Studies of Trypanosoma melophagium (Flu 1908) Noller 1917 (Protozoa Mastigophora)

Sen-Chi Lu
The University of Montana

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STUDIES OF *TRYPANOSOMA MELOCAGIUM*

(FIU, 1908) NÖLLER, 1917

(PROTOZOA: MASTIGOPHORA)

By

Sen-Chi Lu

B.S., National Taiwan University, 1960

Presented in partial fulfillment of the requirements for the degree of

Master of Science

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Approved by:

[Signatures]

Chairman, Board of Examiners

Dean, Graduate School

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CHAPTER I

INTRODUCTION

SYSTEMATICS

According to Hoare (1966), the classification of *Trypanosoma melophagium* is as follows:

- Phylum PROTOZOA Goldfuss, 1818; emend. Siebold, 1845
- Subphylum SARCOMASTIGOPHORA Honigberg & Balamuth, 1963
- Superclass MASTIGOPHORA Diesing, 1866
- Class ZOONASTIGOPHOREA Calkins, 1909
- Order KINETOPLASTIDA Honigberg, 1963
- Suborder TRYPANOSOMATINA Kent, 1880
- Family TRYPANOSOMATIDAE Doflein, 1901; emend. Grobben, 1905
- Genus *Trypanosoma* Gruby, 1843
- Subgenus *Megatrypanum* Hoare, 1964
- Species *Trypanosoma (Megatrypanum) melophagium* (Flu, 1908)
  Nöller, 1917

The synonyms of this parasite are listed below:

- 'Trypanosomenähnliche Flagellaten' Pfeiffer, 1905
- *Crithidia melophagia* Flu, 1908
- 'Leptomonas du Melophage' Roubaud, 1909
- *Leptomonas melophagi* Mesnil, 1909
- *Crithidia melophagi* Swingle, 1909
- 'Crithidia of Melophagus ovinus' Wenyon, 1913

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Leptomonas melophagia Dunkerley, 1913
Trypanosoma (Cystotrypanosoma) melophagi Brumpt, 1913
Trypanosoma woodcocki Brumpt, 1913
Herpetomonas melophagia Doflein, 1916
Trypanosoma (Cystotrypanosoma) melophagia Brumpt, 1922

HISTORICAL REVIEW

Pfeiffer (1905) discovered the presence of a trypanosome-like organism in the gut of sheep keds in Germany. These parasites were later described by Flu (1908) as Crithidia melophagia. Flu (1908) and many subsequent observers, including Swingle (1909), Roubaud (1909), Porter (1910), and Dunkerley (1913), regarded it as a specific insect parasite, transmitted from ked to ked through the ova or by cysts voided with the feces. Woodcock (1909b), on the other hand, suggested that ked-flagellates represented a phase in the life cycle of a sheep trypanosome. This view had been expressed previously by Léger (1902, 1904) and by Woodcock (1909a) for the Crithidia (now Blastocrithidia) of blood-sucking insects in general. In 1910, Woodcock observed a trypanosome in the blood of an English sheep and suggested the possibility that the ked-flagellate was the invertebrate phase of this parasite. His views were severely criticized by Porter (1911a, b) who claimed to have demonstrated transmission of the ked-flagellate by means of cysts or "hereditarily" through the ova of this insect. On the other hand, Woodcock (1911) replied by defending his opinion on theoretical grounds, since the finding of an isolated trypanosome could not be regarded as conclusive proof of the relationship.
The first serious attempt to solve this question experimentally was made by Swingle (1911a, b). He examined the blood of numerous sheep, obtained blood cultures from them, and inoculated other sheep with the gut contents of keds infected with C. melophagia. He also used newly-hatched keds for xenodiagnosis. All of his work produced negative results.

Behn (1911, 1912) observed scanty trypanosomes in the blood of German sheep. By 1912, Chatton and Delanoë (1912) and Cauchemez (1912) had denied the occurrence of this flagellate in the ova of keds.

Nöller (1917) presented an important contribution to this question by demonstrating that the culture forms of both the sheep trypanosome and the ked-flagellate were identical. Thus, he transferred C. melophagia to the genus Trypanosoma Gruby, 1843. Nöller (1919a, b) also demonstrated by hemoculture the presence of trypanosomes in a large proportion of sheep in Germany. Kleine (1919) provided experimental evidence of the connection between the sheep-trypanosome and the ked-flagellate. He demonstrated that laboratory-bred keds became infected only after feeding on sheep that harbored trypanosomes in their blood. He proved that keds were unable to transmit the flagellates to one another and also reported that the ked transmitted the trypanosome to sheep by the inoculative method through the bite. Witzky (1922) and Sprehn (1923), however, examined the proboscis of numerous keds and failed to find the infective stages of the flagellates described by Kleine (1919).

Although there was strong evidence of the connection between the sheep-trypanosome and the ked-flagellate, there still remained the possibility that keds might harbor two kinds of trypanomatid flagellates—one representing the invertebrate phase of the sheep-trypanosome, the
other a specific insect-flagellate (Brumpt, 1922; Sluiter et al., 1922). This problem was finally settled when Hoare (1923) provided morphological and experimental evidence against the existence of "cysts" in infected keds. "Hereditary" transmission of the ked-flagellates was disproved by the inability to find organisms in the ova and larvae of keds, as well as by the absence of infection in newborn insects (Chatton and Delancey, 1912; Cauchemez, 1912; Nöller, 1913; Sikora, 1918; Hoare, 1921). Moreover, the possibility of any monogenetic trypanosomatid being associated with this insect was excluded by the inability of keds to infect each other (Kleine, 1919).

Finally, Nöller (1920) demonstrated the true nature of the ked-flagellates by cultivating them at 37° C, and observing the transformation of epimastigote forms into typical blood forms of *T. melophagium*. Thus, the presence of a trypanosome in the blood of sheep had been definitely established and its connection with ked-flagellates clearly demonstrated. To that date, nothing was known regarding the exact method of its transmission from ked to sheep, nor had its complete life cycle been studied until Hoare (1921, 1922, 1923) carried out experiments on all stages of development of *T. melophagium* in its final and intermediate hosts.

After Hoare's (1923) contribution to the morphology and life cycle studies of *T. melophagium*, eleven papers appeared (Section III) reporting the presence of this parasite in sheep from several countries.

Turner and Murnane (1930) reported that they were able to produce appreciable parasitaemia in a splenectomized lamb from Australia.
Bogdanov and Baldicina (1940) reported a case of intrauterine infection of a still-born lamb from Kazakhstan in which numerous trypanosomes were present in the blood.

Nelson (1956) studied the mortality of sheep keds infected with *T. melophagium*. Nelson (1958) also reported the study of the transfer of sheep keds from ewes to their lambs. He suggested that the infective forms present in ked feces may be airborne and may enter the sheep via the respiratory mucosa as well as by the oral route (1961).

Rodriguez Gómez (1964) reported the complement-fixation test for *Trypanosomiasis melophagia* of sheep in Columbia.

Herbert (1965) reported the cytoplasmic inclusions and organelles of *in vitro* cultured *T. melophagium* and speculated on their function.

Stewart and Beck (1967) found the absence of the DNA-histone antigens in *T. melophagium*.

**GEOGRAPHICAL DISTRIBUTION AND HOSTS**

Little has been published on *T. melophagium* in recent years. Its current prevalence in sheep and its geographical distribution are not clearly delineated. The reason for this uncertainty is because of its harmless nature, non-pathogenicity and its infrequent detection by direct examination of the blood.

Scanty numbers of trypanosomes usually appear in the peripheral blood of sheep. Most authors (Woodcock, 1910; Behn, 1912; Douwes, 1920; Dios, 1928) have detected only single trypanosomes after careful examination of numerous samples of blood, both in fresh preparations and in stained thick smears. Hoare (1923) found only several trypanosomes in...
over a thousand blood smears (fresh and stained) taken from five sheep known infected and thoroughly examined for periods varying from several months to a year. Thus, to date the only reliable method of establishing its presence in sheep is by blood culture.

The prevalence of infection with *T. melophagium* is known for a few countries. In Germany, Nöller (1919), and in England, Hoare (1923), demonstrated upwards of 80 per cent of the sheep examined to be infected. Probably the percentage was near to 100, since the majority of negative cultures were contaminated and were discarded. Few data are available for other countries. These were reported from Southeastern Russia (Bozhenko and Zeiss, 1928), Kazakhstan (Bogdanov and Baldicina, 1940; Cелинкеv, 1946), North Africa (Colas-Belcour, 1931), Canada (Nelson, 1956, 1961), Argentina (Dios, 1928), Australia (Turner and Murnane, 1930; Mackerras, 1959).

*T. melophagium* can be safely assumed to occur in all parts of the world where domestic sheep are kept and where there is evidence that they are infected with keds.

Nothing is known regarding the occurrence of *T. melophagium* in other ovine or non-ovine hosts.

Sheep keds are the only known intermediate host of *T. melophagium*.

**LIFE CYCLE AND MORPHOLOGY**

**Development in Sheep**

In sheep, *T. melophagium* is known from less than a dozen reports of adult blood forms described by several authors (Behn, 1912; Hoare, 1923; Turner and Murnane, 1930). These authors were unsuccessful in
discovering stages of multiplication of this trypanosome in the blood and various organs of sheep.

The trypanosome in the blood of the sheep is of a very large size (Figure 1, a). The measurements (Hoare, 1972) of eight specimens from stained blood smears were as follows: $L = 41$ to $60.5\, \mu\text{m}$, $F = 2.5$ to $7.5\, \mu\text{m}$, body width at the level of the nucleus $= 2.3$ to $4\, \mu\text{m}$, $KN = 2.4$ to $9\, \mu\text{m}$, $PK = 13$ to $21\, \mu\text{m}$, kinetoplastic index $KI = PN/KN = 3.3$ to $6.0$, and the rod-shaped kinetoplast is $1.4\, \mu\text{m}$ in length. (For abbreviations, see Figure 4.)

According to Hoare (1972), the prepatent period in experimentally infected sheep is from six to seven days, while the duration of infection varies considerably, usually about three months. In some cases, infection may last from one week to a month. Infection often terminates in spontaneous recovery, after which time no trypanosomes can be detected in the blood.

**Development in Sheep ked and Transmission**

The entire life cycle of *T. melophagium* in the intermediate host, sheep ked (Figure 2), takes place in its alimentary tract (Hoare, 1923).

The complete life cycle of *T. melophagium* is represented diagrammatically in Figure 3.

Hoare (1923) proved the infection with *T. melophagium* is transmitted to sheep by the contaminative method, *per os*. He used lambs, bred from ewes that had been freed of keds and disinfected before parturition, for the experiment. Nelson (1961) recently suggested that the infective forms present in ked feces may be airborne and may enter the sheep via the respiratory mucosa as well as by the oral route.
Figure 1. Trypanosoma (Megatrypanum) melophagium (1600 X).
a, From sheep's blood. b-e, Epimastigotes in midgut. f and g, Promastigotes in gut. h, Amastigotes in hindgut. i, Metatrypanosomes in hindgut. j and k, Trypomastigotes in primary hemoculture. l and m, Large and small epimastigotes in culture. n and o, Immature metatrypanosomes in old culture. (From Hoare, 1923.)
Figure 2. Sheep ked, *Melophagus ovinus* (left), and its pupa (right). About 137 X.
Figure 3. *Trypanosoma (Nagatrypanum) melophagium*. Life cycle in sheep ked: 1, Trypanosome ingested with sheep's blood. 2, Large epimastigote, giving rise by division, 3, to smaller epimastigotes, 4 and 5, in midgut. 6, Transitional epimastigote in hindgut which is transformed into small one, 8, by division, 7. 9, 9a, 10, Two modes of division of epimastigotes, 8, leading to formation of metatrypanosomes, 10a, in hindgut, 10b, Amastigotes. (From Hoare, 1923, 1972.)
HOST—PARASITE RELATIONS

In Sheep

Hoare (1923) detected no signs of discomfort in naturally and experimentally infected sheep kept under observation for long periods. Thus, no manifestations of disease were produced in his experimental lambs of different ages (from fourteen days to one year) and adult sheep experimentally infected with large doses of flagellates. In addition, there was no evidence that trypanosomiasis in the dam was the cause of abortion (Bogdanov and Balcidina, 1940).

In Sheep Ked

Nelson (1956) reported that large numbers of keds have been observed to die on sheep as a result of blockage of the posterior midgut by large masses of epimastigotes of T. melophagium. In 1958, he also observed that only newly emerged keds are transferred to the lambs. The older keds on the ewes gradually die as a result of infection with T. melophagium.

Experimental Animal Infection

Laveran et al. (1914) found amastigotes in mice fed on the sheep ked infected with T. melophagium. In 1919, these investigators also inoculated six mice intraperitoneally with pure cultures of this parasite. One of the mice remained uninfected, two acquired a light infection, but three were heavily infected. In these mice, intraerythrocytic forms were found in the blood and amastigotes in different organs. All the infected animals showed symptoms of a diseased condition. Galli-Valerio (1923) also claimed to have produced a similar infection in a rat.
On the contrary, Hoare (1921) failed to infect five mice inoculated intraperitoneally with the contents of the infected ked gut. In 1923, he extended the experiments to introduce the stages from the ked gut and cultures both from the ked gut and the sheep blood into laboratory animals (mice, rats, and guinea pigs) per os, intravenously, and intraperitoneally. All the results were again negative. Similar results were obtained by Buchner (1922) who inoculated the contents of ked gut into mice, per os, intraperitoneally, and subcutaneously. Becker (1923) also failed to produce any infection in rats, mice, a rabbit, and a guinea pig inoculated intraperitoneally with *T. melophagium* from the ked. In addition, Bogdanov and Baldicina (1940) inoculated various rodents, two rabbits, three guinea pigs, and four mice, with the blood of a heavily infected lamb, but likewise failed to detect any trypanosomes in the experimental animals.

No other animal experiments have been made since 1940. It is the opinion of Hoare (1972), however, that *T. melophagium* is restricted to sheep as hosts.

**Immunology**

Hoare (1972) believed that numbers of *T. melophagium* in peripheral sheep blood are always kept at a minimum, reflecting upon a certain degree of natural immunity against infection with the flagellates. Turner and Murnane (1930) were able to produce appreciable parasitaemia only in a splenectomized lamb, supporting the views of Hoare (1972). Moreover, the low numbers of trypanosomes normally present in infections are apparently insufficient to elicit a lasting immunity. Hoare (1923) demonstrated that sheep, which had recovered from an infection and were free of
trypanosomes for up to eight months, could be readily reinfected with the second infection lasting two to four weeks. He showed also that as long as sheep are infected with keds, trypanosomes are present in the blood. If the keds are removed, however, the infection diminishes in two to three months, but reappears when sheep are again exposed to keds. The immunity in ovine trypanosomes is, therefore, due to typical premunition (Hoare, 1972).

The immunological test for trypanosomiasis melophagia of sheep so far reported is the complement-fixation test by Rodriguez Gómez (1964).

The only other immunological study was reported by Stewart et al. (1967). They found the absence of DNA-histone antigens in T. melophagium and suggested that this is perhaps linked with the inability of the species to survive in a free-living environment.

CYTOLOGICAL STUDIES

Herbert (1965) studied cytoplasmic inclusions and organelles of T. melophagium cultured in vitro in an enriched N. N. N. medium at 28°C. From electron microscope-histochemistry studies he demonstrated a complex system of organelles (mitochondria, Golgi apparatus, endoplasmic reticulum, ribosomes, and lysosomes) and inclusions (lipid) in T. melophagium. He reported that his study did not leave one certain that structures seen in electron photomicrographs can be accurately matched with the chemical reactivities seen in the histochemical tests. However, analogy with studies on vertebrate cells permit a number of speculations which he felt might have some basis in fact.
The electron photomicrographs and histochemical studies showed increased granulation or globule formation of trypanosomes as the culture medium ceases to provide a favorable environment. He attributed this granulation in some measure to the accumulation of toxic metabolic products and to the aging of individual cells.

From his studies, he suggested that the volutin granules in trypanosomatidae are either several different inclusions or organelles, or inclusions or organelles at differing stages of development and activity.

CULTURE

Nöller (1917) was the first to culture T. melophagium from both sheep blood and ked gut. The culture medium used was a modified N. N. N. (Nicolle's modification of Novy and MacNeal's) medium.

Hoare (1923) used defibrinated sheep blood and slightly alkaline nutritive broth to establish primary culture of T. melophagium. Either Nöller's or Wenyon-Noguchi's medium was used for his subcultures. The latter medium is also a modified N. N. N. medium. For his cultures from ked gut, only Nöller's and Wenyon-Noguchi's media were used both for primary and subsequent cultures.

Nöller (1919) observed that cultures require a minimum temperature of about 30°C, and will not grow at lower temperatures. For this reason, Hoare kept his cultures at 30°C. On the other hand, Herbert (1961) isolated and maintained T. melophagium from ked guts in an enriched N. N. N. medium incubated at 28°C. Hoare (1923) stated that in experiments using Nöller's medium, the flagellates grew rapidly. Maximum development was reached at about nine or ten days then rapidly declined in numbers. On
the contrary, in Wenyon-Noguchi's medium the flagellates were reported to grow very slowly, sometimes taking a month to attain their full development and were found in cultures after two months.

Primary cultures of *T. melophagium* from sheep blood, blood trypomastigotes are transformed into culture trypomastigote forms (Figure 1). These culture trypomastigotes develop into a variety of epimastigotes, which in turn give rise to metacyclic trypomastigote forms. These begin to appear in old Nöller's cultures after about one month. Their numbers increase both with the age of the individual culture and with the period during which the strain was under cultivation. In Nöller's culture medium, *T. melophagium* from ked gut exhibits similar growth patterns as in the ked gut. Primary Nöller's cultures from sheep blood show the same patterns as those from ked gut growing from epimastigotes, the main stages in ked gut, whereas the blood forms begin as blood trypomastigotes. Development of trypanosomes in culture may imitate normal development in the intermediate host.

The works cited above are the only ones known for the culture of *T. melophagium* outside of its hosts. Most of these investigators have not dealt with their data in an interpretable manner. Furthermore, the differentiation of different morphs, pH, and varied temperatures have not been studied. The only primary culture media used previously have an N. N. N. base for ked and blood forms; whereas, broth culture medium has only been used for primary cultures of blood forms.
STATEMENT OF PROBLEM

*Trypanosoma melophagium* seems to exhibit a high prevalence and world-wide distribution in domestic sheep and the common sheep ked, *Melophagus ovinus*. In spite of the fact that *T. melophagium* is a common parasite, very little is known of its cultural characteristics. Its diagnosis in sheep is almost entirely dependent upon its culture from infected blood. For this reason, it was decided to study the growth of *T. melophagium* at different inoculum sizes, temperatures, and pH in Modified Monophasic Medium for Trypanosomes (MMNT) and Medium 199 with ten per cent calf serum containing different percentages of hemolyzed defibrinated rabbit blood. The stage composition of *T. melophagium* at different temperatures in media as well as the morphological studies of trypomastigote forms in MMNT and Medium 199 with ten per cent calf serum containing five per cent hemolyzed defibrinated rabbit blood (199-CS-5) were studied statistically.
CHAPTER II

MATERIALS AND METHODS

GENERAL METHODS AND MATERIALS

Dissection of Sheep Ked Gut

Sheep keds were placed on their backs on a slide under the dissecting microscope. The living ked was cut into two parts along the transverse suture just anterior to the second pair of legs. The portion with the head was discarded. The lateral abdominal margins of both sides of the body were cut carefully with a razor blade to avoid damaging the internal organs. The ventral abdominal wall was then separated with a pair of scissors. One drop of antibiotic-saline (0.7 per cent NaCl with penicillin G, 100 units/ml and streptomycin sulfate, 100 ugm/ml) was placed on the preparation. The ked was held fast with a needle and the abdominal wall was reflexed to the rear with a forceps. The end of the rectum was then held by the forceps and pulled to the rear, causing removal of the entire gastrointestinal tract. The midgut was then cut off for inoculating into the medium (Section III).

Culture Media and Their Preparations

One diphasic and two monophasic media were used for the studies. In Nicolle's modification of Novy and MacNeal's (N. N. N.) Medium, 0.7 per cent NaCl with penicillin G, 100 units/ml and streptomycin sulfate, 100 ugm/ml, was used for overlay. Medium 199-CS was made by adding...
10 per cent inactivated (56° C for 30 minutes) calf serum into Medium 199. It was kept at 4° C in a cold room as a stock medium. Before using, 5, 10, and 15 per cent hemolyzed defibrinated rabbit blood were added to make media 199-CS-5, 199-CS-10, and 199-CS-15. Another primary monophasic medium used was Modified Monophasic Medium for Trypanosomes (M199MT).

The preparations of N. N. N. medium and Medium 199 are as indicated by Novy et al. (1904) and Morgan et al. (1950), respectively.

The medium M199MT described by Wang et al. (1973) was modified in one respect: calf serum replaced the horse serum. The composition and preparation of this medium were as follows:

- Trypticase soy broth ........................................... 16 cm
- Distilled water .................................................. added to 500 ml

The above mixture was heated gently and then autoclaved at 15 lbs. pressure at 121° C for 15 minutes. After cooling, the following materials were added.

- Inactivated (56° C for 30 minutes) and filtrated calf serum ........................................... 30 ml
- Hemolyzed defibrinated rabbit blood (1 vol blood in 3 vols distilled water) .................. 120 ml
- Penicillin G .................................................... 200 units/ml
- Streptomycin sulfate ........................................ 200 ug/ml

The pH values of M199MT were adjusted by adding 10 per cent sodium bicarbonate or 2 N hydrogen chloride to the trypticase soy broth before autoclaving.

The media were prepared and dispensed in such a manner that each culture tube (16 mm X 150 mm, with screw cap) contained a final volume of 5 ml.
Isolation of *T. melophagium* from Sheep Ked

The midgut was dissected from the sheep keds. Only the posterior two-thirds of the midgut was used for inoculation into the medium. The isolated portion of midgut was then teased with two needles and washed continuously in three drops of antibiotic-saline on the slide before inoculation into screw capped tubes containing a N. N. N. medium. The culture tubes were kept in the incubator at 27° C. Seven days after inoculation the tubes were examined for the presence of organisms by removing a bacteriological loop of medium antiseptically. If flagellates were present, they were transferred into MMMT after an additional three days for maintenance of the flagellates.

Maintenance of Cultures

Flagellates were maintained in MMMT at 27° C for ten days by adding 1 ml of culture medium from ten-day-old culture to 10 ml of medium newly prepared or medium thawed after freezing.

For obtaining large numbers of organisms for study, large volumes of medium were used. The medium was prepared in milk dilution bottles containing 100 ml medium or 300 ml in 500 ml bottles. In both cases, the inoculum consisted of 10 ml ten-day-old culture per 100 ml medium.

Preparation of Stained Slides

Giemsa stain was used for tincturing culture smear slides.

Culture smears were prepared by placing one drop of culture medium on a clean slide and spreading evenly with a Pasteur pipette. The preparations were air-dried before and after fixation in absolute methanol for three minutes. The fixed preparations were then tinctured with dilute
Giems stain (stock Giemsa 1 drop:2 ml phosphate buffered water, pH 7.0) for 35 minutes, and washed in distilled water. Preparations were made permanent with coverslip mounted in Permount.

**Counting Methods**

Flagellates from four culture tubes were mixed and shaken equally. A half or one ml of the sample was removed, fixed and diluted with a solution of 10 per cent glacial acetic acid in 10 per cent formalin in a vial. From two to twenty-one different dilutions according to the population of organisms in medium were made. The preparation was then counted in an improved Neubauer hemocytometer after fixing and diluting. All counts were performed in duplicate.

**Measuring Methods**

The measurements of flagellates were made by means of a camera lucida at a magnification of 2000 X. An outline drawing was made of the projected image of each flagellate, including nucleus and kinetoplast. A line was carefully drawn through the midline of the projected figure of organism. The distance was measured by marking with a pair of dividers and correlated with a projected scale corresponding to each drawing. Where necessary, estimates of fractions were made by eye to 0.25, 0.33, 0.50, 0.66, and 0.75 of a micrometer. This method was modified from that of Bruce, Hamenton, and Bateman (1909) by use of a transparent plastic ruler instead of dial calipers.

Figure 4 indicates the six measurements of body size where they were observed.
Figure 4. Diagram of measurements of trypanosomes. \( L \) = total length (including free flagellum); \( PK \) = distance from posterior end to kinetoplast; \( KN \) = from kinetoplast to middle of nucleus; \( PN \) = from posterior end to middle of nucleus; \( NA \) = from nucleus to anterior end; \( F \) = length of free flagellum. (After Hoare, 1970.)
Statistical Methods

Statistical methods were employed on the morphological studies of trypomastigote forms cultured in M9MT and 199-CS-5 at 37° C. The statistical constant calculated included the mean, standard deviation, standard error of the mean. In order to present the statistics for the measurements graphically, Dice squares (Dice et al., 1936) showing the mean, ranges, standard deviation, and twice standard error of the mean were made. In this system if twice the standard error of the mean areas do not overlap, the differences are statistically significant. If standard deviation areas do not overlap, separation of groups is on an 84 per cent level.

EXPERIMENTAL METHODS AND MATERIALS

Growth Studies with Different Inoculum Size

Two different inocula, 4.5 X 10^5 organisms/ml and 8.1 X 10^5 organisms/ml, were used for culturing T. melophagium in M9MT at each temperature of 27° and 30° C.

Growth Studies at Different Temperatures

The temperatures of 27°, 30°, and 37° C were used for growth studies in M9MT; 27° C for 199-CS-5, 199-CS-10, and 199-CS-15; and 37° C for 199-CS-5.

The organisms were counted every two or four days for twenty days.

Growth Studies at Different pH

Cultures were studied for twenty days at pH values of 6.3, 6.66, 7.25, and 7.7 in M9MT. Cultures at pH 5.93, 8.25, and 8.55 were studied for two to eight days.
The growth curves in different inoculum sizes, temperatures, and pH were made according to the data observed.

**Developmental Stage Composition in Culture Smear Slides**

A minimum of 200 organisms were counted from each culture smear slide under oil immersion (1000 x) for percentages of different stages.

**Morphological Studies of Trypomastigotes in MmMT and 199-CS-5 Incubated at 37° C on Day Four**

Fifty trypomastigotes from each of MmMT and 199-CS-5 were measured for PK, KN, NA, and F. PN is the sum of PK and KN, while the total body length is the sum of PK, KN, NA, and F. The body width was measured from the breadth through the center of the nucleus. The nuclear index (NI) was calculated from NI = PN/NA, and the kinetoplastic index (KI) from KI = PN/KN.

The above measurements obtained were studied statistically.
CHAPTER III

RESULTS

GROWTH STUDIES OF T. MELOPHAGIUM IN CULTURE MEDIA

All counts reported throughout this section will be in number of organisms/ml.

Inoculum Size

In order to establish a base line to investigate growth characteristics of T. melophagium in MMNT culture media, the effects of different concentrations of the organisms at varied temperatures were studied.

Figure 5 shows growth characteristics of cultures inoculated with $8.1 \times 10^5$ and $4.5 \times 10^5$ in MMNT at 27° and 30° C. No lag phases were observed in each instance. At 27° C, cultures inoculated with $8.1 \times 10^5$ increased their numbers rapidly to attain a maximum population of $48 \times 10^5$ on day six, six times their initial inoculum size. After this time, the number of organisms decreased rapidly. Cultures initiated with an inoculum of $4.5 \times 10^5$ and incubated at 27° C increased their numbers slowly and peaked at $17 \times 10^5$ on day twelve, 2.6 fold their initial inoculum. These cultures peaked six days later than those inoculated with the larger inoculum size. The number of organisms then decreased gradually during the following days.

Similar results were obtained for cultures incubated at 30° C. However, maximum populations were much more higher than those incubated
Figure 5. Effect of inoculum size on the growth of T. melophagium in MMST at 27° and 30° C.
at 27° C. At 30° C, cultures inoculated with $8.1 \times 10^5$ increased their numbers rapidly and peaked at $66.6 \times 10^5$, about eight fold their inoculum size, on day eight. This was followed by a rapid decrease during the subsequent two days, then more gradually on the following days. Cultures initiated with $4.5 \times 10^5$ increased their numbers rapidly and peaked at $42 \times 10^5$, about nine times the initial inoculum size on day ten, then decreased gradually during the following days. It also took these cultures two days longer to peak than those established with larger inoculum.

Effect of Temperature on MMMT Cultures of T. melophagium

Figure 6 shows that the optimum growth temperature for T. melophagium in MMMT is 30° C.

At 37° C, the organisms attained a population of $16 \times 10^5$ on day two from an initial inoculum of $8.1 \times 10^5$. This was followed by a very slow increase to a peak at day six when $18 \times 10^5$ were counted. The number of organisms decreased during the following six days. From day twelve to sixteen the population of the organisms remained at about the same level. On day twenty the number of organisms decreased below the inoculum size.

 Cultures growing at 27° and 30° C were described under the previous heading.

Effect of pH on MMMT Cultures of T. melophagium Incubated at 30° C

In this study the size of the initial inoculum was $7.9 \times 10^5$. In MMMT held at pH 5.93 the organisms did not multiply, and few sluggish organisms were found in medium taken from the bottom of the culture tube during days two to eight. At pH 8.25 and 8.55 only sediment in about
Figure 6. Effect of temperature on the growth of *T. melophagium* in MMT.
one-third of the medium was found in each culture tube. Occasionally few disintegrated organisms were found on day two.

Figure 7 depicts MMNT cultures held at pH 6.3, 6.33, 7.25, and 7.7. At pH 6.3 the organisms multiplied rapidly in the first two days then increased slowly in numbers to $26 \times 10^5$ on day twenty. At pH 6.66 the organisms increased in numbers rapidly in the first four days to $56 \times 10^5$, then showed a tendency toward a long stationary phase until day twenty. The maximum population at pH 6.66 was seen on day twelve at $64 \times 10^5$, eight fold its inoculum size. At pH 7.7 the cultures increased in numbers rapidly in the first four days then increased slowly to attain a maximum population of $52 \times 10^5$ two days later, 6.5 times their initial inoculum size then decreased more rapidly than at any other pH.

The growth of the organisms in MMNT at pH 7.25 was shown previously in this chapter.

**Growth of T. melophagium in Medium 199-CS Plus Different Concentrations of Hemolyzed Defibrinated Rabbit Blood at 27°C**

Figure 8 shows that 199-CS-15 induced the fastest growth rate of the organisms in the three different media tested. The protozoans increased rapidly in numbers to attain a maximum population of $53 \times 10^5$ on day six, 6.5 fold increase over its initial inoculum. Then the count decreased gradually to $11.5 \times 10^5$ on day twenty. On day ten a second peak in the number of organisms ($39 \times 10^5$) was observed. The second fastest growth was observed in 199-CS-10, in which the number of cells increased rapidly and peaked at $44 \times 10^5$ on day six, 5.5 fold its initial inoculum, but decreased rapidly on the following two days to $32 \times 10^5$ before increasing...
Figure 7. Effect of pH on the growth of T. melophagium in MMNT at 30°C.
Figure 8. Effect of concentrations of hemolyzed defibrinated rabbit blood on the growth of T. melophagium in 199-CS at 27°C.
slowly again to a population of $39 \times 10^5$ on day fourteen. However, the numbers decreased slowly on the following four days then rapidly to $18 \times 10^5$ on day twenty.

Cultures of organisms in 199-CS-5 provided the lowest numbers of organisms of the three different media prepared. Numbers of organisms increased rapidly during the first four days to $30 \times 10^5$. Then the cultures continued the same level to day twelve. On day fourteen the same cultures attained a maximum population of $37 \times 10^5$, 4.5 times their initial inoculum, then gradually decreased to $20 \times 10^5$ on day twenty.

**Growth of T. melophagium in 199-CS-5 at 37° C**

As shown in Figure 9, the organisms attained the maximum population of $26 \times 10^5$ on day four, three times their initial inoculum, then rapidly decreased during the following eight days. From day twelve to sixteen the population of the organisms remained stationary. By day twenty, the number of organisms had slowly decreased to $9.7 \times 10^5$.

**STAGE COMPOSITION STUDIES**

**EDMT at 27° C**

Figure 10 shows that the percentage of epimastigotes increased slightly during the first four days from 92 to 97.5. The percentage of organisms decreased gradually to 45.5 on day sixteen, then increased slowly to 50.5 on day twenty. On the other hand, the percentage of the degenerating forms decreased slightly during the first four days from 8.1 to 2.5. Subsequently, degenerating forms increased gradually to 53.5 per cent on day sixteen, then decreased slowly to 49 per cent on day twenty.
Figure 9. Growth curve of *T. melophagium* in 199-CS-5 at 37°C.
Figure 10. Changes in proportions of stages of *T. melophagium* in culture in MM at 27° C. D, degenerating forms. E, epi­mastigotes. T, trypomastigotes.
A very low percentage of trypomastigotes were observed on days six, eight, sixteen, eighteen, and twenty, varying from 0.5 per cent to 1 per cent.

**MMT at 30° C**

Figure 11 shows that the percentage of the epimastigotes increased slightly during the first two days from 92 to 94.5. The percentage then decreased gradually to 19 on day twenty. On the other hand, the percentage of degenerating forms decreased slightly during the first four days from 8.1 to 2.5, increasing gradually to 77.5 on day twenty. The percentage of the trypomastigotes found was very low, varying from 1.5 on day two to 8.5 on days fourteen and sixteen.

**MMT at 37° C**

Figure 12 shows that the percentage of epimastigotes decreased gradually from 92 on day 0 to 0 on day twenty. On the other hand, degenerating forms gradually increased in the percentage from 8.1 on day 0 to 100 on day twenty. Trypomastigotes attained their peak on day four with a percentage of 23.5. The following four days represented a stationary phase followed by a gradual decrease to 0 on day twenty.

**199-CS-5 at 37° C**

Figure 13 shows the percentage of epimastigotes decreased rapidly from 92 on day 0 to 8.3 on day eight with a gradual decrease to 0 on day twenty. On the other hand, the percentage of degenerating forms increased slowly during the first four days from 8.1 on day 0 to 10.4 on day four. This was followed by a rapid percentage increase to 76.5 on day eight then a slowed rate of increase to 98.5 on day twenty. Trypomastigotes peaked
Figure 11. Changes in proportions of stages of *T. melophagium* in culture in MMNT at 30° C. D, degenerating forms. E, epi-
mastigotes. T, trypomastigotes.
Figure 12. Changes in proportions of stages of *T. melophagium* in culture in MMAT at 37° C. D, degenerating forms. E, epi-mastigotes. T, trypomastigotes.
Figure 13. Changes in proportions of stages of *T. melophagium* in culture in 199-CS-5 at 37° C. D, degenerating forms. E, epimastigotes. T, trypomastigotes.
at 32.5 per cent on day four, then decreased gradually to 15.2 per cent on day eight. From day eight to twelve trypomastigotes decreased slowly to 1.5 per cent on day twenty.

**Culture Forms of *T. melophagium* Observed in the Cultures**

Only the culture forms of *T. melophagium* in MMKT incubated at 27°, 30°, and 37° C, and in 199-CS-5 at 37° C were studied.

Epimastigotes, trypomastigotes, amastigotes, sphaeromastigotes, promastigotes, and degenerating forms have been observed (Figure 14) in both media used. No difference was observed between the kinds of the stages found in both culture media. Epimastigotes were seen abundantly during the exponential phase of cultures at 27°, 30°, and 37° C. Trypomastigotes were found in higher percentage at 37° C, lower at 30° C, and very low at 27° C. Amastigotes, promastigotes, and sphaeromastigotes were rarely seen in the cultures. Degenerating forms increased in the percentage in the later incubation period of the cultures at any temperature studied. Meta-trypanosomes were rarely found in cultures and most of those found were immature.

**MORPHOLOGICAL STUDIES OF THE TRYPOMASTIGOTES IN MMKT AND 199-CS-5 INCUBATED AT 37° C**

Fifty trypomastigotes were measured in each medium used on day four when both cultures peaked. Some of the trypomastigotes studied are illustrated in Figures 15 and 16. The results of measurements are shown in Table 1.
Figure 14. Various stages of *T. melophagium* from cultures in MMT at 27°, 30°, and 37° C, and 199-CS-5 at 37° C. 1-4, Various sizes of epimastigotes. 5 and 6, Aberrant epimastigotes with undulating membranes partly detached or absent. 7, Aberrant epimastigote with two nuclei. 8-10, Dividing epimastigotes. 11-13, Various sizes of trypomastigotes. 14, Dividing trypomastigote. 15, Aberrant trypomastigote with two nuclei. 16-27, Different stages of metacyclogenesis. 28 and 29, Sphaeromastigotes. 30, Amastigote. 31, Promastigote. 32-41, Different stages of degeneration of culture forms.
Figure 15. Trypomastigotes from four-day M M T cultures at 37°C. (Camera lucida drawings; at 2000 X from air-dried Giemsa preparations.)
Figure 16. Trypomastigotes from four-day 199-CS-5 culture at 37° C. (Camera lucida drawings, at 2000 X from air-dried Giemsa preparations.)
Table 1

Mensural Ranges and Means, and Nuclear and Kinetoplastic Indices of the Trypomastigotes Cultured in MLEIT and 199-CS-5 on Day Four of Incubation at 37° C

<table>
<thead>
<tr>
<th>Medium</th>
<th>Total Length</th>
<th>Flagellum</th>
<th>Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLEIT</td>
<td>Mean 34.33±0.87</td>
<td>9.82±0.55</td>
<td>1.88±0.05</td>
</tr>
<tr>
<td></td>
<td>S. D. 6.16</td>
<td>3.88</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Range 20.50-47.75</td>
<td>3.50-17.50</td>
<td>1.25-3.00</td>
</tr>
<tr>
<td></td>
<td>Mean 35.38±0.60</td>
<td>9.46±0.41</td>
<td>2.01±0.06</td>
</tr>
<tr>
<td>199-CS-5</td>
<td>S. D. 4.28</td>
<td>2.93</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>Range 25.66-44.50</td>
<td>3.33-14.75</td>
<td>1.00-3.33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medium</th>
<th>PK</th>
<th>KN</th>
<th>PN</th>
<th>NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLEIT</td>
<td>Mean 9.75±0.33</td>
<td>3.87±0.22</td>
<td>13.63±0.40</td>
<td>10.88±0.48</td>
</tr>
<tr>
<td></td>
<td>S. D. 2.37</td>
<td>1.58</td>
<td>2.84</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>Range 4.50-17.00</td>
<td>1.50-8.50</td>
<td>8.00-21.00</td>
<td>4.25-17.25</td>
</tr>
<tr>
<td></td>
<td>Mean 11.66±0.33</td>
<td>3.97±0.27</td>
<td>15.63±0.46</td>
<td>10.29±0.41</td>
</tr>
<tr>
<td>199-CS-5</td>
<td>S. D. 2.35</td>
<td>1.93</td>
<td>3.26</td>
<td>2.87</td>
</tr>
<tr>
<td></td>
<td>Range 7.75-17.00</td>
<td>1.00-9.00</td>
<td>9.75-25.00</td>
<td>4.50-17.00</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Medium</th>
<th>Nucleus</th>
<th>Nuclear Index</th>
<th>Kinetoplastic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLEIT</td>
<td>Mean 2.43±0.07 X 1.30±0.03</td>
<td>1.40±0.09</td>
<td>3.99±0.29</td>
</tr>
<tr>
<td></td>
<td>S. D. 0.52</td>
<td>0.22</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>Range 1.33-3.50 X 0.75-1.75</td>
<td>0.61-3.41</td>
<td>1.94-8.23</td>
</tr>
<tr>
<td></td>
<td>Mean 2.32±0.08 X 1.25±0.04</td>
<td>1.68±0.09</td>
<td>4.70±0.28</td>
</tr>
<tr>
<td>199-CS-5</td>
<td>S. D. 0.53</td>
<td>0.29</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>Range 1.50-3.33 X 1.00-2.00</td>
<td>0.63-3.92</td>
<td>1.97-11.22</td>
</tr>
</tbody>
</table>

Note: All measurements except indices are in micrometers.
As shown in Table 1 and Figure 17, there was no significant difference between trypomastigotes measured from MMNT and those from 199-CS-5. Parameters measured included total body length, body width, length of free flagellum, NA, and size of nucleus. In MMNT, total body length ranged from 20.5 um to 47.75 um (mean = 34.33 um), body width ranged from 1.25 um to 3 um (mean = 1.88 um), length of free flagellum ranged from 3.50 um to 17.50 um (mean = 9.82 um), NA ranged from 4.25 um to 17.25 um (mean = 10.88 um), and size of nucleus ranged from 1.33 to 3.50 um x 0.75 to 1.75 um (mean = 2.43 um x 1.30 um). Measurements for trypomastigotes cultured in 199-CS-5 were total body length from 25.66 um to 44.50 um (mean = 35.38 um), body width from 1.00 um to 3.33 um (mean = 2.01 um), length of flagellum from 3.33 um to 14.75 um (mean = 9.46 um), NA from 4.50 um to 17.00 um (mean = 10.29 um), and size of nucleus from 1.50 to 3.33 um x 1.00 to 2.00 um (mean = 2.32 um x 1.25 um).

A statistically significant difference between trypomastigotes from MMNT and those from 199-CS-5 was demonstrated (Figure 17) with respect to PK, KN, PN, and nuclear and kinetoplastic indices. Measurements and calculations on trypomastigotes cultured in MMNT were as follows: PK from 4.50 um to 17.00 um (mean = 9.75 um), KN from 1.50 um to 8.50 um (mean = 3.87 um), PN from 8.00 um to 21.00 um (mean = 13.63 um), nuclear index from 0.61 to 3.41 (mean = 1.40), and kinetoplastic index from 1.94 to 8.23 (mean = 3.99). Trypomastigotes cultured in 199-CS-5 yielded the following values: PK from 7.75 um to 17.00 um (mean = 11.66 um), KN from 1.00 um to 9.00 um (mean = 3.97 um), PN from 9.75 um to 25.00 um (mean = 15.63 um), nuclear index from 0.63 to 3.92 (mean = 1.68), and kinetoplastic index from 1.97 to 11.22 (mean = 4.70).
Figure 17. Dice squares for trypomastigotes incubated at 37° C in MMVT and 199-CS-5. Extreme limits of variation shown by length of horizontal line; means marked by vertical lines; dark rectangles represent standard deviation; light areas represent twice the standard error of the mean. Where light areas do not overlap, the differences in mean are statistically significant. Where dark areas do not overlap, separation of population is on an 84 per cent level. (Additional abbreviations: W = body width; LN = nuclear length; WN = nuclear width.)
CHAPTER IV

DISCUSSION AND CONCLUSIONS

Culture media contained hemolyzed defibrinated blood from three different rabbits. Several workers have shown that unknown factors in blood from various rabbits may affect the growth of *Trypanosoma* species in blood-based culture media. For example, Dusanic (1968) found that variations in growth of *Trypanosoma lewisi* were observed in culture media prepared with blood collected from different rabbits. For this reason, he heated defibrinated rabbit blood for thirty minutes at 56° C for use in preparing the modified diphasic medium described by Tobie et al. (1950). Variations resulting from the different sources of rabbit blood were not determined in this study. Calf sera, used for preparing the media in this study, were obtained from the same pool and were also inactivated at 56° C for thirty minutes. Thus, any factors of complement that might have affected the *in vitro* growth of *T. melophagium* could not be detected by comparison of the different cultures prepared with different sera.

The concentrations of penicillin G and streptomycin sulfate in the media may have had an effect on the growth of *T. melophagium*. This possibility was not ascertained in the present problem. In this regard, studies on the effects of blood and/or calf serum from various sources as well as varying the concentration of antibiotics, in culture media will be investigated in the future.

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The size of the inoculum affected the subsequent accretion of *T. melophagium* in MM1 both at 27° and 30° C. Maximum populations in cultures initiated with larger inocula were much greater in numbers than were those initiated with smaller inocula. The former cultures developed maximum numbers two to six days earlier than the latter cultures at 30° C and at 27° C, respectively. Because aging of the culture caused denaturation of the medium, cultures initiated with small inocula were not able to attain peak populations as high as those cultures initiated with larger inocula. However, Dusanic (1968), working with diphasic blood-agar cultures of *T. lewisi*, found that the growth limits were similar regardless of inoculum size. He also stated that limitation of growth may be a result of the diffusion of toxic products and/or availability of substrates and growth factors. Further investigations may indicate a great variation in the limitations of different culture media.

Though no lag period in the accretion curves for *T. melophagium* were found, other workers (Chang, 1947; Dusanic, 1968) have stated them to be present in other trypanosome species in other culture media. Cultures described in this study were not examined before the two-day post-inoculation period, and a minor decrease in counts corresponding to a lag phase may have occurred within this two-day period.

According to Möller (1919), the optimum temperature for culturing *T. melophagium* in Möller's medium was 30° C. Möller (1919) stated further that *T. melophagium* would not grow at lower temperatures. In this study, *T. melophagium* was isolated from the midgut of the sheep ked in N. N. N. medium incubated at 27° C. Herbert (1961) also isolated and maintained sheep trypanosomes in an enriched N. N. N. medium at 28° C. Results
obtained in this study support Nöller's findings since the optimum tempera-
ture for incubation of T. melophagium cultures in MMHT of the three tem-
peratures used in this study (27°, 30°, and 37° C) was 30° C.

Preparing MMHT with a specific hydrogen ion concentration was dif-
ficult since the pH was adjusted in the trypticase soy broth which had a
high buffering property. The addition of inactivated calf serum and
hemolyzed defibrinated rabbit blood to the autoclaved MMHT with trypticase
soy broth altered the pH, as did the inoculum added later. The pH values
reported in this study were obtained as soon as possible after inoculation.
Without adjustment, the pH of MMHT prepared according to the usual formula
was 7.25.

The growth of T. melophagium in MMHT of various pH values showed
marked differences. Growth in media of pH 6.66 and 7.25 was more profuse
than in media of pH 6.3 and 7.7. The culture forms in MMHT having a pH
of 6.3 continued increasing in numbers even by day twenty, while those in
MMHT at pH 6.66 showed a tendency toward a long stationary phase at least
to day twenty. Cultures in MMHT with a pH of 6.3 or 6.66 should be
studied further for periods of more than twenty days.

The growth of T. melophagium in 199-CS preparations having dif-
f erent concentrations of hemolyzed defibrinated rabbit blood and incubated
at 27° C indicated that blood concentrations were very important in their
effects. The most rapid growth was observed in 199-CS cultures containing
15 per cent hemolyzed defibrinated rabbit blood. Further studies are
needed on 199-CS containing concentrations of hemolyzed defibrinated blood
greater than 15 per cent to determine the optimum concentration.
The developmental stages of *T. melophagium* observed in MMT cultures incubated at 27°, 30°, and 37° C, as well as those observed from 199–CS–5 culture incubated at 37° C (Figure 14), were identical to those reported by Hoare (1923) in Nöller's and Wenyon-Noguchi's media incubated at 30° C.

When *T. melophagium* was cultured in MMT medium at 30° or 37° C, the relative proportion of epimastigotes decreased and that of degenerating forms increased with greater incubation time. In MMT cultures incubated at 27° C, however, the relative proportion of epimastigotes increased while that of the degenerating forms decreased during the final four days of incubation. This fact was probably attributable to my inability to detect numerous degenerating forms in the stained smears at days eighteen and twenty. Further evidence for an error in detection was an apparent failure of the cultures incubated at 27° C to increase in numbers as did cultures held at 30° and 37° C. Organisms in MMT cultures incubated at 27° C did not increase during the last four days. This was apparently caused by the increasing of the number of uncountable degenerating forms in the stained smears on days eighteen and twenty.

The stage composition of *T. melophagium* cultured in MMT at pH of 6.3, 6.66, and 7.7 were not studied in this research. Castellani, Ribeiro, and Fernandes (1967) have reported that the optimal pH in HIL medium for induction of metacyclogenesis in *Trypanosoma cruzi* was 6.7 at 28° C. The optimal pH required for metacyclogenesis of *T. melophagium* in MMT and 199–CS has not been determined in this study and remains to be studied.
In this study only 199-CS-5 was used to study the stage composition of *T. melophagium* at 37° C. The other two media, 199-CS-10 and 199-CS-15, were not studied. The relative proportions of different stages of *T. melophagium* in 199-CS-5 at 37° C were similar to those cultured in MMT at 37° C. Possibly, the stage composition of those cultured in 199-CS-10 and 199-CS-15 at 27° C are similar to those found in MMT cultures at 27° C.

The differentiation of epimastigotes to trypomastigotes of *T. melophagium* in cultures in MMT was found to occur at 27°, 30°, and 37° C, and in 199-CS-5 at 37° C. The highest percentage of trypomastigotes was found in MMT and 199-CS-5 at 37° C, both on day four. The trypomastigotes were found in a low percentage in MMT at 30° C while rarely found in the cultures at 27° C. Nöller (1920) was the first to demonstrate the true nature of the ked-flagellates through cultivation in Nöller's medium at 37° C when the epimastigote forms were transformed into blood forms of *T. melophagium*. Hoare (1923) also found trypomastigotes in cultures in Nöller's and Wenyon-Noguchi's medium at 30° C. In his hands, the proportion of trypomastigotes rose from 1 per cent to 11.5 per cent by subculturing these forms through fourteen transfers. In this study, the proportion of trypomastigotes found in MMT was 8.5 per cent on day fourteen and sixteen on after the first subculture of transfer from 27° C to 30° C. Decreased proportion of trypomastigotes may occur in the subsequent transfers at the same temperature.

The metatrypanosomes have been rarely found in the cultures in MMT at three temperatures studied as well as in 199-CS-5 at 37° C. Almost all of these metatrypanosomes were immature. The reason for the
observed immaturity of the metatrypanosomes may be due to the fact that a twenty-day incubation period is not sufficient time for metacyclogenesis.

Significant differences were observed in PK, KN, PN, NI, and KI, but none on total body length, body width, length of free flagellum, and size of nucleus of *T. melophagium* kept in MMNT and 199-CS-5 cultures on day four at 37° C.

Nuclear indices of the trytomastigotes of *T. melophagium* in MMNT (ratio: 1.40) and 199-CS-5 (ratio: 1.68) show that the nucleus in forms cultured in MMNT was much further apart from the anterior end of the body than in those cultured in 199-CS-5. Kinetoplastic indices show the kinetoplast in MMNT forms (ratio: 3.99) to be much further apart from the nucleus than in 199-CS-5 (ratio: 4.70).

If the data observed in this study (Table 2) is compared with the most reliable data of Hoare (1972) that was based on eight specimens from stained blood smears, the trytomastigotes cultured both in MMNT and 199-CS-5 were much shorter and thinner than the blood forms. The flagellar lengths, however, were much longer in cultured than in blood forms. The PK of the culture forms were shorter than those of the blood forms. Averages of KN and KI indices of trytomastigotes in MMNT and 199-CS-5 were within the range of those reported for blood forms. Thus, it is clear that the trytomastigotes cultured in MMNT and 199-CS-5 were much smaller in size than blood forms reported by Hoare (1972). The differences in size and morphs are probably due to the different nutritional elements and/or the growth conditions in artificial media compared to sheep blood. In view of the complexities of the undefined blood system, the problem to obtain size and morphs indicated in all the different media used is difficult to accomplish.
Table 2

Comparison of Blood Form Trypomastigotes of *T. melophagium* with Those Cultured in NMT and 199-CS-5 at 37° C on Day Four

<table>
<thead>
<tr>
<th>Observers</th>
<th>Total Length</th>
<th>Width</th>
<th>Flagellum</th>
<th>PK</th>
<th>KN</th>
<th>KI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoare (1972):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In Blood</td>
<td>41</td>
<td>-60.5</td>
<td>2.3 - 4</td>
<td>13</td>
<td>-21</td>
<td>2.4 - 9.0</td>
</tr>
<tr>
<td>This Paper:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In NMT</td>
<td>20.50-47.75</td>
<td>1.25-3.00</td>
<td>3.50-17.50</td>
<td>4.50-17.00</td>
<td>1.50-8.50</td>
<td>1.94-8.23</td>
</tr>
<tr>
<td></td>
<td>(34.33)</td>
<td>(1.88)</td>
<td>(9.82)</td>
<td>(9.75)</td>
<td>(3.87)</td>
<td>(3.99)</td>
</tr>
<tr>
<td>In 199-CS-5</td>
<td>25.66-44.50</td>
<td>1.00-3.33</td>
<td>3.33-14.75</td>
<td>7.75-17.00</td>
<td>1.00-9.00</td>
<td>1.97-11.22</td>
</tr>
<tr>
<td></td>
<td>(35.38)</td>
<td>(2.01)</td>
<td>(9.46)</td>
<td>(11.66)</td>
<td>(3.97)</td>
<td>(4.70)</td>
</tr>
</tbody>
</table>

Notes: 1. All measurements except KI are in micrometers.
2. Numbers shown in the parentheses are means.
CHAPTER V

SUMMARY

1. The size of inoculum affected the growth of *T. melophagium* in MMNT both at 27° and 30° C.

2. The optimum temperature for culturing *T. melophagium* in MMNT was 30° C.

3. Culture forms of *T. melophagium* survived but did not multiply after eight days in MMNT at pH 5.93 at 30° C. These forms died and disintegrated on day two in MMNT at pH 8.25 and 8.55 at 30° C.

4. Culture forms of *T. melophagium* in MMNT at pH 6.66 and 7.25 increased more profusely than those in MMNT at pH 6.3 and 7.7. Medium MMNT at pH 7.25 provided the most suitable condition but the number of organisms decreased more rapidly than that at pH 6.66 after the culture had peaked.

5. Medium 199-CS-15 induced the fastest growth of the three different media (199-CS-5, 199-CS-10, and 199-CS-15) tested.

6. In MMNT held at 30° and 37° C, the proportion of epimastigotes decreased while that of degenerating forms increased in direct proportion to incubation time. The results were similar at 27° C to cultures held at 30° C except on days eighteen and twenty when the proportion of epimastigotes increased and that of degenerating forms decreased. Similar results were observed in 199-CS-5 held at 37° C as in MMNT at 37° C.
7. The differentiation of epimastigotes to trypomastigotes in MfMT cultures held at 27°, 30°, and 37° C, as well as in 199-CS-5 at 37° C. The highest percentage of trypomastigotes was found in the cultures on day four of incubation in both media incubated at 37° C. Thus, temperature seems to be a critical factor.

8. The stages of epimastigotes, trypomastigotes, amastigotes, sphaeromastigotes, promastigotes, and degenerating forms of T. melophagium have been observed and described for MfMT at 27°, 30°, and 37° C as well as in 199-CS-5 at 37° C.

9. The morphology between the trypomastigotes in MfMT and 199-CS-5 at 37° C on day four of incubation was statistically studied and also compared with blood forms.
CHAPTER VI

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