceptible. Rarely a larger blood vessel was apparently opened and then the fly engorged rapidly. An uninterrupted feed on the writer was timed at approximately four minutes. The whole process of mechanical transmission usually lasted from 10 to 120 minutes depending on the number of flies used. Tabanids were quite prepared to engorge on the fluid in the oedematous scrotum of a rabbit.

In spite of the fact that many flies fed to repletion, in several instances more than once, no eggs were laid under the existing conditions. Oviposition was apparently not stimulated by placing leaves, twigs, stones or the bark of trees into cages. Dissection revealed that considerable ovarian development had occurred in some of the flies that had engorged on cattle or rabbits. In an *A. nigromaculatus* which had three blood meals over a period of three weeks and died nine days after the last, the ova had reached a length of roughly 3 mm and appeared to be ready for laying. Other species, notably *T. denticornis*, also sometimes showed advanced ovarian development, but in the majority it was slight or not in evidence. A striking feature was the lack of ovarian development in newly caught flies.

Some flies were fed on an isotonic sucrose-saline solution containing 10 per cent blood in an attempt to stimulate ovarian development, which in turn, it was thought, might stimulate the need for a blood meal. This had no apparent effect on oogenesis, however, and the flies remained as " unco-operative " as ever. The nature of the biting stimulus remains obscure. When the urge really came it appeared to be overpowering. Some flies were noticed to probe with exposed biting fascicles at the wooden framework of their cages and at cement walls.

The life-span of flies in captivity was usually about two weeks, with a maximum of six in one instance. None were infective to rabbits on which they were fed prior to being used for transmission purposes.

2. *Mechanical transmission of cyst organisms by tabanids*

Transmission to rabbits

Five of the 12 rabbits became infected, four from Bull 688 used so successfully for transmission by *G. brevipalpis*, and one from Cow 934 (Table 8). The incubation periods varied from six to twelve days. Pronounced thermal reactions developed which lasted from four to eleven days and reached a maximum of 105°F. Proliferative forms were found in blood smears of four of the rabbits for one to four days during the febrile period. In the fifth (Rabbit 2894), a rabbit inoculation test was positive. Three developed the typical symptomatology of besnoitiosis. They recovered, however, and cysts were found in the skin of the nose of all five and in the lip of three of them.

TABLE 8.—*Mechanical transmission of cyst organisms from cattle to rabbits by tabanid flies*

Bovine donor No.	Rabbit recipient No.	Date of infection	Tabanid flies			Reaction					Immunity Test			
			Species	Total number bites donor	Total number transfers	Incubation period (days)	Proliferative organisms	Skin cysts	Symptoms	Fate	Time after last infection (days)	Strain	Passage level	Result
688	2865	16.11.60	*A. nigromaculatus*.....	5	5	—	0	n.e.	0	S	42	Schoeman....	25	Susceptible
	2868	18.11.60	*A. nigromaculatus*.....	7	7	—	0	n.e.	0	S	53	Schoeman....	26	Susceptible*
	2894	5. 1.61	*T. denticornis*.....	28	28	10	0†	+	0	S	—	—	—	—
			T. denticornis.....											
			A. nigromaculatus.....											
	2904	16. 1.61	*H. albihirta*.....	6	6	8	+	+	+	S	—	—	—	—
	2905	16. 1.61	*A. nigromaculatus*.....	15	15	6	+	+	0	S	—	—	—	—
		16. 1.61	*A. nigromaculatus*.....											
		17. 1.61	*T. denticornis*.....											
		17. 1.61	*T. denticornis*.....											
	2944	21. 2.61	*A. nigromaculatus*.....	18	1	12	+†	+	+	S	—	—	—	—
	3155	30.10.61	*A. nigromaculatus*.....	13	3	—	0	n.e.	0	S	49	Schoeman....	47	Susceptible
		21.11.61	*A. nigromaculatus*.....											
	3166	2.11.61	*A. nigromaculatus*.....	25	2	—	0	n.e.	0	S	51	Schoeman....	46	Susceptible
		20.11.61	*A. nigromaculatus*.....											
934	2896	6. 1.61	*A. nigromaculatus*.....	9	9	10	+	+	+	S	—	—	—	—
	2899	7. 1.61	*A. nigromaculatus*.....	11	11	—	0	n.e.	0	S	33	Fuls.....	229	Susceptible
			T. denticornis.....											
	2950	2. 3.61	*A. nigromaculatus*.....	10	1	—	0	n.e.	0	S	40	Schoeman....	27	Susceptible
1061	2959	11. 3.61	*A. nigromaculatus*.....	4	4	—	0	n.e.	0	S	39	Fuls.....	235	Susceptible

Legend: * No parasites seen, only pyrexia and monocytosis † Rabbit inoculation test positive Also see Table 5

Because of the difficulties involved in inducing tabanids to feed, the number of flies used at each attempt was small. Nonetheless the results were similar to those obtained with larger numbers of tsetse flies, both as regards the number of rabbits that became infected and the severity of the reactions that resulted. The flies used in this experiment were mainly *A. nigromaculatus*, some *T. denticornis* and one *H. albihirta*. As can be seen in Table 8, from one to four flies were employed for successful transmission. In three instances (Rabbits 2904, 2944 and 2896) a single fly succeeded in transmitting the disease; in two (Rabbits 2904 and 2896), infected by an *H. albihirta* and *A. nigromaculatus* respectively, the conventional approach of undelayed transfer from the donor to the recipient and back was followed until the fly had engorged or refused to co-operate. But in the case of Rabbit 2944 an *A. nigromaculatus* was allowed to bite the donor in 18 different sites first before she was transferred to the recipient, where she penetrated the skin at 18 different sites. A single transfer therefore resulted in transmission. The seven rabbits that did not contract the disease were treated similarly to those that became infected but on the whole received a smaller number of bites (Table 8). They were fully susceptible to the challenging inoculum.

Smears of the drops of blood from bite wounds on the donors revealed numerous cyst organisms in four of the five occasions where transmission occurred. They were, however, also present in four of the seven negative attempts, for example in smears from Bull 1061, a very severe case in which cysts were very numerous but not yet mature, and on which the fly was fed in the lumbar region.

Transmission to cattle

Three of the five recipients showed evidence of successful infection (Table 9). Bull 688 was once again the most suitable donor, serving as the sole source of infection for two of the recipients (Oxen 9398 and 9472) and together with Bull 130 for the remaining Ox 138.

Again the number of flies used in each trial was comparatively small. An initial attempt to infect Ox 9398 with two *A. nigromaculatus* that administered six transfer feeds between them failed to produce a noticeable reaction (not listed in Table 9). A second venture six weeks later with six *A. nigromaculatus* which dispensed 25 alternate bites over a two-day period succeeded. Cyst organisms were plentiful in smears made from the bite wounds on the donor. The recipient was placed in an open paddock where it was exposed to the hot January sun. Thirteen days later it developed a pronounced febrile reaction which lasted 12 days and reached a peak of 107·6°F (Fig. 8). This was accompanied by decided listlessness and inappetence. The ox preferred to lie down and if forced to walk, tired rapidly and showed polypnoea. A marked hyperaemia of the muzzle, skin around the eyes and nasal mucous membrane was evident. At no stage was anasarca or scleroderma noticed. In spite of careful examination of blood smears no proliferative forms were seen during the febrile reaction. Their presence was, however, revealed when five of the eight rabbits inoculated with blood on five consecutive days developed typical besnoitiosis reactions.

Borrelia theileri (Laveran, 1903) was found in small numbers in blood smears on the twelfth day of the abovementioned febrile reaction, viz. 25 days after the animal had been removed from the tick-free stables. It is therefore possible that this parasite was responsible for the latter part of the febrile response. The following day the temperature returned to normal and *B. theileri* was not seen again until five

TABLE 9.—*Mechanical transmission of cyst organisms from cattle to cattle by tabanid flies*

Bovine donor No.	Bovine recipient No.	Date of infection	Tabanid flies			Reaction						Immunity Test			
			Species	Total number bites donor	Total number transfers	Incubation period (days)	Duration fever (days)	Max. temp. °F	Proliferative organisms	Cysts	Rabbit inoculation	Time after last infection (days)	Strain	Passage level	Result
688	9398*	10. 1.61 10. 1.61 11. 1.61 11. 1.61 11. 1.61 11. 1.61	*A. nigromaculatus*.. *A. nigromaculatus*.. *A. nigromaculatus*.. *A. nigromaculatus*.. *A. nigromaculatus*.. *A. nigromaculatus*..	25	25	13	12	107·6	0	+	5/8†	—	—	—	—
	9472	18. 1.61 18. 1.61 18. 1.61 19. 1.61 19. 1.61	*T. denticornis*..... *T. denticornis*..... *A. nigromaculatus*.. *A. nigromaculatus*.. *A. nigromaculatus*..	27	27	13	6	105	0	+	1/1	—	—	—	—
	188*	29. 3.61 4. 4.61	*A. nigromaculatus*.. *T. taeniola*.....	15	3	15?	3?	104	0	0	1/4	82	Schoeman	30	Susceptible
934	9160*	15. 3.61 15. 3.61 17. 3.61 20. 3.61	*A. nigromaculatus*.. *A. nigromaculatus*.. *A. nigromaculatus*.. *A. nigromaculatus*..	14	7	—	—	104·6‡	0	0	n.c.	96	Schoeman	30	Susceptible
688 & 130	138	30.11.61 30.11.61 30.11.61 1.12.61 2.12.61 2.12.61	*A. nigromaculatus*.. *A. nigromaculatus*.. *Haematopota* sp. . . *T. denticornis*..... *A. nigromaculatus*.. *A. nigromaculatus*..	45	45	11	4	104·4	0	0	2/4	57	Schoeman	48	No reaction

Legend: *Exposed to elements in open paddock
†5/8 denotes 5 rabbits reacted out of 8 injected
‡Due to *B. theileri*

? = doubtful reaction
n.c. = not conducted
Also see Table 5.

days later when its presence was again accompanied by mild fever that lasted three days. A single *R. evertsi* adult tick, which is a known vector (Theiler, 1909) of *B. theileri*, was removed from this animal a week after it was placed in the paddock. Since the prepatent period of *R. evertsi*-transmitted *B. theileri* infection may be as short as 24 days (Theiler, 1909) it must be assumed that it had been transmitted by ticks rather than mechanically by tabanids. The absence of this parasite in the other two animals (Oxen 9472 and 138), infected from the same donor but maintained under tick-free conditions, adds weight to this reasoning and at the same time argues against the possibility that ticks were responsible for the transmission of besnoitiosis to Ox 9398.

Four small cysts were seen microscopically in an SC biopsy specimen taken 28 days after the first rise in temperature. At 41 days 10 cysts were clearly visible in the right and five in the SC of the left eye; this situation remained unchanged until the animal was slaughtered two months later. One immature cyst was seen in skin sections 19 days after the first rise in temperature. A week later two were observed but the following week only one was found.

Ox 9472 became infected after 27 undelayed transfer feeds by three *A. nigromaculatus* and two *T. denticornis* flies. Cyst organisms were plentiful in smears made from bite wounds on the donor. Ox 9472, as well as Ox 138 (see below), were kept in a rather dark stable under tick-free conditions and can be regarded as controls for Ox 9398 referred to above. The febrile reaction which developed in Ox 9472 after an incubation period of 13 days was not as pronounced as in the latter animal; it lasted only six days and reached a maximum of 105°F. No other symptoms were observed. Ox 9472 fed normally and did not show any sign of listlessness. Although no proliferative forms were seen during the febrile reaction, their presence was revealed by a biological test on a rabbit. Four rabbits, also inoculated at this time, died from other causes. Three cysts were detected in the SC of both eyes 50 days after the first rise in temperature; none could be found in skin sections.

Bull 688 also served as potential donor of Ox 188. Again cyst organisms were present in blood smears made from bite wounds inflicted on the donor by an *A. nigromaculatus* and a *T. taeniola* used as vectors. These flies were transferred only three times, but bit the donor in fifteen and the recipient in seven different places. The transmission was carried out on two days with a six-day interval. This animal had been placed in an open paddock eight days previously to determine the influence of prevailing climatic conditions in autumn. Ox 188 had a rather undulating temperature which reached a peak of 104°F 15 days after the last feeding session. At this stage blood was inoculated into four rabbits of which one became infected. The animal showed no other sign of illness, no proliferative forms were seen in blood smears and no cysts in the SC or skin. When its immunity was challenged 82 days after infection it nevertheless developed a febrile reaction and its blood was infective to two rabbits. This unexpected result casts doubt on the apparently successful mechanical transmission as indicated by the positive rabbit inoculation test. Hitherto it has been the experience that cattle that have shown signs of infection, no matter how mild, have been solidly immune to challenge. It is certainly strange that only one of four rabbits injected became infected and the possibility of misidentification of the rabbit concerned must be considered. For this reason the results obtained with Ox 188 will be regarded as inconclusive.

Ox 188 developed *A. marginale* infection 29 days after it was first bitten by tabanids. By that time it had been in the paddock for 37 days which falls within the limits of the prepatent period of tick-transmitted anaplasmosis. The possibility that anaplasmosis had been transmitted biologically by ticks rather than mechanically by tabanids therefore has to be kept in mind even though no ticks had been noticed on Ox 188, and the donor was almost certainly a carrier of anaplasmosis. The same arguments apply to erythrocytic stages of a *Theileria* sp., presumably *T. mutans*, which appeared in blood smears three months later.

The attempt to infect Ox 9160 with besnoitiosis from Cow 934 failed despite the presence of cyst organisms in some of the smears prepared from the donor's bite wounds which indicated that cysts had been punctured. In this instance four *A. nigromaculatus* bit the donor in fourteen and the recipient in ten different sites during the seven undelayed transfer feeds in four feeding sessions over a 6-day period. This animal had been placed in the abovementioned paddock 22 days before the first attempt. Three days after the last feed, viz. 31 days after transfer to the paddock, *B. theileri* was seen in its blood smears for one day only. Once again the possibility that it was tick-transmitted cannot be excluded. The *B. theileri* parasitaemia was accompanied by a mild febrile reaction which reached a maximum of 104·6°F. Erythrocytic forms of a *Theileria* sp. appeared 59 days after the animal had been placed in the paddock. When challenged with besnoitiosis 96 days after attempted infection the animal developed a pronounced febrile reaction that lasted 6 days and was accompanied by anorexia, lassitude and hyperaemia of the muzzle. Small numbers of SC cysts appeared 38 days after the first rise in temperature.

The last animal listed in Table 9, Ox 138, received 45 alternate bites inflicted by four *A. nigromaculatus*, one *T. denticornis* and one *Haematopota* sp., which had their infective feeds on Bulls 688 and 130, over a period of three consecutive days. Ox 138 was kept under tick-free conditions and did not contract *B. theileri*, *Theileria* sp. or *A. marginale* infections. After an incubation period of 11 days it developed a short-lived febrile reaction which reached a maximum of 104·4°F but was not accompanied by other symptoms. Although no proliferative forms were seen in smears, blood drawn during the reaction was infective to two out of four rabbits. No cysts could be found in the skin and SC of Ox 138. Further proof of successful infection was, however, provided by its immunity.

Flies had less difficulty in penetrating the comparatively soft and pliable skins of the recipient cattle than the thick, hard, hyperkeratotic skins of the donors. The cattle all gave definite signs of marked discomfort such as jerking of the skin itself and the head and neck, flicking of the tail and kicking when flies punctured the skin.

Longevity of B. besnoiti *in tabanids as determined by feeding*

The object of this study was to determine how long flies remained infective for mechanical transmission purposes, and whether cyclical development of *B. besnoiti* and transmission via the proboscis could possibly occur in them. Bull 688 served as donor in 12 instances; Cow 934 and Bull 1061 each in one instance.

Unfortunately both rabbits, on which flies were fed 30 minutes after biting the donor, died from other causes. However, two of the other twelve attempts were successful. The first (Rabbit 3219) received 30 bites from five *A. nigromaculatus*

and one *T. taeniola* about 3 hours after potentially infective feeds on Bull 688 (Table 10). It developed typical symptoms and both proliferative forms and cysts were demonstrable. A second rabbit (3249) exposed to 17 bites from five *A. nigromaculatus* flies after a 3 hour delay failed to react and was susceptible to challenge. However, suspensions of heads as well as abdominal organs of some of these flies were infective to rabbits. This indicated that mechanical transmission was not a *sine qua non* of penetration of cysts and contamination of the mouthparts and intestinal tract with viable organisms.

TABLE 10.—*Longevity of cyst organisms in tabanids as determined by feeding on rabbits*

Time interval (hours)	Case No.	Number tabanids fed	Result
3	1	6	+
3	2	5	0
1 × 24	3	2	+
1 × 24	4	1	0
1 × 24	5	3	0
1 × 24	6	2	0
4 × 24	7	1	0
5 × 24	8	2	0
6 × 24	9	1	0
7 × 24	10	1	0
12 × 24	11	1	0
24 × 24	12	1	0

Legend: + = transmission 0 = no transmission

One rabbit (2895) exposed to bites by two *T. denticornis* 24 hours after potentially infective feeds also contracted besnoitiosis, developing severe anasarca of the scrotum, prepuce and face. Both proliferative forms and cysts were demonstrated, the latter being very plentiful in the lip and nose. The balance of the rabbits exposed 1, 2, 4, 5, 6, 7, 12 and 24 days after potentially infective feeds failed to become infected in spite of the presence of several flies among them known to have been involved in transmission by undelayed transfer feeding 1, 2 and 7 days previously.

To recapitulate, there was evidence of short-lived survival of *B. besnoiti* in the flies tested, but not of cyclical transmission by the " anterior station ". These results showed good correlation with those obtained by subinoculation (see below).

Longevity of B. besnoiti *in tabanids as determined by subinoculation*

This method to determine the longevity of cyst organisms in flies was much less laborious than the previous one. The results are summarized in Table 11.

Suspensions prepared from the heads of six and four *A. nigromaculatus* three and four hours after they had fed on chronically infected Bull 688, were infective to Rabbits 3230 and 3234 respectively. They developed typical besnoitiosis symptoms and proliferative forms were found in both. Rabbit 3230 died from severe besnoitiosis but Rabbit 3234 survived and cysts were very numerous in the nasal skin. Another rabbit injected with the heads of four tabanids triturated four hours post feeding failed to react in spite of the fact that the abdominal organs of the flies were infective. The same applied to two attempts made 24 hours after feeding with two and five flies respectively. The three rabbits were susceptible to the challenge.

TABLE 11.—*Longevity of cyst organisms in tabanids as determined by subinoculation into rabbits*

Portion of flies	Time interval (hours)	Rabbit recipient No.	Tabanid flies		Reaction				Immunity Test			
			Species	Number triturated	Prolifer-ative organisms	Skin cysts	Symptoms	Fate	Time after last infection (days)	Strain	Passage level	Result
Heads.....	3	3230	*A. nigromaculatus.*	6	+	n.e.	+	D	—	—	—	—
	4	3221	*A. nigromaculatus.*	4	0	n.e.	0	S	67	Schoeman..	52	Susceptible
	4	3234	*A. nigromaculatus.*	4	+	+	+	S	—	—	—	—
	1 × 24	3259	*A. nigromaculatus.*	2	0	n.e.	0	S	43	Fuls.....	267	Susceptible
	1 × 24	3284	*A. nigromaculatus.*	5	0	n.e.	0	S	43	Schoeman..	55	Susceptible
Abdominal organs	0·33	2890	*A. nigromaculatus.*	1	+	n.e.	+	D	—	—	—	—
	3	3251	*A. nigromaculatus.*	1	+	n.e.	+	D	—	—	—	—
	4	3220	*A. nigromaculatus.*	3	+	+	0	S	—	—	—	—
			T. taeniola.....	1								
	4	3233	*A. nigromaculatus.*	4	+	n.e.	+	D	—	—	—	—
	4	3229	*A. nigromaculatus.*	2	+	+	0	S	—	—	—	—
	1 × 24	3258	*A. nigromaculatus.*	1	+	+	+	S	—	—	—	—
	1 × 29	3192	*T. denticornis*....	1	0	+	0	S	46	Fuls.....	267	No reaction
			A. nigromaculatus.	1								
	2 × 24	2945	*T. denticornis*....	1	0	n.e.	0	S	44	Fuls.....	234	Susceptible
Whole flies.....	3	2966	*A. nigromaculatus.*	1	0	+	0	S	30	Fuls.....	235	No reaction
	1 × 24	2946	*A. nigromaculatus.*	2	+	+	+	S	—	—	—	—
	1 × 24	2951	*A. nigromaculatus.*	1	+	+	0	S	—	—	—	—
	2 × 24	3164	*A. nigromaculatus.*	2	0	n.e.	0	S	43	Fuls.....	257	Susceptible
	2 × 24	3169	*A. nigromaculatus.*	2	0	n.e.	0	S	32	Fuls.....	257	Susceptible
			T. denticornis....	1								
	3 × 24	3169	*A. nigromaculatus.*	1	0	n.e.	0	S	32	Fuls.....	257	Susceptible
	14 × 24	2926	*T. denticornis*....	2	0	n.e.	0	S	41	Fuls.....	232	Susceptible

Legend: See Table 5

Cyst organisms apparently remained infective for longer periods in the gut of tabanids. Suspensions prepared from the abdominal organs of one to four flies 20 minutes, 3, 4, 24 and 29 hours after they had fed were all infective to the seven rabbits that were inoculated (Table 11). By 48 hours, however, infectivity had ceased. With the exception of Rabbit 3192, where Bull 130 was used, Bull 688 served as donor throughout. The guts of a total number of thirteen *A. nigromaculatus*, one *T. taeniola* and two *T. denticornis* flies were triturated. In three instances a single fly had been used, which clearly illustrates the ease with which tabanids penetrated cysts and ingested cyst organisms, and is further proof of their excellent vector-potential. Four of the seven rabbits that reacted, developed typical besnoitiosis symptoms. Proliferative forms were seen in six and cysts in four that survived the acute form of the disease. Again cysts were more plentiful in the nasal skin than the lip. Rabbit 3192 (29 hours) in which only cysts were seen was also immune to challenge, whereas Rabbit 2945 (48 hours) that failed to react was susceptible.

Suspensions from whole flies were infective when prepared at three and 24 hours, but not at two, three and 14 days after feeding on the donors, Bulls 688 and 130 and Cow 934 (Table 11). In this experiment a total number of nine *A. nigromaculatus* and three *T. denticornis*, in two instances once again a single fly, were triturated. Only one of the three rabbits that reacted developed typical besnoitiosis symptoms. Proliferative forms were seen in two and cysts in comparable numbers, in the lip and nose in all three of them. Rabbit 2966, in which no proliferative forms could be found, was immune to challenge, whereas Rabbits 3164, 3169 and 2926, that failed to react, were susceptible.

Smears of the suspensions of abdominal organs and whole flies revealed the presence of cyst organisms [Plate 1 (1)] for as long as 24 hours after potentially infective feeds, but not thereafter. With the odd exception when they were quite plentiful, as in an *A. nigromaculatus* inoculated into Rabbit 2926 for instance, cyst forms were rather scarce in the suspensions. Smears were also made from the teased-out mouthparts, salivary glands, proventriculus, crop, midgut, malphigian tubules and hindgut of two *A. nigromaculatus*, of which one was inoculated into Rabbit 2890 within 20 minutes of feeding. In both, cyst forms were plentiful in the blood-filled midgut, in which clumps of organisms were sometimes seen. They were also numerous in smears made from the crop which contained a clear yellowish fluid, presumably sugar water. In one fly a few organisms were present in the mouthparts and hindgut; they were, however, absent in these organs in the second. The other organs were negative in both.

The phenomenon of the presence of organisms in the crop especially, where they were particularly plentiful, was not investigated any further. In the absence of even a trace of blood in this organ, the obvious possibility of it being an artefact of the dissection process must be considered. The only other explanation is that blood passes into the crop during feeding as it does in tsetse flies (Buxton, 1955), from where it is transferred *in toto* to the midgut within minutes of completion of the blood meal. Some cyst organisms may, however, be retained in the crop, as are trypanosomes in tsetse flies. The writer was unable to obtain any information on this point from the literature. Various developmental stages of flagellates were also encountered in some of these smears. The " barley corn " forms were differentiated from cyst forms on the presence of a kinetoplast (see also below). The suspensions were infective to rabbits in two cases (Rabbits 2951 and 3192) where no cyst organisms could be found, but in no instance did the reverse situation apply.

TABLE 12.—*Attempted mechanical transmission of proliferative organisms from rabbits to rabbits by tabanid flies*

Rabbit donor			Rabbit recipient No.	Date of infection	Tabanid flies			Reaction					Immunity Test			
No.	Strain	Passage level			Species	Total number bites donor	Total number transfers	Incubation period (days)	Proliferative organisms	Skin cysts	Symptoms	Fate	Time after infection (days)	Strain	Passage level	Result
2869	Schoeman..	26	2911	23.1.61	*A. nigromaculatus**	5	5	—	0	n.e.	0	S	35	Fuls.....	231	Susceptible
2901	Fuls.....	226	2916	25.1.61	*T. denticornis*†...	5	5	—	0	n.e.	0	S	33	Fuls.....	231	Susceptible
			2920	26.1.61	*A. nigromaculatus*† *T. denticornis*†...	10	10	—	0	n.e.	0	S	35	Schoeman..	29	Susceptible
2946	Bull 688...	0	2961	13.3.61	*A. nigromaculatus*†	12	12	—	0	n.e.	0	S	38	Fuls.....	235	Susceptible

Legend: *Fed on oedematous scrotum of donor †Fed on ears of donor Also see Table 5

It is therefore evident that the maximum survival time of *B. besnoiti* cyst organisms in these flies was 29 hours. There is of course no direct proof that flies that were not infective had definitely imbibed cyst forms during feeds. However, the fact that 80 per cent of the rabbits inoculated with tabanids triturated within 29 hours of potentially infective feeds reacted, and the complete lack of infectivity of those triturated thereafter makes the above conclusion a valid one. Furthermore, three flies among the latter had actually participated in successful mechanical transmission experiments.

3. *Attempted mechanical transmission of proliferative forms to rabbits*

Although they experienced from five to twelve undelayed transfer feeds not one of the four recipient rabbits became infected and they were all susceptible when challenged (Table 12). Proliferative forms were found on only one occasion in smears prepared from the bite wounds but smear examination revealed the customary low-grade parasitaemia in all the donors. It is therefore probably just unfortunate that mechanical transmission did not take place. Had the experiment been more comprehensive it is not unlikely that the results would have been comparable to those obtained with tsetse flies (Chapter 5).

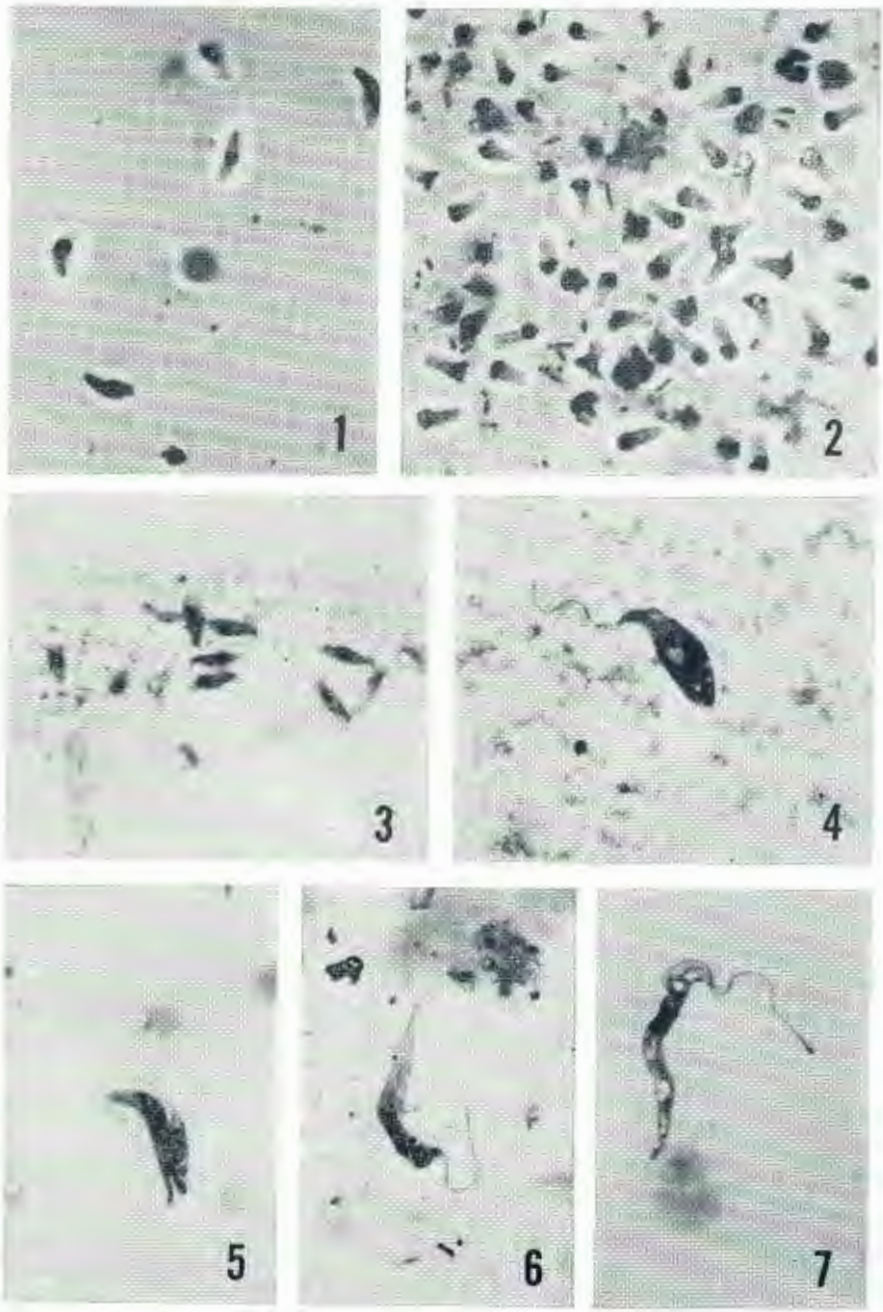
4. *Investigations on flagellates found in tabanids*

Morphology

The most convincing evidence that the flagellates found in the gut of various tabanid species could not possibly be a stage in the life-cycle of *B. besnoiti* came from studies on their morphology. The terminology recently proposed by Hoare & Wallace (1966) for developmental stages of trypanosomatid flagellates is used here.

Flagellates were found in 15 of the *A. nigromaculatus* flies that were screened for infection. The total number examined was not recorded. The most common form encountered in the faeces was the so-called " grain d'orge " (" barley corn ") of Léger (cited by Wallace, 1966) or choanomastigote (Hoare & Wallace, 1966). These were pear-shaped organisms with a kinetoplast usually lying adjacent to the nucleus situated in the wider posterior end, and a short flagellum which sometimes projected slightly from the truncated anterior end [Plate 1 (2)]. They varied from 4·5 to 6·7 microns in length (mean = 5·2) and 1·5 to 3·0 microns in width (mean = 2·1). They could easily be confused with *B. besnoiti* in smears or sections of flies at a cursory glance, since they are rather similar in size and shape. Sometimes the flagellum was longer [Plate 1 (4)] and the chances of confusion therefore smaller. The morphology of cyst forms of *B. besnoiti* has been described in Chapter 4. Bigalke, Van Niekerk, Basson & McCully (1967) recorded their mean size as 8·4 by 1·9 microns. Pols (1960) who described proliferative forms found that they varied from 5 to 9 microns in length and 2 to 5 microns in width. The most obvious distinguishing features between choanomastigote forms and *B. besnoiti* are the absence of the kinetoplast and flagellum in the latter.

Flagellates were also found in smears from suspensions or squash preparations of the guts of 23 of the 74 (29·7 per cent) flies examined. One of them was an *H. albihirta*, three were *T. denticornis* and the rest *A. nigromaculatus*. Since no sections were cut the exact topographical position of the flagellates in the intestinal tract was not determined. Choanomastigote forms were seen in all except one of the flies. They were also more numerous in mixed infections, but epimastigote forms were also encountered [Plate 1 (4)]. Three *A. nigromaculatus* and the *H. albihirta* harboured choanomastigote and epimastigote forms. Two *T. denticornis* contained choanomastigote and the third epimastigote forms only.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Choanomastigote forms were found in flies that, judging from the complete lack of ovarian development and the absence of blood in the gut, had never had a blood meal before suggesting that they were true insect parasites. In two of those containing a mixed infection of choanomastigote and epimastigote forms ovarian development had occurred but this was probably due to a blood meal taken 48 hours previously.

Choanomastigote and epimastigote forms are of course well-known parasites of tabanids, representing developmental stages of *Crithidia* and either *Blastocrithidia* or *Trypanosoma* spp. respectively (see Wallace, 1962, 1966, for a comprehensive review). Epimastigote forms are large, typical flagellates and cannot possibly be confused with cyst or proliferative forms of *B. besnoiti* morphologically.

In no instance were cyst organisms as well as flagellates found in a smear. Some flies that showed flagellates had, however, been fed on chronically infected bovines and had probably ingested some cyst organisms. Furthermore, the flies had all been captured in a camp where chronic cases of besnoitiosis were present, and by some stretch of imagination it could be argued that cyst forms had possibly given rise to the flagellates. Gut suspensions from some recently caught flies that harboured flagellates were therefore injected into animals to see whether they would develop besnoitiosis; the flagellates were tested for viability by cultivation *in vitro* (see below).

Cultivation

Initial attempts to cultivate flagellates from nine flies without the use of antibiotics failed because of contamination with bacteria or moulds. The organisms were still demonstrable after 24 hours in culture but were apparently overwhelmed by the contaminants thereafter. There was usually no contamination when penicillin, streptomycin, neomycin and fungizone were present in the suspending medium, but again flagellates were detected for a short time only because of the trypanocidal effect of the latter antibiotic (see investigations on *T. theileri* below).

With the aid of penicillin, streptomycin and neomycin in the abovementioned concentrations (Materials and Methods), flagellates were isolated from six *A. nigromaculatus* in which choanomastigote forms were demonstrable in the faeces and gut suspensions. In two instances the parasites were passaged seven times in culture over a period of eight weeks.

←—

PLATE 1

1. *B. besnoiti* cyst forms in smear of midgut of *A. nigromaculatus*. × 1200
2. Choanomastigote (= " barleycorn ") forms of a *Crithidia* sp. in smear of hindgut of *A. nigromaculatus*. × 1200
3. Choanomastigote forms with longer flagella in drop of faeces of *A. nigromaculatus*. × 1200
4. Epimastigote in smear of abdominal organs of *T. denticornis*. × 1200
5. Choanomastigote forms of a *Crithidia* sp. isolated in NNN medium from *A. nigromaculatus*. × 1200
6. Epimastigote form that developed in culture after passage of strains isolated from *A. nigromaculatus*. × 1200
7. Epimastigote form of *T. theileri* isolated from Bull 130 and passaged in culture. × 1200

The small choanomastigotes, sometimes lying in rosettes, were the only forms seen in primary isolates [Plate 1 (5)], but in the two passaged strains epimastigote forms [Plate 1 (6)] appeared in spite of the fact that only choanomastigote forms were detectable in the original suspensions. The former may, however, have been present in submicroscopic numbers. In older (14 day) cultures epimastigote forms with kinetoplasts lying immediately behind the nucleus were sometimes observed.

Infectivity studies on rabbits

The only form seen in smears of suspensions was the choanomastigote. The four rabbits (2889, 2926, 2952 and 2953), that were inoculated, failed to show signs of *B. besnoiti* or any other infection and were susceptible to besnoitiosis when challenged.

Infectivity studies on cattle

Injection of cattle served two purposes: Firstly to extend the studies on the relationship of the curious choanomastigote forms with short flagella [Plate 1 (2)] to *B. besnoiti;* and secondly to determine the relationship of the flagellates to *T. theileri.* For this reason two oxen that were respectively immune and susceptible to *B. besnoiti* were chosen.

Ox 9011 inoculated with a gut suspension from an *A. nigromaculatus* that contained choanomastigote forms failed to show any sign of successful infection. No flagellates could be found in blood smears and attempts to isolate them in culture from blood were also fruitless. The animal was immune to *B. besnoiti*, so no besnoitiosis reaction was expected.

Ox 765 inoculated with large numbers of choanomastigote and epimastigote forms grown in culture originating from choanomastigote-containing tabanids, also failed to react. Blood cultures were consistently negative, as was lymph admixed with a little blood aspirated from the regional (prescapular) node 24 hours after injection. The animal was susceptible to subsequent challenge with *B. besnoiti.* Hence, no indications were obtained that tabanids harbouring these flagellates were also carriers of *B. besnoiti.* No attempt was made in this study to relate the flagellates to any of the species of *Crithidia* or *Blastocrithidia* already described from Tabanidae (Wallace, 1962, 1966), and evidence that they might actually be the bovine parasite *T. theileri* was not forthcoming.

5. *Comparative investigations on* T. theileri

Flagellates could only be isolated from one of the eight cattle tested for infection viz. Bull 130. This animal was chronically infected with besnoitiosis, which it had acquired naturally, when purchased three years previously (Table 1) and had been kept in several open paddocks at this Institute. His blood was tested on three occasions over a period of 35 days. It was positive on 13 March and 26 April, but not on 21 March 1962.

The other four animals (Bulls 895, 2035, 2069 and Ox 2068) with naturally acquired, chronic besnoitiosis were negative, as were the two Oxen 9011 and 138, that had been bred at this Institute and infected with besnoitiosis by inoculation with cyst organisms (Bigalke, 1967) and mechanically by tabanids respectively. This also applied to the two Oxen 738 and 739, that had not been exposed to besnoitiosis infection.

Morphology

Typical large epimastigote forms [Plate 1 (7)] were found in smears made from the condensation fluid of young cultures. They were morphologically indistinguishable from those encountered in some tabanid cultures (see cultivation of flagellates found in tabanids above). Usually the kinetoplast lay anterior or lateral, but sometimes also immediately posterior to the nucleus. In older cultures the flagellates had a bizarre bulbous appearance. The strain was passaged in culture for 15 generations. Transparent feathery colonies as described by Nöller (1917) developed along the path of the bacteriological needle in later passages.

The haemoflagellate isolated from Bull 130 was designated *T. theileri* on account of its morphological features in culture and because it is the only trypanosome known to occur in the blood of cattle in these areas.

Influence of antibiotics in vitro

Empirical use of a combination of penicillin, streptomycin, neomycin and fungizone in attempts at the cultivation of flagellates from horse flies indicated that one or more of them might be trypanocidal, because the organisms died (see cultivation of flagellates found in tabanids above). It was therefore decided to test each of the antibiotics individually against the strain of *T. theileri.*

Satisfactory growth occurred at concentrations of 1000, 500 and 250 units of penicillin per ml, and at 1000, 500 and 250 micrograms of streptomycin or neomycin per ml medium. With fungizone, however, no growth occurred in any of the concentrations used. Penicillin, streptomycin and neomycin were therefore subsequently employed in combination at the highest of the abovementioned concentrations to grow flagellates from infected tabanids.

Infectivity studies on cattle

The two Oxen 738 and 739, injected with young and old cultures of the eleventh and fourteenth subcultures respectively of the *T. theileri* strain, failed to show any evidence of infection. No trypanosomes could be found in blood smears, and blood cultured in NNN medium did not show any growth. The reason for this result was not clear, but no further investigations were done along these lines.

Discussion

Following up the positive results obtained with *G. brevipalpis* it was shown that flies of a ubiquitous group like the family Tabanidae are also capable of transmitting *B. besnoiti* mechanically to both rabbits and cattle. The biggest hazard was to obtain these flies in sufficiently large numbers to work with. Placing bait cattle in the vicinity of the trap helped to improve the catches, but flies that were prepared to take a blood meal were never plentiful. Hence they had to be used sparingly to investigate the various parameters relevant to this study. This investigation would have been considerably less laborious and time-consuming, and more comprehensive, had all the tabanids caught been prepared to take a blood meal. The incentive was not determined but it was observed that *T. denticornis* fed more readily than *A. nigromaculatus.* Nieschulz (1930) and Howell, Sanborn, Rozeboom, Stiles & Moe (1941b) in their respective studies on the mechanical transmission of surra and anaplasmosis by tabanids also noticed that species differed in this respect.

Why two such closely related flies with similar sense organs, both capable of maintaining themselves on plant juices and both requiring blood for the maturation of their ova (Gordon & Lavoipierre, 1962) should differ so is obscure. Host preferences and thirst did not appear to play a role. Studies on the nutritional requirements and senses of these flies might open up the pathway to successful breeding of Tabanidae in captivity.

In order to conserve the number of flies, they were sometimes coaxed to bite the bovine donor in several sites before being transferred to the recipient. Thus an *A. nigromaculatus* transmitted the disease to a rabbit by means of only one undelayed transfer feed. This method was not employed with tsetse flies. The smallest number of conventional immediate transfers which resulted in infection of rabbits was six as compared to 15 with tsetse flies. Although observations were not very extensive, it appeared as if tabanids were more suitable for mechanical transmission of cyst forms to rabbits than tsetse flies.

No attempt was made to infect cattle by means of only one fly, but there is no apparent reason why it should not be successful experimentally and in nature if conditions are favourable. The smallest number of transfer feeds that resulted in transmission was 25, again less than the 30 recorded for tsetse flies. Howell *et al.* (1941b) stated that 13 undelayed transfer feeds by *T. oklahomensis* was the smallest number that resulted in the transmission of anaplasmosis from a clinical case to a susceptible bovine, but many more were necessary in most of their experiments. The smallest number of transfers that resulted in transmission from anaplasmosis carriers was 80. The figure obtained in this investigation compares rather well with these.

Rabbits developed typical besnoitiosis reactions as seen after artificial infection or mechanical transmission by tsetse flies. Only one of the three successfully infected cattle showed a fairly severe reaction. But anasarca and scleroderma failed to materialize, even though cysts were eventually not infrequent in the skin. The other two oxen developed a milder form of the disease. The possibility of environmental temperatures influencing the severity of besnoitiosis reactions in cattle is discussed in Chapter 12.

The three oxen exposed to the prevailing climatic conditions in an open paddock became infected with one or more of the known tick-borne pathogens *B. theileri*, *A. marginale* and a *Theileria* sp. Only one of them, however, contracted besnoitiosis. This, together with the fact that the two oxen infected mechanically from the same donor but maintained under tick-free conditions developed besnoitiosis only, argues against the possibility that the latter disease was tick-transmitted. Conversely, it also indicates that organisms other than *B. besnoiti* were not transmitted mechanically, a concept strengthened by calculations based on known prepatent periods of tick-transmitted *B. theileri* and *A. marginale* infections. The most convincing evidence against the possibility of tick-transmission of besnoitiosis is provided by the rabbits that became infected despite adequate precautions against tick infestation.

Flies that penetrated cysts whilst probing for a blood meal did not invariably transmit the infection mechanically. Cyst organisms were for instance found in drops of blood exuding from bite wounds and in midgut smears of flies involved in unsuccessful experiments. Furthermore, triturated heads from tabanids that had failed to transmit besnoitiosis by bite, three hours after potentially infective feeds, were infective, indicating that mechanical transmission could fail even when viable organisms were present somewhere in the upper digestive tract.

Assuming that organisms which are transmitted mechanically are not regurgitated from lower down in the intestinal tract, the most likely site for their adherence in large numbers seems to be the serrated and roughened edges of the mandibles and maxillae. Other structures such as the labrum with its food canal and the hypopharynx may also be implicated. The forces involved in deposition of organisms in the skin are probably basically similar to those mentioned when discussing transmission by tsetse flies (Chapter 5).

The main emphasis of this investigation was on mechanical transmission. It soon became apparent from dissections, however, that flies ingested large numbers of cyst organisms and that there was an excellent opportunity for cyclical development to occur if it were possible. In the longevity studies lack of infectivity was taken to mean non-survival, and imply non-multiplication, with full realization that parasites such as *Trypanosoma* spp. and *Plasmodium* spp., undergoing cyclical development in a vector, are only infective when the cycle has been completed. The absence of organisms other than flagellates in smears from flies kept for periods exceeding 24 hours was accepted as additional proof that no biological development had occurred.

The feeding programme used to study longevity gave comprehensive cover over shorter periods and fairly good coverage over longer periods extending up to 24 days. The maximum period of infectivity was 24 hours. Hence there was no indication of cyclical transmission by the " anterior station ". The subinoculation programme gave good coverage up to 72 hours post-feeding, but then there was a big gap until the final attempt at 14 days. Here the maximum was 29 hours. Good agreement was therefore obtained between longevity as determined by feeding, subinoculation of triturated flies and smear examination. None produced evidence of anything but short-lived survival of *B. besnoiti* in tabanids. It would, however, be advisable to do a more comprehensive study beyond the 72 hour post-feeding period before stating categorically that no cyclical development occurs in tabanids.

Failure to transmit besnoitiosis mechanically from acutely diseased rabbits does not necessarily mean that tabanids are incapable of picking up proliferative forms. In fact there is every reason to believe that they will transmit at any stage of infection provided a sufficiently large number of organisms are present in the skin and/or blood. But for reasons outlined elsewhere acute cases with their transitory bout of parasitaemia are regarded as being of little epidemiological importance.

No shred of evidence could be obtained contrary to the concept that the curious parasites found in the gut of captured tabanids not yet used for experimental purposes, as well as in some that had been used, were anything but flagellates. They had the typical morphological features of the well-documented family Trypanosomatidae and could be cultivated in cell-free media. Susceptible animals inoculated with these organisms showed no sign of besnoitiosis although they must have received infective forms of the parasite concerned. They were apparently transmitted by the " posterior station " since the choanomastigote forms were plentiful in the faeces and absent in the upper alimentary tract. It also seems highly improbable that a flagellate would alter its morphology and physiology so markedly when entering a vertebrate host as to take on the appearance and metabolic requirements of a *Besnoitia*, which is an obligate intracellular parasite.

Wallace (1962, 1966) has reviewed the flagellates found in Tabanidae. He regarded the " barley corn " (= choanomastigote) stage as belonging to the genus *Crithidia* Leger, 1902, which are parasites of arthropods only. Wallace agreed with Laird (1959) that it was incorrect to classify the insect flagellates which have an undulating membrane under the genus *Crithidia*, as had originally been done by Lühe (1906, cited by Wallace, 1966), and which subsequently became standard practice. He suggested that flagellates with undulating membranes found in female tabanids either belonged to the genus *Blastocrithidia* Laird, 1959, or constituted developmental stages of a blood-inhabiting trypanosome, possibly *T. theileri.*

If this concept is correct it would mean that some of the flies in this study were harbouring more than one species of flagellate since choanomastigote and epimastigote forms were present in either mixed or apparently pure infections, judging from smears. In culture, however, some suspensions containing choanomastigote eventually produced epimastigote forms which were identical to those seen in *T. theileri* cultures. Wallace (1962) believes that Nöller (1916, 1917, 1925) probably had the same experience when cultures from *T. glaucopis* and *H. pluvialis* containing " resting stages " (= " barley corn " or choanomastigote forms), which he was convinced were stages in the life-cycle of *T. theileri*, produced epimastigote forms. Wallace suggests that the phenomenon may be due to overgrowth of the *Crithidia* by a cryptic *Blastocrithidia* or *Trypanosoma* infection. It is obvious that the only way to determine whether choanomastigote can give rise to epimastigote forms in culture would be to study clones.

Attempts to infect cattle with choanomastigote forms in triturated tabanids, and epimastigote plus choanomastigote forms of tabanid origin in culture, failed. The work of Nöller (1925) who claimed to have infected cattle with epimastigote and choanomastigote forms, isolated from flies in culture, could therefore not be repeated. Seeing that ventures to transmit a strain of *T. theileri* isolated *in vitro* back to cattle were also unsuccessful, the experiments done to determine whether the tabanids harboured *T. theileri* must be regarded as inconclusive. More experience is needed regarding the infectivity of cultured *T. theileri.* The presence of choanomastigote forms in flies that showed no sign of ever having had a blood meal before is, however, good circumstantial evidence in favour of the viewpoint (Wallace, 1962) that they are not developmental stages of *T. theileri* as stated by Nöller (1925).

It is obvious that further studies are necessary on the biological properties of these flagellates before any attempt be made to identify them specifically. The choanomastigote (*Crithidia* sp.) and epimastigote forms (*Blastocrithidia* sp. or *T. theileri*) found in *T. denticornis* are apparently the first record of flagellates in this host.

7. Mechanical Transmission of *B. besnoiti* by Stable Flies

Introduction

The muscid subfamily Stomoxyinae is another group of blood-sucking flies of which members are wide-spread in S.Afr. Species of the genera *Stomoxys*, *Siphona* and *Haematobia* are well-represented in the enzootic regions. Of these *S. calcitrans* was an obvious choice because it had been noticed to be particularly plentiful in one of the paddocks in which natural transmission of besnoitiosis had occurred (Chapter 4).

Stable flies have been bred successfully at a number of laboratories all over the world (Jones, 1966). It was therefore decided to establish a colony in order to have a continuous supply of large numbers of newly-emerged flies, known to be clean from infection with *B. besnoiti*, available all the time.

Again the main objective was to determine whether stable flies would be capable of penetrating cysts harboured in the skin of chronically infected cattle and transmitting cyst organisms mechanically. Indications of possible cyclical development of the parasites were also sought. Finally an attempt was made to isolate *B. besnoiti* from recently engorged flies caught in an open paddock in which a few chronic cases were housed. A positive result could be regarded as a good indication that flies also penetrated cysts under natural conditions.

Materials and Methods

Stable flies

A colony was established from engorged flies caught at random on the premises of this Institute, using a modification of the method described by Doty (1937). The flies were placed in a large ($\pm 0{\cdot}21$ m^3) breeding cage covered with plastic gauze which was kept in the same room as the tabanids (Chapter 6). The flies were fed twice daily on cotton wool soaked with warmed ($\pm 40^\circ$C) citrated bovine blood obtained from cattle maintained under tick-free conditions. One sheet of cotton wool was placed on top of the cage and another, rolled into a plug, on an approximately 40 cm high platform in a Petri dish. This was placed in a larger dish containing larval medium in which most of the eggs were laid.

The larval medium consisted of a mixture of 47 per cent lucerne hay meal, 47 per cent wheat bran and 6 per cent oat hulls on a weight basis, moistened with tap water. Medium which contained eggs was removed thrice weekly into rectangular glass jars and mixed with fresh medium. The jars were closed with organdie-covered wooden frames and placed in an insectary running at 26°C and 80 per cent relative humidity. Jars with pupae were transferred to a special cage in which the flies emerged. Except for a rectangular window in its roof on which a small, non-return, removable catching cage with a transparent perspex lid was fitted, the emerging cage was made of non-transparent materials. A light directly above the cage served to attract newly-emerged flies into the catching cage. The latter was replaced once or twice daily and the newly-emerged flies returned to the breeding cages.

If flies were required for experimental purposes the catching cage was enclosed in a plastic bag and exposed to a slow trickle of CO_2 for about 10 minutes. The anaesthetized flies were then counted and placed in small (± 375 cm^3) gauze-covered cages in numbers of up to 250. Single flies were restrained in 2×5 cm flat-bottom glass tubes of which the open end was covered with gauze.

Mechanical transmission of cyst organisms by stable flies

Donors: Bulls 688 and 2035 served as donors for the rabbits and Bull 688 for the ox. The history of Bull 688 is given in Table 1. Bull 2035 came from Mara and was a severe chronic case of about 5½ months standing when first used (see Chapter 11 for details).

Transmission to rabbits: The donors were restrained in a crush and the flies fed on convenient sites as described in Chapter 6. Initially flies were fed singly and the actual number of transfer bites counted. Soon, however, when cages containing large numbers of flies were used, the number of transfers of the cage from the donor to the recipient was recorded. The cage was pressed against the donor for a few minutes and then immediately transferred to the ears of the recipient where the process was repeated. In most cases attempted transmission extended over several days (Table 13). The rabbits were examined and challenged as described in Chapter 3.

Transmission to an ox: The recipient, Ox 9703, was $2\frac{3}{4}$ years old. Restraint and preparations for feeding were as described in Chapters 5 and 6. From 75 to 220 flies were used per feeding session of which there were twelve over a period of 28 days. The cage was transferred from the lumbar region of the donor to that of the recipient as described above. The ox was examined for signs of infection.

Longevity of B. besnoiti *in stable flies as determined by feeding:* Flies (950), fed one hour previously on Bull 688 and Rabbits 3144 and 3184, that had been transferred 24 times from the donor to the recipients with a final feed on the former, were allowed to engorge on Rabbit 3183 an hour later.

Longevity of B. besnoiti *in stable flies as determined by subinoculation:* Batches used for mechanical transmission purposes were kept in the insectary used for tsetse flies, etc. (Chapter 5). They were anaesthetized with CO_2 at increasing intervals of time after potentially infective feeds and triturated in saline containing antibiotics (Chapter 6). The resulting red, pink or light brown suspensions, from which smears were made, were injected subcutaneously into rabbits (Table 14). Most of the rabbits received the suspensions over several days. With the exception of the batch injected into Rabbit 3493, which was fed on citrated blood, flies held over for 24 hours and longer were fed on sugar solution. The wastage was, however, considerable. The rabbits were examined and challenged, and the results compared with those obtained with mechanical transmission.

Attempted mechanical transmission of proliferative forms to rabbits

Rabbits 2960 and 2988 artificially infected with the 237th passage-level of the Fuls strain and a strain obtained from Ox 188 (Chapter 6) respectively served as donors. Thirteen flies were fed on the oedematous vulva or swollen upper lip of the donors and transferred 26 times to the recipient, Rabbit 2997. The flies were thereafter triturated and inoculated into Rabbit 2998 as described above.

Isolation of B. besnoiti *from feral stable flies*

During the late summer and early autumn of 1961 and summer of 1966/67, engorged and semi-engorged flies were caught from the walls of a stable standing in the paddock which harboured the cattle in Group 1 (Chapter 4). The majority were collected between 8.00 and 9.00 a.m., others in the late fore-noon and some at 3.30 p.m. They were either anaesthetized with CO_2 or immobilized at 4°C for about an hour, triturated in saline or Hanks' solution and inoculated into rabbits by the subcutaneous route (Table 15). Rabbit 4906 thus infected was exsanguinated at the height of the acute reaction. A suspension was prepared from its testes in ice-cold serum-free Hanks' solution, which was strained through a double layer of gauze. The three-year old Heifer 3088 was inoculated intravenously with 100 ml blood and 12 ml testes suspension to test the virulence of the strain.

Results

1. *Some observations on the breeding of stable flies*

It was noticed that flies fed more readily on blood-soaked sheets of cotton wool placed on top of the cage than on the plugs inside it. Oviposition was largely confined to the larval medium but there were always some eggs attached to the cotton wool as well.

If the water content of the larval medium was too high, fluid accumulated at the bottom of the jars and the larvae refused to work that layer. The optimum water content was just moist through. If very many larvae were present in a jar they collected at the surface of the medium and tried to scale the walls in an attempt to escape from what they regarded as an unfavourable environment. This phenomenon was apparently due to an overpopulation in the flask. The medium was invariably still quite fresh. Replenishment failed to curb their " wanderlust ". Even division into smaller numbers was not effective immediately. It took them about 24 hours to settle down again. Possibly an excess of excretory or secretory products was responsible.

It was necessary to keep the larval jars tightly covered to prevent house and fruit flies from ovipositing in the medium. The latter had a nuisance value only but the vigorous house fly larvae outgrew their stable fly contemporaries and the colony rapidly changed into a *M. domestica* Utopia. Fungi multiplied prolifically, as also unidentified saprophytic mites, but they did not appear to hinder the larvae to any extent.

A large parasitic mite of the family Macrochelidae, provisionally identified as *Macrocheles muscadomesticae* (Scopoli)*, proved to be an important obstacle to the establishment of a vigorous colony. It is a well-known phoretic parasite (Kinn, 1966) of *M. domestica*, but this is apparently the first record of spontaneous predation on *S. calcitrans*. In the laboratory these mites (Kinn, 1966) have been shown to feed on the eggs and first instar larvae of *M. domestica*, and on eggs of *S. calcitrans*.

Although no predation on eggs was noticed, there was a marked drop in the number of larvae in this colony. Mites could be seen moving about in the larval jars but they were never noticed to attack the larger larvae or pupae; first instar larvae were not examined closely enough to exclude the possibility of their predation by mites. When the flies emerged, however, they were often literally covered with mites and the mortality rate among them was appallingly high. The result was a rapidly waning colony that had to be abandoned on two occasions and started afresh. Before doing so the glassware, which was not heat resistant, was washed with an aqueous solution of 4 per cent formaldehyde and the cages painted with xylol. Mites were occasionally noticed on feral stable flies and were apparently introduced into the colony in this way.

* Kindly identified by Dr. P. A. J. Ryke, Institute of Zoological Research, University of Potchefstroom

TABLE 13.—*Mechanical transmission of cyst organisms from cattle to rabbits by stable flies*

Bovine donor No.	Rabbit recipient No.	Date of infection	Number daily feeding sessions	Stable flies			Reaction				Immunity Test			
				Total number flies	Total number transfers	Total number bites recipient	Prolife-rative organisms	Skin cysts	Symp-toms	Fate	Time after last infection (days)	Strain	Passage level	Result
688	2881	7.12.60	1	9	16	16*	0	n.e.	0	S	46	Schoeman.....	26	Susceptible
	2882	8.12.60	1	12	27	27*	0	n.e.	0	S	40	Fuls.....	227	Susceptible
	2883	15.12.60	1	18	37	37*	0	n.e.	0	S	34	Fuls.....	227	Susceptible
	2995	1. 5.61	3	14	21	21*	0	n.e.	0	S	39	Fuls.....	239	Susceptible
	3048	29. 6.61	2	170	12	2,040†	0	n.e.	0	S	46	Schoeman.....	34	Susceptible
	3055	4. 7.61	2	200	12	2,400†	0	n.e.	0	S	39	Schoeman.....	34	Susceptible
	3120	12. 9.61	3	600	37	22,200†	0	n.e.	0	S	40	Fuls.....	251	Susceptible
	3144	18.10.61	5	1,150	76	87,400†	0	n.e.	0	S	49	Schoeman.....	46	Susceptible
	3184	22.11.61	2	600	15	9,000†	0	n.e.	0	S	52	Fuls.....	258	Susceptible
	3273	20. 3.62	9	1,520	98	148,960†	0	n.e.	0	S	35	Fuls.....	261	Susceptible
2035	3402	30. 8.62	13	2,250	130	292,500†	0	+	0	S	—	—	—	—
	3420	1.10.62	9	1,300	70	91,000†	+	n.e.	+	D	—	—	—	—
	3480	3.12.62	6	870	60	52,200†	0	+	0	S	—	—	—	—

Legend: *Flies fed singly; every bite recorded †Flies fed in batches; number of bites = number of flies × number of transfers Also see Table 5

2. *Mechanical transmission of cyst organisms by stable flies*

Transmission to rabbits

This proved to be more difficult than had been anticipated in spite of the fact that flies fed very readily. The laborious technique of feeding flies individually, as had been done with tabanids and tsetse flies, was abandoned after vain attempts to infect the first four rabbits listed in Table 13. It was realized that large numbers of flies would probably have to be used in batches for successful transmission. Even so the whole feeding process usually lasted from 1 to 1½ hours, and after as many as 17 undelayed transfers the majority of the flies were still prepared to feed, which meant that they had not engorged fully. Some flies probably bit the donor and recipient more than once during each transfer whereas others failed to do so. The calculated total number of bites received by each rabbit is therefore only a rough estimate.

Not one of the ten rabbits for which Bull 688 served as donor became infected, even though up to 1520 flies were transferred 98 times delivering an estimated 148,960 bites. Short-lived febrile reactions occurred in two of the rabbits but they were apparently not due to besnoitiosis because no proliferative forms or symptoms were seen and the rabbits were all susceptible to challenge (Table 13).

Large numbers of flies were used from the outset with the other donor, Bull 2035. The smallest number employed was 250 and the largest 2250, which were transferred 60 and 130 times respectively (Table 13). All the rabbits became infected. Since the feeding sessions extended over 10 to 20 days the incubation periods could not be determined accurately. In Rabbits 3402 and 3480 febrile reactions were noticed 14 and 12 days after the first feeding sessions. The temperature was irregular and unreliable in Rabbit 3420, but parasites were detected in blood smears 20 days after the first feed. The thermal reactions reached peaks of 105·2, 104·6 and 105·6°F and lasted 18 and 9 days in the former two rabbits. Proliferative forms and typical symptoms were only seen in Rabbit 3420 that died. Cysts were, however, found in small numbers in the lip and nose of the other two.

These results suggest that the more recent case, Bull 2035, was a better donor for mechanical transmission by stable flies than the long-standing one, Bull 688. This was not because flies failed to penetrate cysts in the latter. Cyst forms were present in smears made from blood welling from bite wounds, and rabbits inoculated with triturated flies became infected (see below), indicating that they had penetrated cysts and ingested organisms.

Transmission to an ox

A total of 2175 flies transferred 167 times in this experiment fed rather well on both donor (Bull 688) and recipient (Ox 9703), causing great discomfort manifested by kicking, sweeping movements of the head and tail and almost continuous twitching of the skin. It was calculated that Ox 9703 received 145,725 bites during the 12 feeding sessions.

TABLE 14.—*Longevity of cyst organisms in stable flies as determined by subinoculation into rabbits*

Time interval (hours)	Bovine donor No.	Rabbit recipient No.	Number stable flies triturated	Reaction				Immunity Test			
				Proliferative organisms	Skin cysts	Symptoms	Fate	Time after last infection (days)	Strain	Passage level	Result
0·25	688	3004	140	+	n.e.	0	D	—	—	—	—
	688	3080	200	+	n.e.	+	D	—	—	—	—
	2035	3403	510	+	+	+	S	—	—	—	—
	2035	3425	450	+	+	+	S	—	—	—	—
0·33	688	3049	100	+	n.e.	0	D	—	—	—	—
	688	3050	70	+	+	+	D	—	—	—	—
	688	3056	200	+	n.e.	+	D	—	—	—	—
	688	3066	435	+	+	+	S	—	—	—	—
0·5	688	2996	13	+	+	+	S	—	—	—	—
	688	3079	140	+	n.e.	0	D	—	—	—	—
1·0	688	3152	400	+	n.e.	0	S	—	—	—	—
3·0	688	3274	670	0	n.e.	0	S	35	Fuls.....	269	Susceptible
1 × 24	688	3081	600	0	n.e.	0	S	35	Fuls.....	247	Susceptible
1 × 24	688	3185	280	0	n.e.	0	S	53	Fuls.....	258	Susceptible
2 × 24	688	3086	400	0	n.e.	0	S	35	Fuls.....	247	Susceptible
4–6 × 24*	688	3151	250	0	n.e.	0	S	35	Fuls.....	254	Susceptible
6–7 × 24	688	3128	90	0	n.e.	0	S	44	Fuls.....	252	Susceptible
1–11 × 24	688	3493	500	0	n.e.	0	S	27	Schoeman	72	Susceptible

Legend: *4–6 × 24 denotes 4 to 6 days elapsed between feeding on donor and trituration

Also see Table 5

Twenty-six days after the first exposure Ox 9703 developed a thermal reaction that lasted five days and reached a maximum of 105·4°F. The animal failed to develop any other signs of ill health and no proliferative forms were detectable. Their presence was, however, revealed when six rabbits inoculated with blood on three consecutive days during the febrile response all developed typical besnoitiosis reactions in which both proliferative forms and cysts were demonstrable. Three cysts were observed in the SC of the left and one in the right eye 39 days after the elevation in temperature. They increased to seven in the left and to two in the right eye a week later. A single cyst was also seen in skin sections 48 days after the rise in temperature.

The ox developed a second bout of fever 36 days after the first exposure to *S. calcitrans* and five days after cessation of the first thermal response. This was due to *B. bigemina* and was treated successfully with acaprin (Bayer). Two days later the temperature was elevated and *B. theileri* and *E. wenyoni* were found in smears. Blood failed to produce besnoitiosis in four rabbits and two sheep and they were susceptible to the usual challenge. The latter were also susceptible to blood-induced heartwater [*Cowdria ruminantium* (Cowdry, 1925)] infection. After treatment with 5 mg per Kg terramycin (Pfizer) the temperature immediately subsided, with disappearance of the abovementioned two parasites to which the reaction was ascribed.

The flies used to infect this animal produced besnoitiosis reactions when triturated and inoculated into rabbits (see below).

Longevity of B. besnoiti *in stable flies as determined by feeding*

Rabbit 3183 on which a batch of flies was fed one hour after a potentially infective feed on Bull 688 failed to contract besnoitiosis. This was not surprising seeing that the same batch had failed to transmit the disease by immediate transfer.

Longevity of B. besnoiti *in stable flies as determined by subinoculation*

The eleven rabbits inoculated with batches of flies triturated from fifteen minutes to one hour after potentially infective feeds all contracted besnoitiosis (Table 14), indicating that the flies still contained infective organisms. In cases where accurate calculations were possible the incubation periods varied from six to thirty-one days. Typical symptoms were seen in seven, proliferative forms in all eleven, and cysts in five of the rabbits. Six died from a severe form of the disease.

Seven rabbits that received flies triturated at 3 hours, 1, 2, 3, 4, 5, 6, 7, 8, 10 and 11 days after potentially infective feeds did not show any sign of successful infection (Table 14), and were susceptible to challenge.

The only protozoan parasites demonstrable in smears of the suspensions of laboratory bred and feral flies (see below) were " trypanosome "(= opisthomastigote) forms of what is probably a *Herpetomonas* sp.

It is interesting to note that in five cases, where mechanical transmission had failed, subinoculation revealed that cysts had in fact been penetrated and organisms ingested. Rabbit 2996, for instance, that developed a typical reaction, was inoculated with only 13 flies 30 minutes after a vain attempt to infect Rabbit 2995 by undelayed transfer feeding. Once the three hour viability " barrier " was reached the situation was, however, reversed when flies, known to have transmitted the disease mechanically, were not infective by injection.

TABLE 15.—*Isolation of* B. besnoiti *from feral stable flies in rabbits*

Rabbit No.	Stable flies			Reaction				Immunity test			
	Date of capture	Number of flies	Dose (ml)	Incubation period (days)	Prolife-rative organisms	Skin Cysts	Symp-toms	Time after last infec-tion (days)	Strain	Passage level	Result
2954	9.3.61	40	5·0	—	0	n.e.	0	34	Schoeman	25	Susceptible
2960	14.3.61	47	4·0	—	0	n.e.	0	38	Fuls.....	235	Susceptible
2970	23.3.61	80	5·0	—	0	n.e.	0	50	Schoeman	27	Susceptible
2974	27.3.61	22	5·0	—	0	n.e.	0	35	Schoeman	27	Susceptible
	7.4.61	22	2·5								
4904	29.12.66	57	5·7	—	0	n.e.	0	39	Fuls.....	534	Susceptible
4905	30.12.66	50	5·0	—	0	n.e.	0	37	Fuls.....	534	Susceptible
4906	31.12.66	130	10·0	32	+	n.e.	+	—	—	—	—
4907	2.1.67	200	20·0	26	+	+	+	—	—	—	—
4925	8.2.67	112	5·0	—	0	n.e.	0	41	Fuls.....	538	Susceptible
4929	14.2.67	177	10·0	—	0	n.e.	0	36	Fuls.....	538	Susceptible
4930	15.2.67	210	10·0	—	0	n.e.	0	47	Fuls.....	539	Susceptible
4937	27.2.67	50	9·0	26	+	+	+	—	—	—	—
	28.2.67	70	10·0								
	2.3.67	66	12·0								

Legend: See Table 5

3. *Attempted mechanical transmission of proliferative forms to rabbits*

The attempt to transmit besnoitiosis by undelayed transfer feeding of thirteen flies failed. The rabbit showed no reaction and was susceptible to the disease when challenged. This was not due to failure to encounter viable organisms since a suspension prepared from the flies 30 minutes later produced besnoitiosis in the recipient rabbit. It developed a typical reaction accompanied by proliferative forms in blood smears twelve days later and died from the disease five days after the first symptoms appeared.

4. *Isolation of* B. besnoiti *from feral stable flies*

The majority of flies had probably engorged on the animals in the paddock where they were caught. These cattle consisted mainly of clinical and inapparent cases of chronic besnoitiosis. There were, however, other cattle close by on which some of them may have fed.

Three of the twelve attempts to isolate the parasite were successful. The rabbits involved had been inoculated with a suspension of 130, 200 and 186 flies respectively (Table 15). In two cases (Rabbits 4906 and 4907) the flies had been caught in the early morning, probably very shortly after engorging, since they are diurnal feeders (Gordon & Lavoipierre, 1962); in the third instance (Rabbit 4937), one to two hours later. The exact interval between feeding and capture could obviously not be determined. It was fairly recent because many of them had greatly distended abdomens full of fresh red blood.

The incubation periods were rather protracted, i.e. 32, 26 and 26 days in Rabbits 4906, 4907 and 4937 respectively, suggesting a low-grade infection (Chapter 13). During the acute febrile stage of the disease all three rabbits developed parasitaemia and typical symptoms. Anasarca was confined to the scrotum in Rabbits 4906 and 4937, but in Rabbit 4907 the prepuce was also involved. In addition the nasal region was swollen and the skin hyperaemic. The scrotum eventually became cyanotic and finally necrotic. Rabbit 4906 was exsanguinated six days after the first rise in temperature which reached 106·4°F. After febrile reactions which lasted eleven and two days and reached peaks of 105 and 104·6°F respectively, Rabbits 4907 and 4937 recovered. Young cysts were plentiful in the skin of the nose collected 19 and 23 days after the first rise in temperature.

The other nine rabbits failed to show signs of besnoitiosis and were susceptible to the challenge. Seven had received less than 100 triturated flies but for the rest the numbers were comparable to those of the three successful attempts.

Since the flies were caught in a paddock harbouring chronic besnoitiosis cases it was reasonable to assume that they became infected from cysts penetrated accidentally. However, the remote possibility that they were harbouring cyclically developing *B. besnoiti* and had contracted the infection elsewhere had to be considered. Hence the pathogenicity of one of the strains for a bovine was also investigated by inoculation of Heifer 3088 with blood and a testes suspension from Rabbit 4906. She developed a fairly pronounced form of besnoitiosis, but not as severe as some cases produced with strains isolated directly from cattle (Chapter 12). Proliferative forms were fairly plentiful in blood smears and the same applied to cysts in the SC and skin. More details on the symptomatology are given in Chapter 12.

Discussion

Stable flies succeeded in transmitting cyst organisms of *B. besnoiti* mechanically to rabbits and an ox under the existing experimental conditions. Compared to tabanids and tsetse flies very large numbers were, however, required and the question arises whether they would be effective vectors in nature. Comparison of the results obtained by immediate transfer feeding with those of subinoculation of the same batches of flies indicates that although stable flies had no difficulty in penetrating cysts and ingesting organisms, they were not very efficient vectors. No rabbits could be infected from Bull 688 for instance, whilst he was concurrently being used for mechanical transmission by tabanids with much success.

The most likely explanation for these discrepancies is that *S. calcitrans* makes smaller, shallower bite wounds than tabanids, rupturing less cysts in the process and liberating fewer organisms to contaminate its mouthparts and transmit the disease. Another possibility is that cysts are penetrated quite readily but the proboscis does not offer suitable sites for the attachment and subsequent dislodgement of a sufficient number of parasites for the fly to be highly infective.

The total number of potentially infective bites experienced by the recipient was calculated on the assumption that every single fly bit the donor as well as the recipient each time the cage was transferred. Except for the four instances where flies were fed individually, these figures are probably not very accurate. They nevertheless give some indication of the number of transfer feeds an animal would have to experience under natural conditions to contract the disease. Thus it was calculated that Ox 9703 experienced 12,144 daily bites. A more realistic figure would probably be a quarter of this, or even less, which could be within the limits of what might occur in nature. At the height of the *S. calcitrans* season cattle may be seen with their legs, in particular, literally covered with these flies and they probably experience several thousands of bites daily. How many of these would be immediate transfer feeds is open to speculation, but it must be a good proportion since flies may continually be seen flying from one animal to another. If a severe case of chronic besnoitiosis happens to be present among a group of susceptible animals at such a time, transmission will most certainly occur sooner or later.

The isolation of *B. besnoiti* from stable flies caught in a paddock containing chronically infected cattle corroborates the above argument. Judging from negative smears of suspensions and the long incubation periods in rabbits the flies could not have contained very many parasites, i.e. there was no indication that they had multiplied in the vectors. In experimental animals the strains behaved like those isolated directly from chronically infected cattle. Hence there was no evidence to suggest that the parasites in wild-caught flies were anything but cyst organisms ingested accidentally whilst feeding on chronic cases shortly before capture.

This is substantiated by the studies on the longevity of *B. besnoiti* in flies infected experimentally. Survival was extremely short. Flies still harboured infective organisms after an hour, but from three hours to the endpoint of the experiment at eleven days they were conspicuous by their absence. The conclusion is drawn that there was no evidence of cyclical development of *B. besnoiti* in *S. calcitrans* under the existing experimental conditions.

Apart from differences in hosts and feeding sites, the fact that cyst organisms are rapidly inactivated or digested probably goes a long way to explain why the infection-rate in feral flies is so much lower than in those fed experimentally and triturated soon afterwards. Stable flies start feeding early on warm summer days and unless they are captured and subinoculated in less than three hours of having engorged the chances that they still harbour viable cyst organisms are slight.

Stable flies have already been proved to be mechanical vectors of surra (Leese, 1909; Mitzmain, 1913; Nieschulz, 1930), nagana (summarized by Zumpt, 1939) and anaplasmosis (Sanders, 1933). Their feeding habits make them ideally suited to mechanical transmission. Compared to tsetse flies they are slow feeders, gradually forcing the rather blunt labium into the skin with the aid of the labellar teeth and much pressure resulting in a crescendo of pain. It stands to reason that they are likely to be interrupted in the process.

From the experimental evidence it can safely be concluded that *S. calcitrans* plays a role in the mechanical transmission of *B. besnoiti* in nature. If the severity of the disease depends mainly on the magnitude of the infective inoculum, as is postulated in Chapter 12, very large numbers of flies will probably be required to produce a typical clinical case. But they should have no difficulty in producing milder cases. They are regarded as the most likely vectors of the cattle in Groups 1, 3 and 6 that became inapparently infected under conditions of cohabitation (Chapter 4). Other members of the subfamily Stomoxyinae may prove to be even more important and efficient vectors than their better known relation *S. calcitrans*.

Laboratory-bred stable flies had this advantage over tabanids that large numbers known to be free from *B. besnoiti* infection were available. Although some flies used to initiate the colony had been caught in a paddock where chronic cases of besnoitiosis were present, the flies were bred through several generations before they were used for transmission purposes. In contradistinction to the position in ticks, no protozoan parasites are known to pass through the eggs of insects. These experiments did not give any indications that *B. besnoiti* might be an exception to this rule.

The appearance of *B. bigemina* and *B. theileri* in the ox infected with *B. besnoiti* mechanically does not necessarily mean that they were also transmitted by stable flies. Despite the apparent absence of ticks, the possibility of the animal having picked up a few in the process of being transferred to and from the crush in which the feeding was done cannot be excluded with absolute certainty. The arguments put forward in Chapter 6 against the possibility that benositiosis was transmitted by such ticks also hold here.

8. Ingestion of *B. besnoiti* by Mosquitoes

Introduction

The flies used hitherto to prove that bovine besnoitiosis has a great potential for being transmitted mechanically from chronically infected carriers were all well-equipped for this purpose. Not only were they diurnal feeders so that they could be seen but they also had most painful bites. This would elicit a violent response resulting in the required interruption of the feeding process.

Female mosquitoes also suck blood. The majority are, however, nocturnal feeders and their bites not sufficiently painful to attract much attention. From an anthropomorphic point of view their singing is infinitely more formidable. They are, however, very plentiful at this Institute and in some parts of the enzootic regions, and may play a role in mechanical transmission even if only on account of their numbers. They have been incriminated as mechanical vectors of other diseases such as surra (Kelser, 1927; Nieschulz, 1930) and anaplasmosis (Howell, Stiles & Moe, 1941a). Very large numbers were required in both instances and hence they were not regarded as being particularly important vectors in nature.

Actual mechanical transmission of besnoitiosis by means of mosquitoes was not attempted. This study was only concerned with their ability to penetrate cysts and the longevity of the imbibed organisms within them.

Materials and Methods

Mosquitoes

A colony of *Culex simpsoni* Theobald*, was established as follows: Four unidentified culicine mosquito females caught in a light trap were placed in the same type of cage ($\pm 0{\cdot}21$ m^3) used for stable flies in the basement of the insectary (Chapter 7). They were allowed to feed overnight on a shaven guinea pig restrained in a small cage made of stout 1 cm mesh wire. Six days later a single egg raft laid in a dish of water placed inside the cage was used to start the colony.

The method used for breeding larvae was based on that described by Swart & Reinecke (1962) for the propagation of *Bulinus tropicus* Krauss in small aquaria. The egg raft was placed in a plastic dish into which tap water dripped via a thin rubber tube from a reservoir raised above the level of the tray. The tray had an outlet connected to a drain by rubber tubing of identical bore. The outflow was regulated to equal the inflow by means of thumb screws and the depth maintained at about 4 to 6 cm. Escape of larvae was prevented by guarding the outlet with an organdie-covered perspex barricade reinforced with clean, coarse river sand which permitted unhindered flow. Larvae were limited to about 200 per tray. As the colony grew the number of dishes was increased. The larvae were fed daily on powdered milk with a low fat-content (Semilko-Nestlé).

Pupae were transferred to a dish in the mosquito cage from which the adults emerged and in which eggs were laid. The adults were fed on a cotton wool plug soaked with sugar solution. If eggs were required the plug was removed for 24 hours and the females allowed to feed on a guinea pig overnight as described above.

When mosquitoes were required for transmission studies pupae were placed in a separate cage and a sufficient number of adults subsequently aspirated and anaesthetized for release in a small (± 375 cm^3) gauze-covered cage.

Unidentified *Culex* spp. caught in a light trap were also used occasionally.

Longevity of B. besnoiti *in mosquitoes*

In a preliminary trial five trapped mosquitoes, starved for 24 hours, were fed individually on chronically infected Bull 2035 (Chapter 7). They were anaesthetized with CO_2 15 minutes later, triturated in saline containing antibiotics (Chapter 6) and the resulting suspension injected subcutaneously into a rabbit. A second rabbit received a suspension of a batch of 20 mosquitoes fed on Bull 688 and triturated 20 minutes later (Table 16).

* Kindly identified by Dr. R. du Toit, Veterinary Research Institute, Onderstepoort

These observations were extended with laboratory-bred *C. simpsoni*. Two small cages containing approximately 200 mosquitoes each were secured to the carefully clipped loins of Bull 2035 with the aid of a large piece of cloth into which two windows just large enough to accommodate the cages were cut. The cloth was stuck onto the hair by means of Unnas paste (Milks, 1949) and the cages tied down tightly with tapes attached to the sides of each window (Plate 2). The host was tied up short to prevent him from dislodging the cages.

PLATE 2.—Attachment of cages containing mosquitoes to loins of Bull 2035, chronically infected with besnoitiosis

The cages were attached at 6.00 pm and removed at 8.00 am the next morning. Batches of engorged and semi-engorged mosquitoes were kept in an insectary running at 26°C and 80 per cent relative humidity and fed on sugar solution. They were anaesthetized and triturated at increasing intervals of time and inoculated into rabbits (Table 16) that received from two to four batches each. Smears prepared from the suspensions were examined as usual, and rabbits that failed to react were challenged.

Ingestion of proliferative forms by mosquitoes

Two batches of sixteen and eight trapped mosquitoes were fed on the scrotum and ears of Rabbit 2897 infected with the 29th passage-level of the Schoeman strain. They were triturated immediately thereafter and each batch inoculated into a rabbit as described above.

TABLE 16.—*Longevity of cyst organisms in mosquitoes as determined by subinoculation into rabbits*

Time interval (hours)	Bovine donor No.	Rabbit recipient No.	Number mosquitoes triturated	Reaction				Immunity test			
				Prolife-rative organisms	Skin Cysts	Symp-toms	Fate	Time after last infection (days)	Strain	Passage level	Result
0·25	2035	3646	5*	0	n.e.	0	D†	—	—	—	—
0·33	688	2984	20*	+	n.e.	+	D	—	—	—	—
2·0	2035	3592	188**	+	+	+	S	—	—	—	—
24	2035	3609	450**	0	n.e.	0	S	53	Fuls.....	283	Susceptible
50	2035	3610	250**	+	+	+	S	—	—	—	—
74	2035	3649	70**	0	n.e.	0	S	30	Fuls.....	284	Susceptible
122–146	2035	3671	100**	0	n.e.	0	D‡	—	—	—	—

Legend: *Trapped unidentified *Culex* sp.
**Laboratory bred *C. simpsoni*
†Died of unknown cause 52 days after injection
‡Died of unknown cause 25 days after first injection
Also see Table 5.

Results

1. *Some observations on the mosquitoes*

Laboratory-bred mosquitoes were preferred to trapped ones because they were known to be free from *B. besnoiti* infection. A single egg raft was used to establish the colony to ensure that the strain was pure. The running water used in the larval pans prevented a surface film from being formed. The larvae grew well under the existing conditions and completed their development in 11 to 15 days provided they were not overpopulated. In contradistinction to trapped *Culex* spp. that usually fed fairly readily the laboratory-bred mosquitoes were not very keen to engorge while being handled, and fed rather poorly overnight. No attempt was made to transmit the disease mechanically.

2. *Longevity of* B. besnoiti *in mosquitoes*

The preliminary investigation indicated that mosquitoes were quite capable of penetrating the cysts in the bovine donor (Table 16). The suspension of 20 mosquitoes fed on Bull 688 produced a severe besnoitiosis reaction in Rabbit 2984 after an incubation period of 15 days. Apart from microscopically detectable parasitaemia, the animal developed severe anasarca and died from the acute disease a day after the rise in temperature. The suspension of five mosquitoes fed on Bull 2035 apparently failed to infect, for although Rabbit 3646 was not challenged it showed no sign of besnoitiosis over 52 days of close observation.

Since the laboratory-bred *C. simpsoni* mosquitoes were left on the donor overnight the exact time-interval between engorgement and trituration could not be determined. Consequently the time when the cages were removed from the donor was taken as zero hour. In actual fact the time-intervals may have been anything up to 14 hours longer than those recorded in Table 16. There was considerable wastage among the mosquitoes as time passed, hence the marked differences in their numbers.

The results obtained were somewhat erratic. The incubation periods could not be determined accurately in the two rabbits that became infected because they received the suspensions over several days. It was at least 18 days in Rabbit 3592 and 15 days in Rabbit 3610, inoculated two and fifty hours after potentially infective onvernight feeds respectively. Both developed typical symptoms. Proliferative forms were seen in smears and cysts in the skin of the lip and nose. Hence it was strange that Rabbit 3609 inoculated with three different batches totalling up to 450 mosquitoes which had fed 26 hours previously failed to react and was fully susceptible to the challenge. Rabbit 3649 injected at the 74 hour level also failed to contract the disease. No reaction was seen in Rabbit 3671 during the 25 days that it was kept under observation before it died from another cause. As it had been inoculated with four batches over a period of 13 days, the final one only 12 days before death, this negative result at the 122 to 146 hour (five to six d ys) level cannot be regarded as conclusive.

Nothing resembling *B. besnoiti* could be found in smears made from suspensions of triturated mosquitoes. Schizonts and spores of an unidentified microsporidian parasite, and opisthomastigote and other forms of what is probably a *Herpetomonas* sp. were, however, present in all the smeais made from *C. simpsoni.*

3. *Ingestion of proliferative forms by mosquitoes*

Rabbits 2935 and 2937 inoculated with suspensions of mosquitoes that had fed on rabbits with acute besnoitiosis both contracted a severe form of the disease. They developed typical anasarca which was accompanied by proliferative forms in blood smears, and cysts could subsequently be demonstrated in sections of the lip of Rabbit 2935 only and those of the nose of both rabbits.

Some of the mosquitoes that had been fed on the oedematous scrotum were light yellow in colour after engorging, indicating that they had imbibed the serum-like fluid.

Discussion

This experiment has indicated that even mosquitoes are capable of penetrating the relatively thick, resilient walls of *Besnoitia* cysts and ingesting organisms whilst they are probing for a blood meal. If they are disturbed in the process it is quite conceivable that they will resume feeding on a neighbouring animal and transmit the disease as was the case with all the other flies tested hitherto. Seeing that their mouthparts are basically similar to those of tabanids, though much less robust, they may even prove to be more efficient mechanical vectors than stable flies. The writer is of opinion that their importance should not be underestimated (Chapter 14).

Although no signs of cyclical development could be obtained, the longevity studies were not very comprehensive and should be extended over longer periods before stating categorically that it does not occur. It is difficult to explain why 450 mosquitoes held over for 26 hours were not infective, when viable organisms were still present in 250 mosquitoes 50 hours after potentially infective feeds. Perhaps the explanation lies in the element of chance involved in penetrating a sufficient number of cysts to ingest the large numbers of organisms probably required to remain infective for longer periods.

9. Artificial Transmission of *B. besnoiti* by non-parenteral Routes

Introduction

Every conceivable method that might be involved had to be considered in these attempts to elucidate the mode of transmission of bovine besnoitiosis. The likelihood of insect transmission has been investigated. The possibility of venereal transmission was excluded on circumstantial evidence obtained from the experiment recorded in Chapter 4. Most obvious of the remaining possibilities were the digestive and respiratory tracts, and eye.

This investigation has already presented a considerable amount of evidence in favour of the concept that chronic cases of besnoitiosis serve as reservoirs of the disease. So the main emphasis here as elsewhere was on the transmissibility of cyst forms of the parasite; the oral, nasal and conjunctival routes were employed. Some attention was, however, also paid to the infectiousness of proliferative forms by these routes.

Since *Besnoitia jellisoni* Frenkel, 1953, (Jellison, Fullerton & Parker, 1956) and *Toxoplasma gondii* (Nicolle & Manceaux, 1908) (reviewed by Jacobs, 1956) are transmissible by the oral route, it was considered that the closely related parasite *B. besnoiti* should be able to do likewise, despite claims to the contrary by previous workers (Besnoit & Robin, 1912; Franco & Borges, 1916 cited by Pols, 1960; Cuillé & Chelle, 1937a; Pols, 1960).

Initially cattle and hamsters were used for this purpose. At the time (1959) it was not yet known that hamsters were susceptible to parenteral infection (Neuman & Nobel, 1963) with cyst and proliferative forms of *B. besnoiti*, with the result that this possibility was investigated prior to oral infection in both instances. Frenkel (1953, 1965) has shown that the hamster is a satisfactory host for *B. jellisoni*.

Hamsters proved to be susceptible but were not as good models as rabbits for investigating the bovine parasite. Rabbits were therefore used in studies on the transmissibility of cyst forms via the nostrils and conjunctiva, and cattle were exposed to proliferative and cyst forms by mouth and cyst organisms via the nostrils. Their susceptibility to proliferative (Pols, 1954a, 1960) and cyst forms (Bigalke, 1960, 1967) by inoculation has been established. Brief reference has already been made to the results obtained (Bigalke, 1966a, 1966b).

Materials and Methods

1. *Transmission to hamsters*

Proliferative forms parenterally: A hamster was injected subcutaneously with 4 ml blood of a rabbit harbouring the 160th passage-level of the Fuls strain. Nine days later it was killed and a suspension of viscera in normal saline inoculated into a rabbit, and when it reacted blood was injected intraperitoneally into further hamsters. By intraperitoneal subinoculation of 0·5 to 2 ml of blood and viscera suspensions the strain was passaged in hamsters, with a reverse rabbit passage at the fourth level. Most passages were carried out at six to eleven day intervals. From the fifth passage some hamsters received cortisone acetate or prednisolone intramuscularly a day or two after infection in doses varying from 8·3 to 25 mg. Viscera and blood from a hamster of the sixth passage-level was injected intravenously into Ox 8092.

A second group was inoculated intraperitoneally with 1·5 ml rabbit blood of the seventh passage-level of the Lamprechts A strain (Chapter 2), and with cortisone as above.

Proliferative forms orally: Four cortisonized hamsters of the seventh hamster passage-level of the Fuls strain were killed *in extremis* on consecutive days. Their carcasses were opened and four hamsters starved overnight allowed to feed on the viscera. A second group was fed on the carcasses of five hamsters injected with the Lamprechts A strain. A third group was fed on testes and viscera of a rabbit of the fifth passage-level of the Schoeman strain. Some of the hamsters in each group received cortisone.

Cyst forms parenterally: Cysts were dissected from the skin of a chronic bovine case (Ox Lamprechts—see Table 1, Bigalke, 1967), crushed in normal saline containing antibiotics (Chapter 6) and 0·1 ml injected intraperitoneally into each of three hamsters which were cortisonized 24 hours later.

Cyst forms orally: Three pieces of skin about 1 cm in diameter, removed by biopsy from Bull 130 (Table 1), were cut into smaller pieces and fed to four starved hamsters, three of which received cortisone 24 hours later.

Hamsters were examined for signs of successful infection as described in Chapter 3. Rabbit inoculation tests were conducted with blood and a suspension of viscera collected within two weeks of infection in all except one of the above-mentioned groups.

2. *Transmission of cyst forms to rabbits via the eyes and nostrils*

A suspension was prepared in normal saline from a piece of biopsied skin from Bull 130, strained through gauze, and 0·75 ml slowly dripped into the conjunctival sac of a rabbit.

A similar suspension was prepared in Hanks' solution from skin of Bull 3329, a severe case obtained from the Warmbad district of Transvaal in 1964, a few months after it had contracted besnoitiosis. The cyst forms were counted and 1 ml dripped onto the eyes of two rabbits. The same dose was instilled into the nostrils of two rabbits held on their backs to facilitate the operation.

On both occasions rabbits were inoculated subcutaneously with the same doses to determine the toxicity of the suspensions. Subsequently rabbits were examined, biological tests conducted if necessary and finally challenged.

3. *Transmission to cattle*

Proliferative forms orally: The viscera of two cortisonized rabbits harbouring the seventh and first passage levels of the Lamprechts A and B strains respectively (Chapter 2), were removed at the height of the reactions. They were cut up into smaller pieces and rubbed vigorously onto the glossal and oral mucous membranes of the three-year old Ox 8576 and two-year old black Friesian-cross, Ox 9607. Finally the animals were forced to swallow the remaining pieces.

Cyst forms orally: Skin taken by biopsy from Bull 130 was cut into narrow strips and ground in a small volume of normal saline in a mortar with a pestle thereby liberating cyst organisms into suspension. Pieces of partially crushed skin were wrapped in gauze, soaked in the suspension and dosed to the yearlings Ox 205 and Heifer 204 as described above.

Cyst forms via nostrils: A suspension was prepared in Hanks' solution from skin of Bull 3329, strained through gauze, and the cyst forms counted. The 2½-year old Ox 3204 was tranquilized with 25 mg acetylpromazine (Boots) and 6 ml of the suspension administered slowly via each nostril. The three-year old Ox 2924 was infected similarly.

The cattle were examined for signs of infection and challenged if none materialized.

4. *Smears from the nasal mucous membranes of chronic cases*

Smears were prepared from cotton wool swabs introduced into the nostrils of six chronically infected cattle, taking care not to damage the surface of the epithelium. The smears were stained and examined. The animals involved were all natural cases. Their respective histories are given elsewhere: Bull 3329—Chapter 9; Bull 2035—Chapters 7 and 11; Bull 2068, Bull 2073 and Ox 2069—Chapter 11; Ox 3865—Table 1, Bigalke, 1967.

Results

1. *Transmission to hamsters*

Proliferative forms parenterally

Hamsters were susceptible to parenteral infection with both the highly passaged Fuls and the recently isolated Lamprechts A strains of *B. besnoiti* (Table 17). Although their distribution in the viscera was similar, proliferative forms were much more numerous in cortisonized than non-cortisonized hamsters. They were found in only four (15·4 per cent) of the latter 26 animals injected. In negative cases it was usually possible to reveal their presence by rabbit inoculation. Extra- and intracellular proliferative forms were, however, found in large numbers in smears from 20 (91 per cent) of the 22 cortisonized hamsters in one or more of the following sites: peritoneum, spleen, liver, lungs, kidneys and blood; they occurred to a lesser extent in the adrenal, brain and testes. Some hypertrophied macrophages contained up to 60 organisms in their cytoplasm. All told, proliferative organisms were therefore observed in 24 (50 per cent) of the 48 hamsters.

The respective mortality rates provided further evidence that proliferation was augmented by cortisone. Thirteen (59·1 per cent) of the 22 cortisonized hamsters died or were killed *in extremis* from the fourth to eleventh days after injection. Only one (3·8 per cent) of the 26 untreated animals was killed *in extremis*, and none died. Six died from undetermined causes which may or may not have been related to besnoitiosis, because no parasites could be demonstrated in them.

Furthermore, hamsters injected with cortisone developed severe symptoms from one to two weeks after infection. Apart from inappetence, listlessness, weakness, diarrhoea and conjunctivitis manifested by " gumming up " of the eyes, some of them developed severe nervous symptoms. A couple held their heads askance. The majority showed either partial or complete paralysis (Plate 3) and invariably died. No nervous symptoms were noticed in untreated animals; the one killed *in extremis* showed listlessness and diarrhoea, the rest nothing.

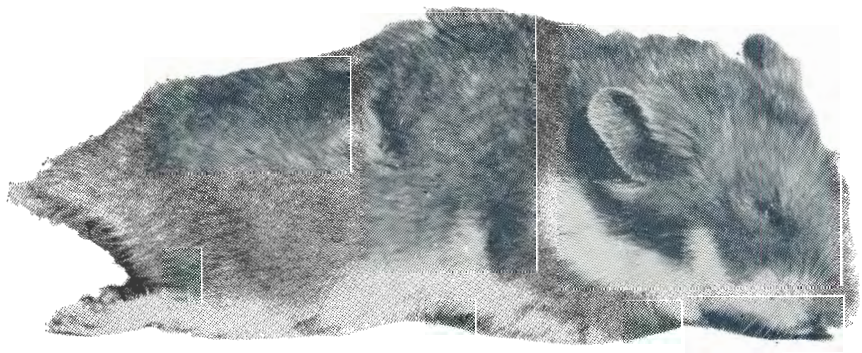

PLATE 3.—Cortisonized hamster infected with *B. besnoiti* showing complete paralysis

Infection took place by both the intraperitoneal and subcutaneous routes. The spectrum of organs harbouring proliferative forms was also identical. No cysts could be demonstrated in the skin of four hamsters that were examined two (1), three (2) and four (1) weeks after injection.

Ox 8092 injected intravenously with 1 ml blood and 3 ml of a suspension of viscera developed a febrile reaction 10 days later lasting 10 days. Although no proliferative forms could be found in blood smears, their presence was revealed by rabbit inoculation. The reaction was of the mild type usually seen in cattle and there was no indication that the virulence of the parasite had been enhanced.

The Fuls strain was lost after the ninth passage (j in Table 17) when viscera from two hamsters injected 15 days previously were subinoculated. No parasites could be found in the recipients nor in those of a second blind passage eight days later, despite cortisone treatment. A rabbit inoculation test with blood and viscera from the last group was also negative, whereas rabbits injected from the first and fifth groups became infected.

The Lamprechts A strain was not passaged in hamsters.

TABLE 17.—*Transmission of proliferative forms to hamsters*

Route	Group No.	Strain	Proliferative forms in viscera			Rabbit inoculation test
			+ Cortisone	− Cortisone	Total	
Parenteral...	1a*	Fuls.....	0	0/1	0/1	+
	b		0	0/3	0/3	+†
	c		0	1/4‡	1/4	n.c.
	d		0	0/4	0/4	n.c.
	e		0	1/4	1/4	+
	f		3/3	1/3	4/6	n.c.**
	g		3/3	0/3	3/6	n.c.
	h		5/6	0	5/6	n.c.
	i		2/2	1/2	3/4	n.c.
	j		1/2	0/2	1/4	n.c.
	2	Lamprechts A.....	6/6	0	6/6	+
			20/22	4/26	24/48	
Per os.....	3	Fuls.....	0/2	0/2	0/4	+
	4	Lamprechts A.....	1/4	0	1/4	+
	5	Schoeman.....	0/1	0/4	0/5	0
			1/7	0/6	1/13	

Legend: *a–j = serial passages
†no parasites seen, only pyrexia and monocytosis
‡1/4 denotes 1 hamster positive out of 4 injected
**Ox 8092 reacted
n.c. = not conducted

Proliferative forms orally

Two of the three attempts were successful (Table 17).

The abovementioned symptoms were absent in members of the group fed on hamster viscera containing numerous proliferative forms of the Fuls strain, irrespective of whether or not cortisone had been administered. Smears of the viscera of the three hamsters killed 7 (1) and 13 (2) days after exposure were negative for proliferative forms, but a rabbit inoculation test revealed their presence. The skin of the fourth animal killed at 21 days was negative for cysts.

The group that ingested viscera containing numerous proliferative forms of the Lamprechts A strain also contracted rather mild infections in spite of injection of cortisone. Proliferative forms were found in lung and heartblood smears of one of the two hamsters killed ten days after exposure, and a rabbit inoculation test was positive. None were, however, demonstrable in two killed 11 and 12 days after infection despite the fact that one showed the abovementioned symptoms including paralysis.

The third group fed on viscera of a Schoeman strain infected rabbit, of which only the testes showed severe parasitosis, failed to become infected. No proliferative forms were found in smears of the viscera when the hamsters were killed nine and ten days after exposure and rabbit inoculation tests were also negative.

Cyst forms parenterally

The two cortisonized hamsters inoculated with cyst organisms died from besnoitiosis on the seventh and eighth days after infection. Large numbers of extra- and intracellular proliferative forms were found in smears of the peritoneum, spleen, liver, lungs, kidneys and adrenals. Although the animals were obviously ill and very weak prior to death, no nervous symptoms occurred. The third animal died from pyometra 13 days after injection. No parasites could be demonstrated in the viscera (Table 18).

TABLE 18.—*Transmission of cyst forms to hamsters*

Route	Group No.	Donor	Proliferative forms in viscera			Rabbit inoculation test
			+ Cortisone	— Cortisone	Total	
Parenteral.	1	Ox (Lamprechts)	2/2‡	0/1	2/3	n.c.
Per os....	2	Bull 130.....	1/3	0/1	1/4	+

Legend: See Table 17

Cyst forms orally

Three of the hamsters fed on cyst-bearing bovine skin and injected with cortisone 24 hours later were killed 11 days after exposure. One of them, showing symptoms of diarrhoea and weakness, had proliferative forms in the peritoneum and lungs; the other two were negative. A rabbit inoculated with pooled blood and viscera developed a typical reaction. Proliferative forms and cysts were absent in the fourth hamster when it was killed 17 days after exposure.

2. *Transmission of cyst forms to rabbits by non-parenteral routes*

Rabbits 2662 and 4859, injected as controls for the toxicity of the inocula, died within 24 hours (also see Bigalke, 1967). No evidence of toxaemia was seen in the rabbits that received the same inocula by non-parenteral routes.

TABLE 19.—*Transmission of cyst forms to rabbits by non-parenteral routes*

Route	Bovine donor No.	Rabbit recipient No.	Date of infection	Reaction						Immunity Test			
				Incubation period (days)	Prolife-rative organisms	Skin cysts	Symptoms	Fate	Rabbit inocu-lation	Time after infection	Strain	Passage level	Result
Conjunctival sac...	130	2663	23. 3.60	13	0	+	0	S	1/1*	—	—	—	—
	3329	4860	12.10.66	—	0	n.e.	0	S	n.c.	34	Fuls.....	526	Susceptible
		4861	12.10.66	—	0	n.e.	0	S	n.c.	34	Fuls.....	526	Susceptible
Nostrils.....	3329	4862	12.10.66	10	+	n.e.	0	S	n.c.	34	Fuls.....	526	No reaction
		4863	12.10.66	10	+	n.e.	0	S	1/1	34	Fuls.....	526	No reaction

Legend: *1/1 denotes 1 rabbit reacted out of 1 injected n.c. = not conducted Also see Table 5

Via the eyes

Rabbit 2663, for which Bull 130 served as donor, was the only one that contracted the infection (Table 19). The reaction was very mild. A slight thermal response was noticed 13 days after exposure. It lasted two days, reached 104°F and was accompanied by a monocytosis. No anasarca was noticeable. A rabbit inoculation test revealed the presence of circulating proliferative forms. Small numbers of cysts were detected in the skin of the lip. The number of cyst forms in the original inoculum was not determined. However, in wet preparations many more were visible per microscopic field than in the suspension from Bull 3329, which contained about 455,000 cyst forms per ml, and failed to infect Rabbits 4860 and 4861 when administered by the same route. The latter showed no sign of besnoitiosis and were susceptible to challenge.

Via the nostrils

The same inoculum that failed to infect via the conjunctival sac was infective when administered into the nostrils (Table 19). Rabbits 4862 and 4863 developed mild febrile reactions that lasted six days and reached 106·2 and 104·8°F respectively. Proliferative forms were seen in blood smears and a rabbit inoculation test was positive. Again no anasarca was noticeable. The rabbits made an uneventful recovery and were immune to challenge. They were not examined for cysts.

3. *Transmission to cattle*

Proliferative forms orally

The rabbits used as donors had received 25 mg cortisone acetate intramuscularly on five consecutive days from the day before infection to augment proliferation. Smears of their viscera revealed very large numbers of proliferative forms.

Ox 8576 developed a fairly pronounced febrile reaction eleven days after infection, which lasted five days and reached a ceiling of 105°F (Table 20). Except for some inappetence, the typical symptoms of besnoitiosis were, however, not seen. No proliferative forms were detected in blood smears, but their presence was revealed by inoculation of rabbits. Those injected during the first four days of the reaction died from a severe form of besnoitiosis. But one inoculated on the last day of fever and two injected nine days after the temperature had returned to normal failed to become infected indicating that parasitaemia had ceased. No cysts could be found in the SC or skin sections of Ox 8576. All of this suggests that the animal had experienced a mild infection. Immunity to challenge provided further proof of successful transmission.

The second recipient, Ox 9607, developed a milder thermal reaction after an incubation period of 14 days. Fever rarely exceeded 104°F although it also lasted five days (Table 20). Once again concomitant parasitaemia was only revealed by injection of rabbits on the second and fourth days of fever. Large numbers of cyst organisms were found in blood smears on the 73rd and 80th days after exposure.

TABLE 20.—*Transmission of* B. besnoiti *to cattle by non-p renteral routes*

Route	Form of *B. besnoiti*	Donor No.	Bovine recipient No.	Date of infection	Reaction						Immunity Test			
					Incubation period (days)	Duration fever (days)	Max. temp. °F	Proliferative organisms	Cysts	Rabbit inoculation	Time after infection (days)	Strain	Passage level	Result
Per os..	Proliferative organisms	Rabbit 2469.	8576	18. 9.59	11	5	105	0	0	5/8*	144	Fuls.....	195	No reaction
		Rabbit 2537.	9607	7.12.59	14	5	104·2	0	+	2/2	67	Lamprechts, A.	8	No reaction
	Cyst organisms.	Bull 130.....	205	2. 8.60	0	2	105·4†	0	0	0/4	37	Schoeman.....	18	Susceptible
		Bull 130.....	224	2. 8.60	0	2	103·6†	0	0	0/4	37	Schoeman.....	18	Susceptible
Nostrils.	Cyst organisms.	Bull 3329....	3204	23.11.66	12	9	105	0	+	2/2	—	—	—	—
		Bull 3329....	2924	20.12.66	13	6	105	0	+	2/2	—	—	—	—

Legend: *5/8 denotes 5 rabbits reacted out of 8 injected †Fever due to inoculation of anthrax and blackquarter vaccines Also see Table 5

Smears prepared from jugular blood on the 73rd day did not reveal any parasites and two rabbits inoculated with this blood failed to contract besnoitiosis indicating the absence of parasitaemia. Small numbers of cysts were seen in sections prepared from a biopsy taken from the tip of the tail on the 81st day. The organisms had therefore obviously originated from cysts punctured accidentally in the process of routine preparation of blood smears (see also Chapters 4 and 12). Six cysts were visible in the SC of the left and three in that of the right eye when the animal was examined on the 81st day, and he was immune to challenge.

Cyst forms orally

Wet preparations of skin suspensions used for attempted oral infection revealed large numbers of motile cyst organisms. They were also plentiful in stained smears. However, neither Ox 205 nor Heifer 224 showed any clinical signs that could be ascribed to besnoitiosis. The maximum temperatures illustrated in Table 20 were the peaks of two-day febrile episodes due to annual administration of anthrax and quarter evil vaccines. Rabbit inoculation tests were negative on both days. When both animals were challenged 37 days after exposure they developed typical mild besnoitiosis reactions unaccompanied by skin lesions. Proliferative forms could only be demonstrated by rabbit inoculation. Small numbers of SC cysts appeared 55 and 54 days post-challenge respectively. Cysts were also found in sections of the upper eyelid of Heifer 244. It was therefore concluded that both bovines had failed to contract besnoitiosis when dosed with cyst organisms.

Cyst forms via nostrils

The doses received by the cattle were comparatively small (Chapter 12). It was calculated that Oxen 3204 and 2924 received a total number of approximately $4{\cdot}2 \times 10^6$ (350,000 per ml) and $2{\cdot}22 \times 10^6$ (185,000 per ml) cyst organisms respectively.

Ox 3204 developed a thermal reaction which exceeded 102°F for nine days (Table 20) and reached its peak of 105°F on the sixth day. Fairly marked inappetence accompanied by hyperaemia of the muzzle and skin around the eyes was noticed, but at no stage was there evidence of anasarca or scleroderma. No proliferative forms could be found in smears but its blood was infective to rabbits on the second, third and fifth days of the thermal reaction. A single cyst was visible in the SC of the left eye 43 days after infection. When the animal was slaughtered 57 days later there were three in the right and two in the left eye. About 50 cysts were present in the facial veins and about 20 in the peripheral veins of the limbs. None were noticed in the mucous membranes of the nasal cavities.

Ox 2924 developed a shorter febrile response which reached its peak on the fourth day (Table 20). Pyrexia was accompanied by slight anorexia and hyperaemia of the muzzle and hairless skin around the eyes. Again concomitant parasitaemia was only demonstrable by rabbit inoculation on the second and third days of fever. Two cysts were clearly visible in the SC of the left eye on the 50th day after exposure and a third was found when the animal was slaughtered 36 days later. There were none in the right eye and nasal mucous membrane, but a few were present in the jugular, facial and peripheral veins of the limbs.

4. *Smears from the nasal mucous membranes of chronic cases*

Cysts in the nasal mucosa of severe chronic cases are not situated very deeply and can be seen quite readily with the naked eye. These smears were made with the object of determining whether such cysts ever rupture spontaneously and discharge their contents onto the surface, which might then be responsible for transmission of the disease. To avoid rupturing cysts accidentally the smears were made as carefully as possible from the anterior portion of the nasal cavity close to the nasal orifice.

Ox 3865 was the only animal in which cyst organisms were found, and they were not plentiful. Cysts were very numerous in the region of its nasal orifice and the possibility exists that the parasites originated from cysts ruptured accidentally whilst securing the animal. Despite the precautions taken mild seepage of blood indicative of mechanical injury was noticed. The smears also revealed the presence of large numbers of eosinophils, smaller numbers of neutrophils and some epithelial cells. Smears from the other five animals showed the latter only.

Discussion

This investigation has shown that *B. besnoiti* need not necessarily be inoculated into the tissues to be infective. Syrian hamsters, for instance, were not only susceptible to parenteral infection with proliferative and cyst forms, but also to oral infection. The observations on injected hamsters are largely in agreement with those recorded by Neuman & Nobel (1963), except that the pathogenic effects of the parasites were less severe unless cortisone was used to augment the infection. They recorded an incidence of nervous and other symptoms in about 60 per cent of hamsters, whereas only one out of 34 (2·9 per cent) that had not received cortisone showed ill health in this investigation. No nervous symptoms occurred in the absence of cortisone and proliferative forms were not easy to detect. The parasite was difficult to passage in hamsters. The Fuls strain, that had been passaged 160 times in rabbits, was lost after only nine serial transfers. Had cortisone and a couple of reverse passages into rabbits not been employed, the strain would almost certainly have died out sooner. Brain, which according to Neuman & Nobel (1963) may harbour parasites for as long as a year, was not included amongst the tissues utilized for subinoculation purposes.

A really profuse peritoneal exudate was never seen. Parasites were sometimes quite plentiful in smears made from the peritoneum, peritoneal washings, or the small volume of exudate which was occasionally present. The peritoneal cavity was, however, never consistently rich enough in parasites to warrant the use of cortisonized hamsters as a reliable source of supply of large numbers of organisms.

The actual site where parasites penetrated the tissues of the alimentary tract of hamsters was not determined. If intra- and extracellular organisms are destroyed by the gastric juices, as occurs with *T. gondii* (Jacobs, 1956), it must be assumed that infection occurred higher up in the tract when proliferative forms were ingested. The possibility of penetration posterior to the stomach must, however, be considered in hamsters fed on cyst-bearing skin because the walls of cysts are probably resistant to gastric juices like those of *T. gondii* cysts. The reactions were milder than in the inoculated hamsters, probably because less parasites invaded the tissues.

Hamsters were particularly suitable for studies on the oral transmission of *B. besnoiti* since they fed quite readily on mammalian tissues. However, even with massive parasitaemia and parasitosis they failed to develop anasarca or other signs of skin involvement and cysts could not be demonstrated in the skin. The possibility that cysts are unable to develop due to the absence of a suitable host cell must be considered. The pathogenesis of *B. besnoiti* in hamsters is apparently very different from that in cattle and rabbits. Hence hamsters cannot be regarded as good models for studies on bovine besnoitiosis.

The conjunctival sac of rabbits appeared to be a less reliable route of infection with cyst forms than the nostrils, probably because part of the inoculum inevitably escaped. For reasons outlined above the site of penetration was probably anterior to the stomach seeing that the inoculum consisted of free cyst organisms only. Apart from the possibility of penetration of the conjunctiva, organisms administered into the conjunctival sac may traverse the greater part of the nasal cavity with a larger surface area affording better opportunities of invasion. This applies to an even greater extent to organisms dispensed via the nasal orifice and it is tempting to assume that this delicate vascular membrane was the actual port of entry. However, organisms will eventually reach the digestive tract by both these routes and since infectivity of organisms by the oral route was not studied in rabbits, it remains a supposition only.

The results obtained with cattle were somewhat perplexing. Whereas both bovines given proliferative forms by mouth contracted besnoitiosis, neither of those that received skin suspensions, which must have contained many millions of cyst organisms as well as intact cysts in partially crushed skin, became infected. On the other hand the two head dosed with cyst forms via the nostrils contracted the disease, and although the reactions were not very severe, they were comparable to those encountered in cattle injected with cyst organisms (Bigalke, 1967). From these results it seems reasonable to assume that the latter penetrated the nasal mucous membrane and not lower down in the digestive tract. But this fails to explain the discrepancy between the results obtained with cyst and proliferative forms given orally. There is reason to believe that cyst organisms in skin suspensions are less infective than proliferative forms (Chapter 12). The number of experimental animals was small and further attempts to transmit cyst forms orally may therefore well be successful. It seems unlikely that proliferative forms would have been able to penetrate the keratinized, stratified squamous epithelium of the mouth, but they may have invaded the tissues via small lesions in the mucous membrane.

The most important question raised by this investigation is whether besnoitiosis is transmissible by non-parenteral routes in nature. Strong evidence incriminating chronically infected cattle as a source of infection under natural conditions is presented in Chapters 4 and 11. In order to make a contaminative type of transmission to cattle feasible, a mechanism for the release of viable parasites from such carriers would have to be found. Studies on the distribution of cysts in the body (McCully *et al.*, 1966) indicate that the chances of excretion of cyst organisms originating from ruptured cysts via the faeces or urine are slim. Even if they should be excreted they will be so short-lived that their importance as a source of infection by ingestion would be negligible. Equally remote are the chances that cyst forms liberated by accidental mechanical abrasion of the skin or occasional rupture through the epidermis as reported by Schulz (1960) will be ingested by a sufficient number of cattle

to account for the high incidence of the disease. Besides, this investigation has shown that even when cyst forms are ingested in very large numbers infection does not necessarily follow. The very fact that the incidence of besnoitiosis is extremely low in unweaned calves as compared to older animals (Chapters 10 and 11) is a strong argument against the possibility that suckling is of any importance in the transmission, even though cysts have been detected in the mucous membrane of the teat canal (Giesecke & Tustin, Veterinary Research Institute, Onderstepoort, personal communication, 1967).

This leaves the respiratory and more particularly the nasal mucous membrane as a potential source as well as route of infection. A severe rhinitis associated with the presence of large numbers of parasites starts in the acute and extends well into the chronic stage of the disease. It is possible that proliferative forms may escape from the acutely inflamed mucous membrane and be transferred to a recipient in droplet form to be inhaled or ingested. Once again it is stressed, however, that the acute stage is transitory.

In cattle cysts lie much closer to the surface in the nasal membrane than in the skin. They can be seen since they are covered by a thin transparent layer of pseudo-stratified epithelium only. The possibility of spontaneous or mechanical rupture of cysts by the rough tongue of the beast or some other means is probably not so far-fetched. Schulz (1960) claims to have occasionally seen evidence of rupture onto the surface in sections of the nasal mucosa, and to have found free and phago-cytosed organisms in nasal secretions. Cyst forms were also found in the nasal mucus of one of the animals studied here. The fact that they probably came from cysts ruptured accidentally only serves to emphasize that cysts in this position are very prone to mechanical influences.

Hofmeyr (1945) has postulated that large numbers of organisms may be liberated from nasal mucosa cysts which could be released into water troughs during drinking and infect animals slaking their thirst subsequently. It is, however, very unlikely that organisms will survive in water for even brief periods. Moreover, ingestion, as described above, does not encourage infection. Droplet infection via the upper respiratory tract would be a more plausible alternative.

There are, however, several good reasons why droplet infection, if it does occur at all, is probably not the usual mode of transmission in nature. The observations made in this study, albeit limited, suggest that rupture of cysts in the nasal mucosa is a rarity. Organisms will therefore never be very plentiful in the nasal mucus and their longevity outside the protective cyst wall limited. It is extremely doubtful if the relatively small numbers of organisms that could be disseminated by droplets would be sufficient for the infection of a bovine, even if they were fully viable. If infection does take place the reaction will probably be very mild.

The best argument against transmission by droplets or any other non-parenteral method is an epidemiological one, i.e. the seasonal incidence of the disease. It has been mentioned previously that the majority of clinical cases occur in summer and there is some evidence (Chapter 4) that clinically inapparent cases follow the same pattern. This implies that arthropods are responsible for the transmission. If a

contaminative method were involved one would have expected a random distribution of cases throughout the year, unless a non-biting insect like *M. domestica* acts as a purely mechanical carrier of organisms already liberated. To clarify these questions it will, however, be necessary to allow affected and susceptible animals to cohabit under completely insect-free conditions to see whether or not transmission will occur.

10. A Survey on the Incidence of Bovine Besnoitiosis

Introduction

The number of animals that develop clinical besnoitiosis on farms in the enzootic regions is never very high. This led Pols (1960) to believe that mainly very young and possibly some mature stock might contract clinically inapparent infections with subsequent development of premunity which would protect them from severe forms of the disease in later life. This hypothesis was, however, never substantiated because no known reliable method existed of making a diagnosis in the absence of clinical signs.

Hofmeyr (1945) drew attention to the fact that SC cysts become macroscopically visible in clinical cases. Pols (1960) conducted a limited investigation on their diagnostic value when he examined the SC of fifteen chronic, clinical cases with cysts in skin sections. He found SC cysts in only nine of them. Four had nodules which were not *B. besnoiti* cysts as determined microscopically, and in two there was nothing at all. Apart from visual inspection it would appear that Pols cut sections of portions of the SC (Pols, like Hofmeyr uses the term sclera for what can only be the SC) of the animals concerned. In instances when SC cysts are rare experience has shown that their detection is difficult even with the aid of a stereoscopic microscope. Hence a positive diagnosis by Pols' method would depend on the remote possibility of a section striking a cyst, which makes it unreliable.

The results of a more extensive study conducted along similar lines have been recorded (Bigalke & Naudé, 1962). Contrary to Pols' experience it was found that examination of the SC for cysts was a very reliable diagnostic procedure in chronic clinical cases and that it also revealed the presence of a sizeable proportion of inapparent infections. Out of a group of thirty animals detected as infected by demonstration of SC cysts, twelve (40 per cent) showed typical signs of scleroderma and five (16·7 per cent) had excessive wrinkling of the skin that may have been due to besnoitiosis. The rest were clinically inapparent cases with perfectly normal skins. Cysts were found in serial skin sections of only twenty-three (76·7 per cent) of the whole group. A close correlation existed between the severity of the clinical picture and the number of cysts in the SC and skin. It was therefore concluded that SC examination was a more reliable diagnostic procedure than serial sectioning of skin biopsies taken from the neck, and that the method should prove useful to conduct surveys on the incidence of the disease.

This was put to the test on two farms in the enzootic region of Transvaal (Bigalke & Naudé, 1962). The survey confirmed the reliability of this method in clinical cases. It also showed that the majority of the animals contracted an inapparent form of the disease. More details on the abovementioned survey and full particulars on similar surveys conducted on five other farms are recorded in this chapter.

Materials and Methods

Farms

Seven farms were included in this survey. With the exception of Doornpoort which was situated in the Bankenveld, the farms were situated in the Bushveld and Lowveld of Transvaal where the disease is known to be enzootic (Pols, 1960). The distribution of these farms is illustrated in Fig. 2.

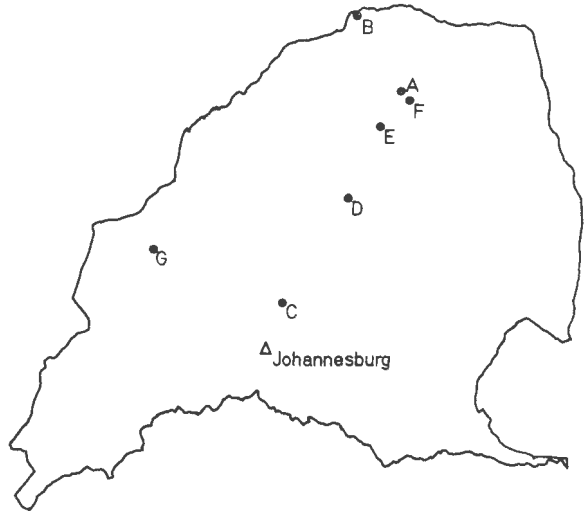

FIG. 2.—Map of the Transvaal province showing the distribution of seven farms surveyed for bovine besnoitiosis. A = Mara, B = Parma, C = Doornpoort, D = Somerset Estate, E = Springforbi, F = Blijdschap, G = Klipkuil

Method of examination

The cattle were restrained in a crush and examined for cysts in the SC of both eyes as described in Chapter 3. When large numbers of cysts were present it was usually not difficult to distinguish them from similar whitish nodules sometimes encountered. With the exception of the studies on Mara and Parma, the identity of doubtful cysts was verified or refuted microscopically, particularly when only one or two were present. Presumed cysts were removed from the eyes of 35 Mara cattle after slaughter and examined likewise. Skin specimens taken by biopsy from some of the animals at Mara and Parma were examined for cysts (Chapter 3).

The animals were also examined for skin lesions. Only unequivocal clinical cases were recorded as such. Animals with what appeared to be excessive wrinkling of the skin were classified as inapparent cases because some cattle, especially Afrikaners, normally have wrinkly skins.

Rainfall figures

These were obtained from the S. Afr. Weather Bureau, Pretoria, or from the farmers themselves if they happened to keep records.

Observations

Mara Agricultural Research Station

This experimental farm is situated in the Soutpansberg district and is about 25,392 acres in size. The cattle were examined in September and October 1960. Since research on the adaptability of certain indigenous and exogenous breeds of cattle and crosses between them to hot climates is being done, a variety of breeds and crosses is present which are divided into groups. The mean annual rainfall is 419·1 mm. The main supply of drinking water for the animals is from bore-holes which feed drinking troughs. Wild life is fairly plentiful and arthropods of veterinary importance abound, as on most Bushveld farms. More details on ecological factors which may be relevant to the epidemiology of besnoitiosis and the full history of the disease are given in Chapter 11. It would appear that the first clinical cases of besnoitiosis were seen on this farm only two years before the survey was conducted.

The survey showed that 114 cattle, representing 4·6 per cent of the total population of 2,471, had SC cysts (Fig. 3). Only 15 (0·6 per cent) of these showed typical symptoms of scleroderma of a lesser or more severe degree, viz. clinical chronic besnoitiosis. The other 99 (4 per cent) had apparently normal skins and would never have been recognized as infected on a purely visual inspection, unless the SC had been examined. Although there was no history of ill health it does not necessarily mean that they had never shown any signs whatsoever. They were referred to as inapparent, subclinical or mild cases (Chapter 2). The ratio between inapparent and clinical cases was 6·1: 1.

A close correlation was observed to exist between the number of SC cysts and the severity of the disease, and this was the experience throughout the survey. With few exceptions clinical cases all had large numbers of cysts in the SC but none of them were without SC cysts. In some of the inapparent cases the cyst numbers were comparable to those seen in clinical cases, and they may well have shown mild skin lesions at an earlier date. Conversely, a few clinical cases had a lesser number of SC cysts than these animals. But in the majority of subclinical cases the numbers were small. Sometimes a single cyst in only one eye was the only indication of infection. Rarely as many as six or seven cysts were visible in one eye whilst the other was negative. In most cases cysts were present in both eyes in roughly equal numbers.

Ninety of the 114 infected animals were cows, representing 7·5 per cent of the total number of 1,195 cows on the farm; 14 (1·2 per cent) were clinical and 76 (6·3 per cent) inapparent cases. Eighteen bulls, constituting 17·5 per cent of the total population of 103, were infected; only one (0·97 per cent) was a clinical case, the rest (16·5 per cent) inapparent—most of the clinical cases had been slaughtered and some had succumbed (Chapter 11). Four (1·3 per cent) of the 300 oxen over two years of age had a clinically inapparent infection; the clinical cases had all been disposed of. Two subclinical cases were also seen among the 8 to 10-month old heifers, representing 0·65 per cent of the total number of 310. No sign of infection was found among the 373 tollies and recently weaned bull calves, and the 190 unweaned calves. The youngest animals detected as infected were in the 8 to 10-month age-group (Chapter 11, Fig. 5).

Parma

The greater part of this farm-complex, which is about 20,314 acres in size, is situated in the Limpopo river valley, in the Soutpansberg district, very close to its confluence with the Shashi river that forms part of the boundary between Rhodesia and Botswana. The farm Cherbourgh, which is about 30 miles inland, also had to be used for grazing purposes as result of the severe drought. Big game is quite plentiful along the Limpopo river. For the rest the fauna, inclusive of arthropods, is similar to that of Mara. This portion of the Lowveld is known as Mopani-veld because of the predominance of this tree [*Colophospermum mopane* (Kirk)]. The annual rainfall on Parma is about 305·8 mm. Boreholes supplying troughs are the main source of drinking water but the river and dams are also used. The herd consists almost exclusively of Afrikaners and bulls run with the cows constantly.

The history of besnoitiosis was not recorded accurately. Apparently the first five (clinical) cases were seen late in 1957 and their number increased to about 20 per annum in the 1959/1960 summer season despite a severe drought. At this stage cases were rather plentiful amongst recently weaned claves (see below). No serious attempt was made by the owners to control the disease.

Only 155 of the approximately 630 head of cattle were examined in September 1960. Groups known to have large numbers of clinical cases among them were usually singled out for examination. The sample was therefore heavily biased towards the clinical side. Hence it was not surprising that 37 (48·1 per cent) of the 77 cases with cysts in the SC or skin sections (one case) showed symptoms of scleroderma. Thus 23·9 per cent (37) of the total number examined were clinical and 25·8 per cent (40) inapparent cases, giving a total infection-rate of 49·7 per cent (Fig. 3). The ratio between inapparent and clinical cases was 1: 1. Forty-five of the 77 cases were cows; 18 were clinically and 27 subclinically infected. In a group of 35 cows with no clinical cases among them, perhaps the only random sample examined, 18 had small numbers of cysts, the rest none. The trend of predomination of inapparent cases seen at Mara therefore also existed in this group. Two of the five infected bulls showed scleroderma. A skin biopsy from a bull with diffuse generalized alopecia but no scleroderma or SC cysts revealed a few cysts in sections. They were, however, so rare that they could not have been responsible for the skin condition. The case was classified as clinically inapparent without SC cysts, the first confirmed in an animal infected in the field (Chapter 4). Ten clinical and five inapparent cases were encountered in a group of 12 to 18-month old heifers that had been running with some severely affected cows. The situation among the tollies was similar where seven of the twelve with SC cysts were clinical cases. The youngest animal detected as infected (subclinically) was an eight-month old unweaned calf.

Doornpoort

This farm is situated on the fringe of the northern suburbs of Pretoria and is fairly close to this Institute. Prior to the survey besnoitiosis had not been known to occur quite so far south in this region. Only the smaller kinds of wild animals occur here, but arthropods are plentiful. The mean annual rainfall is 711·2 mm, and drinking water is supplied by bore-holes and a large dam.

The history was that an Afrikaner bull purchased in June 1958 from Mara had contracted a severe form of the disease which persisted for six weeks and terminated fatally on the day the herd was examined (12 June 1961). It had shown typical symptoms of anasarca and fever initially and subsequently developed a very severe scleroderma. Since this was the first case ever seen on the farm, an excellent opportunity was provided to study a new outbreak.

A total number of 147 animals was examined, 18 Friesian cows, 70 Afrikaner cows and calves, the abovementioned bull and 59 grade Afrikaner oxen. The latter had been purchased 12 months previously in the Soutpansberg district, *inter alia* on the farm Blijdschap referred to below. The other animals had been on the farm for many years.

Twenty-four (16·3 per cent) of the 147 animals had SC cysts. The dead bull was the only clinical case (0·7 per cent) whereas 23 (15·6 per cent) were clinically inapparent infections (Fig. 3). The ratio between inapparent and clinical cases was 23: 1. A significant feature was the high infection-rate in the 59 recently acquired oxen. Nineteen (32·2 per cent) had SC cysts. Some had more than 50 in each eye indicative of quite massive infections, but they did not show signs of scleroderma. On the other hand only four cows (4·6 per cent) out of the 87 animals, that had been on the farm for a long time, were subclinically infected with only small numbers of cysts in each eye. Thus, animals that had been on the farm for many years constituted only 20·8 per cent of the total number infected, and the introduced oxen 79·2 per cent.

Skin sections of the dead bull revealed large numbers of well-developed cysts. They were also visible in the SC.

Somerset Estate

This farm is about 20,000 acres in size, and lies on the northern verge of the "Springbok Flats" in the Potgietersrus district. The wild life and arthropod position is similar to that pertaining at Mara. The approximately 3,000 head of cattle consist of Afrikaners, Herefords and crosses between them, and are divided into various groups. There are summer and winter breeding seasons every year. The rainfall for 1962 was 297·4 mm. Drinking water is supplied from bore-holes.

Besnoitiosis has been a problem on this farm since 1951 when eight Hereford bulls with severe scleroderma were sent to this Institute. Since then the policy has been to sooner or later slaughter all clinical cases. The owner stated that subsequently from three to six cases have occurred annually. The annual incidence remained at this level despite the severe drought during the 1961/62 summer season.

About a third (1,013) of the whole herd was examined in June 1962. The groups were selected more or less at random and therefore constituted a fairly good sample of the whole population. It was found that a total number of 106 (10·5 per cent) of the cattle had SC cysts, of which only three (0·3 per cent) showed signs of a relatively mild scleroderma accompanied by large numbers of cysts in the eyes. The ratio between inapparent and clinical cases was 34·3: 1. Four of the inapparent cases showed more than 50 cysts in each eye. The rest (10·2 per cent) had the typical inapparent form with smaller numbers to very few cysts (Fig. 3).

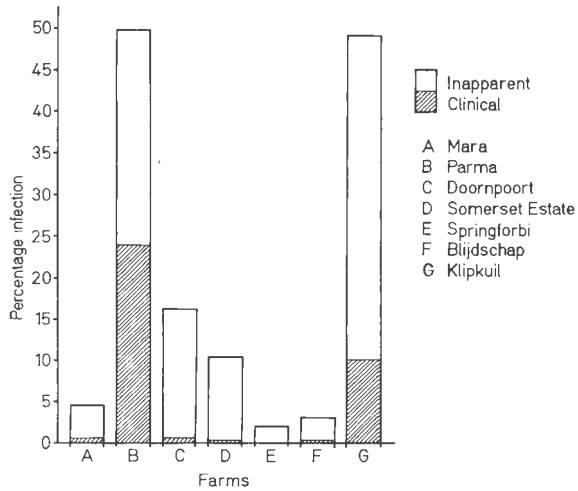

FIG. 3.—Incidence of clinical and inapparent cases of bovine besnoitiosis on seven farms in the Transvaal

The majority of the infected animals were cows, i.e. 94 (18·3 per cent) of the 515 examined. Of these, 91 (17·7 per cent) were subclinical and three (0·6 per cent) clinical cases. Not a single infected bull was encountered. Those that were examined were mainly stall-fed pedigreed young animals, whereas only a few herd bulls were seen. Two (2·5 per cent) of the 81 older steers that were being fattened, six (3·6 per cent) of the 167 tollies and four (2·4 per cent) of the 168 newly weaned 1961/1962 summer calves were inapparently infected. No cysts were observed in any of the 82 unweaned calves. The youngest animals with SC cysts were in the 8 to 10-month age-group.

Springforbi

This farm is about 7,194 acres in size and is situated in the Pietersburg district. The herd consists mainly of South Devons, Bonsmaras and crosses between them. The wild life and arthropod situation is similar to that at Mara. The same applies to the rainfall and water supply.

The first clinical case of besnoitiosis was seen in a bull in 1960. Subsequently the diagnosis was verified in this and six other South Devon bulls by means of skin sections. In 1961 and 1962, using the SC technique which had been demonstrated at Mara, the owner picked out 58 animals with cysts of which four (three cows and a bull) were clinical cases. These animals were isolated and slaughtered when they had put on condition. Since the owner was not always confident about the diagnosis, he sought the writer's assistance.

Only 11 (2·1 per cent) of the 516 animals examined in August 1962 were subclinically infected (Fig. 3). The maximum number of SC cysts seen was about 20 in both eyes, and the smallest, two in one eye. Ten of the animals were cows and one a two-year old heifer. No cysts were observed in the bulls and unweaned calves.

Blijdschap

This farm is situated close to Mara in the Soutpansberg district and is about 8,252 acres in size. The herd consists mainly of Afrikaners, some Bonsmaras, various crosses and a few Brahmans. Wild life, arthropods, the rainfall situation and the source of drinking water are similar to that pertaining at Mara.

The first clinical cases of besnoitiosis were seen in 1960. After having been shown what SC cysts of inapparent cases look like in 1961, the owner examined his whole herd by this method in August 1961 and again in April 1962. He found one clinical and nine inapparent cases at the first, and five of the latter at the second examination.

Seventeen (3·1 per cent) cases were detected by the writer amongst the 557 head of cattle examined in August 1962 (Fig. 3). At least five cases had been picked out previously by the owner and were isolated from the breeding stock, but not disposed of. Fifteen (2·7 per cent) of these were clinically inapparent cases harbouring from one to about 30 cysts in each eye, whereas two (0·4 per cent), a cow and an ox, showed typical scleroderma. The ratio between inapparent and clinical cases was 7·5: 1. Seven of the eight infected cows, six of the seven oxen and two infected bulls were inapparent cases.

Klipkuil

This farm, which is about 18,515 acres in size, is situated in the Rustenburg district and administered by the Department of Bantu-administration and Development. The herd consists mainly of Afrikaners. Klipkuil's fauna is also similar to that encountered at Mara. It's mean annual precipitation is 609·6 mm and the main water supply via boreholes.

It is not known when bovine besnoitiosis first appeared on the farm, but an appreciable number of cases were present in 1959 and their numbers were said to be increasing every year. The situation was alleged to be particularly alarming because a sizeable proportion of valuable purebred Afrikaner bulls used to improve Bantu-owned stock, were contracting the disease and being rendered sterile. Apart from disposing of sterile bulls, no serious attempt was being made to control the disease.

Only 159 of the 398 cattle were examined in February 1963. The object was to find as many infected animals as possible, and only groups that contained clinical cases were studied, with the result that the sample was biased.

Seventy-eight (49·1 per cent) of the 159 animals had SC cysts. Of these 16 (10·1 per cent) were obvious chronic clinical cases. The rest (39 per cent) showed no skin lesions (Fig. 3). Most of the clinical cases contained more than 50 cysts in both eyes. Some exceptions to this rule were, however, encountered. One bull showed mild scleroderma and cysts were quite plentiful in skin sections, but none could be found in the SC. Cysts in sections were large and should therefore have been visible to the naked eye. This is the only clinical case amongst the 74 encountered in the entire investigation in which no SC cysts were visible. Another animal with only four cysts in the left, had about 50 in the right eye.

Of the 43 cows examined, 22 (51·2 per cent) were infected; eight (18·6 per cent) clinically and 14 (32·6 per cent) inapparently. Twenty-eight (59·6 per cent) of the 47 bulls had SC cysts; three (6·4 per cent) were clinical and 25 (53·2 per cent) inapparent cases. The infection-rate was also high amongst the oxen, i.e. 28 (50·9 per cent) of the 55 examined; five of them were clinical and 23 (41·8 per cent) inapparent cases. The only group in which no cysts could be found was the 14 unweaned calves. The ratio between inapparent and clinical cases was 3·9:1.

Discussion

It has already been stated that initial trials had indicated that inspection of the SC for cysts was a useful and reliable method of conducting surveys on the incidence of the disease. This impression has been strengthened by the additional surveys recorded above, if not by comparison of the technique with other diagnostic procedures, then by the magnitude of the results and the relative ease with which they were obtained. It is conceded, however, that the method is not sensitive enough to detect all the infected cattle. Cysts take about eight weeks to become macroscopically visible (Chapter 13), and early subclinical cases will not be diagnosed. Furthermore, the experiment in which cattle were exposed to conditions of cohabitation (Chapter 4) indicated that up to 50 per cent of animals that contract mild besnoitiosis naturally may not develop SC cysts. Some cattle infected artificially also failed to do so. Hence there must be many more inapparent cases on these and other farms in the enzootic regions whose detection will have to await the development of an even better diagnostic method.

If a sufficient number of serial sections are cut through pieces of skin collected by biopsy it might be possible to make a positive diagnosis in many very mild cases with no SC cysts. It is possible that a more reliable site for the detection of cysts in sections may be found than the skin of the neck generally used (Chapter 12). Serial sectioning is, however, such a laborious technique compared to SC examination that it is doubtful if it will replace this more practical and at the moment more reliable method. The close correlation which, with few exceptions, exists between the number of SC cysts, the severity of the disease and the number of cysts in skin sections, show that the semi-transparent SC serves as a convenient window through which the cyst-situation in the skin may be ascertained.

A disadvantage of this method is that it is not possible to scrutinize more than a small sub-division of the entire SC, i.e. the greater part of the dorsal portion. The section under the third eyelid and the ventral segment are hardly visible. The presence of cysts at these sites has been confirmed at autopsy. Eyes vary in their accessibility from beast to beast. The more eyes bulge in their sockets, the greater the expanse of SC that can be seen. Cows of European breeds are easily examined; old Afrikaner bulls with their massive eyebrows are particularly difficult. The amount of light that falls into the eye is very critical. In bad light cases with only a few small cysts may be overlooked entirely. Sometimes cysts are atypical and may be confused with other white or yellowish foci which occasionally occur in the SC, or vice versa. The non-conformity of the latter with cysts needs to be verified microscopically time and again.

The seven farms included in this survey represent a very small proportion of the farming units in the enzootic regions. The 5,018 cattle examined constitute only 0·23 per cent of the estimated total of 2,152,947 (based on June 1965 census figures) head present in the districts of Transvaal where the disease is known to occur. There is, however, good reason to believe that besnoitiosis is present on most farms in this region and that the trend is similar throughout.

The survey provided further evidence (Bigalke & Naudé, 1962) that the majority of cattle contract the inapparent form of the disease. Of the 5,018 head examined, 427 (8·5 per cent) had SC cysts. Only 74 (1·5 per cent) had typical skin lesions. The rest (353 or 7 per cent) showed no clinical sign other than the cysts mentioned. Thus 17·3 per cent of the animals were clinically infected and 82·7 per cent subclinically, giving an overall ratio of 1: 4·8 between the two categories.

Composite figures like the above obscure the fact that there were marked differences between the incidence on individual farms, as a glance at Fig. 3 will show. On two of these the samples were biased towards the clinical and infected side. Had the entire herd been examined the percentage infection would undoubtedly have been lower and the ratio between clinical and inapparent cases larger. This in turn would have accentuated the overall predomination of inapparent over clinical cases.

The policy that each farmer adopts towards besnoitiosis must have a considerable influence on the number of cases on his farm. Since there is as yet no effective treatment or prophylactic vaccine, and because there is good evidence that survivors are carriers, many farmers eliminate the severe cases from their properties. The cumulative totals of clinical cases would therefore tend to be reduced all the time. Some even dispose of their inapparent cases, as on Springforbi and Blijdschap referred to above. The marketing of " healthy " animals would in turn tend to reduce the number of inapparent cases on a farm. Depending on the type of farming practised, cases would be eliminated from certain groups such as steers, whereas they would be retained in others such as breeding stock. On five of the farms this was in fact the case. The two exceptions were Doornpoort, where, as will be argued below, the majority of oxen were already infected when introduced, and Klipkuil which was not a productive unit and where oxen were therefore retained.

The general trend for inapparent to outstrip clinical cases in numbers was evident in all the age-groups. The highest incidence occurred amongst the cows and bulls. Only at Mara was the overall incidence in bulls considerably higher (17·5 as opposed to 7·5 per cent) than in cows. Some stock-owners maintain that bulls are more susceptible than cows. The figures obtained in this survey were, however, too limited to verify or repudiate this belief. The incidence was low in heifers and tollies and very low in unweaned calves where only one inapparent case was encountered, a phenomenon that is difficult to explain (Chapter 14).

The allocation of cases into the two categories of clinical and inapparent, solely on the strength of a visual inspection for the presence or absence of skin lesions, is probably somewhat artificial. It is very likely that at least some of the inapparent cases with fairly large numbers of cysts had previously shown mild anasarca and scleroderma, apart from other symptoms of illness. These signs had probably not been noticed since they were ranch cattle, and being mild the animals soon recovered (cf. Oxen 2431, 3894, 3565 and Heifer 3088—Chapter 12). This means that there is probably a continuous gradation between the two extremes of very severe clinical cases with large numbers of cysts in the usual sites, to very mild inapparent cases in which cysts are so few that they cannot be demonstrated by any of the methods used in this investigation. On the one hand it is therefore possible that the preponderance of inapparent over clinical cases may not be as marked as recorded here. On the other hand the large number of inapparent cases without SC cysts, that must also be present on such farms, would probably more than cancel out the discrepancy.

Because of the besnoitiosis policies adopted by farmers it is obviously not possible to get a reliable impression of the sequence of events if besnoitiosis were allowed to progress unhindered in an area. The situation at Doornpoort is probably a good illustration of what sometimes happens in the very early stages. Being on the fringe of the enzootic area the owner was blissfully unaware of the existence

of such a disease until the first clinical case appeared on the farm. The low incidence (5·7 per cent) in cattle that had been on the farm for a long time as compared to the high incidence (32·2 per cent) in the introduced oxen suggests that the latter were responsible for the importation of besnoitiosis. The fact that they had all been purchased from a well-known enzootic area, some actually from a farm where a survey was made, strengthens the argument.

An opportunity arose recently to conduct a survey on a farm well outside the well-established enzootic region (Bigalke & Schoeman, 1967a). Clinical besnoitiosis had been present for 18 months but had not been recognized as such, so that no active human intervention occurred. The incidence was found to be rather similar to that recorded in this chapter. Thirty-six (10·8 per cent) of the 333 head of cattle examined had SC cysts. Five (1·5 per cent) showed scleroderma. The remaining 31 (9·3 per cent) were inapparent cases. The ratio between the two forms was 1: 6·2.

With the exception of Doornpoort where the epidemiological history could be reconstructed rather clearly, it was not possible to establish exactly when besnoitiosis had first appeared on the farms. The only clue was the appearance of clinical cases and, as indicated above, it is very likely that these were not recognized initially. The writer believes that in most instances, where only one or two carrier animals are introduced, inapparent infections will precede clinical cases by several years (Chapter 14). This would mean that besnoitiosis may have invaded the farms five years or more before the dates mentioned, viz. in the late forties on Somerset Estate and middle fifties on Mara and Parma for instance. Hence there had been sufficient time for the disease to become well-established before the surveys were made.

Another important aspect of the discovery of large numbers of inapparent cases, first envisaged by Pols (1960), is the effect their immune status might have on the epidemiology of the disease. There is good experimental evidence that artificially produced and natural inapparent cases are immune to challenge (Pols, 1960; Bigalke, 1967; Chapters 4, 5, 6 and 7). Although it is conceded that bovine controls did not develop the very severe reactions sometimes seen in the field, it seems reasonable to assume that inapparent cases would be immune to even a severe natural challenge. This would imply that all the inapparent cases in the field, inclusive of those that cannot be detected as yet, are protected from the severe form of the disease, which would account for the relatively small number of clinical cases seen annually on farms in the enzootic regions.

Cysts were seen in most breeds and crosses but the majority of infected animals were Afrikaners. Since they form the bulk of the cattle population in the ranching areas, there is no reason to believe that they are more susceptible than other breeds.

No acute cases of besnoitiosis were encountered during the surveys. This is not surprising because of the short duration of the anasarca as compared to the long scleroderma stage and the virtually exclusive confinement of acute cases to the warmer months of the year instead of the winter when most of the surveys were done.

The nematode *Thelazia rhodesii* (Desmarest) was very common in the eyes of both infected and non-infected animals, i.e. there was no correlation between their presence and besnoitiosis.

11. The Influence of Quarantine Measures on the Incidence of Bovine Besnoitiosis

Introduction

Most of the experimental evidence presented hitherto incriminates chronically infected cattle as carriers of bovine besnoitiosis. It was reasoned that if cattle were the only reservoirs, and the disease transmitted mechanically or contaminatively, the removal of such animals from a farm where the disease is prevalent should result in a reduction in its incidence. Depending on the reliability of the method used for the detection of carriers, and providing there was no contact with infected cattle on neighbouring farms, anything from complete elimination to a noticeable decrease in the incidence could be expected.

This working hypothesis was put to trial on Mara where the disease was becoming so prevalent that it was interfering with the breeding programmes. The method used to detect carriers was examination of the SC for cysts. It is the most reliable method available at present, but as stated previously cannot detect cases before they are about eight weeks old and will not diagnose all the chronic cases present on a farm. Cattle with SC cysts were immediately isolated on a remote corner of the farm and the severely affected ones subsequently slaughtered. Two-and-a-half years later all the isolated animals were slaughtered. Examination of the herd was repeated annually over a period of seven years. These observations are outlined below.

Materials and Methods

Examination of the Mara herd

This experiment was initiated in 1960 when 1,622 of the herd of 2,471 cattle were examined in mid-September and the balance in early October (Chapter 10). With the exception of mid-November of the same year and early February 1961 when a small number of animals that had been in contact with two fresh clinical cases were studied, the herd was examined annually towards the end of winter or early spring.

The method of examination of the SC for cysts has been described in Chapters 3 and 10. Prior to February 1961 when the technique of SC biopsy was first introduced, the diagnosis was verified either by removal of cyst-bearing SC and skin after slaughter at an abattoir, or by skin biopsy, the latter a less reliable procedure.

Quarantine measures

All cattle with SC cysts were removed from the herd within a few days of having been examined. They were isolated in a well-fenced camp on a remote corner of the farm with a buffer zone consisting of a large camp on one and a fenced off road on the other side. All the oxen and the more severely affected cows and bulls—those with more than about 20 cysts in each eye—were slaughtered within a month of their isolation. Weaned calves of the remaining cows were only reintroduced into the herd if they showed no SC cysts after an interval of ten weeks. The isolated animals were also examined annually until they were all slaughtered on 26 March 1963. All clinical cases that developed after the first examination were railed to this Institute as soon as possible after they were recognized.

Rainfall figures

Mara is a S.Afr. Weather Bureau sub-station and accurate records are available. The values illustrated in Fig. 4 represent the annual precipitation in mm from October 1 of the previous year to September 30 of the year concerned.

Observations

Ecological conditions existing at Mara

Ecological conditions regarded as being of possible relevance to the epidemiology of the disease are outlined below.

Mara is situated about 7 miles south of the Soutpansberg mountain range between 23° 3′ and 23° 10′ latitude S and 29° 33′ and 29° 40′ longitude E. It is about 900 m above sea-level. The climate is essentially subtropical. The mean annual rainfall over 23 years is 448 mm, maximum precipitation usually occurring from mid-December to the end of February. The total annual rainfall may vary considerably from year to year as it did during the course of this investigation (Fig. 4).

Mara is about 25,392 acres in size and divided into more than 60 camps by standard 1·37 m, 7-strand barbed-wire fences. It is traversed from south to north by the Sand river, a rather small stream which is fairly consistent during the rainy season, but ceases to flow during winter and long dry spells. Water may, however, still be found at such times by digging into the sandy bed and there is a single pool that contains water constantly. There are no dams. Pans are plentiful but shallow and retain water for short periods only after good rains. The cattle are furnished with drinking water from 24 bore-holes, each of which supplies a number of camps so constructed as to converge at the watering points. From the bore-holes water flows into round concrete drinking troughs. Although there is no direct contact between the groups of cattle from different camps that congregate at such points during the day, they are all in fairly close proximity to each other. Those of a particular group are of course very close to each other at certain times. The cattle of one or more groups are often confined to kraals at central points during the day for weighing, dipping, inoculating or dosing purposes.

The grazing is predominantly sweet and can support approximately 2,500 head of cattle during good seasons but appreciably less during mediocre and bad ones. On the average about 16·8 acres are required per beast. It is typical bushveld country. The dominant tree is the haak-en-steek (*Acacia heteracantha* Burch.), and rooibos (*Combretum apiculatum* Sond.) is also quite plentiful on some parts of the farm.

Various exogenous and indigenous beef breeds are being tested for their adaptability to hot climates. Hence a wide variety of breeds such as Shorthorns, Herefords, Simmentalers, Santa Gertrudis, Bonsmaras, Afrikaners and Ngunis, and various crosses between them are present. A small herd of Jerseys is also kept. The animals have been allocated to a large number of groups. Since pasture rotation is practised they are transferred quite frequently from one camp to another.

There are usually two breeding seasons, viz. from February to April, and August to October when the stud bulls run with the cows. For the rest of the year the bulls rejoin the younger ones, the majority of which are kept in a camp close to the Sand river and a Bantu settlement. Calves are weaned when they are approximately eight months of age and the sexes are then separated from each other. The herd is not closed.

The only other domesticated animals, apart from poultry and pets, were a few horses and mules. In April 1963 and August 1966 76 sheep and 15 goats were respectively introduced. The number of sheep has remained more or less constant but the goats have dwindled to three. They run in camps with the Jersey cattle.

The bushveld is cattle ranching country *par excellence*. Mara is surrounded by farms with similar ecological conditions and the abovementioned fence is the only boundary for the greater part of its circumference. Three fenced-off public roads run through the farm, one of which forms part of its southern boundary. Considerable movement of cattle occurs along these roads and there is an outspan at the Sand river where such animals sometimes pass the night.

Game enjoys protection and is quite plentiful, as it is on many bushveld farms. A herd of impala [*Aepyceros melampus* (Lichtenstein)], estimated at 700 in 1960, increased to 1,000 head in 1967. Kudu [*Tragelaphus strepsiceros* (Pallas)], bushbuck [*Tragelaphus sylvaticus* (Sparrman)] and waterbuck [*Kobus ellipsyprymnus* (Ogilby)] are much less plentiful. Smaller antelopes such as steenbuck [*Raphicerus campestris* (Thunberg)] and duiker [*Sylvicapra grimmia* (L)] as well as warthog [*Phacochoerus aethiopicus* (Pallas)] are fairly numerous. A variety of rodents and smaller carnivores are well-represented. There is also a profuse bird life.

This region with its warm climate is a paradise for arthropods of veterinary importance. Ticks are very plentiful. Control measures are aimed at keeping the population at a level which will ensure continued transmission of pathogens like *B. bigemina*, *B. bovis* and *C. ruminantium* in order to maintain the state of premunity in the cattle without exposing them unduly to the direct deleterious effects of tick parasitism. Ticks are controlled by dipping in Toxaphene, DDT, BHC or Dieldrin, but shortly before the start of this investigation the bulls were switched to an arsenical dip.

Blood-sucking insects are also plentiful. Tabanid flies are seen in considerable numbers as early as September and they are also present at other times of the year. Their larvae are found in the sand of the trickling river which is obviously a breeding site. *H. rufipes*, *S. calcitrans*, mosquitoes and ceratopogonids are present. Another fly said to be very plentiful at times is *Siphona uniseriata* (Malloch) (Stomoxyinae), or "mopanievlieg", a few of which were caught for identification purposes.

Mara lies in an enzootic region and scleroderma cases have been noticed on the majority of neighbouring farms. Similar cases are seen from time to time amongst cattle trekking through the farm.

History of besnoitiosis on Mara

The first clinical cases were recognized early in 1958 when four bulls varying from three to eight years in age contracted the disease. A year later, in January 1959, two bulls and an ox went down and the diagnosis was confirmed histologically. In the following year six bulls, seven cows and an ox became infected during the summer and autumn, the last case being noticed in early June. Most of them were in the scleroderma stage of the disease when first observed, viz. they must have been ill for at least a few weeks. The majority were disposed of shortly after they were seen.

The incidence of besnoitiosis on Mara up to August 1967

These observations are summarized in Fig. 4.

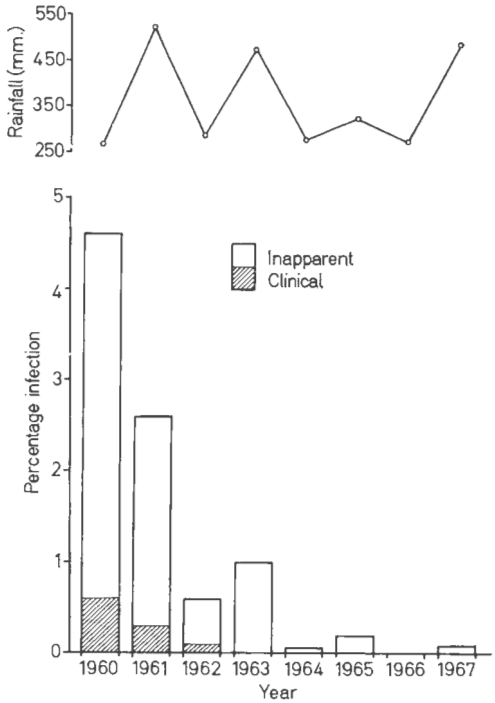

FIG. 4.—Annual incidence of clinical and inapparent cases of bovine besnoitiosis on Mara from 1960 to 1967 as detected by SC examination, together with the annual precipitation over that period

Observations in September and early October 1960

As indicated in Chapter 10 this initial survey revealed that 114 head of cattle or 4·6 per cent of the total population were chronically infected with besnoitiosis, using the presence of SC cysts as criterion. Of these 15 or 0·6 per cent showed typical sclerodermatitis, viz. they could be recognized quite readily by a visual inspection. There was considerable variation in the severity of the skin lesions. The other 99 animals (4 per cent), however, showed no sign of skin involvement, and no symptoms that could be associated with besnoitiosis had been noticed in them. The presence of SC cysts was the only evidence of infection.

A break-down of the abovementioned figures outlining the contributions made by the various sexes and age groups has been made in Chapter 10. It will suffice to mention that 7·5 per cent of the cows, 17·5 per cent of the bulls, 1·3 per cent of the older oxen and 0·65 per cent of the 8 to 10-month old heifers were infected, whereas no sign of infection was seen in the newly weaned bulls and tollies and the unweaned calves.

The age-distribution of 107 cases of which the age at the time cysts were seen could be determined is illustrated in Fig. 5. Cysts remain visible for many years, so these figures may not be an accurate reflection of the age at which cattle are likely to contract the disease and have to be interpreted with care. The highest incidence (44·8 per cent) occurred in the over $4\frac{1}{2}$ to 6 (22·4 per cent) and over 3 to $4\frac{1}{2}$-year (22·4 per cent) age-groups. There were also lesser peaks in the over $10\frac{1}{2}$ to 12 (8·4 per cent) and over 12 to $13\frac{1}{2}$-year (9·3 per cent) levels. The incidence of clinical cases was much lower but appeared to follow the same pattern.

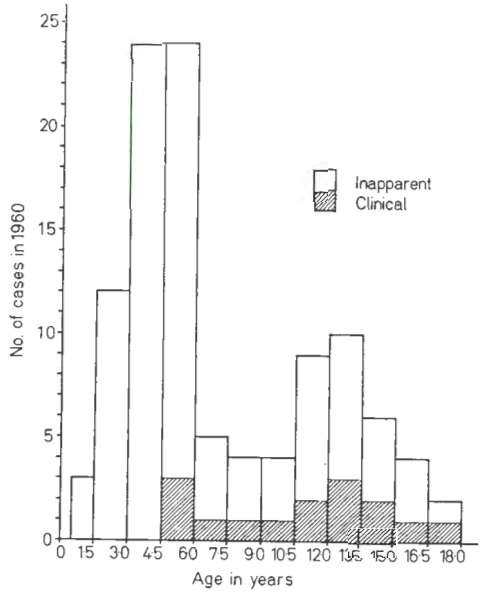

FIG. 5.—The age-distribution of clinical and inapparent cases of bovine besnoitiosis on Mara at the initial examination in 1960

The animals with cysts were isolated as described above and 45 subsequently dispatched to the Pretoria abattoir where they were slaughtered. In 35 of the milder cases the diagnosis was verified microscopically by demonstration of SC cysts, and in nine of these also by histological examination of skin specimens.

There was no indication that breeds varied in their susceptibility. Cysts were seen in the eyes of Afrikaners, Herefords, Shorthorns, Bonsmaras, Ngunis, Jerseys, crosses between them and later also in Simmentalers.

The total rainfall for the year was 363·6 mm.

Observations during the year ending 13 *August* 1961

A 13-year old Hereford cow (Cow 934, Chapter 6) was noticed to be suffering from fairly severe besnoitiosis on 12 November 1960. Numerous SC cysts were visible. She was isolated and railed to this Institute. On arrival seven days later she showed fairly severe scleroderma with sit-fasts over both hip joints. Large numbers of SC cysts were present. Since cysts take approximately eight weeks to become visible it was deduced that the animal was either in the incubation period when examined originally on 15 September or had contracted besnoitiosis soon

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thereafter. The carrier position in 267 cattle in the same and adjacent camps was studied shortly after this case was seen. Two inapparently infected cows were found among the animals in the adjoining paddocks.

On 30 January 1961 a 2½-year old Hereford bull (Bull 1061, Chapter 6) was observed to be suffering from severe, fairly acute besnoitiosis. On arrival at this Institute four days later it showed marked generalized thickening and hardening of the skin, slight oedema of the brisket and necrosis of the ventral portion of the scrotum. No SC cysts were visible and their maximum diameter in skin sections, in which they were not very plentiful at this stage, was 29·8 microns. On the basis of the symptomatology, and the size of skin cysts in comparison to those in systematically studied experimental cases (unpublished), it was estimated that the case was between two and three weeks old on arrival. If an incubation period of thirteen days (Chapter 13) is added it means that the animal contracted the infection in late December or early January. The skin became progressively thicker and harder and deep cracks, from which a serosanguinous fluid oozed, appeared in the breech and along the sides of the body. The necrotic tip of the scrotum was eventually shed completely. There was an irregular intermittent thermal reaction and persistent phlegmosis of the right foreleg. The animal fed very poorly, became progressively more emaciated and was killed *in extremis* on 14 March, viz. about 9½ to 10½ weeks after presumed infection. Minute SC cysts were first seen eight to ten weeks after infection and six to eight weeks after the first symptoms are thought to have developed. At autopsy those in the SC were small and the maximum diameter of 20 cysts measured in skin sections, in which they were now very plentiful, was only 197 microns, i.e. they were obviously immature (Pols, 1960).

This animal had been in a camp bordering on two public roads with 14 other young bulls for a few months when it developed the disease. A group of neighbouring Jerseys and all the bulls on the farm were examined on 6 and 7 February 1961. One mild case was found among the in-contact and ten among the 81 other bulls; none had more than ten cysts in each eye. Most of the Jerseys had curious white foci in the SC which proved not to be cysts—the SC biopsy technique was used for the first time—but one had about eight cysts in both eyes. Two groups of cattle with the largest number of cases during the initial survey were also examined and three inapparent cases with a few cysts were found.

The third clinical case was a 15-year old Hereford cow first noticed to have a thickened skin on 1 March 1961. When examined nine days later at this Institute no unequivocal SC cysts could be seen but they could be detected quite readily in a biopsy specimen by means of the stereoscopic microscope. The animal had therefore apparently contracted the infection less than eight weeks previously, viz. towards the end of January. It had been in camps bordering on public roads for approximately two months prior to showing symptoms.

The SC of a nine-year old Hereford cow, first noticed with scleroderma on 2 March 1961, was examined 14 days later. Nine cysts were visible in the right and three in the left eye. Although this animal, like the previous one, was immediately slaughtered, it is very likely that being a clinical case, cysts had appeared fairly recently and that the full complement was not yet present. This would mean that she had contracted besnoitiosis at about the same time as the previous animal though she had not been in contact with the latter, and had been nowhere near a public road or boundary at this time.

Two more cows had large numbers of SC cysts when scleroderma was first noticed on 12 April 1961, indicating that they had contracted the infection in early February at the latest. Only one of them had been in a boundary camp at the time.

No further clinical cases occurred and the whole herd, which now consisted of 2,135 animals, was examined from 6 to 13 August 1961. The figures mentioned below refer to the total number of cases seen since the first examination of the whole herd and therefore include the inapparent cases detected in November 1960 and February 1961 as well as the abovementioned six clinical cases.

A total number of 56 animals, representing 2·6 per cent of the whole population, were found to be infected (Fig. 4). Six (0·3 per cent) showed typical symptoms as described above. The rest (2·3 per cent) showed nothing but a few SC cysts which in only one case exceeded 12 in either eye. The ratio between inapparent and clinical cases was 8·3: 1. Thirty-three (4·0 per cent) of the 825 cows were infected, of which five (0·6 per cent) were clinical cases. Fourteen (10·6 per cent) of the 132 bulls were affected, one (0·8 per cent) clinically. Only two (0·7 per cent) mild cases were found among the 298 older oxen, four (1·3 per cent) among the 311 12 to 18-month old heifers, and three (1·1 per cent) among the 271 newly weaned 8 to 10-month old calves. The 242 unweaned calves and a group of 56 2-year old heifers showed no sign of infection. Thus cows contributed 1·6, bulls 0·6, older oxen 0·1, 12 to 18-month old heifers 0·2 and 8 to 10-month old calves 0·1 per cent to the total of 2·6 per cent infected.

The age-distribution of 47 of the animals that became infected during the year is illustrated in Fig. 6. Since the approximate date of infection of inapparent cases cannot be calculated the margin of error may be as much as a year for some of them. But this is small compared to the possible error in the cattle examined for the first time in 1960 (Fig. 5). The highest incidence (20, or 42·6 per cent) occurred in the over 3 to 6-year old animals, but the over 6 months to 3-year (nine) and over 6 to 9-year (seven) age-groups also accounted for a fair proportion of the cases. There were six in the over 15 to 18-year age-groups of which one was a clinical case. The oldest animal that became infected was a cow that was 17 years and 10 months old. The clinical cases showed a random distribution.

In all except two animals of the isolated group consisting of 51 head of mildly infected cattle, the number of SC cysts was apparently unchanged. In both no cysts could be detected.

The total rainfall during the current year was 523·5 mm.

Observations during the year ending 15 *August* 1962

No clinical cases were seen until 9 April 1962 when a 39-month old Hereford bull with scleroderma titis and necrosis of the epidermis was noticed and isolated. When seen five weeks later cysts were fairly plentiful, but seeing that the animal had not been examined in the interim, they had probably appeared sooner. The bull had therefore apparently contracted the infection early in March at a time when it was kept in a camp bordering on a public road. A 40-month old Jersey bull that had been running with this animal showed similar symptoms and numerous SC cysts on 30 June. It must therefore have contracted the infection towards the end of April, or the first week in May at the latest.

An aged mule slaughtered on 12 March 1962 was found to be infected with *Besnoitia bennetti* Babudieri, 1931. This animal had been on Mara for 28 years. Although it harboured numerous cysts in the skin, mucous membranes of the lip, nose, pharynx, larynx, the superficial and deep fascia, no scleroderma was apparent. *B. bennetti* is, however, a parasite of equines and not involved in the epidemiology of the bovine disease (Chapter 14).

The herd consisting of 2,100 cattle was examined from 13 to 15 August 1962. Only ten (0·5 per cent) of the animals were subclinically infected. This brought the total number for the year to 12 (0·6 per cent) (Fig. 4). The ratio between inapparent and clinical cases was 5: 1. With two exceptions that showed about 20 and 25 in either eye, the inapparent cases had very few cysts. Seven of the subclinical cases were cows, two were oxen and one a 14-month old heifer.

The age-distribution of 11 of the animals that became infected during the year is illustrated in Fig. 6. Most of the cases (eight) were in the over three to nine-year age-groups but the peak was not as pronounced as in the previous year when many more infected animals were present.

No cysts were seen in the horses and mules.

The cyst-situation in the 48 isolated cattle—three had been disposed of—appeared to be unchanged.

The total rainfall during the current year was 283·1 mm.

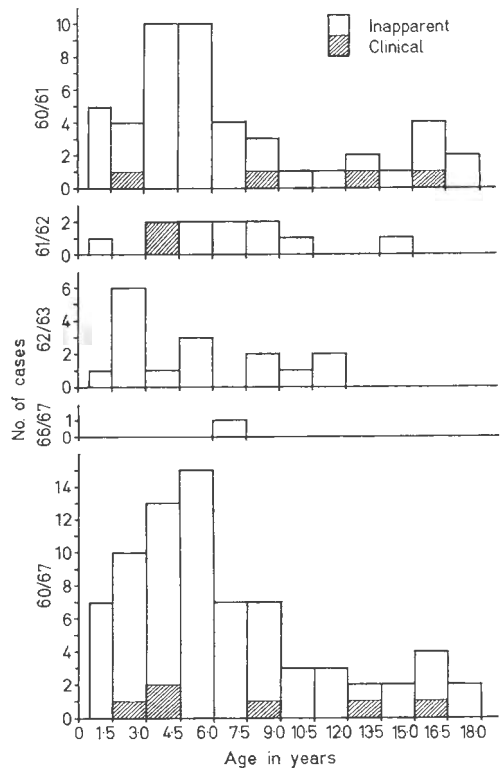

FIG. 6.—The age-distribution of clinical and inapparent cases of bovine besnoitiosis at Mara after the disposal of infected animals in 1960

Observations during the year ending 8 *August* 1963

No clinical cases of besnoitiosis occurred during this year. The herd which had been reduced to 1,610 animals as result of the drought was examined from 4 to 8 August 1963. Sixteen inapparent cases constituting 1·0 per cent of the herd were found (Fig. 4). The infected animals comprised eigth cows, six bulls, one ox and a 20-month old heifer.

It is possibly significant that 11 of the 16 cases occurred among two groups containing only 130 head. Here the infection-rate was 8·5 per cent as compared to the 1·0 per cent of the whole herd. One of the groups consisted of 65 bulls of which the six infected ones were between 2 and 4 years of age and had not been used for breeding purposes. They had all been running in a camp bordering on a Bantu settlement where a considerable number of cattle were present. Four of the bulls had from 10 to 20 cysts per eye, the others less. The second group consisted of 52 cows and 13 calves, of which five cows were infected. One showed about 25 cysts in both eyes, the rest only a few in one or both eyes. Cysts could also be seen quite readily in the nasal mucous membrane, suggesting a fairly severe infection which might have caused noticeable clinical signs at an earlier stage. This group had been running in a camp bordering on a privately owned farm during the current year. Three inapparent cases had been detected in the same group the previous year.

The age-distribution of the 16 animals that became infected during the year can be seen in Fig. 6. This time the highest incidence was in the over 1½ to 6-year old animals (10 cases), to which the above-mentioned six young bulls made the biggest contribution.

The total rainfall during the year was 283·1 mm.

Observations during the year ending 15 *September* 1964

Again no clinical cases of besnoitiosis occurred. Only two cases of doubtful nature were encountered when the whole herd consisting of 1,486 head was examined.* Although not confirmed by biopsy, both were sufficiently atypical to be regarded as negative. One was subsequently slaughtered and in the other no cysts were detected when examined in 1965.

A cow that had been transferred from Mara to Roodeplaat Agricultural Research Station on 8 September 1964 had one cyst when examined on the latter farm on 7 April 1965. Roodeplaat is situated on the perimeter of the enzootic region. Hence the possibility exists that the animal contracted the infection there and not at Mara. As the true source of infection is obscure this case will be included as one at Mara in Fig. 4.

It would thus constitute 0·07 per cent of the population. Because of the element of uncertainty the case has been excluded from Fig. 6.

It was a very dry year, the rainfall totalling only 275·0 mm.

* Due to overseas leave of the writer the herd was examined by Mr. G. J. Davel only

Observations during the year ending 21 *October* 1965

Because of the persistent drought—the total precipitation was 317·8 mm—the cattle population was reduced even further. During the winter months some of the cattle were confined to small paddocks and fed.

There were no clinical cases. The herd consisting of 1,517 animals was examined from 18 to 21 October 1965. Three subclinical cases (0·2 per cent of the population) were found, all among a group of Simmentaler cows introduced from farms near Windhoek, South West Africa in November 1964 (Fig. 4). Due to an oversight they had not been examined for besnoitiosis prior to their introduction. Bovine besnoitiosis is known to occur in Ovamboland, South West Africa (De Boom, 1947, cited by Pols, 1960), and although the disease has not been recorded in the Windhoek region, it may well be that the animals had been infected on the farm of origin. As this is uncertain they have been regarded as having contracted the infection at Mara. Because of this element of doubt, the animals have been excluded from Fig. 6.

Two of the cows were slaughtered at the Pretoria abattoir ten days later. One with about ten cysts in both eyes had fairly large numbers in the facial veins, appreciably less in the veins of the limbs and a few in the nasal mucous membrane. The other with four SC cysts in both eyes had a few in the veins of the legs only.

Observations during the year ending 6 *October* 1966

In this year the rainfall reached its lowest ebb, i.e. 270·5 mm. The grazing became so poor that the cattle population had to be reduced by about a third from November 1965 to February 1966. With the exception of the bulls and dairy animals, the cattle were confined to small paddocks situated more or less in the centre of the farm and fed, often on maintenance rations only.

No animals with SC cysts could be detected when the 910 head were examined from 5 to 6 October 1966 (Fig. 4).

Observations during the year ending 23 *August* 1967

Again no clinical cases of besnoitiosis occurred but a single inapparent case (0·08 per cent of the herd) was detected when the 1,228 head were examined from 21 to 23 August 1967 (Fig. 4). This animal, a seven-year old Afrikaner bull (Fig. 6), with one cyst in the right eye, had been running in a camp with other bulls on the northern border of the farm, close to the Bantu settlement referred to previously, for the greater part of the year. During the breeding season he was used for hand service at the paddocks. No cases were found among the animals that had been confined to the feeding paddocks for the entire year.

The total rainfall for the year was 467·2 mm, viz. well above the mean.

Overall age-distribution of cases detected after the disposal of chronic cases in 1960

A fairly accurate indication of the age at which the animals on Mara were likely to contract besnoitiosis under the existing conditions could be obtained after the herd had been cleared of visibly infected animals in October 1960. It stands to reason that this would be influenced by the presence of undetected inapparent cases that were immune.

It was possible to determine the exact age at the time of detection, which is not synonymous with the time of infection as indicated previously, of 75 of the 89 animals that developed signs of infection after the elimination of chronic cases. The data for each individual year and a composite figure for the whole period of observation are illustrated in Fig. 6. Most of the animals (59 or 78·7 per cent) became infected between the ages of 6 months and 9 years. The highest incidence (15 cases or 20 per cent) occurred in the over $4\frac{1}{2}$ to 6-year age-group. Close behind were the over 3 to $4\frac{1}{2}$ and over $1\frac{1}{2}$ to 3-year groups with 17·3 (13 cases) and 13·3 (10 cases) per cent respectively. The over 6 months to $1\frac{1}{2}$-year, over 6 to $7\frac{1}{2}$-year and over $7\frac{1}{2}$ to nine-year groups all had seven cases. There was a sprinkling of cases among the older animals. Clinical cases were few and widely dispersed.

Generally speaking the overall age-distribution showed a striking resemblance to that prior to the disposal of chronic cases.

Discussion

The ecological conditions existing at Mara, that are potentially relevant to the epidemiology of bovine besnoitiosis, are very similar to those occurring on the majority of Bushveld farms. In essence they are a hot, rather dry climate, relatively low-lying country sparsely covered with somewhat stunted trees and sweet grasses. An ample, probably ubiquitous potential reservoir exists in the form of chronically infected cattle, and an abundant, largely unexplored one in the wild vertebrate animals. Other domesticated mammals are sometimes present but not plentiful. Potential vectors in the form of arthropods are very numerous. Because of the relative aridity, centrally placed bore-holes are the only consistent supply of drinking water. This gives rise to a daily diurnal congregation of cattle at the watering points thus creating conditions conducive to spread of infectious and parasitic diseases.

The chronically infected beast was regarded as the most important of the above-mentioned factors and singled out for this study. Mara is, however, not well-isolated from the point of view of control of an infectious disease by quarantine measures. It is traversed by public roads and virtually surrounded by farms on which besnoitiosis is known to occur. Its barbed-wire, boundary fence keeps large domesticated animals but very little else out. The method of isolating mild besnoitiosis cases on a corner of the farm, used until 1963, would have prevented spread by contact but not by cyclically infected arthropods for example, if such existed. The experiment was, however, based on the assumption that besnoitiosis was transmitted mechanically by immediate transfer feeding of bloodsucking insects, in which case a fenced-off road might well serve as a fairly effective barrier by keeping infected animals a considerable distance away from susceptible ones. It was reasoned that most insects in a " blood rage " would tend to remain with a particular group of cattle until replete. Even if the odd one did fly off, it is unlikely that it would produce anything but a mild case. Since most parasites, that are transmitted mechanically, do not survive long on the mouth-parts, flies would probably not be capable of transmitting the infection if they were to feed again a few days later on a different group of animals (see Chapters 5, 6, 7 and 8).

Some of the other epidemiological features were influenced to a considerable extent by the prevailing drought. The mean annual rainfall over the relevant period was only 372·7 mm as compared to 448 mm over 23 years. As can be seen in Fig. 4 there was a sustained drop in rainfall until it reached its lowest level in 1966, returning to normal in 1967. Because of the severe deterioration of the grazing the authorities were eventually forced to reduce the cattle population by more than half. Arthropod reproduction must undoubtedly have been curbed very considerably. Thus some uncontrolled variables that cannot be ignored were introduced into the experiment. This probably confused the main issue somewhat, which was unfortunate as it was not possible to have a neighbouring farm, where besnoitiosis was allowed to rage unchecked, available as a control.

There was undoubtedly a concurrent drop in rainfall and besnoitiosis incidence, which was probably not coincidental. Closer scrutiny of the individual figures shows that the correlation was, however, sometimes far from close. Obviously the 1960 figure obtained when the herd was examined for the first time represents a cumulative total accrued from the time besnoitiosis first appeared on the farm. Most of the 56 cases diagnosed during the first year of observation, when the rainfall was above the average, must have contracted the infection shortly before or after the elimination of chronic cases. The former are those that contracted besnoitiosis before they were examined but in which cysts or typical symptoms were not yet visible at the time of examination. It is not possible to estimate the number of inapparent cases that fell into this category but as the animals were examined at a time when the clinical incidence was low, it is reasonable to assume that they could not have been many. This also applies to mild cases with only one or two cysts that may have been overlooked. The above considerations hold for the duration of the experiment.

Twelve cases occurred during the second year when the rainfall was extremely low, and only a slight increase to 16 took place the following year when it was above the average. The incidence dropped to one and three in the fourth and fifth years when the rainfall was well below the mean. No cases were detected in the sixth year when the rainfall reached its lowest level, but there was only one in the seventh year when it was again above average. If the precipitation has been very intimately involved the correlation should have been much closer. The complete cessation of clinical cases in 1962 does not reflect the rainfall figures either. On Somerset Estate (Chapter 10), where quarantine is not practised, clinical cases continued to occur during 1961/1962 when the rainfall was very low. Potential vectors such as ticks and blood-sucking insects continued to be active at Mara although they were probably reduced in numbers.

Hence the drought was not responsible for, but contributed to the favourable outcome, which, in the opinion of the writer, was primarily due to the isolation and eventual removal of all detectable bovine besnoitiosis cases from the farm. This experiment has provided further evidence that chronically infected cattle act as important reservoirs of the disease.

It may well be asked what information was obtained about the mode of transmission. The reduction in the incidence occurred rather slowly, especially in the first year when it decreased from 4·6 to 2·6 per cent, and clinical cases continued to occur into 1962. This may mean that the chronic bovine case is not a direct source of infection for mechanical (or contaminative) transmission but only a link in a chain of events leading to transmission. Conversely, mechanical transmission may still have operated from an undetected, waning source.

As indicated previously (Chapters 4 and 10) not all infected animals could be detected by SC examination. Cases with very few cysts are probably of little or no significance as carriers. It is, however, very likely that some animals with fairly large numbers of cysts in the skin were also retained. They may have been eliminated from the farm, or the number of cysts in them may have decreased over the course of years. Seeing that the odd degenerating cyst is almost invariably found in tissue sections of chronically infected animals and no new ones are being formed (Chapter 13), it stands to reason that cysts must be diminishing very slowly all the time. Cattle passing along public roads or on adjoining farms may have been involved. Six of the eight fresh clinical cases, for instance, had been in camps bordering on such roads or neighbouring farms at the time of probable infection. All the bulls that contracted inapparent infections in 1963 and 1967 had also been on the border for the greater part of the year. Furthermore, in 1967 no cases occurred among the cattle confined to feeding paddocks in the centre of the farm far from the boundaries. This presupposes a concomitant decrease in the occurrence of infected animals on neighbouring farms, otherwise the incidence on Mara would not have reached such a low level. According to popular opinion of farmers there was an overall decline of clinical besnoitiosis in the drought-stricken areas. An adverse factor like that could well be responsible for a temporary remission on a country-wide scale due to a decreased transmission-rate and elimination of carriers by slaughter or death.

Wild life in general maintained their position remarkably well. Impala even increased in numbers. Had they served as reservoirs many cases would have continued to occur. For the same reason ticks and insects could not have served as carriers by transovarian transmission of the pathogen, for instance. It can still be argued that they require infected cattle to become cyclically infected, and that hereditary transmission does not occur indefinitely or not at all. Whatever the reason for the gradual subsidence in the incidence of besnoitiosis may be, the possibility that mechanical transmission is the only method has not been ruled out.

The eight clinical cases provided useful information on the time of the year that transmission usually occurs at Mara, because the actual time of infection could be calculated with fair accuracy. One animal apparently became infected in spring, six in summer and one in autumn. Hence the majority of cases occurred in summer which agrees with less precise observations made in S. Afr. (Hofmeyr, 1945; Pols, 1960; Bigalke & Schutte, 1960), France (Cuillé & Chelle, 1937b; Barrairon, 1938, cited by Pols, 1960) and the Congo (Kin.) (Herin, 1952 cited by Pols, 1960). The time of infection of the large number of inapparent cases could not be determined. The marked seasonal incidence prompted the decision to carry out the annual inspection of the herd in the late winter or early spring. It was reasoned that most of the animals which had acquired subclinical infections prior to the winter would have had sufficient time to develop SC cysts and that the chances of any new cases being added during the winter would be low. It is nevertheless possible that some overlap occurred every year.

The isolated group of cattle provided field evidence that the number of SC cysts is unlikely to change much once they are clearly visible. With the exception of a few very mild cases that became negative, and this is quite possible since a very limited number of cysts are slowly dying off all the time, there was no obvious decrease and certainly no increase in their numbers. With few exceptions, therefore, cattle with SC cysts will be detectable for many years after infection. This observation not only has a bearing on the cumulative total referred to previously, it also

helps to rule out the possibility that inapparent cases seen after the isolation and disposal of infected animals had contracted the infection many months or even years previously with the cysts gradually increasing in numbers or size to become visible in the SC (see Chapters 12 and 13).

Not only has this experiment furnished some useful information on the epidemiology of bovine besnoitiosis but it has also provided a possible method of control, i.e. the elimination or isolation of carriers. The former may be too costly to apply on farms with a high incidence because it means disposing of many perfectly healthy, mildly infected, often valuable animals such as bulls and stud cows. An alternative would be to keep an infected and a "clean" herd that are well-separated geographically. Most of the calves in the infected herd would probably be ostensibly cyst-free at weaning. Such calves would have to be isolated in a second quarantine camp and withstand a second examination about ten weeks later before being introduced into the "clean" herd, always remembering that some mild infections will not be detected anyway.

On the farm Springforbi, for instance (Chapter 10), all the infected cattle were disposed of after the 1962 examination. The owner has subsequently examined his stock annually for SC cysts and states that no new infections have been seen up to April 1967. The owner of Blijdschap also marketed his infected animals. No new clinical cases have occurred but when he examined the whole herd again for the first time in August 1967, ten inapparent cases were detected.

In the light of the observations made hitherto, and for reasons outlined previously, it seems improbable that permanent eradication would be obtained in the enzootic areas unless the method of slaughter or isolation is practised over the entire region for a long time. If such a campaign were to be launched, it would be advisable to examine each animal at least twice initially, with an interval of ten weeks between the two examinations, and thereafter annually. Furthermore, all new acquisitions would have to withstand two negative examinations as explained above for the two-herd system. It would therefore be an expensive campaign and a rather difficult one to enforce in practice.

The rule applicable to new introductions could, however, be enforced with regard to importation of cattle from enzootic to non-enzootic regions in order to prevent wide-spread dissemination of the disease.

12. Various Specific Attempts to produce Clinical Cases of Bovine Besnoitiosis

Introduction

One of the most puzzling features of besnoitiosis research, commented on by Pols (1960) and Bigalke (1967), is the mildness of the reactions seen in the majority of experimentally infected cattle. Although the riddle remained unsolved, the discovery of a marked preponderance of mild over severe cases in nature (Bigalke & Naudé, 1962) made it a little less mysterious. It was evident that under both field and laboratory conditions one or more factors must be operative which determine or influence the severity of the reactions. It was reasoned that the determining force might reside with the parasite, the host, the vector(s), the environment, or possibly even with a combination of one or more of these variables. A wide variety of pilot tests was therefore conducted to obtain parameters worthy of use as a basis for more detailed investigations. These experiments are recorded here.

In their pioneering studies on the artificial transmission of bovine besnoitiosis in France, Cuillé *et al.* (1936) succeeded in reproducing the severe form of the disease by subinoculating 100 to 1,000 ml blood from natural bovine cases, that were in the acute anasarca stage into three susceptible cattle. Pols (1954a) repeated this work in S. Afr. By transfusing 1,000 and 250 ml blood he produced two moderately severe cases with slight anasarca, scleroderma and depilation, as well as two mild cases. Mild reactions also occurred in an ox and two bulls inoculated with blood from artificially infected rabbits (Pols, 1960).

The transfusion technique was also employed in this investigation. The other methods of attempting to produce severe cases, i.e. organisms grown *in vitro*, organisms in testes and blood of artificially infected rabbits, cyst organisms from a natural bovine case, and cyst forms transmitted mechanically by tabanids, have either never been used before, or not in the same context. Thirteen bovine cases are considered in this chapter of which two have been referred to previously (Chapters 6 and 7).

Materials and Methods

1. *Inoculation with rabbit tissues harbouring proliferative forms*

With a recently isolated strain: The testes were removed from four acutely diseased rabbits harbouring the second passage-level of a strain isolated from cyst-bearing skin obtained from Bull 895 (Table 1). They were homogenized in ice-cold, serum free Hanks' solution, strained through gauze and 42 ml of the suspension injected intravenously into the 44-month old Ox 2052.

Heifer 3088 was inoculated intravenously with blood and a testes suspension prepared as above from a rabbit harbouring a strain isolated from feral *S. calcitrans* flies (Chapter 7).

With a high passage-level strain: The 3½-year old Ox 2923 was inoculated with 45 ml of a heparinized testes suspension prepared as described above from four rabbits harbouring the 542nd passage-level of the Fuls strain (Chapter 2).

2. *Inoculation with organisms grown* in vitro

With a recently isolated strain: Three Roux flasks containing confluent monolayers of a line of lamb kidney cells were seeded with organisms of the second *in vitro* passage-level of a strain isolated from the testes of acutely diseased rabbits inoculated with a suspension of cyst-bearing skin from Bull 3329 (Chapter 9). Three days later the medium was discarded and the culture, which showed fairly pronounced cytopathogenic changes, harvested with a trypsin-versene mixture (Bigalke, 1962), resuspended in ice-cold, serum-free, heparinized Hanks' solution, and 19 ml injected intravenously into the 28-month old Ox 3894.

Sixteen 500 ml McCartney roller bottles containing monolayers of primary lamb kidney cells were seeded with organisms of the eighth *in vitro* passage-level of the same strain. Two days later the cultures were harvested as above and 42 ml inoculated intravenously into the 3-year old, splenectomized Ox 3515.

With a high passage-level strain: Three Powitsky flasks containing monolayers of primary lamb kidney cells were seeded with the fourth, sixth and ninth *in vitro* passage-levels of the Fuls strain originally isolated from the testes of an acutely diseased rabbit harbouring the 275th passage-level. The cultures were harvested seven days later and 50, 28 and 32 ml respectively injected into the 27-month old Oxen 738 and 739 by the intravenous and the aged Ox 1502 by the subcutaneous routes.

3. *Inoculation of large volumes of blood from acute experimental bovine cases*

With a recently isolated strain: On the fifth day of its febrile response, four litres of blood were transfused from Ox 2052 into the 39-month old Ox 2431 via a canula, having first withdrawn 4 litres from the recipient.

The 32-month old Ox 3565 received 3·5 litres of blood from Ox 3894 on the fourth day of its reaction.

With a high passage-level strain: The 39-month old Ox 3588 likewise received 4 litres of blood from Ox 2923 on the fourth day of its reaction.

4. *Inoculation with large numbers of cyst forms*

Cyst-bearing veins and nasal, pharyngeal and laryngeal mucous membranes were harvested immediately after slaughter from a severely affected Friesian cross, Bull 4711 that had acquired the disease naturally. Portions were placed in the cleaning fluid, crushed in a mortar with a pestle, as well as in an MSE homogenizer, and finally strained through gauze. The 2-year old Ox 3259 was inoculated with 10 ml of the suspension by both the intravenous and subcutaneous routes.

Mechanical transmission by tabanids

Ox 9398, infected mechanically from a chronic bovine case by horse flies and exposed to the elements subsequently (Chapter 6), is also discussed here.

Whenever possible the number of parasites in the inoculum was determined with the aid of a haemocytometer. Smears were prepared, stained and examined.

Oxen 2923, 3894, 3515, 3565 and 3588 received 1 to 2 ml 1: 1,000 adrenaline intravenously shortly before infection to counteract possible shock. The cattle were examined for signs of infection as described in Chapter 3. Rabbit inoculation tests were conducted on the majority. Those in which no parasites could be demonstrated were challenged. Skin specimens were collected at regular intervals from the more severe cases for studies on the development of cysts and pathogenesis. Skin specimens of roughly equal size were collected from various sites on the right side of the body from six of the cases when they were slaughtered, to make a quantitative study on the distribution of cysts in the skin. The total number of cysts seen in a single section was counted, the length of the epidermis measured with the aid of an ocular or stage micrometer and the number of cysts per unit mm of epidermis calculated.*

* A modification of a method used by R. M. McCully, Major, USAF, V.C., staff member of Geographic Pathology Div., Armed Forces Institute of Pathology, Washington D.C.

Results

1. *Inoculation with rabbit tissues harbouring proliferative forms*

With a recently isolated strain

Great difficulty was experienced in counting the organisms in undiluted testes suspensions. At one in ten dilutions extracellular parasites were more clearly visible but the heads of spermatozoa, other testicular elements and debris still confused the issue. Doubtful parasites were ignored hence the counts are under rather than over estimates. Intracellular organisms were not counted because they could not be unequivocally distinguished in wet preparations. They were very rare in stained smears. Parasites in the blood were not counted because they were very rare at the best of times.

Ox 2052 received approximately $27{\cdot}3 \times 10^6$ organisms. Four days later it developed a reaction which was classified as fairly mild (Table 21). The thermal reaction was quite pronounced, lasted eight days (Fig. 7) and was accompanied by inappetence, listlessness and slight hyperaemia of the muzzle. Small numbers of extra- and intracellular proliferative forms could be detected in blood smears on the third, fourth, fifth and eighth days. No anasarca developed and when the temperature subsided the animal made an uneventful recovery. Two SC cysts were seen 47 days after the first fever (see Chapter 13) and when the animal was slaughtered, 75 days after infection, cysts could be found in small numbers in the facial and leg veins.

The number of organisms in the blood and testes suspension received by Heifer 3088 was not determined. Smears revealed fair numbers of parasites in both tissues. Her reaction was classified as fairly severe (Table 21). After a prodromal rise in temperature, the animal developed pronounced fever two days later. It exceeded 106°F for five days and took a further eight days to return to normal (Fig. 7). It was accompanied by marked anorexia, lassitude, dragging of the hind limbs and pronounced hyperaemia of the muzzle and skin around the eyes. On the fifth day of fever the face became markedly swollen. A tight-fitting halter used to handle the animal left a deep notch across the nasal bones that took some time to fill out again. On the fifth and sixth days oedematous swellings were noticed along the ventro-lateral aspects of the thorax and abdomen. On the seventh day this had gravitated ventrally and the breach was also swollen. Thereafter oedema fluids collected in the inter-mandibular, brisket, sternal, abdominal and hock regions, whereas the swellings of the face, thorax and breech rapidly disappeared. Feeding was resumed and the loss in weight regained. Hyperaemia of the muzzle, etc. faded but slight oedema was still noticeable along the ventral line until the sixteenth day of the reaction.

Despite the rather pronounced anasarca, subsequent skin lesions were mild. Small patches of epidermis were shed below both eyes on the 24th day of the reaction. Fairly extensive nodule formation of the skin developed along the sides of the face and in the intermandibular region. These nodules had the same consistency as the skin, were up to about 5 mm in diameter and often devoid of hair. On the 32nd day the skin of the gaskin was observed to be thickened and wrinkled with loss of hair. Jagged pieces of necrotic skin could be detached over the hip joints leaving hairless scars. There was no further increase in the severity of skin lesions.

TABLE 21.—*Various specific attempts to produce clinical cases of bovine besnoitiosis*

Recipient	Infection					Reaction					
	Date	Source infective inoculum	Total number parasites	Dose in ml	Route	Incubation period (days)	Duration fever (days)	Max. temp. °F	Proliferative organisms	Cysts	Severity
2052	28.2.66	Rabbit testes.....	$27{\cdot}3\times10^{6}$	42	i.v.	4	8	106	+	+	FM
3088	6.2.67	Rabbit testes + blood	n.d.	12+100	i.v.	7	13	106·8	+	+	FS
2923	5.5.67	Rabbit testes.....	$141{\cdot}75\times10^{6}$	45	i.v.	1	9	106·6	+	?	S*
3894	8.6.67	Cell culture.....	$5{\cdot}985\times10^{5}$	19	i.v.	3	10	106·6	+	+	FS
3515	6.9.67	Cell culture.....	1068×10^{3}	40	i.v.	1	28	106·6	+	+	S
738	26.10.62	Cell culture.....	$46{\cdot}75\times10^{6}$	50	i.v.	1	7	105·6	0	0	M
739	31.10.62	Cell culture.....	$66{\cdot}4\times10^{5}$	28	i.v.	1	4	104	0	0	M
1502	3.12.62	Cell culture.....	$106{\cdot}56\times10^{6}$	22	s.c.	4	4	104·8	0	0	M
2431	8.3.66	Bovine blood....	n.d.	4000	i.v.	7	15	106·4	+	+	FS
3565	15.6.67	Bovine blood....	n.d.	3500	i.v.	5	10	106·6	+	+	FS
3588	9.5.67	Bovine blood....	n.d.	4000	i.v.	2	10	106·6	+	+	FS
3259	13.5.66	Bovine tissues....	2300×10^{3}	10+10	i.v. & s.c.	3	9	104·8	0	0	M
9398	10–11.1.61	Tabanids.....	n.d.	—	25 bites	13	12	107·6	0	+	FS

Legend:
* The only fatal infection
n.d. = not determined
i.v. = intravenous
s.c. = subcutaneous
? = possible (immature) cyst

M = Mild
FM = Fairly Mild
FS = Fairly Severe
S = Severe
Also see Table 5.

Proliferative forms were quite plentiful in blood smears from the third to fifth days of the febrile response (Fig. 7). The peak occurred on the fourth day when one intra- and 14 extracellular organisms were found during a 15 minutes search. Young cysts were first detected in sections of the neck skin taken 12 days after the start of fever. Thereafter they were fairly plentiful in sections of the neck and gaskin respectively. SC cysts were clearly visible on the 37th day after the rise in temperature when about 25 were detected in both eyes (see Chapter 13). From the 31st day onwards cyst organisms were almost invariably present in tail blood smears. This was not surprising if one considers the rather large number of cysts found in sections of the tip of the tail (Table 22).

When the animal was slaughtered 70 days after infection, cysts were very numerous in the facial veins and nasal mucous membrane, but less so in the jugular and peripheral veins of the limbs. They were fairly plentiful in the subcutaneous fascia of the ventral portion of the thorax and abdomen, and forearms. Sections revealed that more cysts occurred in the skin below the lower eyelid, over the hip joints, in the breech, gaskin and flank fold than in any of the other sites sampled (Table 22).

With a high passage-level strain

Ox 2923, which received approximately $141 \cdot 75 \times 10^6$ organisms, developed a very severe reaction with a fatal termination (Table 21). The day after inoculation its temperature was 102°F. From there it climbed steeply to reach a peak of 106·6°F on the fourth day (Fig. 7). It was accompanied by rapidly increasing anorexia and hyperaemia of the muzzle, skin around the eyes and nasal mucous membrane. The animal also showed polypnoea and pronounced weakness in the hindquarters. The latter was manifested by progressive difficulty in rising, a slow swaying gait and dragging of the hind feet. The head was held depressed. On the seventh day of the reaction the animal initially made several fruitless attempts to rise. It eventually succeeded, but could hardly move and reeled about when forced to do so. At this stage the muzzle, periorbital skin and nasal mucous membrane were almost purple in colour and the face was slightly swollen. The next day the face, eyebrows and jowl were very oedematous and polypnoea pronounced. The animal was unable to rise and supported its head on the floor. On the ninth day it stopped feeding and expiration became forced. Anasarca became more extensive from the ninth day onwards and by the eleventh, when the animal lay flat on its side, virtually the entire body was involved. Oedema was most pronounced in the neck, jowl and sternal regions of the left side on which it was lying. The animal expired on the same day.

Proliferative forms were demonstrable in blood smears from the third day of fever until the day of death (Fig. 7). Peak parasitaemia occurred on the third day when 26 intra- and 13 extracellular organisms were observed during a 15 minute search. Hypertrophied macrophages with basophilic cytoplasm, probably destined to become cysts, were recognized in skin collected after death. Two rabbits inoculated with blood drawn on the sixth and seventh days developed typical besnoitiosis reactions. One survived and small numbers of cysts were found in the skin of the nose.

2. *Inoculation with organisms grown* in vitro

With a recently isolated strain

It was calculated that Ox 3894 received about $5 \cdot 985 \times 10^6$ organisms. Smears of the three-day old cultures showed fairly large numbers of well-stained parasites; no degenerating or intracellular organisms were seen. The animal developed a fairly severe reaction (Table 21). Three days after infection its temperature started rising, then climbed rapidly to reach 106·4°F on the fourth day of the reaction. It remained above 106°F until the seventh, then dropped steeply to reach normal limits by the eleventh day (Fig. 7). Pronounced polypnoea accompanied the thermal reaction, and forced exertion outside the stable gave rise to breathlessness. The ox ate very little at the height of the reaction and lost condition considerably. It was listless, hung its head with its back slightly arched, was stiff behind and inclined to lie down. Slight hyperaemia of the muzzle and periorbital skin, and moderate anasarca of the face was noticed on the fifth day. No oedematous swellings were observed elsewhere. But micrometer measurements of skin folds in the middle of the neck, cheek and thoracic region just behind the scapula showed increases of 60, 53 and 52·5 per cent respectively from the eighth to ninth days of the reaction indicating an increase in thickness of the skin.

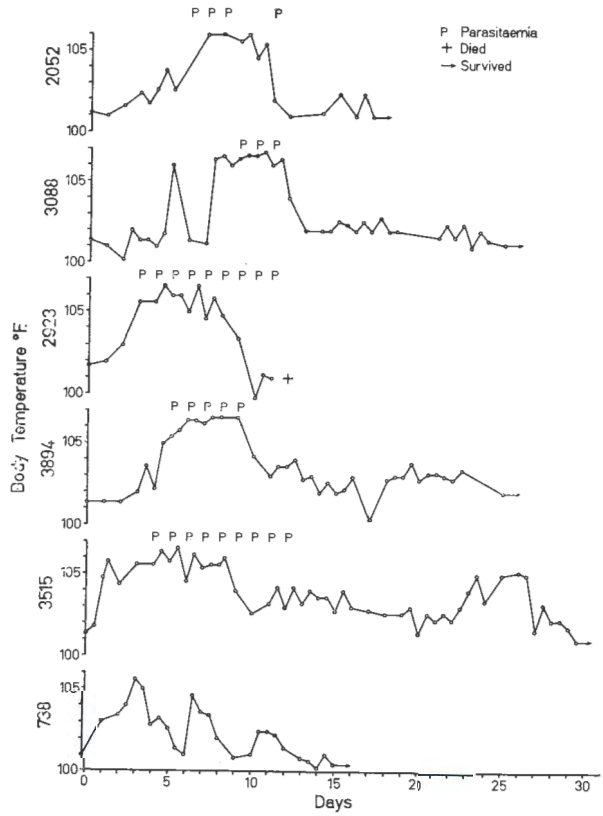

FIG 7.—Febrile reactions and parasitaemia in six head of cattle infected with *B. besnoiti* by various methods outlined in text

Proliferative forms appeared in blood smears from the third to seventh days of pyrexia (Fig. 7) with peak parasitaemia occurring on the fifth day when 13 extra- and 15 intracellular organisms were seen. Mitotic figures of leucocytic cells were fairly frequent in smears, a feature also noticed in most of the other animals. An immature cyst was seen in skin sections taken on the fourteenth day of the reaction. SC cysts were clearly visible 35 days after the first rise in temperature (see Chapter 13). Cyst organisms were found in tail blood smears on the 36th day and occasionally thereafter. When the animal was slaughtered 71 days after infection cysts were plentiful in the facial and fairly numerous in the peripheral veins of the limbs and nasal mucous membrane, although they appeared to be smaller in the latter site. The relative frequency of skin cysts is illustrated in Table 22. The five most favoured sites were the skin over the hip joint, the face, upper eyelid, gaskin and front coronet in descending order of frequency.

The splenectomized Ox 3515 received a massive inoculum of approximately 1068×10^6 organisms that appeared viable and healthy in both wet and stained preparations of the two-day old cultures. Intracellular organisms were also quite plentiful in the latter.

The animal developed a very severe reaction from which it almost succumbed (Table 21). Within 24 hours of infection the temperature rose to 105·8°F to reach a peak of 106·6°F on the fifth day of the reaction (Fig. 7). It remained above 105°F until the ninth day when it subsided to fluctuate at a slightly lower level. The ox which was a carrier of *B. bovis* developed a very severe stress-induced relapse requiring acaprin (Bayer) treatment on the 25th day of the reaction. Although the temperature thereafter slowly returned to normal levels it was irregular and often exceeded 102°F.

Pyrexia was accompanied by progressive inappetence leading to complete anorexia by the eighth day, whereafter feeding was gradually resumed. Other early symptoms were polypnoea and increasing weakness of the hindquarters manifested by a slow swaying gait, dragging of the feet and progressive difficulty in rising from the recumbent position usually adopted. When standing, the head was held depressed and the legs tucked in underneath the body. Hyperaemia of the muzzle and periorbital skin became very pronounced on the seventh day. Petechiae were visible in the latter site, and larger haemorrhages above the muzzle and left eye. The entire skin was sensitive to touch and the coronets and other hairless sites also slightly hyperaemic. Deliberate exposure to sunlight caused much distress, viz. signs of photophobia. After prolonged exposure it would stand or lie with its tongue protruding and panting like a dog. Body temperatures of up to 109°F were recorded on such occasions.

Anasarca of the face, which was most pronounced around the muzzle, and slight thickening of the skin folds of the neck were noticed on the seventh day. On the tenth day the root of the tail, breech, sternum and forearms were also oedematous. The lower half of each cornea was opaque on the eleventh day but this soon cleared. Thereafter anasarca of the dorsal regions subsided with accumulation of fluid in the lower reaches of the body. By the 17th day of the reaction the legs were so swollen below the carpus that they resembled tree-stumps. Micrometer measurements of skin folds revealed maximum increases of 126·7, 113·5, 136·7 and 47·5 per cent in the neck, cheek, dewlap and thoracic regions between the ninth and tenth days. By the 26th day these measurements were virtually back to normal.

On the 21st day of the reaction, when there was still slight oedema of the brisket region, the skin over the carpus was noticed to be necrotic, and unsightly granulating wounds developed that failed to heal over the three months of observation. From about the 25th day onwards the hair around the eyes and mouth became sparse and nodule formation as described for Heifer 3088 occurred. Eventually the skin of these areas was a mass of these wart-like nodules, which extended to involve the cheeks and was accompanied by moderate scurfiness. Sections showed that the nodules were due to massive accumulations of cysts in the dermis. The prescapular lymph nodes were markedly enlarged by the fourth week. The skin of the face, neck, breech, inner thighs and tail which had shown fine wrinkling after the oedema had subsided, increased in thickness and consistency to form large numbers of prominent, hard folds which lacked elasticity, lost hair progressively and showed nodule formation. This was clearly noticeable by the fifth week but became much more pronounced as time progressed. The face and neck regions started itching rather badly which resulted in some self-mutilation with scab formation. At 12 weeks the face and neck skin again showed increases in thickness of 51·7 and 34·8 per cent, whereas the throat and thoracic regions were virtually normal. Thinning of the haircoat and puckering of the skin were also noticeable on the fetlocks and pasterns. Bare patches with nodule formation appeared over the points of the hips and lateral aspects of the hocks. Ox 3515 lost a considerable amount of weight which it failed to regain. From a robust, rather unmanageable animal it turned into a weak, docile, dull beast.

Proliferative forms were detected in blood smears from the fourth to twelfth days of the reaction (Fig. 7). The remarkably high count of 51 parasites in 200 oil-immersion fields was recorded on the seventh day; in all 100 extra- and 14 intracellular parasites were seen during a 15 minute search. Rabbit inoculation tests conducted at roughly bi-weekly intervals were positive until the 19th day of the reaction, but 20 ml blood ceased to be infective from the 22nd until the 42nd day when biological tests were discontinued.

Young cysts were first detected in skin sections on the 13th day of the reaction. About 20 SC cysts were fairly distinctly visible on the 36th day (see Chapter 13) and more or less doubled their numbers in two weeks, but remained rather small. Another peculiar feature was that these cysts were inclined to appear in clusters and sometimes became surrounded by an opaque tissue which made recognition difficult. Cyst organisms were invariably present in blood smears from the 27th day onwards, but always absent in smears made concurrently from the ears. Negative rabbit inoculation tests that coincided with positive tail blood smears on three occasions provided additional proof that there was no parasitaemia.

When the animal was slaughtered 106 days after infection very large numbers of cysts were found at the usual sites such as the facial veins, nasal mucous membrane, larynx, trachea and subcutaneous and intermuscular fascia. There was a tendency for them to occur in clusters as in skin nodules. Skin cysts were much more numerous in the majority of the sites sampled than in the other animals (Table 22). The five most favoured regions in descending order of frequency were the tip of the tail, face, breech, upper eyelid and flank fold.

With a high passage-level strain

Ox 738 received the smallest inoculum, i.e. approximately $46 \cdot 75 \times 10^6$ organisms. Smears of the one-week old cultures revealed large numbers of well-stained extracellular, but also many contracted, obviously degenerating parasites; no intracellular organisms were seen. The same applied to Oxen 738 and 1502 that received about $66 \cdot 4 \times 10^6$ and $106 \cdot 56 \times 10^6$ organisms respectively.

Ox 738 developed a very mild reaction (Table 21). The temperature was elevated on the day after infection and reached the maximum of 105·6°F after three days. There was no other sign of ill health, however, and five days later the temperature was back to normal (Fig. 7). No proliferative forms could be found in blood smears, and the SC and skin sections were negative for cysts. The animal was nevertheless immune when challenged 22 days after infection with the 67th passage-level of the Schoeman strain.

Ox 739 developed a similar but even milder reaction (Table 21 and Fig. 8). Again no parasites were seen, and immunity to challenge 26 days post-infection with the 286th passage-level of the Fuls strain was the only other indication of successful infection.

Ox 1502 that was injected subcutaneously, instead of intravenously, developed a very similar reaction to that of Ox 739, the only difference being the slightly longer incubation period (Table 21). Apart from the febrile response (Fig. 8), the only evidence of successful infection was immunity to challenge 36 days after infection with the 287th passage-level of the Fuls strain.

3. *Inoculation with large volumes of blood from acute experimental bovine cases*

With a recently isolated strain

Ox 2431 contracted a considerably more severe form of besnoitiosis than its donor Ox 2052 (Table 21). After a prodromal rise on the fourth day, it developed a pronounced febrile reaction three days later which remained at a high level for six and required a further nine days before it returned to normal (Fig. 8). Pyrexia was accompanied by pronounced inappetence, listlessness, lassitude, grinding of the teeth and marked hyperaemia of the muzzle and nasal mucous membrane. As in the case of Ox 3894, exertion quickly caused exhaustion.

Marked thickening of the skin of the neck and oedema of the brisket region were noticed on the sixth day of the reaction. Due to gravitation of fluid the latter became progressively more pronounced until the tenth day when there was a large swelling. A needle was inserted and about 5 ml of clear yellow serum-like fluid expressed. A rabbit was inoculated subcutaneously but failed to react. The rest was centrifuged and smears prepared from the slight deposit. A few extra- and intracellular organisms as well as small numbers of large mononuclear cells were found. The oedema then started subsiding, the animal resumed feeding and started regaining condition.

Subsequent skin lesions were milder than expected. Patches of detachable necrotic epidermis and sitfasts were noticed at the point of the brisket on the 22nd day. At 29 days similar patches were seen on both gaskins. On the 36th day the skin of the gaskins was observed to be thickened, markedly wrinkled and covered with sparse hair only.

TABLE 22.—*Topographical distribution and frequency of skin cysts*

Bovine No.	Ox 2431		Heifer 3088		Ox 3588		Ox 3894		Ox 3565		Ox 3515		Total number/ mm epidermis
Skin cysts	Total number	Number/ mm epidermis	Total number	Number/ mm epidermis	Total number	Number/ mm epidermis	Total number	Number/ mm epidermis	Total number	Number/ mm epidermis	Total number	Number/ mm epidermis	
Upper eyelid.....	26	2·6	244	7·2	0	0	24	0·9	72	2·8	413	16·1	29·6
Lower eyelid.....	1	0·1	36	2·0	0	0	1	0·05	3	0·2	90	4·5	6·85
Face.....	50	4·1	80	3·3	0	0	35	1·7	25	1·3	534	23·8	34·2
Muzzle.....	5	0·4	6	0·4	0	0	2	0·1	7	0·5	254	12·7	14·1
Neck.....	0	0	19	0·7	0	0	9	0·4	0	0	170	8·5	9·6
Brisket.....	7	0·5	64	4·8	0	0	5	0·2	28	1·6	275	11·5	18·6
Posterior to scapula	0	0	24	1·0	0	0	2	0·1	0	0	64	5·9	7·0
Flank fold.....	1	0·1	136	4·9	0	0	7	0·4	8	0·2	170	12·9	18·5
Gluteal region.....	0	0	19	1·6	0	0	5	0·2	5	0·3	30	3·1	5·2
Over hip joint.....	79	5·4	130	7·2	0	0	39	2·3	12	0·5	90	5·9	21·3
Breech.....	5	0·3	175	7·2	0	0	15	0·7	3	0·1	383	18·8	27·1
Gaskin.....	109	7·1	86	5·2	0	0	32	1·6	19	0·9	170	9·4	24·2
Front coronet.....	143	12·9	50	2·3	0	0	20	0·9	12	0·5	50	3·5	20·1
Hind coronet.....	28	2·0	102	3·8	0	0	17	0·6	27	1·3	135	7·5	15·2
Tip of tail.....	30	2·1	102	2·3	0	0	12	0·7	5	0·5	364	33·7	39·3

Proliferative forms were seen in blood smears from the first to fifth days of the reaction (Fig. 8). On the fourth day four litres of blood were drawn for inoculation into another ox. The latter, however, died of shock ten minutes after the transfusion. An immature cyst was found in the neck skin collected on the 14th day. Approximately 20 small SC cysts were visible 36 days after the rise in temperature (see Chapter 13). Cyst organisms were frequently encountered in blood smears from the 42nd day onwards. There were none in smears prepared from jugular blood drawn on the same day for inoculation into a rabbit, and the latter failed to contract besnoitiosis.

When Ox 2431 was slaughtered 11 months after infection, cysts were plentiful in the facial veins and nasal mucous membrane but rare in the peripheral veins of the limbs. Their distribution and relative frequency in the skin is illustrated in Table 22. Most favoured of the sites sampled were the coronet of the front leg, gaskin, skin over the hip joint, face and upper eyelid in descending order.

Ox 3565 contracted only a slightly more severe form of the disease than its donor (Table 21). Five days after injection it developed a pronounced febrile reaction that remained high for eight days, reaching a maximum of 106·6°F on the seventh and returning to normal limits on the 11th day (Fig. 8). It was accompanied by polypnoea, progressive inappetence, slight drooling from the mouth, slight hyperaemia of the muzzle, periorbital skin and nasal mucous membrane and slight coughing. Although the animal did not lie down excessively, it was listless, stood with its head hanging, dragged its hind feet and tired rapidly. Slight swelling of the face was noticed on the sixth day, and there were increases of 40, 45 and 43 per cent in the thickness of skin folds of the neck, cheek and thoracic regions. As the temperature subsided these symptoms disappeared and the condition of the animal improved. Thickening and slight alopecia of the skin of the gaskin, and alopecia and hardening over the hip joint was noticed on the 28th day.

Proliferative forms were seen in blood smears on the second, fifth and seventh days of the reaction (Fig. 8). They were scarce; the maximum count was one extra- and four intracellular parasites. Cysts were first seen in skin sections on the 23rd day of the reaction. About 20 SC cysts were clearly visible 34 days after the rise in temperature (see Chapter 13). Cyst forms were detected in blood smears on the 41st and 57th days only. When the animal was slaughtered 74 days after infection the incidence and distribution of cysts were found to be very similar to those of its donor. The most favoured sites in the skin were the upper eyelid, brisket, face, hind coronet and gaskin in descending order of frequency (Table 22).

With a high passage-level strain

Ox 3588 developed a much milder reaction than its fatally infected donor (Table 21). The thermal rseponse, that developed after two days, was pronounced and lasted ten days (Fig. 8). It was, however, not accompanied by anasarca. The only symptoms shown were fairly severe anorexia, loss of weight, a slightly swaying gait, rapid exhaustion, slight lassitude and slight hyperaemia of the muzzle, nasal mucous membrane and periorbital skin. The animal made a rapid recovery after its temperature had subsided. A couple of intracellular proliferative forms were seen in blood smears on the fifth day of the reaction only (Fig. 8), but blood was fatally infective to rabbits on both the fifth and ninth days. No SC cysts appeared and only one was detected in a section of the neck skin collected 20 days after the initial rise in temperature (Tables 21 and 22). When the animal was slaughtered 66 days after infection ten cysts were found in the facial veins and one in a peripheral vein of a hind leg but none in the nasal mucous membrane.

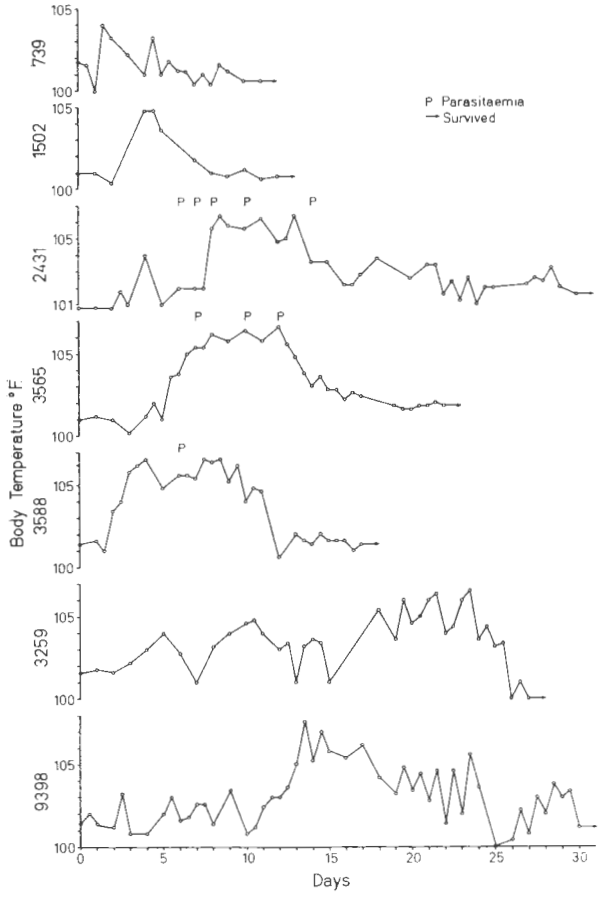

FIG. 8.—Febrile reactions and parasitaemia in seven head of cattle infected with *B. besnoiti* by various methods outlined in text

4. *Inoculation with large numbers of cyst forms*

Ox 3259 received a very massive inoculum of about 2,300 × 10^6 cyst organisms. The febrile response that developed after three days was of rather intermittent nature and never very pronounced even though it extended over nine days (Table 21). It was not accompanied by any of the other symptoms seen in some of the more severe cases described above, except for some inappetence. No proliferative forms were seen in blood smears. The only proof that the fever reaction was due to *B. besnoiti* was the fact that blood drawn on the sixth and eighth days was infective to two rabbits and two sheep. Proliferative forms were demonstrable in both rabbits. A fairly large number of cysts were found lying fairly deeply in the nasal mucosa over the turbinate bones of the sheep when they were slaughtered seven months after infection; a few were also present in the cornea of one of them. No cysts could be detected in their veins.

Two days after the initial febrile episode had subsided Ox 3259 developed a second, but much more pronounced one, that lasted 14 days and reached a maximum of 106·6°F (Fig. 8). It was accompanied by severe pyrexia, initial polypnoea, and final dyspnoea and prostration. In this instance the rabbit inoculation test was negative. The animal was killed *in extremis*. The autopsy revealed a severe purulent pneumonia, from which *Pasteurella multocida* (Lehman & Neumann) was isolated, thrombosis of the pulmonary veins and a purulent endocarditis and arthritis. Hence it was concluded that the severe secondary reaction was due to a fulminating bacterial infection introduced with the original inoculum.

Cattle inoculated with small numbers of cyst forms were not included in this study. Such cases have, however, been produced previously and the mild nature of the reactions stressed (Bigalke, 1967).

5. *Mechanical transmission by tabanids*

Full particulars of the infection and subsequent reaction of Ox 9398 have been given in Chapter 6. The animal is, however, included here, firstly because it was infected with cyst organisms transferred mechanically by tabanids, and secondly because a specific attempt was made to produce a severe case by exposing the animal to the hot mid-summer sun during the incubation period and the reaction.

Briefly, Ox 9398 developed a severe fever that remained elevated for 12 days (Table 21 and Fig. 8). The animal presented marked anorexia, polypnoea, lassitude and pronounced hyperaemia of the muzzle and nasal mucous membrane. No anasarca or scleroderma was noticed and proliferative forms were only demonstrable by inoculation of rabbits. Cysts were found in the SC 41 days after the rise in temperature and in addition also in skin sections (see Chapters 6 and 13).

It so happened that the weather was rather cool and wet during the early stages of the reaction, which confused the issue of the influence of the environment. However, Oxen 188 and 9546 infected by tabanids and tsetse flies respectively (Chapter 6 and 5) and exposed similarly, developed slightly milder reactions than Ox 9398, indicating that the fairly severe reaction in the latter probably had nothing to do with the environment.

Discussion

The main incentive behind these deliberate attempts to produce severe clinical cases with pronounced anasarca and scleroderma was to gain insight into the mechanisms responsible for their production. It was hoped that the results would help to elucidate several teasing questions, viz.: (1) Whether mechanical transmission could be responsible for the production of typical clinical cases; (2) whether it would be possible to grade the severity of the reactions of artificially infected cattle at will, thus creating the machinery for a safe method of immunization, as prompted by the solid immunity that follows even low-grade parasitosis; in addition a method would be provided for offering a severe challenge in immunological studies. Studies on the pathogenesis of the disease in relation to the developmental cycle of the parasite from start to finish could be supported by macroscopical, histological, histochemical and electron-microscopical methods; (3) whether it would be feasible to develop a standard method for comparison between the degree of virulence of bovine and antelope strains for a specific host. It is not anticipated that bovine strains of different origins will differ in their pathogenicity.

Although only some of these objectives have been realized, useful guiding principles have emerged. Subinoculation of large volumes of blood from experimental cases produced fairly severe reactions comparable to those recorded by Pols (1954a, 1960), but not as severe as those noted by Cuillé *et al.* (1936), where one animal that received 1,000 ml from a natural case died 15 days later. In this experiment moderate anasarca and slight scleroderma developed in both cattle inoculated with recently isolated strains. The mildest reaction occurred, rather unexpectedly, in an animal transfused from a donor fatally infected with a high passage-level strain. Even in the most severe of the three cases the lesions could only be compared to those of the milder type of clinical besnoitiosis seen in the field. The actual dose of organisms received by the animals was not calculated. If it is, however, considered that 13 proliferative forms were found in only a portion of a smear of a small drop of peripheral blood from one of the donors, the total number of organisms injected must have been quite considerable.

The situation was reversed when tissues from acutely diseased rabbits served as a source of infection. The most severe case ever produced at this Insitute was seen in an ox that received a massive dose of proliferative forms of the frequently passaged Fuls strain in a suspension of rabbit testes. It developed a remarkably high parasitaemia and showed extensive anasarca when it died 12 days after infection. The reaction in the heifer inoculated with a strain isolated via rabbits from stable flies was perhaps slightly more pronounced than that of the most severe case produced by blood transfusions. In spite of developing fairly extensive anasarca, subsequent scleroderma was rather localized. The number of organisms in the inoculum was not determined but it could not have been nearly as great as that of the previous animal. The mildest reaction in this category occurred in an ox that received a fairly big dose of a strain isolated recently from a chronically infected bovine. These reactions were, however, considerably more severe than most of those encountered hitherto when susceptible cattle were injected with smaller doses of rabbit blood (Pols, 1954a, 1960; Bigalke *et al.*, 1967).

The results obtained with young cultures of recently isolated strains were encouraging. The ox that received the biggest dose developed a very severe reaction with extensive anasarca and scleroderma comparable to that seen in nature. The possibility that the animal owed its severe reaction to the fact that it was splenectomized has to be considered. A previous investigation, however, provided no evidence that susceptibility to besnoitiosis was enhanced by splenectomy in the sense that it would turn a potentially mild reaction into a severe one (Bigalke, 1967). An appreciably smaller inoculum than the above was still capable of producing mild anasarca and scleroderma in an ox.

The reactions in the three animals inoculated with cultures of the high passage-level Fuls strain were much less severe in spite of the fact that they received bigger doses than the latter animal. The results are, however, not directly comparable with the above because much older cultures were used which contained many dead and degenerated organisms and probably many others that had died recently or lacked viability, even though they were normal in appearance.

In spite of the considerable amount of direct and circumstantial evidence presented in this and other investigations (Bigalke, 1967; Bigalke & Schoeman, 1967) that chronically infected cattle serve as a source of infection, not a single typical clinical case has been produced in the 14 head infected with cyst organisms by a variety of methods to date. Ox 3259 injected with an astronomical dose of cyst forms was no exception to this rule. It is therefore significant that Ox 9398 infected mechanically by only 25 alternate tabanid bites, and hence undoubtedly by a much smaller number of organisms, developed a fairly severe reaction accompanied by obvious ill health, even though anasarca and scleroderma were not recognizable. The writer is of opinion that not much more by way of pathogenic effects of the parasites would have been required to turn the case into a typical clinical one.

The train of reasoning adopted in this investigation was that if all other things were equal—and it is conceded that individuals will vary in their susceptibility—the severity of the disease would be determined by the number of organisms injected. Hence they were counted whenever possible. Reference has, however, already been made to instances where the number of parasites bore no relation to the severity of the disease produced. A young culture of *B. besnoiti*, for instance, caused a more severe reaction than numerically superior, older ones, and cyst forms transmitted by tabanid bites were more pathogenic than tremendous numbers injected. On the other hand there were definite indications that numbers were of crucial importance in the pathogenesis. A big dose of proliferative forms in rabbit testes caused a fatal infection whereas smaller doses produced moderately severe or mild disease. The same applied to large and small inocula of young cultures of organisms grown *in vitro*. This means that a direct count cannot be regarded as a suitable measure of their viability or pathogenicity. A more reliable basis for comparison in experiments of this nature would be to determine their infectivity, a term that includes the concepts of quantity as well as quality.

It is for instance possible that the infectivity, and therefore pathogenicity, of cyst forms in a suspension prepared from cyst-bearing tissues may be reduced by the action of humoral antibodies, or some other antagonistic agent present in such tissues. Cyst organisms transmitted mechanically might not be exposed to these substances to the same extent. The interesting problem of pathogenicity requires considerable further investigation in which titrations of infectivity in cell culture or rabbits, each fraught with its own particular problems, must play an important part.

This investigation has nevertheless shown that very large doses of proliferative forms may cause very severe, and smaller doses much milder reactions. It therefore seems as if it might be feasible to grade the severity of the reactions by the size of the inoculum. Hence the possibility of making a living vaccine containing a relatively small number of organisms per dose does not seem too far-fetched. Vaccinated animals will, however, become cyst-bearing carriers if recently isolated strains are used.

After 14 years of passage the Fuls strain (Chapter 2) is still highly virulent for both cattle and rabbits. The fatal infection of Ox 2923, and the fact that it has killed 67·6 per cent and produced microscopically detectable parasitaemia in 78 per cent of the rabbits in which it was passaged from 1966 to 1967 corroborate this statement. The tendency referred to by Pols (1954b, 1960) for the disease to take " a more protracted and less severe course as serial passages progressed " has apparently disappeared. On the contrary, if anything, the virulence has been enhanced.

One of the striking features of the reaction in Ox 3588, the recipient of blood from Ox 2923, was the virtual absence of cysts in the skin and their sparseness in sites like the veins where they usually abound, in spite of the fact that its reaction was not very mild. Cysts were also rather rare in a rabbit that survived a positive blood inoculation test. It appears as if the Fuls strain may be in the process of losing its cyst-producing ability, much like *B. jellisoni* is known to do after about 30 acute passages in mice (Frenkel, 1965). This may mean that proliferation continues for a slightly longer period than with cyst-producing strains (Chapter 13), which might result in an increase in virulence. If this is so it apparently manifests itself with very massive doses only. The small volumes of blood used for immunity tests have never produced typical clinical cases. Hence small standardized doses of the Fuls strain could possibly also be safely used as vaccine, with the added advantage that such animals would have negligible numbers of cysts.

It is still an open question whether environmental factors like the scorching summer sun play a part in the pathogenesis. Limited experimental evidence (Chapters 5 and 6) combined with that of the presence of large numbers of clinically inapparent natural cases in the hot enzootic regions clearly indicates that the sun's rays are incapable of turning a mild reaction into a severe one. If an animal is, however, already in the acute anasarca stage of the disease, it is quite possible that prolonged exposure to sunlight will cause the skin lesions to become more pronounced.

The study on the topographical frequency-distribution of cysts in the skin was limited to only six animals infected artificially by the intravenous route, and the following remarks should be interpreted with due recognition thereof, since they may not necessarily be applicable to natural cases. Considerable variation in the number of cysts from one site to another was noticed in the same animal. Cysts were most plentiful in regions that had shown, or were showing, lesions. The correlation with scleroderma appeared to be closer than with anasarca. Although there was some variation from one animal to the next, the skin of the tip of the tail, face, upper eyelid, breech and gaskin emerged as the most favoured sites when the number of cysts per mm epidermis were added up (Table 22). These figures were, however, dominated by Ox 3515 which harboured many more cysts generally.

When dealing with less severe natural cases the abovementioned sites should be more suitable for biopsy purposes than the skin of the neck usually removed which was a rather poor site in most instances. It is certain that the frequency of cysts in the tip of the tail would have been higher had they not been punctured in the process of making blood smears. Many regions were not sampled and it is quite possible that more reliable sites exist.

13. Observations on the Time-interval between Infection and the Appearance of Cysts in the Scleral Conjunctiva

Introduction

During the course of this investigation it was noticed that the time-interval between infection and the ocular demonstration of the full complement of SC cysts was remarkably constant from one animal to another. It was reasoned that if this were so it should be possible to calculate the time of infection of natural cases fairly accurately by regular inspection of the SC for cysts to become visible, a most useful tool in epidemiological studies as can be seen in Chapters 4 and 11.

A number of the experimental cases in this investigation are analyzed below in terms of this period. Factors which might have had some influence on it such as the method of infection, length of the incubation period and severity of the reaction are also considered.

Materials and Methods

Detailed experimental protocols on the cattle listed in Table 23 have been given in previous chapters.

Briefly, with regard to the six " natural " cases, Ox 9575 was infected mechanically by *G. brevipalpis* (Chapter 5); Oxen 9398 and 9472 mechanically by tabanids (Chapter 6); Ox 9703 mechanically by *S. calcitrans* (Chapter 7); and Oxen 3204 and 2924 via the nostrils with cyst organism-containing skin suspensions (Chapter 9).

Regarding the " artificial " cases, Ox 9160 was challenged with the 29th passage-level of the Schoeman strain after tabanids had failed to infect him mechanically (Chapter 6); Ox 2052 with testes suspensions from rabbits infected with a recently isolated strain and Ox 2431 with 4 litres of blood from the latter (Chapter 12); Heifer 3088 with rabbit blood and a testes suspension harbouring a strain isolated from *S. calcitrans* (Chapters 6 and 12); Oxen 3894 and 3515 with a recently isolated strain grown *in vitro* and Ox 3565 with 3·5 litres of blood from the former (Chapter 12).

In addition to the usual examination for signs of infection (Chapter 3), the animals were inspected at weekly intervals for SC cysts until their numbers had reached a constant level and thereafter less frequently until slaughter. In cases where cysts were rare their identity was confirmed microscopically (Chapter 3). The maximum diameter of cysts in sections of skin biopsy specimens taken on the day that they became clearly visible in the SC, was determined with the aid of an ocular micrometer in five cases.

Results

The cattle that were infected by a method that might occur in nature have been classified as " natural " and those inoculated with a syringe as " artificial " cases.

In the 13 head of cattle listed in Table 23 the first sign of a besnoitiosis reaction invariably was a rise in temperature persisting from five to 28 days. Some showed a prodromal, one-day elevation, usually a day or two before the reaction proper, which was not included when calculating the time-interval that elapsed from the day the first symptom was seen until SC cysts were clearly distinguishable. In six of the animals minute protuberances were just discernible a week before the actual time listed. They were so small, however, that they would not have been recognized as such had their appearance not been expected.

The period varied from 35 to 49 days with an overall mean of 39, viz. almost six weeks. There was no suggestion that it was influenced by the method of infection. The difference between the means of six " natural " and seven " artificial " cases was only 2·79 days (Table 23). Furthermore, no clear-cut evidence could be obtained that the time-interval was influenced by the severity of the reaction. Although a difference of 4·47 days was noted between the means of the seven mild (41·14 days) and the six more severe cases (36·67 days) it cannot be regarded as significant seeing that the examinations were conducted at weekly intervals. In addition there was marked overlapping between individual figures of the two groups.

TABLE 23.—*Chronological data of the appearance of SC cysts in experimentally infected cattle*

Type of case	Bovine No.	Infection			Reaction			Time taken for SC cysts to appear	
		Date	Method	Route	Severity	Incubation period (days)	Duration fever (days)	After infection	After first fever
" Natural ".	9575	23.9.60	Tsetse flies.....	bites	M	13	7	53	40
	9398	10–11.1.61	Tabanids.....	bites	FS	13	12	54	41
	9472	18–19.1.61	Tabanids.....	bites	M	13	6	62	49
	9703	13.7–14.8.61	Stable flies.....	bites	M	n.d.	5	n.d.	39
	3204	23.11.66	Skin suspension.....	nostrils	M	12	9	49	37
	2924	20.12.66	Skin suspension.....	nostrils	M	13	6	50	37
				Mean values.....		12·8	7·5	53·6	40·5
" Artificial "	9160	19.6.61	Rabbit blood.....	i.v.	M	7	6	45	38
	2052	28.2.66	Rabbit testes.....	i.v.	FM	4	8	51	47
	2431	8.3.66	Bovine blood.....	i.v.	FS	7	15	43	36
	3088	6.2.67	Rabbit blood + testes.....	i.v.	FS	7	13	44	37
	3894	8.6.67	Cell culture.....	i.v.	FS	3	10	40	36
	3565	15.6.67	Bovine blood.....	i.v.	FS	5	10	40	35
	3515	6.9.67	Cell culture.....	i.v.	S	1	28	36	35
				Mean values.....		4·87	12·86	42·71	37·71
				Overall mean values.		8·84	10·18	47·25	39·0

Legend: See Table 21

The situation is somewhat different if one considers the time-interval between infection and the appearance of distinct SC cysts, which presents a more useful concept in epidemiological studies. It varied from 36 to 62 days with an overall mean of 47·25 in 12 of the animals. The thirteenth (Ox 9703) was not included in the calculations because the transmission process was so drawn out that the probable time of infection could not be determined with any accuracy (Table 23). In the case of Oxen 9398 and 9472 calculations were based on the assumption that they had contracted besnoitiosis on the last day of exposure. The mean was 53·6 days, or almost eight weeks, in the five " natural " cases, whereas it was only 42·71 days in the seven " artificial " ones, viz. a difference of 10·89 days. The incubation period was largely responsible for the disparity. It was long (mean 12·8 days) in the " natural " and short (mean 4·87) in the " artificial " cases, with a difference of 7·93 days which is fairly close to the abovementioned figure.

" Artificial " cases with their shorter incubation periods were by and large more severely affected than their " natural " counterparts. However, this does not necessarily mean that an inverse relationship between the severity of the disease and the length of the incubation period is a general characteristic of bovine besnoitiosis. Oxen 738, 739 and 1502 for example (Chapter 12) reacted mildly despite the fact that their incubation periods, after artificial infection, were only one day in the former two, and four days in the latter animal. The route of administration and the size and nature of the infective inoculum are apparently all implicated (Table 21) and probably more important.

In most of the animals SC cysts were not very plentiful. Hence their numbers could be estimated fairly accurately. Noteworthy features were the marked degree of synchronicity in their materialization and their persistence in more or less constant numbers over relatively long periods of observation. In the Animals 9398, 9472, 2924, 2431 and 3088 there was little if any change in the estimated number from the time they were first seen until slaughter after 13, 48, 35, 343 and 34 days respectively. The rest (Oxen 9575, 9703, 3204, 9160, 2052, 3894, 3565 and 3515) showed from slight to about four-fold increases over a period of seven to 21 days after cysts were first seen. Thereafter no obvious alterations occurred over 121, 32, 100, 26, 13, 18, 28 and 42 days respectively.

Synchronicity also featured strongly in skin biopsies taken on consecutive days from animals like Oxen 2431, 3515 and Heifer 3088. The uniformity in morphological appearance of cysts in a single section as regards their size, the developmental state of the cyst wall, the staining properties and appearance of the foamy cytoplasm, the number and position of the increasing complement of host cell nuclei en route to the periphery, and in older cysts, the size of infected vacuoles and the number of parasites within such vacuoles, substantiate this viewpoint.

It was assumed that the maximum diameter of cysts in skin collected concurrently would be a fairly accurate reflection of their size in the SC on the day they became visible. The diameter of 17 of the largest cysts in the skin of the gaskin of Ox 2431 varied from 157 to 189 microns; in Heifer 3088 the corresponding values for nine cysts were 205 to 236 microns in the skin of the neck and gaskin, where sizes were very similar; in Oxen 3894 and 3565 it was 173 to 252 and 204 to 221 microns respectively for four and two cysts in the neck; and in Ox 3515 these measurements were 173 to 228 microns for five of the largest cysts. With the exception of Ox 2431 the maximum values were therefore remarkably similar. Mean values were not calculated because the measurements represented cross-sections at various levels through spherical or subspherical objects.

Discussion

From the approximated figure of eight weeks derived from the mean for this period in " natural " cases it should be possible to calculate the probable time of infection of field cases fairly accurately, providing affected animals are examined regularly for the first appearance of SC cysts. Similarly the time when the first symptoms develop can be estimated with even greater accuracy from the figure of six weeks derived from the overall mean for " natural " and " artificial " cases. Also useful in early cases, is the mean incubation period of 13 days obtained from " natural " cases.

Observations on a severe field case indicated that cyst growth may sometimes be slightly slower than in the experimental cases on which these figures are based. The subject (Bull 1061, Chapter 11) was in the late anasarca stage of the disease when first noticed on 30 January 1961. When killed 43 days later, SC cysts were just visible. At this stage the maximum diameter of skin cysts was 197 microns. If the abovementioned figure of six weeks is used it would mean that the first symptoms developed on 31 January which is not possible. Even the extreme value of seven weeks (Table 23) would still be about a week short of the mark, judging mainly from the clinical picture. A possible explanation for this is that the myriads of cysts and cyst organisms, competing for space and metabolic requirements in a host becoming progressively weaker every day, grew and multiplied exceptionally slowly.

This means that the abovementioned figures may not always be precisely applicable to very severe cases in particular. They have been put to use in Chapters 4 and 11 to calculate the probable time of infection of mild and clinical natural cases. Provided incubation periods of field cases are not ultrashort, as in some of the " artificial " cases above, or ultralong, as in rabbits, where a maximum of 35 days has been recorded, the figures should, however, be reasonably close to the mark.

The synchronous appearance of SC cysts deserves some comment. The expression is not used in the sense that cysts were all in exactly the same stage of development and became visible simultaneously, but that the whole complement appeared in a remarkably short space of time. This feature is compatible with the opinion expressed by Pols (1960) that proliferation in macrophages ceases after a relatively short spell to be superseded by cyst formation in histiocytes. It also tallies with the transitory parasitaemia observed in acutely diseased cattle (Cuillé *et al.*, 1936; Pols, 1954a, 1960; Chapter 11), and the absence of circulating parasites in (Bigalke, 1967), and immature cysts in histological material from (Schulz, 1960), chronic cases. It also provides indirect evidence in favour of the concept expounded in Chapter 12 that the severity of the disease is determined by the number of infective organisms that gain entrance to the tissues, each of which proliferates intracellularly for a limited period only, and in the process causes the damage responsible for the early symptoms. The whole complement of cysts is apparently formed in the relatively short period coinciding with and contiguous to parasitaemia, from organisms supplied by active proliferation. As soon as proliferation ceases, however, new cysts are no longer formed. Thereafter the only parasitological developments are a limited period of growth and the occasional death of cysts. If differences in the rate of growth of contemporary cysts exist they cannot be great. It is, however, possible that some cysts may eventually be larger than others.

There must obviously be some mechanism that regulates the processes of proliferation and cyst formation. The earliest that young cysts have been detected in skin sections is 12 days after the beginning of the reaction. It is still an open question whether parasitized histiocytes destined to become cysts are present immediately after infection. If not, and supporting evidence to this effect is outlined below, there must be a mechanism(s) which either initially inhibits or initiates cyst development when proliferation has proceeded far enough. Be it as it may, extracystic proliferation soon ceases completely. A similar phenomenon in *T. gondii* has been ascribed to the emergence of immunity (Jacobs, 1956), but immunity is apparently not a prerequisite to the formation of cysts (Stahl, Matsubayashi & Akao, 1966). The fact that the cyst-producing ability of a *Besnoitia* sp. can be lost by rapid passage (Frenkel, 1965), does not exclude the possibility that cyst-production is an inherent property of the parasite. During acute passages selective pressure would favour proliferation, which implies that there must be some organisms that never produce cysts.

Evidence in favour of the viewpoint that cyst-formation is preceded by some proliferation is provided by the fact that the time-interval between the first symptoms and the appearance of cysts is remarkably constant irrespective of the mode of infection and the incubation period. This suggests that cyst formation does not start immediately after infection but is closely related to the reaction when marked proliferation occurs. It also implies that cyst formation would be synchronous even if infection occurred over more than one day, as might well happen with mechanical transmission in nature.

If a reliable method for measuring the exact diameter of developing cysts from day to day in skin sections could be devised, it should be possible to obtain mean values whereby vital epidemiological statistics could be pinpointed with even greater accuracy. It might even be feasible to make use of measurements and the morphological appearance of structures inside the cysts in this respect.

14. General Conclusions

The primary objective of this investigation has been to unravel the mystery of the mode of transmission and the maintenance of *B. besnoiti* of cattle in nature. Inseparable from the outset, and gaining in stature as the study progressed, has been the omnipresent, indestructible, chronically infected beast. It has emerged as a colossus of reliability both as regards its potential as a life-long reservoir of infection as observed under conditions of cohabitation and the antithesis, quarantine experiments, and as a direct source of infection by " natural " and " artificial " methods (Chapter 13).

Foremost among the " natural " methods has been mechanical transmission of the disease from heavily parasitized cattle by several species of biting flies. The seasonal incidence implies that arthropods serve as vectors. Furthermore, localization of many millions of cysts in the dermis where they are easily accessible to the probing probosces of a wide variety of blood-sucking arthropods suggests that this parasite has adapted its developmental cycle rather remarkably to the requirements for transmission by a vector. Apart from contaminative transmission which for reasons outlined previously is regarded as very unlikely, mechanical transmission is the only type that has emerged from this study. The possibility of cyclical development of the parasite in an invertebrate host has, however, not necessarily been exluded since investigations along these lines have not been exhausted.

It is not known for what length of time *B. besnoiti* has been adequately exposed to the opportunity for adaptation to development in invertebrate hosts, assuming of course that it was not a parasite of arthropods originally. The disease itself has been documented since 1884 when Cadéac (cited by Besnoit & Robin, 1914) described the symptomatology so accurately that there can be no doubt that he was dealing with it. From his report it transpires that the condition was known in Southern France as early as 1859. But the association of the parasite with cattle probably reaches back much further than this.

Other parasites worthy of consideration because of their close relationship to *B. besnoiti* of cattle are the strains isolated from blue wildebeest and impala (Bigalke *et al.*, 1967). Despite the fact that the parasites from cattle, blue wildebeest and impala are morphologically and immunogenically indistinguishable and have therefore been classified under a single species, their biological behaviour is so different that they are regarded as separate strains or biological races of a single species, *B. besnoiti*. The most important of these differences, i.e. the dermatotropism of bovine and the viscerotropism of blue wildebeest strains—it has not been possible to passage impala strains indefinitely—have been retained through over 550 and 80 serial rabbit passages respectively, and are apparently genetically determined. Thus there is good evidence of both common and specific genes, which suggests that some evolutionary divergence has taken place since the strains evolved from a common ancestor, presumably occurring in a wild vertebrate host. The divergence has progressed far enough to make it impossible for antelopes infected with their strains to act as reservoirs of the bovine disease as we know it today. In an evolutionary sense, however, the separation must have occurred fairly recently since the strains still share genes responsible for the production of the important immunogenic antigens.

If the higher degree of pathogenicity of the bovine strain for its host is used as criterion, cattle must be more recent hosts of *B. besnoiti* than antelopes where considerable adaptation between parasite and host has apparently occurred. Hitherto only low-grade infections of cysts in the cardiovascular system, subcutaneous lymphatics and some viscera have been encountered in antelopes (Basson, Van Niekerk, McCully & Bigalke, 1965; McCully *et al.*, 1966). The form of parasitism in antelopes is probably ancestral and the same applies to the mode of transmission, which is at present unknown. It must be quite efficient though because the incidence is fairly high. The chances of cyst organisms being ingested and transmitted mechanically is, however, small because cysts are apparently not as readily accessible as those of the bovine parasite. Presumably parasitaemia will be on a low level and fleeting as in cattle so the opportunities for mechanical transmission would be equally slight in the acute stage. Exposure of a wide variety of arthropods to infection with *B. besnoiti* therefore probably only became really common when the parasite acquired its dermatotropism. When this occurred is obscure. But the time-interval, that has elapsed, has probably been long enough to allow for contact with an arthropod with a metabolic make-up suitable for growth and multiplication of the parasite, if such exists. However, the evidence provided by the experiment where cattle were exposed to conditions of cohabitation and the rather effective control obtained by quarantine measures suggest that transmission operates over relatively short distances only and makes biological transmission of bovine besnoitiosis very unlikely.

There are many facets to mechanical transmission of bovine besnoitiosis. They are all interrelated and must all function smoothly for it to be really effective. For reasons outlined previously cattle in the transitory parasitaemic stage are not regarded as being of much importance in the epidemiology. In the chronic stage organisms are confined strictly to cysts and their accessibility to the probing mouthparts will be of vital importance. It stands to reason that the greater the number of cysts in the more superficial layers of the dermis, where they are usually most prevalent (McCully *et al.*, 1966), the greater the odds in favour of one or more being penetrated, and the more suitable such an animal will be as reservoir.

The number of flies attacking a carrier must also be extremely important. The chances of cysts being penetrated, and feeding insects interrupted, will increase proportionally with their numbers. Another cardinal point is the kind of fly involved and its feeding habits. The chances of an insect being disturbed during the feeding process will depend on the painfulness of the bite. The depth of penetration and the type of wound produced will determine to what extent the mouthparts will be exposed to contamination with cyst forms from damaged cysts, and their structure to what extent organisms will adhere to them for purposes of mechanical transmission.

These studies have clearly shown that some insects are more efficient vectors than others. Tabanids appeared to be more proficient than tsetse flies and both were infinitely better than stable flies, judging from the respective numbers required to transmit the disease. Tabanids are, however, rather slow breeders, and although they may be quite plentiful at times, their incidence is even more strictly seasonal than that of besnoitiosis (Gordon & Lavoipierre, 1962; Fig. 1).

It is, however, most unlikely that a single species of fly is responsible for the mechanical transmission of *B. besnoiti*. There are many other less prepossessing biting insects that may play an important role, if not by their quality as vectors, then by virtue of their profusion. *S. calcitrans* and other members of the subfamily Stomoxyinae such as *Siphona* spp. and *Haematobia* spp. also have most painful bites. The latter are plentiful in the enzootic regions at various times of the year. Their association with cattle is rather close and they tend to fly in swarms from one animal to another. More suitable potential mechanical vectors are difficult to imagine. *Hippobosca* spp. are very numerous in some parts of this country. Their virtually permanent association with their hosts places them in the same category. Midges and various species of mosquitoes in particular may be more important than is generally imagined, again on account of their numbers. Depending upon their distribution one insect may take over from another as the seasons, climate and their breeding cycles vary. Simultaneous involvement of more than one species is also possible.

The outbreak of bovine besnoitiosis discovered recently in the Orange Free State (Bigalke & Schoeman, 1967a), which is ecologically very different from the well-known enzootic regions, strengthens the argument that the same or other species of haematophagous insects are probably involved. The occurrence of the disease in countries as divergent in climate and biotope as Africa (Pols, 1960), Israel (Neuman & Nobel, 1960; Neuman, 1962a), Southern France (Besnoit & Robin, 1912), Portugal (Franco & Borges, cited by Pols, 1960), Venezuela (Vogelsang & Gallo, 1941) and Kazakhstan (Vsevolodov, 1961), supports this view.

Information obtained from pilot tests recorded in Chapter 12 suggests that the number of proliferative forms is probably responsible for the degree of severity of the reaction. Although convincing evidence is still lacking it seems likely that this will also apply to cyst organisms transmitted mechanically by flies. It is postulated that, depending upon the existing conditions, mechanical transmission from chronic carriers will be capable of producing anything from inapparent to very severe clinical cases of besnoitiosis.

Indications that skin cysts are more plentiful in certain sites than others may also have a bearing on the transmission. It is known that insects have preferential feeding sites. Tsetse flies are inclined to feed on the limbs and abdomen, *Hippobosca* spp. in the escutcheon and perineal region, *S. calcitrans* on the limbs, *Siphona* spp. on the back line and *Simulium* spp. on the head, legs and abdomen (Lapage, 1962). Should the distribution of cysts coincide with these sites it stands to reason that the flies concerned will have a better opportunity of transmitting the disease.

A question very relevant to this discussion is the origin of bovine besnoitiosis in S.Afr. If ascertainable it would give an indication to what extent and how quickly it is likely to spread in this and other countries. Although it is thought that *B. besnoiti* of cattle evolved from a parasite which has much in common with the strains found in blue wildebeest and impala this probably occurred long before cattle were first introduced into S.Afr. No evidence has yet been forthcoming that there are wild animal reservoirs of bovine strains of *B. besnoiti*, and it is highly significant that the outbreak in the Orange Free State (Bigalke & Schoeman, 1967a) occurred in the complete absence of antelopes. In the light of the available evidence it is therefore contended that the abovementioned antelopes were neither responsible for its introduction, nor of any importance in the epidemiology of the disease in S.Afr.

The only other *Besnoitia* sp. found hitherto in this country is the equine parasite, *B. bennetti*, which has been seen in several horses (Schulz & Thorburn, 1955; Pols, 1960), a mule, a few donkeys (Bigalke & Schutte, 1967) and a zebra [*Equus burchelli* (Gray)] (McCully, Basson, Van Niekerk & Bigalke, 1965). Although it is morphologically virtually indistinguishable from *B. besnoiti* it is nevertheless regarded as a separate species (Babudieri, 1932, cited by Pols, 1960). Recent studies on the pathogenicity have provided good evidence in favour of *B. bennetti* being host specific. On the basis of our present knowledge there is no evidence that cattle, rabbits, mice and hamsters are susceptible to *B. bennetti* (Bigalke & Schutte, 1967) and domestic solipeds are susceptible to *B. besnoiti* (Pols, 1960; Bigalke & Schutte, 1967). It is therefore quite legitimate to state that equines could not have been rseponsible for the introduction of bovine besnoitiosis into S.Afr. either.

Pols (1960) showed that sheep and goats were susceptible to artificial infection with bovine *B. besnoiti* strains and found cysts in skin sections of the latter. Reference has already been made to the presence of cysts in the cornea and nasal mucous membranes of inoculated sheep (Chapter 12). *Besnoitia* cysts have recently been found in the skins of Kenyan goats that acquired the disease naturally (Bwangamoi, 1967). If the parasite proves to be indistinguishable biologically from bovine *B. besnoiti*, the possibility that goats and possibly sheep are sometimes involved in the epidemiology will have to be considered.

Chronically infected cattle are therefore the only known carriers. Good evidence has been presented in this and another investigation (Bigalke & Schoeman, 1967a), that under suitable ecological conditions they can meet all the requirements for maintenance and spread of a disease in a country. It can therefore be assumed that they were responsible for its introduction into S.Afr.

According to Hofmeyr (1945) farmers have expressed the belief that bovine besnoitiosis was introduced into the Rustenburg district from the " North " in the middle thirties, but proof is lacking. The infection may for instance have been present in stock owned by the Bantu migrating into this country from about the fifteenth century A.D. onwards, or even by the Hottentots who preceded them by a few centuries (Epstein, 1955). Their stock may have acquired the infection in Africa or Asia Minor where the latter had evolved over many centuries from a " wild " ancestor.

It is perhaps significant that the disease is most common in the Afrikaner (Chapter 10), a breed thought by most authorities to have been derived from Hottentot stock (Epstein, 1955). Others believe that the Afrikaner originated from crosses between cattle imported into S.Afr. from Portugal and India in the fifteenth century by the Portuguese navigators revictualling at the Cape of Good Hope (Martinho, 1955). It is interesting to note that besnoitiosis has been known in Portugal since 1885 (Franco & Borges, 1916, cited by Pols, 1960), particularly in cattle from the province Alentenjo. It has been suggested that the Afrikaner has been derived from the Alentejana breed. Whatever their origin, if Afrikaners were responsible for the introduction of the disease, it would go a long way to explain the high incidence of besnoitiosis in ranching areas of S.Afr. where this hardy breed predominates.

The writer believes that bovine besnoitiosis is insidious and slow in its spread, but it seems rather unlikely that it could have gone unnoticed for five or six centuries. A more recent introduction in the nineteenth or eighteenth centuries at the earliest, appears more realistic. Bantu stock would then be the main contenders. The likelihood of it having been introduced by European breeds seems remote because they came from countries where the disease is unknown. French cattle have only been imported since the discovery of besnoitiosis in S.Afr. Even if introduced in the nineteenth century, it would still mean that spread to involve most of the cattle-ranching areas of Transvaal with extensions into Zululand (Bezuidenhout, State Veterinarian, Nongoma, Natal, personal communication, 1967), and an apparently isolated focus in the Orange Free State, had been very slow compared to other infectious diseases.

It is this insidiousness which also makes it so difficult to determine how long besnoitiosis has been present on an individual farm. The sudden appearance of clinical cases does not mean that the disease has just been introduced and the animal concerned is the very first case. It may have been present in a subclinical form for some time, possibly even years. On the other hand it might appear quite quickly after the introduction of a number of good carriers, as apparently happened on the farm Doornpoort (Chapter 10).

If allowed to proceed unchecked, the course of events that would follow the introduction of a single subclinically infected animal into a clean herd would probably be very similar to what happened when the disease was first brought into the country.

One would expect a population of inapparent cases with varying degrees of parasitosis to be built up slowly before clinical cases start appearing which would initially pass unrecognized, if not suspected. There would always be many more inapparent than clinical cases. Since all carriers possess an immunity the incidence of clinical besnoitiosis will never be high. Eventually most of the older animals will be infected, whereafter besnoitiosis should only occur in younger animals or in new introductions. No farm has yet been encountered where the latter situation prevails.

A curious epidemiological feature which does not fit in with the latter expectation and is difficult to explain, is the very low incidence of besnoitiosis in animals under a year of age and its virtual absence in unweaned calves. To date it has been encountered in only two of the latter. Both were about four months old when they contracted the infection. One showed mild symptoms and the other was an inapparent case associated with fairly pronounced parasitosis (Bigalke & Schoeman, 1967a). If the explanation for this phenomenon is a natural protection or passive immunity acquired from the dam, as apparently occurs in babesiosis of cattle (reviewed by Riek, 1963), one would expect many calves to contract a mild infection before weaning, thereby rendering them actively immune for life. This does not usually happen, however, because many animals born on farms where besnoitiosis is enzootic contract the disease when they grow older. Perhaps the latter are the progeny of non-immune dams, or alternatively fail to become infected for some reason with subsequent loss of protection. Obviously a more sensitive test to detect infection and immunity is required to unravel some of the unexplained epidemiological features.

Cattle trade will ensure that carriers are transferred to other farms where ecological conditions suitable for transmission also exist, whereas neighbouring ranches may become infected over the fence. This will lead to a slow but certain dissemination of besnoitiosis over extensive areas as has happened in S.Afr., where the disease is still relentlessly enlarging its domain. The bovine carrier will also be responsible for its spread over wide regions of the globe as it is probably already doing surreptitiously.

15. Summary

(1) The central theme of this investigation was the part played by the chronically infected beast in the epidemiology of bovine besnoitiosis. The incentive was provided by the discovery that cyst organisms of *B. besnoiti* were transmissible to rabbits and cattle by inoculation. It brought the long-neglected, indomitable chronic case into the picture as a life-long reservoir.

(2) Transmission was observed to occur if susceptible cattle were allowed to cohabit with chronic cases in open paddocks. All the animals contracted a clinically inapparent form of the disease. About 50 per cent developed relatively small numbers of SC cysts, and in one-fifth of them cysts were detected in skin sections. In the other half immunity to challenge was the only evidence of infection. Transmission was almost exclusively confined to cattle that were in direct contact with the carriers, indicating a mode of transmission that operated over relatively short distances only. Venereal transmission could be excluded because infection occurred irrespective of whether or not bulls were present.

(3) These results, together with the well-known summer seasonal incidence, implied that mechanical transfer of the disease by a blood-sucking arthropod was a very likely mode of transmission. The feasibility of mechanical transmission was therefore investigated in rabbits using *G. brevipalpis* as tool and chronically infected cattle as donors. The majority became infected and developed typical reactions. The experiments were extended to cattle. They all contracted a rather mild form of the disease with no anasarca or scleroderma. Tsetse flies were no longer infective when tested three hours after potentially infective feeds, and there was no indication of cyclical development of the parasite within them. They also transmitted proliferative forms mechanically from rabbit to rabbit.

(4) The study was extended to flies that inhabit enzootic regions. Tabanid flies were also capable of transmitting cyst forms mechanically. Even a single fly could infect a rabbit. Cattle developed mild to fairly severe reactions but again no anasarca and scleroderma were evident. Tabanids remained infective by bite for 24 hours and were shown by rabbit inoculation with triturated flies to harbour viable organisms in their bodies for 29 hours after feeding; there was no sign of cyclical development in the three species tested. Gut smears of newly caught horse flies revealed the presence of flagellates, the " barleycorn " (choanomastigote) forms of which were remarkably similar to *B. besnoiti* at a cursory glance. Morphological, cultural and infectivity studies on rabbits and cattle indicated that they bore no relationship to *B. besnoiti*. The flagellates were apparently developmental stages of *Crithidia* and *Blastocrithidia* or *Trypanosoma* spp. Attempts to relate them to *T. theileri* were inconclusive.

(5) *S. calcitrans* also transmitted cyst organisms mechanically to rabbits and an ox. Subinoculation of small numbers of triturated flies into rabbits revealed that they had no difficulty in penetrating cysts and imbibing organisms, but large numbers of flies were required to transmit the disease mechanically. After ingestion organisms remained infective for an hour only and no biological development occurred. Three of the twelve attempts to isolate *B. besnoiti* from recently engorged stable flies that had been caught in an open paddock containing some chronically infected cattle were successful, which indicated that they also penetrated cysts when feeding naturally.

(6) Even *C. simpsoni* and unidentified *Culex* spp. penetrated cysts and ingested organisms, which retained their infectivity for up to 50 hours.

(7) Successful transmission in the experiment where cattle were exposed to conditions of cohabitation indicated that the transmissibility of *B. besnoiti* by various natural openings should be reinvestigated. Hamsters were susceptible to infection by ingestion of, as well as by inoculation with, tissues harbouring proliferative forms and cysts. Proliferation was enhanced, and symptoms of paralysis were seen in hamsters injected with cortisone. Rabbits could be infected quite readily by intranasal instillation of cyst organisms, but the conjunctival sac was a less reliable route. Cattle were susceptible to proliferative but not to cyst forms administered by mouth. Cyst organisms were, however, infective if dosed via the nostrils. Cattle developed fairly mild but quite distinct reactions with no anasarca or scleroderma. It is, however, difficult to envisage how cattle could be exposed to infection by these routes in nature.

(8) Seven farms in the enzootic region of Transvaal were surveyed for bovine besnoitiosis by examining the SC for cysts. The incidence varied from 2·1 to 49·7 per cent. With one exception where the ratio was 1: 1, inapparent cases were from four to thirty-four times more frequent than clinical ones. No cysts were encountered in calves under six months of age. The highest incidence occurred in animals that were from three to six years of age.

(9) The concept that chronically infected cattle act as carriers was put to trial on a farm in the enzootic region over a seven-year period, by isolation and eventual slaughter of all cattle with SC cysts. The incidence dropped from 4·6 per cent in 1960, to zero in 1966, and rose to 0·08 per cent in 1967. A severe concurrent drought, which was only alleviated during the seventh year, probably contributed to the favourable outcome. Initially the decrease was rather slow, and clinical besnoitiosis continued to occur during the first two years. But thereafter only small numbers of inapparent cases were encountered. The age-incidence before and after intervention by isolation and slaughter was remarkably similar.

(10) In order to determine why some cattle contract a severe infection while the majority develop a mild form of the disease, deliberate attempts were made to produce severe cases of bovine besnoitiosis by a variety of methods. Definite indications were obtained that the quantity as well as quality of the parasites concerned were involved and that direct counts were not necessarily a gauge for their infectivity and pathogenicity. An ox inoculated with a massive dose of a high passage-level strain died 12 days later in the anasarca stage of a very severe form of besnoitiosis. Another animal which received an even bigger dose of a low passage-level strain very nearly succumbed and developed pronounced anasarca and scleroderma.

(11) Analysis of information obtained from a number of experimental bovine cases provided some useful statistical data for epidemiological studies. The time-interval between infection and the materialization of SC cysts was remarkably constant in the different animals, the average being approximately eight weeks. And cysts took about six weeks after the first rise in temperature to appear. The average incubation period of cattle that were infected by methods, which could conceivably occur in nature, was about thirteen days. These figures can be utilized to calculate the approximate date of infection and other vital statistics of natural cases. Another significant feature was the synchronicity in the materialization of cysts in individual animals. If related to information obtained from histopathological studies, this meant that the full complement of cysts was formed during the primary parasitaemic stage of the disease, apparently from organisms supplied by the active but short-lived extra-cystic proliferative activities of the parasites.

(12) Finally the epidemiology of bovine besnoitiosis is discussed in terms of the knowledge gained from these studies. The chronically infected beast has emerged as a very efficient carrier and potential disseminator of the disease. All the evidence points towards the fact that bovine besnoitiosis is self-contained. The only other requirement for transmission to occur is apparently a mechanical vector in the form of a blood-sucking insect.

16. Acknowledgements

The writer wishes to express his gratitude to the following: The Chief, Veterinary Research Institute, Onderstepoort, Prof. Dr. B. C. Jansen for facilities to conduct this investigation and for guidance as promotor; Prof. Dr. W. O. Neitz, head of the Department of Protozoology, for advice and encouragement during the course of these studies and for correcting the manuscript; Mr. J. H. Schoeman for technical assistance and for drawing the graphs; Mr. J. A. Roos, and the staff of the Department of Pathology, for preparation of histopathological material; Technical Services section for keeping records; Mr. H. M. Eloff and Dr. J. D. Skinner for assistance, information and advice regarding the experiment at Mara; Mr. G. J. Davel for assistance in the examination of the cattle at Mara; Mr. A. M. du Bruyn for the photographs and photomicrographs; Mrs. W. M. Willemse for typing the manuscript.

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