Metacyclogenesis of *Leishmania in vitro* and the axenic culture of *Leishmania donovani* amastigotes

Thesis submitted in accordance with the requirements of The University of Liverpool for the degree of Doctor in Philosophy by

Haytham Ahmed Zakai

October 1996

Dedication

This work is affectionately dedicated to my wife, my daughter,

my parents, my sister and my brothers.

Table of contents

List of Figures i

List of Tables v

List of abbreviations vi

Acknowledgment vii

Abstract vii

CHAPTER ONE

1. INTRODUCTION 1

1.1 The parasite 2

1.1.1 The amastigote 2

1.1.2 The promastigote 3

1.2 The vector 5

1.3 The life cycle 5

1.4 Disease and pathology 7

1.4.1 Visceral leishmaniasis 7

1.4.2 Cutaneous leishmaniasis 8

1.5 Diagnosis 9

1.5.1 Parasitological diagnosis 9

1.5.2 Serological diagnosis 9

1.5.3 Diagnosis by molecular techniques 10

1.6 Epidemiology and geographical distribution 11

1.7 Prevention and control 18

1.7.1 Treatment of leishmaniasis 18

1.7.1.1 Treatment of visceral leishmaniasis 18

1.7.1.1.1 Pentavalent antimonials 19

1.7.1.1.2 Aminosidine 19

1.7.1.1.3 Amphotericin B 19

1.7.1.2 Treatment of cutaneous leishmaniasis 19

1.7.1.2.1 Physical treatment 20

1.7.1.2.2 Chemical treatment 20

1.7.2 Control of sandfly vectors 20

1.7.3 Control of reservoir hosts 21

1.7.4 Protection of individuals 21

1.7.5 Vaccination against *Leishmania* 21

1.8 Objectives of this study 23

CHAPTER TWO

2. AXENIC CULTIVATION OF AMASTIGOTES 24

2.1 Introduction 24

2.2 Materials and methods 31

2.2.1 *Leishmania* parasites 31

2.2.2 Storage of parasites in liquid nitrogen 31

2.2.3 *In vitro* cultures 32

2.2.4 Liquid media 32

2.2.5 Manipulation of parasites 33

2.2.5.1 Manipulating the temperature for the optimum transformation and growth of amastigote-like forms 34

2.2.5.2 Determining the optimum pH for the transformation

and growth of amastigote-like forms 34

2.2.5.3 Examination of different culture media for the optimum growth of amastigote-like forms 34

2.2.5.4 Cultivation of amastigote-like forms in different FCS concentrations 35

2.2.5.5 Cultivation of amastigote-like forms under different gas conditions 35

2.3 Results 36

2.3.1 Manipulating the temperature for the optimum transformation and growth of amastigote-like forms 36

2.3.2 Determining the optimum pH for the transformation and growth of amastigote-like forms 36

2.3.3 Examination of different culture media for the optimum growth of amastigote-like forms 38

2.3.4 Cultivation of amastigote-like forms in different FCS concentrations 38

2.3.5 Cultivation of amastigote-like forms under different gas conditions 42

2.4 Discussion 44

2.5 Conclusion 46

CHAPTER THREE

3. CHARACTERIZATION OF AXENIC AMASTIGOTES 47

3.1 Introduction 47

3.2 Materials and methods 51

3.2.1 Morphological characterization 51

3.2.2 Ultrastructural characterization 51

3.2.3 Biochemical characterization 52

3.3 Results 53

3.3.1 Morphological characterization 53

3.3.2 Ultrastructural characterization 53

3.3.3 Biochemical characterization 63

3.4 Discussion 65

3.5 Conclusion 67

CHAPTER FOUR

4. METACYCLOGENESIS IN *LEISHMANIA* 68

4.1 Introduction 68

4.2 Materials and methods 72

4.2.1 The parasite 72

4.2.2 The determination of the optimum pH for metacyclogenesis 72

4.2.3 Examination of the ability of promastigotes to undergo metacyclogenesis in different culture media with different FCS concentrations 73

4.2.4 Effect of culture ventilation on the pH of the medium 73

4.2.5 Effect of long term culture on metacyclogenesis 74

4.2.6 Complement mediated lysis 74

4.2.7 Effect of constant exposure of promastigotes to changes in pH on their ability to undergo metacyclogenesis 75

4.3 Results 76

4.3.1 The determination of the optimum pH for metacyclogenesis 76

4.3.2 Metacyclogenesis in different culture media with different FCS concentrations 79

4.3.3 Effect of culture ventilation on the pH of the media 79

4.3.4 Effect of long term culture on metacyclogenesis 83

4.3.5 Resistance of metacyclic promastigotes to complement mediated lysis 83

4.3.6 Effect of constant exposure to changes in pH on metacyclogenesis 83

4.4 Discussion 88

4.5 Conclusion 93

CHAPTER FIVE

5. CHARACTERIZATION OF METACYCLIC PROMASTIGOTES 94

5.1 Introduction 94

5.2 Materials and methods 97

5.2.1 Ultrastructural examination 97

5.2.2 Infectivity of *L. donovani* promastigotes to BALB/c mice 97

5.2.3 Infectivity of *L. mexicana*, *L. major* and *L. braziliensis* promastigotes to BALB/c mice 98

5.3 Results 99

5.3.1 Ultrastructural examination 99

5.3.2 Infectivity of *L. donovani* promastigotes to BALB/c mice 112

5.3.3 Infectivity of *L. mexicana*, *L. major* and *L. braziliensis*

promastigotes to BALB/c mice 115

5.4 Discussion 120

5.5 Conclusion 125

CHAPTER SIX

6. DISCUSSION 130

6.1 General discussion 130

6.2 Future directions 137

References 140

Appendix 1. 166

List of Figures

Figure 1.1. Light micrograph of *L. donovani* amastigotes. 4

Figure 1.2. Light micrograph of *L. donovani* promastigotes. 4

Figure 1.3. Life cycle of *Leishmania.* 6

Figure 1.4. Geographical distribution of visceral leishmaniasis. 13

Figure 1.5. Geographical distribution of cutaneous leishmaniasis. 16

Figure 2.1. Growth of *L.donovani* axenic amastigotes in HO-MEM medium supplemented with 20% FCS at different pH. 37

Figure 2.2. Growth of *L.donovani* axenic amastigotes in HO-MEM medium and Schneider's *Drosophila* medium both supplemented with 20% FCS. 39

Figure 2.3. Growth of *L.donovani* axenic amastigotes in HO-MEM medium, M199 both supplemented with 20% FCS, and in 100% FCS. 40

Figure 2.4. Growth of *L.donovani* axenic amastigotes in different FCS concentrations. 41

Figure 2.5. Growth of *L.donovani* axenic amastigotes in different gas conditions. 43

Figure 3.1. Light micrograph of promastigotes, axenic amastigotes, and tissue amastigotes. 54

Figure 3.2. Transmission electron micrograph of a longitudinal section of a promastigote showing flagellum extending beyond cell surface. 55

Figure 3.3. Transmission electron micrograph of a cross section in a promastigote showing paraxial rod. 56

Figure 3.4. Transmission electron micrograph of tissue amastigote showing flagellum terminating at cell surface. 57

Figure 3.5. Transmission electron micrograph of tissue amastigote showing the absence of paraxial rod. 58

Figure 3.6. Transmission electron micrograph of axenic amastigote showing the round to oval shape and the flagellum does not extend beyond the cell surface. 59

Figure 3.7. Transmission electron micrograph of axenic amastigote showing the absence of paraxial rod. 60

Figure 3.8. Scanning electron micrograph of promastigotes. 61

Figure 3.9. Scanning electron micrograph of axenic amastigotes. 62

Figure 3.10. Coomassie Blue staining of SDS-PAGE of lysates of axenic amastigotes, promastigotes and tissue amastigotes using non-reducing sample buffer. 64

Figure 4.1. Light micrograph of procyclic and metacyclic promastigotes. 77

Figure 4.2. Determination of the optimum pH for metacyclogenesis. 78

Figure 4.3. Size destribution of log and stationary-phase promastigotes cultured at acidic and neutral pH. 80

Figure 4.4. The percentage of metacyclic promastigotes obtained in stationary-phase populations in different culture media with different FCS concentrations. 81

Figure 4.5. Effect of culture ventilation on the growth of promastigotes and the pH of the medium. 82

Figure 4.6. Effect of lcng term cultivation on the ability of promastigotes to undergo metacyclogenesis. 84

Figure 5.1. Transmission electron micrograph of a longitudinal section in a metacyclic promastigote cultured at pH 5.5. 100

Figure 5.2. Transmission electron micrograph of a longitudinal section in a procyclic promastigote cultured at pH 7.0. 101

Figure 5.3. Transmission electron micrograph of a longitudinal section in the flagellar pocket of a metacyclic promastigote of *L. donovani* cultured at pH 5.5 . 102

Figure 5.4. Transmission electron micrograph of a cross section in a procyclic promastigote of *L. donovani* cultured at pH 7.0 . 103

Figure 5.5. Transmission electron micrograph of a cross section in a metacyclic promastigote of *L. mexicana* cultured at pH 5.5 . 104

Figure 5.6. Transmission electron micrograph of a cross section in a procyclic promastigote of *L. mexicana* cultured at pH 7.0 . 105

Figure 5.7. Transmission electron micrograph of a longitudinal section in the flagellar pocket of a metacyclic promastigote of *L. major* cultured at pH 5.5 . 106

Figure 5.8. Transmission electron micrograph of a cross section in a procyclic promastigote of *L. major* cultured at pH 7.0 . 107

Figure 5.9. Transmission electron micrograph of a cross section in a metacyclic promastigote of *L. braziliensis* cultured at pH 5.0 . 108

Figure 5.10. Transmission electron micrograph of a cross section in a procyclic promastigote of *L. braziliensis* cultured at pH 7.0 . 109

Figure 5.11 Scanning electron micrograph of a metacyclic promastigote. 110

Figure 5.12 Scanning electron micrograph of a procyclic promastigote. 111

Figure 5.13. Light micrograph of a liver immpression smear showing a liver cell containing amastigotes of *L. donovani*. 113

Figure 5.14. Leishman donovan units (LDU) of infectivity of *L.donovani* procyclic and metacyclic promastigotes. 114

Figure 5.15 A photograph of *L. mexicana* lesion in BALB/c mouse. 116

Figure 5.16 *L. mexicana* lesion development in BALB/c mice. 117

Figure 5.17 *L. major* lesion development in BALB/c mice. 118

Figure 5.18 *L. braziliensis* lesion development in BALB/c mice. 119

Figure 5.19 A photograph of *L. braziliensis* lesion in the footpad of a hamster. 124

List of Tables

Table 4.1. Resistance of promastigotes grown at different pH to complement mediated lysis. 85

Table 4.2. Increase in resistance of promastigotes grown at acidic pH to complement mediated lysis 86

Table 4.3. Effect of culture ventilation on resistance to complement mediated lysis and metacyclogenesis of promastigotes after long term culture. 87

Table 5.1. Infectivity of *L. donovani* promastigotes cultured at neutral and acidic pH to BALB/c mice. 126

Table 5.2. Infectivity of *L. mexicana* promastigotes cultured at neutral and acidic pH to BALB/c mice. 127

Table 5.3. Infectivity of *L.major* promastigotes cultured at neutral and acidic pH to BALB/c mice. 128

Table 5.4. Infectivity of *L.braziliensis* promastigotes cultured at neutral and acidic pH to BALB/c mice. 129

List of abbreviations

cDNA	Copy DNA
DAT	Direct agglutination test
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
HBSS	Hank's balanced salts solution
IFAT	Indirect fluorescent antibody test
kDa	Kilo dalton
kDNA	Kinetoplast DNA
LDU	Leishman donovan units
LPG	Lipophosphoglycan
M 199	Medium 199
PCR	Polymerase chain reaction
PCR-SHELA	Polymerase chain reaction-solution hybridization enzyme linked immuno assay
RE I	Rolf and Erica I medium
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TC 199	Tissue culture 199
WHO	World Health Organization

Acknowledgments

I am thankful to Dr Paul A. Bates and Dr Michael L. Chance who supervised this study for their invaluable help, excellent guidance, considerate suggestions and moral support. I am very grateful for their patience and support throughout the study.

I am also grateful to Mr Peter Young and Mrs Paula Stafford of the Electron Microscopy unit at the School of Tropical Medicine for their help. Also to the Medical Illustration and the Animal Unit staff. Also I thank Mr. David Rynold for his help.

Also I would like to express great thanks and appreciation to my wife, Nadia, who has very patiently supported and encouraged my work. Also to my daughter for giving me the happiness in watching her drawings on the draft of this thesis.

I am also grateful to my father, mother, sister and brothers for their constant support and prayers.

I also extend my thanks to my friends in Liverpool, Mr Zamil Attar, Mr Muftah Ali and Mr Ibrahim Alkhalife for their help and support throughout the period of my stay in Liverpool.

Finally my thanks go to King Abdulaziz University, Jeddah, Saudi Arabia for the financial support of this work.

Abstract

This study has investigated the effects of temperature, pH, and gas conditions on the growth and transformation of *Leishmania donovani* promastigotes and axenic amastigotes. Promastigotes of *L.donovani* were cultured in HO-MEM medium (pH 7.2) supplemented with 10% foetal calf serum (FCS) at 27°C. When these promastigotes were cultured in HO-MEM medium supplemented with 20% FCS at pH 5.5 at 37°C in 5% CO_2 and 95% air, they transformed into round, aflagellate, amastigote-like forms (ALFs). These forms were able to grow as ALFs and to transform back to promastigotes when cultured in neutral HO-MEM at 27°C. The size and morphology of axenically cultured ALFs resembled those of lesion amastigotes. Electron microscopy revealed that ALFs lack the paraxial rod that was present in promastigotes. Promastigotes, ALFs, and lesion amastigotes were compared using SDS-PAGE and Coomassie blue staining of protein bands. A variety of bands were observed in all forms including one prominent band. The molecular mass of this band was approximately 51.3 kDa in promastigotes and 66.1 kDa in ALFs and lesion amastigotes.

Promastigotes of *Leishmania donovani, L. mexicana, L. major* and *L. braziliensis* were cultured in Schneider's *Drosophila* medium supplemented with 20% FCS at an initial pH of 5.5 at 26°C. Such cultures produced more metacyclic promastigotes in stationary-phase than those grown at an initial pH of 7.0. Metacyclic promastigotes possessed a short (≤8 μm), narrow (≤1.5 μm) cell body and a long flagellum which is twice or more the length of the cell body. They were also more resistant to complement mediated lysis than the promastigotes grown at pH 7.0. Ultrastructural examination of metacyclic promastigotes, obtained from stationary-phase population of promastigotes cultured at acidic pH, of *L. donovani* and *L. mexicana* revealed that these promastigotes possessed a fuzzy cell coat measuring 18 nm and 11 nm respectively. This coat was not seen in procyclic promastigotes, obtained from stationary-phase population of promastigotes cultured at neutral pH, of *L. donovani* or *L. mexicana* and also was not seen in promastigotes of *L. major* or *L. braziliensis*. Metacyclic promastigotes of all four species were more infective *in vitro* than procyclic promastigotes.

CHAPTER ONE

1. INTRODUCTION

Leishmaniasis is a disease caused by infection with parasites of the genus *Leishmania*.

The disease can be subdivided into two main categories according to clinical manifestations: visceral and cutaneous leishmaniasis (the latter including mucocutaneous disease). Another useful subdivision is based on the geographical occurrence of the disease. New World leishmaniasis occurs in America, while Old World leishmaniasis occurs in other parts of the world.

Cutaneous leishmaniasis was described in the Holy Bible (New International Version, Zondervan Publication House, Grand Rapids, Mitchigan). The Arabian scientist, Abo Bakr Al Razi described cutaneous leishmaniasis in his famous book "Khulaset Al Tajarib = Essence of Experiments". He described the disease as being endemic in Baghdad city and gave it the name "Habet Al Sana = The sore remaining up to one year" (Morsy, 1996). Since that time and to date, scientists have been investigating this disease with respect to its causative organisms, transmission, pathology, and treatment.

1.1 THE PARASITE

Leishmania is classified as a member of the family Trypanosomatidae. The classification of the family according to Molyneux and Ashford (1983) is as follows:

Kingdom	Protista
Subkingdom	Protozoa
Phylum	Sarcomastigophora
Subphylum	Mastigophora
Class	Zoomastigophora
Order	Kinetoplastida
Suborder	Trypanosomatina
Family	Trypanosomatidae

Leishmania parasites exist in two main morphological forms: flagellated promastigotes in the gut of female phlebotomine sandflies and intracellular amastigotes within the macrophages of the mammalian host. Species of *Leishmania* have a nucleus, kinetoplast, and a flagellum.

Leishmania donovani, L. infantum, and *L. chagasi* mainly cause visceral leishmaniasis. *L. major, L. tropica, L. aethiopica, L. mexicana* and *L. braziliensis* mainly cause the cutaneous and mucocutaneous forms of the disease. Other species of *Leishmania* have been identified and some of them are non-pathogenic to humans.

1.1.1 THE AMASTIGOTE

Amastigotes are the non-motile (aflagellated) form of the parasite. They are round or oval in shape with a size of 3 - 5 μm on the main axis. Under light microscopy, Giemsa-stained preparations reveal a round nucleus and a rod-shaped kinetoplast

(Fig.1.1.). Ultrastructural examination of amastigotes reveals a conventional unit surface membrane underlined by microtubules which serve as a cytoskeleton. The flagellar pocket is formed by an infolding of the surface membrane. The main function of the pocket is thought to be as a site of endocytosis and exocytosis (Webster and Russell, 1993). The flagellum is found inside the pocket and contains nine pairs of microtubules arranged in a circle surrounding one pair of microtubules, i.e. the normal nine plus two structure. In amastigotes, the flagellum is not functional, as it does not extend beyond the cell surface. The kinetoplast lies below the origin of the flagellum. This is a dense mass of mitochondrial DNA. The single mitochondrion surrounds the kinetoplast and extends throughout the cell body. The cytoplasm contains organelles such as the Golgi complex, rough and smooth endoplasmic reticulum, lysosomes and glycosomes. Details of the ultrastructure will be discussed in chapter 3.

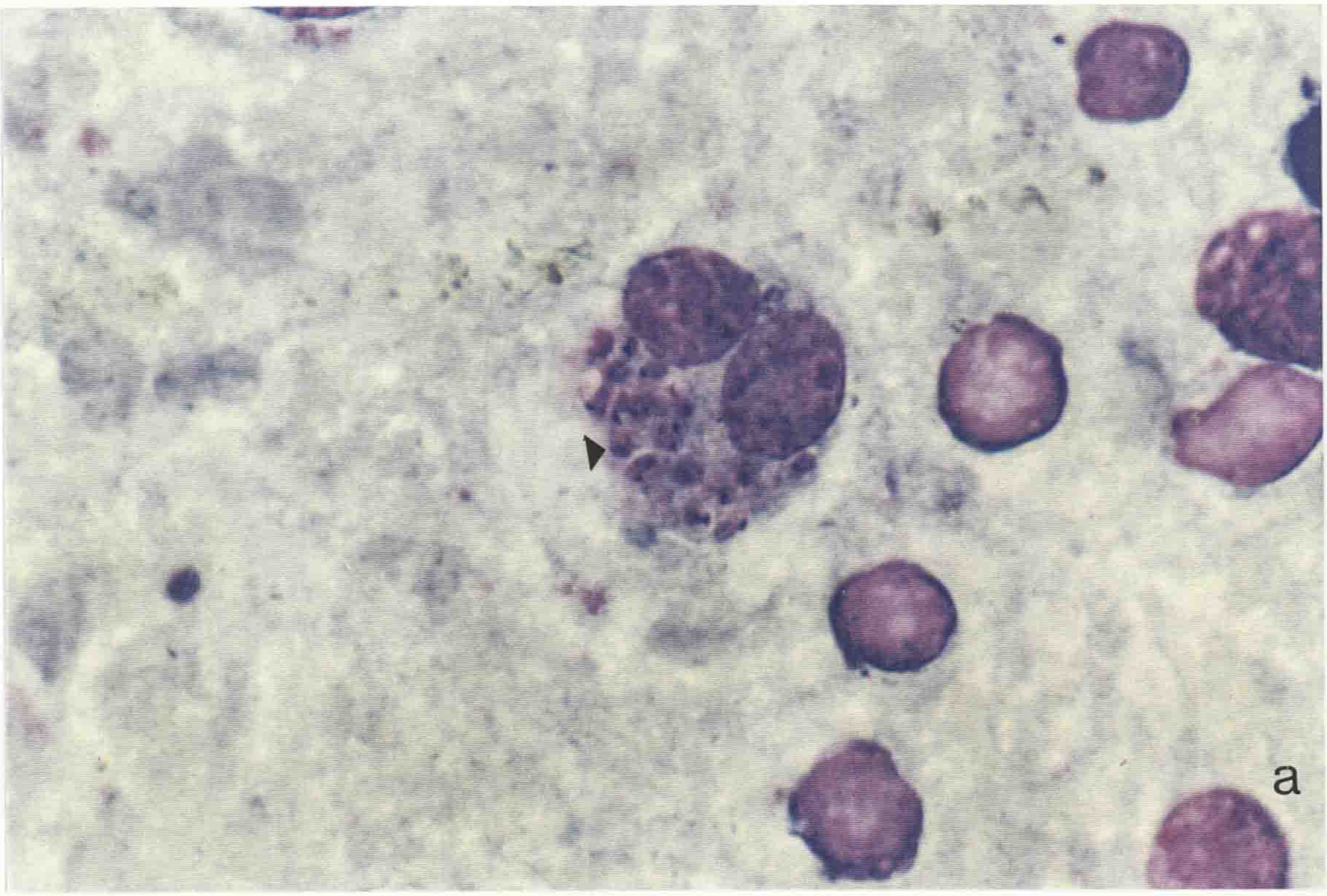
1.1.2 THE PROMASTIGOTE

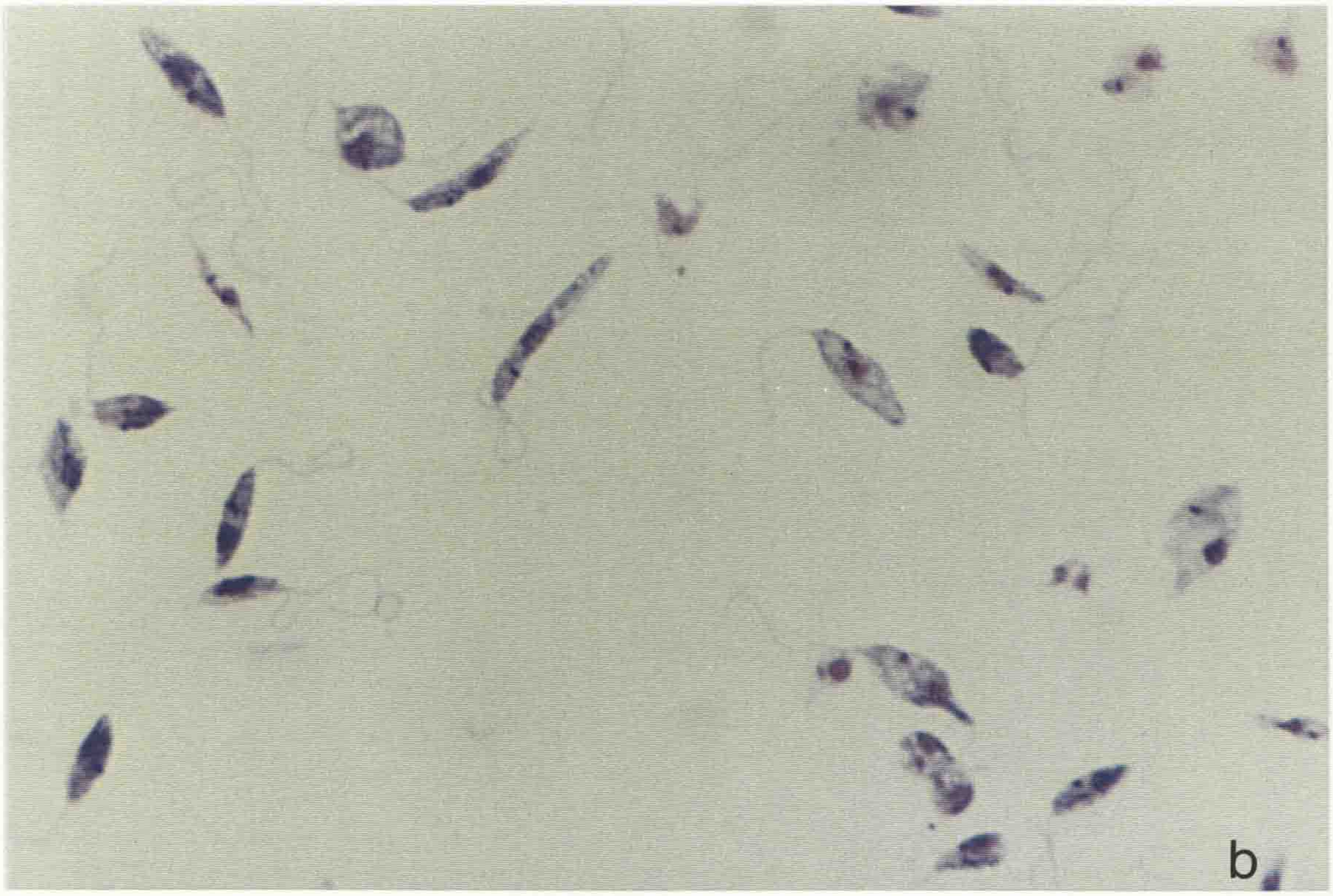
The main structural elements of promastigotes are similar to those described for amastigotes (section 1.1.1.) with a few differences (Fig.1.2). The cell body is elongated measuring 7 -18 μm. The flagellum is functional, as it extends beyond the cell body, and is used in motility. The flagellum has a paraxial rod, a paracrystalline structure, which runs parallel to the microtubules. The function of the paraxial rod is not known. It begins in the part of the flagellum inside the flagellar pocket and extends along the length.

Figure 1.1. Light micrograph of *L. donovani* amastigotes (arrow).

Figure 1.2. Light micrograph of *L. donovani* promastigotes.

Bar = 5 μm.


a


b

Metacyclic promastigotes have a shorter body length than other types of promastigotes and, frequently, a posterior trailing strand of membrane and cytoplasm; they are also highly motile (Howard *et al.*, 1987; Howard *et al.*, 1989; Pimenta *et al.*, 1991).

In sandflies, these forms, although they can be isolated from the midgut, are usually found in the anterior midgut and/or foregut, and are more infective to mammals than other forms (Sacks and Perkins, 1985).

1.2 THE VECTOR

Sandflies belonging to the genus *Phlebotomus* are the vectors of leishmaniasis in the Old World. In the New World, sandflies of the genus *Lutzomyia* act as vectors of the disease. Adult sandflies are recognized by their minute size (1.3 - 3.5 mm in length), hairy appearance, large black eyes and long stilt-like legs (Service, 1986). Only female sandflies obtain blood meals from vertebrates and biting is usually nocturnal. They are weak fliers and usually rest on dry surfaces in dark and humid places.

The first reports on the transmission of leishmaniasis by bites of sandflies were those of Shortt *et al.* (1931) and Adler and Ber (1941).

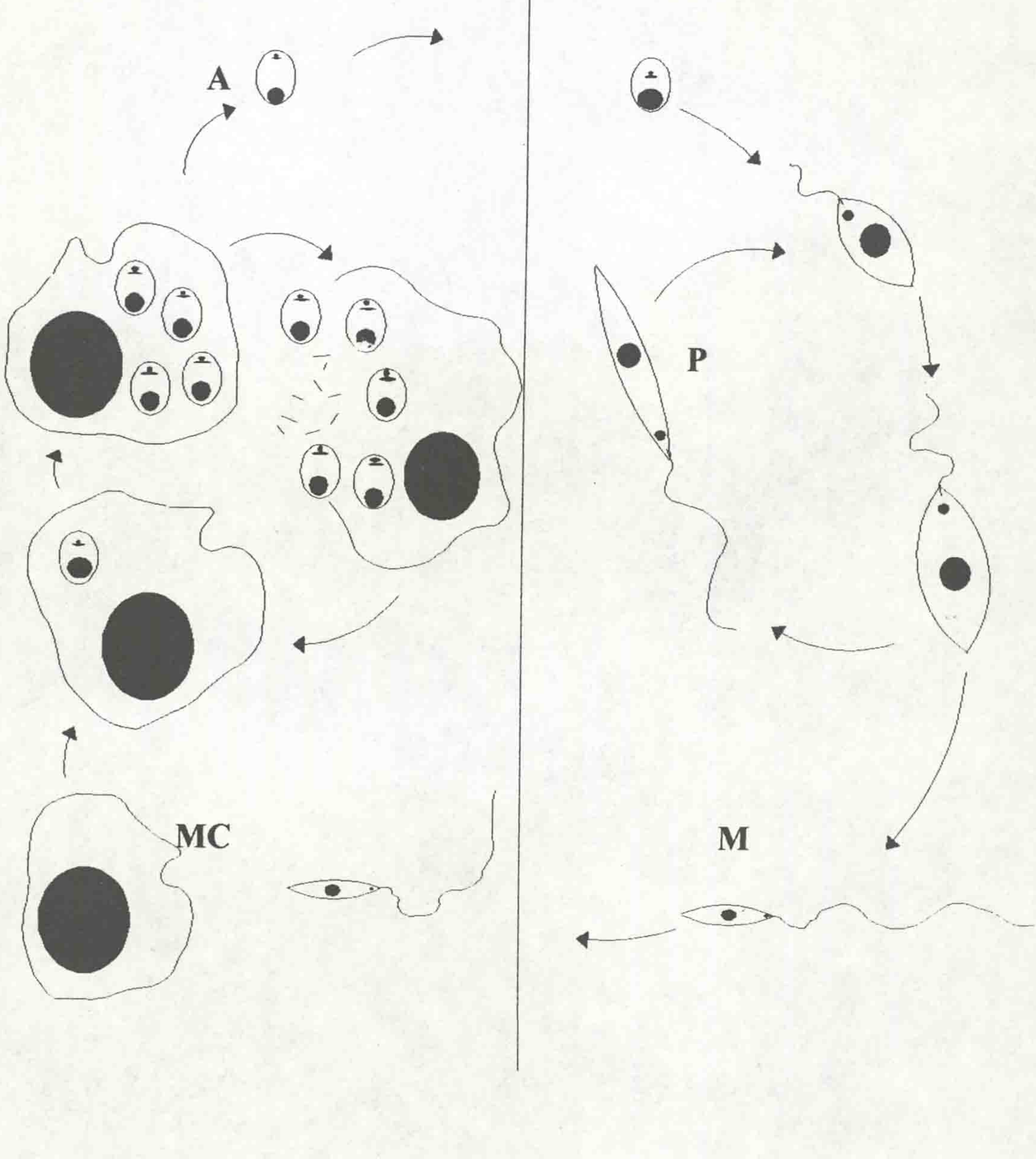
1.3 THE LIFE CYCLE

The life cycle of *Leishmania* is illustrated in Figure 1.3 (reviewed by Molyneux and Killick-Kendrick, 1987). It is composed of two main parts: that in the sandfly and that in the mammalian host.

Figure 1.3. Life cycle of *Leishmania*. A=amastigote, P=promastigote, M=metacyclic promastigote, MC=macrophage.

Vertebrate

Sand fly



When the sandfly obtains a blood meal from the mammalian host, amastigotes are ingested and they transform into promastigotes within the blood meal. Promastigotes multiply in the gut of the sandfly and, eventually, some promastigotes differentiate into metacyclic promastigotes, the infective stage for mammals. Metacyclic promastigotes migrate to the anterior parts of the sandfly and are inoculated in the mammalian host as the sandfly obtains another blood meal.

In the mammalian host, promastigotes are engulfed by reticuloendothelial cells in which they transform into amastigotes. These forms multiply by simple binary fission until the host cell is destroyed whereupon they parasitize new macrophages. The location of infected host cells correlates with the type of disease. In simple cutaneous leishmaniasis, the parasites remain in skin lesions located where the sandfly bite had taken place. In the visceral form of the disease there may be an initial skin lesion but the disease is not pronounced until the parasite is established in the viscera and clinical signs and symptoms of visceral leishmaniasis appear.

1.4 DISEASE AND PATHOLOGY

1.4.1 VISCERAL LEISHMANIASIS

There are about 30 - 100 subclinical infections for every case of visceral leishmaniasis in endemic areas (Bryceson, 1996). Cellular and biochemical changes in the blood usually accompany infiltration and parasitization of the reticuloendothelial cells by the parasites. Globulin levels usually increase, especially IgG and IgM, with a reduction in the levels of complement. Levels of the hepatocellular enzymes are usually normal, but the plasma albumin is reduced. Patients usually suffer from pancytopenia. Sequestration of erythrocytes in the spleen and the reduction of their half life due to

haemolysis contributes to anaemia. Haemoglobin levels usually fall to 7 g/dl and lower values are not uncommon.

The incubation period of the disease is 2 - 4 months on average but may vary from several weeks to 2 years (Bryceson, 1996). Common symptoms are acute fever, malaise, headache, dizziness, and anorexia. Splenomegaly occurs in more than 90% of cases and hepatomegaly in more than 75% (Bryceson, 1996).

1.4.2 CUTANEOUS LEISHMANIASIS

The incubation period for cutaneous leishmaniasis varies from a few days to several months or even a year (Bryceson, 1996). This depends on the species of *Leishmania* and the size of the inoculum. Lesions in early stages are characterized by proliferation of macrophages that contain numerous amastigotes and the overlying skin shows acanthosis and hyperkeratosis. This may be followed by ulceration and necrosis (Beaver *et al.*, 1984).

Lesions caused by *L. major* necrose rapidly and tend to be inflamed and exudative. They are most common on the limbs and if they are found on the lips or nose, they do not spread to the mucosa. In the case of *L. tropica* infections, lesions appear and heal at a slower rate than those of *L. major* and are common on the face.

In cases of *L. aethiopica*, satellite papules accumulate to form a nodule. Nodules usually ulcerate and heal slowly over 2 - 5 years (Bryceson, 1996).

L. braziliensis usually cause a single, deep lesion that rapidly develops an ulcer. The lesion may heal within one year but commonly takes a long time to heal, up to 10 years (Bryceson, 1996).

Mucocutaneous leishmaniasis may be caused by *L. braziliensis, L. guyanensis* or *L. panamensis* and about 15 % of cases have a history of previous cutaneous lesion. Mucosal lesions may appear within 2 - 10 years of the ulcer and the nasal mucosa is the most commonly infected area. Lesions initially appear as a nodule and when the nasal cartilage collapses, lesions spread to involve the oronasopharangeal mucosa down to the larynx. Death may occur due to obstruction in the nasal, pharyngeal, or laryngeal site or due to secondary infections (Bryceson, 1996).

1.5 DIAGNOSIS

1.5.1 PARASITOLOGICAL DIAGNOSIS

In visceral leishmaniasis, parasites can be found in bone marrow aspirates, splenic aspirates, lymph node biopsy and , less commonly, in the blood (Williams, 1995). In cutaneous leishmaniasis, parasites can be found in biopsies from lesions. Stained smears and cultures should be made from any material obtained.

1.5.2 SEROLOGICAL DIAGNOSIS

The specific humoral response in visceral leishmaniasis allows diagnosis using several serological tests, e.g. indirect fluorescent antibody test (IFAT), direct agglutination test (DAT), and enzyme linked immunosorbent assay (ELISA). Serological diagnosis in cases of cutaneous leishmaniasis is of little use. This is because few antibodies can be demonstrated and the patient may show a positive reaction for life even though lesions have been cured.

One of the oldest immunological tests for the diagnosis of leishmaniasis is the leishmanin test. A suspension of promastigotes in 0.5% phenol in saline is prepared

and 0.1 ml of the suspension is injected intradermally, the results are read 48 - 72 hours later (Bryceson, 1996).

IFAT has the advantage of testing for short-lived antibodies that become negative 6-9 months after the patient is cured. Titres above 1/128 are diagnostic. The disadvantage is the difficulty in estimating the end-point titre and the possibility of cross-reaction with trypanosomiasis (Walton, 1972).

ELISA is highly specific (100%) and its sensitivity is in the range of 98% (Williams, 1995). It has the advantage that it can be carried out on serum, plasma or even blood spots collected on filter papers but the problem of false positive reactions make it less useful. Several studies has focused on the evaluation of this serological test (Reed *et al.*, 1990; Gupta *et al.*, 1993) and its application in the field (Okong'o-Odera *et al.*, 1993). This method of diagnosis can also be directed to detect antigens of *Leishmania* rather than antibodies (Azazy *et al.*, 1994).

The DAT was first described by Harith *et al.* (1986) and was later improved to increase its sensitivity and specificity (Harith *et al.*, 1988; Harith *et al.*, 1995). It has the advantage of being simple (El Safi *et al.*, 1989) and has a high specificity and sensitivity. It does not require sophisticated equipment and can be performed in the field (Harith, *et al.*, 1988; Kager *et al.*, 1989). However, serological methods have their own disadvantages such as cross-reactivity and the failure to differentiate between old and active infections.

1.5.3 DIAGNOSIS BY MOLECULAR TECHNIQUES

Diagnosis by molecular biology techniques, such as those using polymerase chain reaction (PCR), are being actively developed (Rodgers, *et al.*, 1990; Piarroux *et al.*,

1994). Diagnosis of leishmaniasis using molecular techniques has great promise. This is because conventional diagnostic methods are complicated and often take a long time to perform. The use of kinetoplast DNA (kDNA) seems to be a promising tool for the diagnosis of leishmaniasis (Barker, 1989; Van Eys *et al.*, 1989). A polymerase chain reaction-solution hybridization enzyme-linked immunoassay (PCR-SHELA) has been developed for the detection of parasites of the *L. donovani*-complex (Qua *et al.*, 1995) and work is still proceeding to develop, improve and simplify molecular methods for the diagnosis of leishmaniasis (Williams, 1995).

1.6 EPIDEMIOLOGY AND GEOGRAPHICAL DISTRIBUTION

It is difficult to estimate the number of cases of leishmaniasis and the number of people at risk of acquiring the disease world wide. This is because so many cases are unreported or misdiagnosed. Also, the data obtained from the few studies that have been carried out to determine the incidence of human infection in specific areas cannot be extrapolated to wider areas because of the focality of the disease. However, it is estimated that 12 million people are infected with leishmaniasis and 350 million people are at risk of acquiring infection in the world (WHO, 1990). Ashford *et al.* (1992) estimated that 200 million people are at risk of visceral leishmaniasis with at least 100 000 new cases annually, and 200 million at risk of cutaneous leishmaniasis with 300 000 new cases every year. Recently, WHO estimated that the annual incidence is 1 to 1.5 million cases of cutaneous leishmaniasis and 500 000 cases of visceral leishmaniasis. Almost 90% of visceral leishmaniasis cases occur in Bangladesh, India, Nepal and Sudan. On the other hand, 90% of cutaneous

leishmaniasis cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria (WHO, 1995).

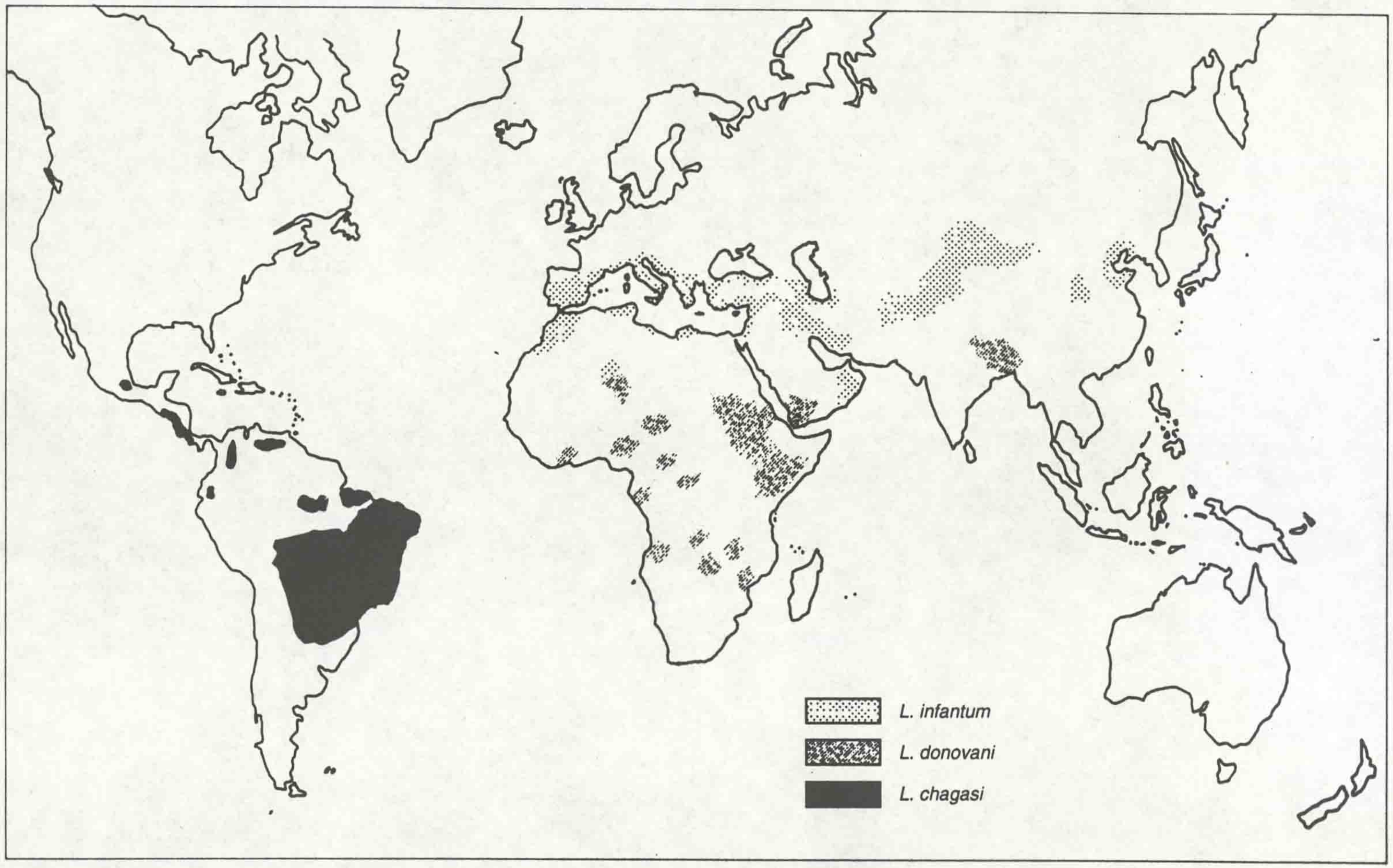
The behaviour of the human population in a given area contributes to the incidence of leishmaniasis in that area. The risk to the population of acquiring infection depends on several factors including:

- Proximity of residence to sandfly breeding and resting sites.
- House type.
- Occupation.
- Exposure to sandfly bites.
- Age.
- Resistance which may be genetic or acquired.
- Prevalence of reservoir hosts (Bryceson, 1996).

Visceral leishmaniasis is due to *L. donovani* and *L. infantum* in the Old World, and *L. chagasi* in the New World (fig. 1.4).

L. donovani causes anthroponotic visceral leishmaniasis in the Indian subcontinent and some parts of China. The disease is transmitted by *Phlebotomus argentipes* and affects mainly the 10-29 year old age group with males more commonly infected than females in a ratio of 6:1 (Bryceson, 1996). This is, perhaps, because males in this age group are the ones who work in fields and areas inhabited by infected sandflies.

Figure 1.4. Geographical distribution of visceral leishmaniasis (adapted from Bryceson, 1996).


L. infantum
L. donovani
L. chagasi

L. donovani causes enzootic visceral leishmaniasis in sub-Saharan Africa especially in southern Sudan, the borders of Ethiopia, Somalia, and northern Kenya. *P. martini* and *P. orientalis* have been incriminated in the transmission of the disease. Reservoir hosts include rodents and dogs. Rare cases have been recorded from Senegal, Gambia, Malawi, Chad, Zambia, Angola, Niger and Zaire where nothing is known of their epidemiology (Bryceson, 1996). In Saudi Arabia, visceral leishmaniasis was reported in infants in the south-west region and the parasite was identified as *L. donovani sensu lato* (Al-Zahrani *et al.*, 1988 and 1989; Ibrahim *et al.*, 1992).

L. infantum causes visceral leishmaniasis in the Mediterranean basin, western Asia and eastern China. It is also found in the south-west region of Saudi Arabia (Al-Zahrani *et al.*, 1988 and 1989; Ibrahim *et al.*, 1992). There are large unaffected areas that separate the affected areas. Leishmaniasis due to *L. infantum* is most commonly found in children under 5 years of age. Dogs are the main reservoir host. *P. chinensis* is the transmitting vector in China (Bryceson, 1996) while *P. perniciosus* and *P. ariasi* are the vectors in other regions (Ashford and Bettini, 1987).

Visceral leishmaniasis in the New World is caused by *L. chagasi*. A controversy exists as to whether *L. chagasi* was recently introduced to the New World or has an ancient origin. Enzyme electrophoretic evidence for the importation of *L. infantum* into the New World was reported by Momen and Grimaldi (1989). Furthermore, a comparison between the susceptibility of *Lutzomyia longipalpis*, the sandfly vector of *L. chagasi*, to a strain of *L. infantum* from southern France with that of the natural vector in the same area (*P. ariasi*) revealed no notable differences (Killick-Kendrick *et al.*, 1980). North-east Brazil is the main endemic area and the disease is transmitted by *Lu.*

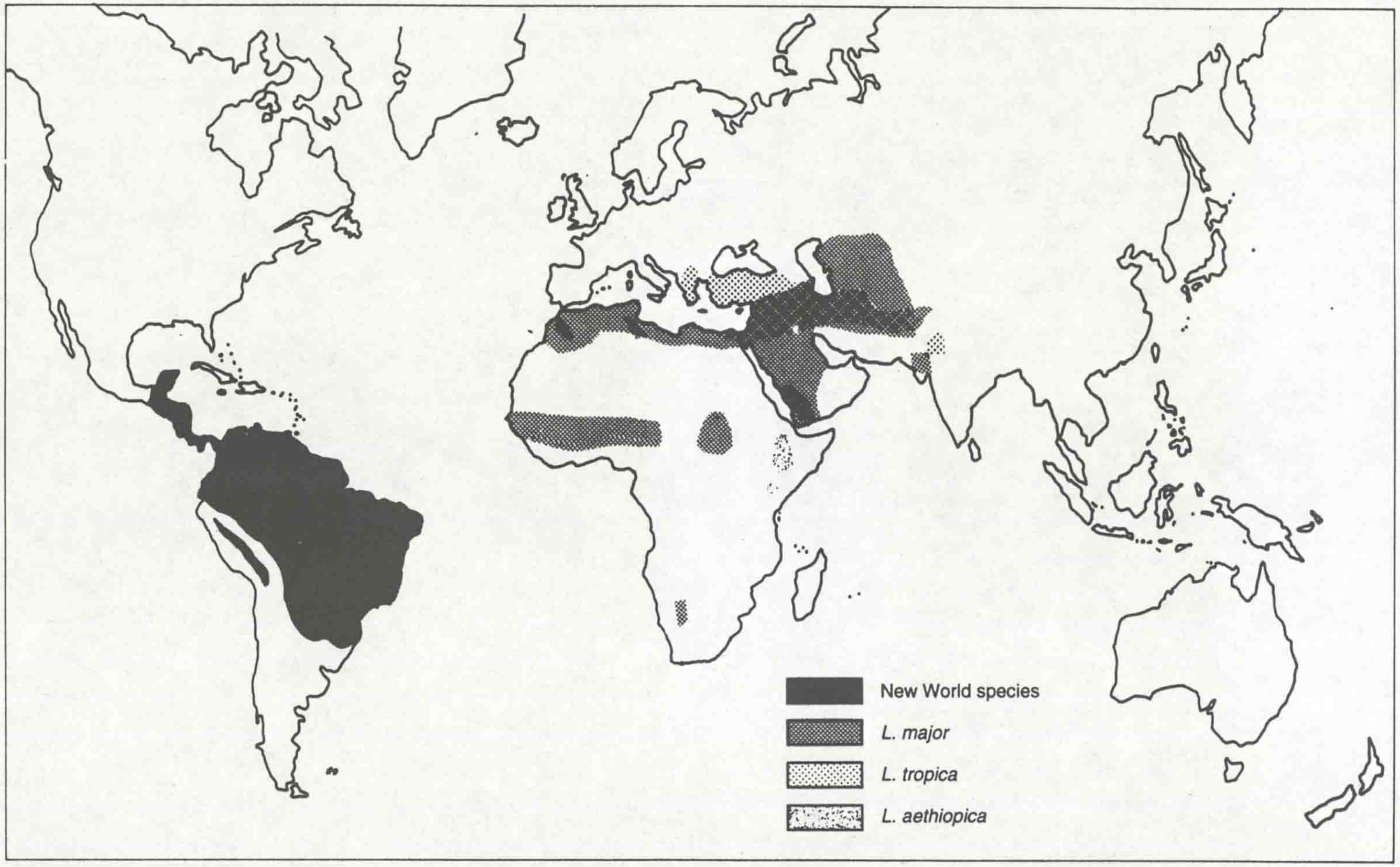
longipalpis. Man, dogs and foxes act as reservoir hosts. Again the disease is most common in children under 5 years of age. Other parts where leishmaniasis caused by *L. chagasi* has been reported include Bolivia, Colombia, El Salvador, Honduras, Mexico and Venezuela. The first case of visceral leishmaniasis in Nicaragua was reported by Duarte *et al.* (1994) but the causative species was not identified.

Old World cutaneous leishmaniasis is caused by *L. tropica*, *L. æthiopica* and *L. major*, which is responsible for most zoonotic cutaneous leishmaniasis (Fig 1.5). *L. infantum* is also responsible for the European cutanneous leishmaniasis.

L. tropica causes anthroponotic cutaneous leishmaniasis and is found around the Mediterranean basin from Greece eastward, as far north as Serbia and Romania, Turkey, North Africa, the Middle East, west Asia to Afghanistan, Pakistan and India. Al-Zahrani *et al.* reported the occurrence of *L. tropica* in the Asir region of south-west Saudi Arabia and *P. sergenti* was incriminated as the vector (Al-Zahrani *et al.*, 1988 and 1989). Although *L. tropica* has been isolated from rats and dogs (Bryceson, 1996), man remains the principle reservoir host. *P. papatasi* and *P. sergenti* act as vectors for the disease.

L. æthiopica is responsible for cutaneous leishmaniasis in Ethiopia, eastern Uganda and western Kenya and hyraxes act as the reservoir host. The disease is transmitted by *P. longpipes* and *P. pedifer*.

Figure 1.5. Geographical distribution of cutaneous leishmaniasis (adapted from Bryceson, 1996).


New World species
L. major
L. tropica
L. aethiopica

L. major is endemic throughout the semi deserts and dry silt valleys of North Africa, west subsaharan Africa, central Sudan, northern Kenya, the Middle East extending to India, Turkmenia, Uzbekistan, Kazakhstan and Tadjikistan. It is also found in the eastern province of Saudi Arabia (Killick-Kendrick *et al.*, 1985; Peters *et al.*, 1985; Elbihari *et al.*, 1987; Ibrahim *et al.*, 1994; Mustafa *et al.*, 1994). Gerbils, rats and jirds act as reservoir hosts (Elbihari *et al.*, 1987; Ibrahim *et al.*, 1994; Bryceson, 1996). The disease is transmitted mainly by *P. papatasi* (Killick-Kendrick *et al.*, 1985; Peters *et al.*, 1985; Mustafa *et al.*, 1994) and, to a lesser extent, *P. sergenti*. The incidence of infection varies from nil to 100 % and all ages are affected.

New World cutaneous leishmaniasis is caused by at least six species of *Leishmania*, three of which can also cause mucocutaneous leishmaniasis. Their geographical distributions overlap (Fig 1.5).

The most common and most serious cause of cutaneous leishmaniasis in Central and South America is *L. braziliensis.* It is found in Argentina, Bolivia, Brazil, Colombia, Costa Rica, Panama, Paraguay, Peru and Venezuela. Although dogs and equines are probable reservoir hosts in the suburban setting, the natural forest reservoir hosts have not been identified despite extensive searches (Bryceson, 1996). Many species of sandfly vectors transmit the disease but most importantly *Psychodopygus welcomei, Lu. whitmani* and *Lu. intermedia.* The disease appears to affect mainly young adult males but there is little reliable data (Bryceson, 1996).

L. panamensis is dominant in Central America. It is found in Costa Rica, Honduras, Nicaragua, Panama, Colombia and Ecuador. The reservoir host is the two-toed sloth

and the principle vectors are *Lu. ylephiletor, Lu. trapidoi,* and *Lu. shannoni*. The incidence rate in endemic areas is 20 to 200 per 100 000 (Bryceson, 1996).

L. amazonensis is found throughout Bolivia, Colombia, Ecuador, Peru, Venezuela and the Amazon forests of Brazil. It is transmitted by *Lu. flaviscutleta* and rodents act as reservoir hosts.

L. guyanensis is found in the Amazonian forests of Brazil, French Guiana, Colombia, Surinam, and Guyana. It is transmitted between man and the natural host, the arboreal sloth, by *Lu. umbratilis* and *Lu. anduzei*.

L. mexicana is prevalent in Mexico and extends through Guatemala, Honduras, Colombia and Panama. Rodents act as reservoir hosts and the disease is transmitted by *Lu. olmeca.*

1.7 PREVENTION AND CONTROL

Control of leishmaniasis can be achieved by treatment of cases, controlling the sandfly vector and controlling the reservoir host. Prevention can either be to individuals or to the community, i.e. vaccination.

1.7.1 TREATMENT OF LEISHMANIASIS

1.7.1.1 TREATMENT OF VISCERAL LEISHMANIASIS

Successful treatment of visceral leishmaniasis requires the use of an effective drug for an adequate duration. Different species of *Leishmania* have different responses to

different drugs and, hence, the adequate regimens may be different in different endemic areas.

1.7.1.1.1 PENTAVALENT ANTIMONIALS

These are regarded the drugs of choice because of their efficacy, availability, familiarity and relative low cost. The two forms of the drug currently used are sodium stibogluconate and meglumine antimoniate.Despite the long period of treatment required to cure the disease, this drug is safe as 90% of it is excreted in the urine within 8 hours (Bryceson, 1996).

1.7.1.1.2 AMINOSIDINE

This is a conventional aminoglycoside antibiotic that has a unique action against *Leishmania* (Bryceson, 1996). Its only drawback is that it has the potential for renal toxicity common to all aminoglycosides and ototoxicity.

1.7.1.1.3 AMPHOTERICIN B

This drug is 400 times more potent than antimonials against *Leishmania.* The side effects are anaemia, thrombosis and renal glomerular and tubular damage.

1.7.1.2 TREATMENT OF CUTANEOUS LEISHMANIASIS

Treatment is usually not needed in cutaneous leishmaniasis as most lesions heal spontaneously. However, treatment accelerates healing and reduces the severity of scarring.

1.7.1.2.1 PHYSICAL TREATMENT

Physical treatment can be achieved by surgical removal of lesions. Heat can also be applied on the lesion to kill the amastigotes and accelerate natural healing. This method usually involves heating the lesion to 39 - 41°C for many hours over several days which can be difficult to achieve. Another alternative is cryotherapy using liquid nitrogen or carbon dioxide snow. This method can only be applied on well defined lesions with little surrounding inflammation.

1.7.1.2.2 CHEMICAL TREATMENT

The application of chemical drugs on localised lesion has been practiced for the treatment of cutaneous leishmaniasis. Aminosidine ointment and a preparation of aminosidine and methylbenzethonium are used but may cause unacceptable inflammation. Systemic application of antimonials remains the most generally useful method of treatment.

Mucocutaneous leishmaniasis is difficult to treat as it responds slowly to drugs. Antimonials remain the drug of choice for treating mucocutaneous leishmaniasis in patients that have not been previously treated by antimonials and without laryngeal involvement. Amphotericin B is used to treat patients relapsing after a previous course of antimonials or in laryngeal involvement.

1.7.2 CONTROL OF SANDFLY VECTORS

Sandflies are very susceptible to most insecticides. The only case of resistance is to DDT in India (Service, 1986). The use of DDT as residual house-spray or fogging of outdoor resting sites of adult sandflies gives excellent control.

1.7.3 CONTROL OF RESERVOIR HOSTS

Unfortunately this method is not very practical. It involves screening and treating or eliminating domestic canine reservoir hosts which is usually costy. It also involves the control of rodents which is almost impossible in places such as the forests of Latin America.

1.7.4 PROTECTION OF INDIVIDUALS

People travelling to endemic areas during the transmission season should take measures to avoid sandfly bites. These include the use of impregnated bed nets, not camping or sleeping near rodent burrows, covering their skin as much as possible and applying insect repellent cream to exposed skin.

1.7.5 VACCINATION AGAINST *LEISHMANIA*

Vaccination against cutaneous leishmaniasis has been practiced from as early as the 19th century. Children in Baghdad were vaccinated against oriental sore by inoculating material from active human lesions into the skin of the arm or thigh (Wenyon, 1911). It is worth remembering that leishmaniasis is a group of quite separate disease syndromes each of which is caused by a distinct agent.This makes the development of a universal vaccine unlikely. However, a cocktail of vaccines may be worth considering. Developing a vaccine for each endemic area is, probably, more practical. Vaccines are either live, using infective virulent or attenuated organisms, or dead, using killed organisms, parts of organisms or released products of organisms. Attenuated organisms have generally not been shown to induce protective immunity and it seems that the organism needs to be infective in order for the host to acquire

protection (Schnur, 1989). Nevertheless, protection against a virulent strain of *L. major* was established by inoculation of an avirulent, lipophosphoglycan deficient *L. major* (Kimsey, *et al.*, 1993). Protection against infection with *L. mexicana* was induced by the injection of plasma membrane antigens of *L.mexicana* reconstituted into liposomes (Alexander and Russell, 1989). Protection against *L. donovani* was induced in mice by immunization with a protein, dp72, purified from *L. donovani* promastigotes (Rachamim and Jaffe, 1993). Several vaccination trials using killed *Leishmania* have been made in Brazil, Venezuela and Iran. The Special Programme for Research and Training in Tropical Diseases (TDR) is actively involved in these trials and their results are expected in 1 - 2 years. Other methods of vaccination such as using genetically manipulated live *Leishmania*, recombinant bacteria or viruses expressing *Leishmania* antigens, or using synthetic vaccines are still in developmental state and are not expected to reach clinical trials for at least 3 years (Modabber, 1995).

1.8 OBJECTIVES OF THIS STUDY

To study *Leishmania*, amastigotes can be obtained from infected animals or from infected macrophages grown *in vitro*. Isolation of these forms can be quite difficult and laborious. In addition, there is always a doubt regarding the purity of such amastigotes. This justifies the need for pure, large amounts of amastigotes which can be obtained by culturing amastigotes axenically. Such amastigotes can then be used in different studies, e.g. their cellular structure, enzyme activities, molecular activities and their response to drugs. Another important stage of the parasite is the metacyclic promastigote, the stage infective to humans. This form of the parasite is usually found in the anterior parts of the sandfly. Also, a small population of stationary-phase promastigotes cultured *in vitro* differentiate to metacyclic promastigotes. To investigate the host-parasite relationship, it is important to study such promastigotes. Therefore, metacyclogenesis and production of metacyclic promastigotes *in vitro* is essential to undertake studies on their biochemical activities, molecular structure, cell entry and establishment of an infection. The specific objectives of this study are:

1. To investigate the effects of different external cues on transformation from the promastigote stage to the amastigote stage and the growth of the latter form.
2. To characterize and compare axenically grown amastigotes, lesion amastigotes and cultured promastigotes in terms of morphology and sizes, protein expression and ultrastructure.
3. To investigate the process of metacyclogenesis in *Leishmania donovani, L. mexicana. L. major* and *L. braziliensis* and the effect of pH on metacyclogenesis.
4. To characterize metacyclic promastigotes in terms of morphology and sizes, resistance to complement-mediated lysis, ultrastructure and infectivity *in vivo*.

CHAPTER TWO

2. AXENIC CULTIVATION OF AMASTIGOTES*

2.1 INTRODUCTION

Leishmania parasites cause a wide range of human diseases from localized self-healing cutaneous lesions to fatal visceral disease. These parasites have a life cycle characterized by the presence of flagellated promastigotes in the sandfly and non-motile amastigotes within the phagolysosomes of macrophages in the mammalian host.

To study these parasites, promastigotes and amastigotes have been cultured under different *in vitro* laboratory conditions and have been the subject of numerous biological and biochemical studies.

Different culture media have been used to grow *Leishmania* parasites. Biphasic media (such as NNN, USMARU and Evans modified Tobie's medium) are used to culture *Leishmania* parasites (Evans, 1987).

---

* In this study the terms "amastigote-like forms" and "axenic amastigotes" are used interchangeably and to denote cultured forms that may or may not resemble those obtained from mammalian hosts; the latter are termed amastigotes often with an appropriate adjective eg. lesion amastigotes , spleen amastigotes or tissue amastigotes.

Monophasic media are more popular for the large scale cultivation of these parasites because of the difficulties of producing such cultures with biphasic media. For example, HO-MEM medium (Berens *et al.*, 1976) is a monophasic medium currently in use by many researchers to culture haemoflagellates. Other examples of such media are: Schneider's *Drosophila* medium which is commercially available and, although it is more expensive, has been widely used for the isolation and bulk cultivation of *Leishmania* (Childs *et al.*, 1978; Evans, 1987); tissue culture Medium 199 (Hendricks, *et al.*, 1978); and medium RE I (Steiger and Steiger, 1976;1977).

Promastigotes of *Leishmania* species are usually cultivated *in vitro* in culture media of neutral pH at 25-27°C. They are easily grown and most biochemical research on *Leishmania* has dealt with this form.

Such cultured promastigotes are more likely to share properties with those found in infected sandflies than with amastigotes in phagolysosomes of macrophages in the mammalian host. Therefore, knowledge of the properties of promastigotes may not improve understanding of the disease itself.

This can be overcome by studying the amastigotes that are responsible for the pathology of leishmaniasis. Amastigotes are an intracellular stage in macrophages and can be obtained from infected animals or from *in vitro* cultures in macrophage-like cell lines. However, there is often a doubt regarding the purity of amastigotes derived from such sources. This can be overcome by the cultivation of amastigotes in a cell-free medium, i.e. axenically, (Bates, 1993a).

In one of the earliest reports a biphasic medium consisting of a blood agar base overlaid by physiological salt solution was used to culture amastigotes of *L. donovani, L. tropica* and *L. braziliensis* axenically (Lemma and Schiller, 1964). However, the first reliable method for cultivation of amastigote-like forms in liquid medium was that described by Pan (1984). Amastigote-like forms of *Leishmania pifanoi* were cultured in a cell-free medium at 33° C and 35° C. The medium used consisted of Medium 199 fortified with a high concentration of water-soluble vitamins, nucleotides, and heat-inactivated foetal calf serum (FCS) at a concentration of 25% (v/v). The initial pH of the medium was 7.2 and the culture was started with an inoculum of promastigotes at a density of 5×10^6 promastigotes per ml. These were subpassaged every 4-10 days and by the end of the incubation period of the second subpassage 99% of the organisms were amastigote-like forms. These could be cultured indefinitely by serial transfers and typically reached a final density of 8×10^7 parasites per ml. When these amastigote-like forms were transferred to a medium with an initial pH of 8.0 and incubated at 26°C, the entire population was transformed into promastigotes after three serial passages (Pan, 1984). By this method, they were able to isolate large numbers of axenic amastigotes which were used to raise monoclonal antibodies for studying stage differentiation and monitoring transformation (Pan and McMahon-Pratt, 1988).

Using a similar methodology, other workers have been able to culture a variety of other species as amastigote-like forms. Eperon and McMahon-Pratt (1989b) succeeded in growing amastigote like forms of *L. panamensis* and *L. braziliensis*. They used Schneider's *Drosophila* medium supplemented with 20% heat inactivated FCS and cultures were incubated at 32° C for *L. panamensis* and 28° C for *L. braziliensis*.

These amastigote like forms were morphologically different from promastigotes. They appeared as round or oval forms that were smaller than promastigotes. Their ultrastructure revealed that their flagella lacked the paraxial rod which was present in flagella of promastigotes. Although they were infective to macrophage cell lines, their infectivity was not compared with that of promastigotes. These amastigote like forms, and lesion amastigotes, were also recognized by monoclonal antibodies which failed to bind promastigotes (Eperon and McMohan-Pratt, 1989a).

Doyle *et al*. (1991) cultured *L.donovani* axenically at 37°C in a 95% air and 5% CO_2 humid atmosphere in 100% FCS. Heat shock was applied to *in vitro* cultured promastigotes which lead to their transformation to amastigotes. The latter forms were cultured long-term at 37° C. They were metabolically active, as evidenced by the release of acid phosphatase in the culture medium, and their ultrastructure was identical to that of intracellular amastigotes (Doyle *et al*., 1991).

Bates *et al*. (1992) cultured amastigote-like forms of *L.mexicana*. Unlike others who adapted promastigotes *in vitro* to transform to amastigotes, amastigotes from lesions of infected animals were directly cultured in Schneider's *Drosophila* medium supplemented with 20% FCS at a pH of 5.4 and incubated at 32-33°C. These amastigote like forms were similar to lesion amastigotes. In addition to the morphological similarity with lesion amastigotes, their ultrastructure revealed their possession of megasomes, the characteristic lysosomes of *L. mexicana* amastigotes, and their cysteine proteinase activities and gelatin SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) banding pattern were also similar to that

of lesion amastigotes. Finally, their infectivity to CBA mice was significantly higher than that of promastigotes.

L. donovani and *L. major* were also cultured axenically in RBLM with a pH of 7.4 and 10% foetal calf serum, using a temperature of 35°C for *L.major*, and 37°C for *L.donovani*, both in an atmosphere of 95% air and 5% CO_2. The infectivity of the axenically cultured amastigotes to BALB/c mice was higher than that of promastigotes (Al-Bashir *et al.*, 1992).

Pan *et al.* (1993) cultured amastigotes of various different species of *Leishmania* axenically. Amastigotes of different species of *Leishmania* were adapted to grow axenically, after being transformed from promastigotes, in different culture media, at low pH, and elevated temperatures. These axenically grown amastigotes resembled lesion amastigotes based on morphological, biological, immunological and biochemical evidences. They also appeared to be different from heat shocked promastigotes.

Castilla *et al.* (1995) were able to grow *L. donovani* amastigotes axenically in TC-199. Promastigote cultures were originally incubated at 28° C and when the temperature was raised to 38° C parasites transformed to amastigotes. These were grown continuously in axenic conditions for more than 3 months. They retained their antigenic capacity, as being recognized by the sera of naturally infected patients, and were able to multiply in macrophages *in vitro*. When these forms were transferred to

lower temperatures, 28° C, they were able to transform into and multiply as promastigotes.

Mohareb *et al.* (1995) incorporated the two factors of lowering the pH and elevating the temperature to transform promastigotes of *L. tropica* to amastigotes axenically. The continuous long-term culture of these amastigotes was never reported. Although these forms were able to establish an infection in macrophages *in vitro*, their infectivity was never compared to that of promastigotes or lesion amastigotes and no other methods of characterization were reported.

The first attempt to reproduce a complete developmental cycle of *Leishmania* in axenic culture was made using *L. mexicana* (Bates, 1994a). Amastigotes obtained from lesions from infected animals were transformed to promastigotes in HO-MEM medium supplemented with 10% FCS at a pH of 7.5 at 25° C. Promastigotes were then subpassaged to Schneider's *Drosophila* medium supplemented with 20% FCS at a pH of 5.5 at 25° C to induce metacyclogenesis. The resulting forms were transformed and grown as axenic amastigotes by subpassaging them in the same medium at a raised temperature of 32° C. The three main stages of the cycle (multiplicative promastigotes, metacyclic promastigotes and amastigotes) were characterized by their morphology, total protein content, proteinases, nucleases and secretory acid phosphatase and each stage exhibited the expected suite of biochemical properties.

This study investigated the axenic cultivation of amastigotes of *L. donovani*. The chapter describes experiments that have been conducted to find the optimum conditions including culture media, FCS concentration, temperature, pH and gas requirements for the transformation to and axenic cultivation of amastigotes.

2.2 MATERIALS AND METHODS

2.2.1 *LEISHMANIA* PARASITES

Infections of *Leishmania donovani* (MHOM/ET/67/HU3;LV9) were maintained in hamsters by intraperitoneal inoculation of amastigotes. Amastigotes from hamsters were isolated as follows. The hamster was killed, dissected aseptically in a laminar flow hood and the liver and spleen were removed and placed individually into sterile petri dishes containing 10 ml of culture medium (see 2.2.4). Small parts of the spleen and liver were used to prepare impression smears which were air dried, then later fixed in absolute methanol and stained in 10% Giemsa's stain for 25-30 minutes. A sterile pair of scissors were used to cut the liver and spleen into small fragments which were then passed through a 19G needle with the aid of a syringe to break down large clumps. The homogenate was centrifuged at 1000g for 5 minutes to remove large clumps, erythrocytes and host cells. The pellet was discarded and the supernatant was retained and further centrifuged at 2000g for 5 minutes. The resulting supernatant was discarded and the amastigote pellet resuspended in a fresh 10 ml of the culture medium.

2.2.2 STORAGE OF PARASITES IN LIQUID NITROGEN

Stabilates of the parasite, amastigotes or promastigotes, were made and stored in liquid nitrogen to keep a constant supply of parasites as follows. Parasites were counted using an Improved Neubauer haemocytometer under phase-contrast microscopy and their density was estimated. Cells were washed three times in Hank's balanced salts solution (HBSS) and resuspended in culture medium at a density of $1x10^7$ cells/ml. These were then mixed in cryotube at a ration of 1:1 with 16% glycerol in culture

medium to yield a final cell density of $5x10^6$ cells/ml. Labelled cryotubes containing 1 ml aliquots were placed in a Nalgene cryo-pot containing isopropyl alcohol and the pot placed in -70° C freezer overnight. Tubes were then stored in liquid nitrogen until used.

2.2.3 *IN VITRO* CULTURES

Leishmania parasites were cultured (subpassaged) as follows. Cells were counted using an Improved Neubauer haemocytometer under phase-contrast microscopy and their density was estimated. The quantity of parasites to be inoculated into the new culture medium was estimated by the following equation: V1 x D1 = V2 x D2, where V1 is the volume of inoculum, D1 is the density of the cells in the stock culture, V2 is the volume of the new medium (usually 10 ml) and D2 is the required density (usually 5×10^5 per ml). Ten ml of fresh medium were pipetted into a 25 cm^3 culture flask and a sterile plastic pipette was used to deliver the inoculum into the fresh medium. Cultures were incubated at 26° - 27° C for promastigotes and 32° C, 35° C or 37° C for amastigote-like forms.

2.2.4 LIQUID MEDIA

Three liquid media were used. HO-MEM medium, Schneider's *Drosophila* medium (GIBCO) and Medium 199 (GIBCO) which was supplemented with Basal Medium Eagle Vitamin Solution (GIBCO).

HO-MEM medium was prepared essentially as described by Berens *et al.*, (1976) as follows:

- SMEM Minimal Essential Medium for suspension cultures with Spinner's salts 10.6g
- MEM Amino Acids 50x 10.0 ml
- MEM Non-essential amino acids 100x 10.0 ml
- Sodium Pyruvate 100x 11.0 ml
- Sodium bicarbonate 2.2 g
- Glucose 1.0-2.0 g
- Biotin 0.1 mg
- Para-aminobenzoic acid 1.0 mg
- Hemin solution (2mg/ml) in NaOH 3.0 ml

Constituents were dissolved in double distilled deionized water and the pH of the medium was adjusted to 7.2 to 7.4. Distilled water was used to adjust the final volume to 1 litre, the medium was filter sterilized and stored at 6 - 8° C.

Heat inactivated foetal calf serum (Gibco) was added, according to the required concentration, just before using the fresh medium. All media were supplemented with 25 μg/ml gentamicine sulphate.

2.2.5 MANIPULATION OF PARASITES

All experiments were conducted using axenic promastigotes in their first subpassage. Promastigotes were counted and their density was estimated. Cultures were initiated at a density of $5x10^6$ cells/ml.

2.2.5.1 MANIPULATING THE TEMPERATURE FOR THE OPTIMUM TRANSFORMATION AND GROWTH OF AMASTIGOTE-LIKE FORMS

Promastigotes were either transferred from 26° C to 37° C or adapted to grow at 32° C, then 35° C and then transferred to 37° C. HO-MEM medium supplemented with 10% FCS at a pH of 7.2 was used to grow cells at 26, 32 and 35° C. Adaptation was performed by leaving the cells to grow for two subpassages at each temperature before transferring to 37° C. For cultures at 37° C, 20% FCS was used and the pH was adjusted to 5.5 using 1*M* HCl. Cell densities were estimated daily.

2.2.5.2 DETERMINING THE OPTIMUM pH FOR THE TRANSFORMATION AND GROWTH OF AMASTIGOTE-LIKE FORMS

HO-MEM medium supplemented with 20% FCS was prepared at different pH values ranging from 4.0 to 6.5. Amastigote-like forms were inoculated into a 10ml of culture medium and cultures were incubated at 37° C in the presence of 5% CO_2. Cell densities were estimated daily over a period of 8 days.

2.2.5.3 EXAMINATION OF DIFFERENT CULTURE MEDIA FOR THE OPTIMUM GROWTH OF AMASTIGOTE-LIKE FORMS

HO-MEM medium, Schneider's *Drosophila* medium and Medium 199 were used to grow axenic amastigotes originally transformed in HO-MEM medium supplemented with 20% FCS at a pH of 5.5 at 37° C. All media were supplemented with 20% FCS.

Also FCS was used on its own as a culture medium.

Amastigotes were inoculated into 10 ml of fresh medium in 25 cm^2 tissue culture flasks. Cultures were incubated at 37° C in the presence of 5% CO_2. Cell densities were estimated every 48 hours.

2.2.5.4 CULTIVATION OF AMASTIGOTE-LIKE FORMS IN DIFFERENT FCS CONCENTRATIONS

Axenic amastigotes were passaged into HO-MEM medium containing 10%, 20%, 25% and 30% FCS, all at pH 5.5. Amastigotes were inoculated into 10 ml of fresh medium in 25 cm^2 tissue culture flasks. Cultures were incubated at 37° C in the presence of 5% CO_2. Cell densities were estimated every 48 hours.

2.2.5.5 CULTIVATION OF AMASTIGOTE-LIKE FORMS UNDER DIFFERENT GAS CONDITIONS

Freshly transformed amastigotes were cultured in HO-MEM medium supplemented with 20% FCS pH 5.5 in different gas conditions: 100% CO_2 (anaerobic); air; 5% CO_2/air; and 6% O_2- 3% CO_2- 91% N_2.

For the anaerobic conditions, cultures were incubated in an anaerobic jar. For air as the gas phase, culture flasks were closed during incubation in the 5% CO_2 incubator and opened daily in a laminar flow hood to allow gas exchange. For 5% CO_2/air gas phase, the lid of the culture flask was left slightly open inside the incubator to allow gas exchange. For the 6% O_2- 3% CO_2- 91% N_2 gas phase, a sterile stream of the gas mixture was blown inside the culture flask daily. Cultures were incubated at 37° C and the cell densities were estimated every three days.

2.3 RESULTS

For the purposes of this chapter amastigote-like forms were defined purely on morphological grounds by light microscopy. More detailed characterization is described in the following chapter.

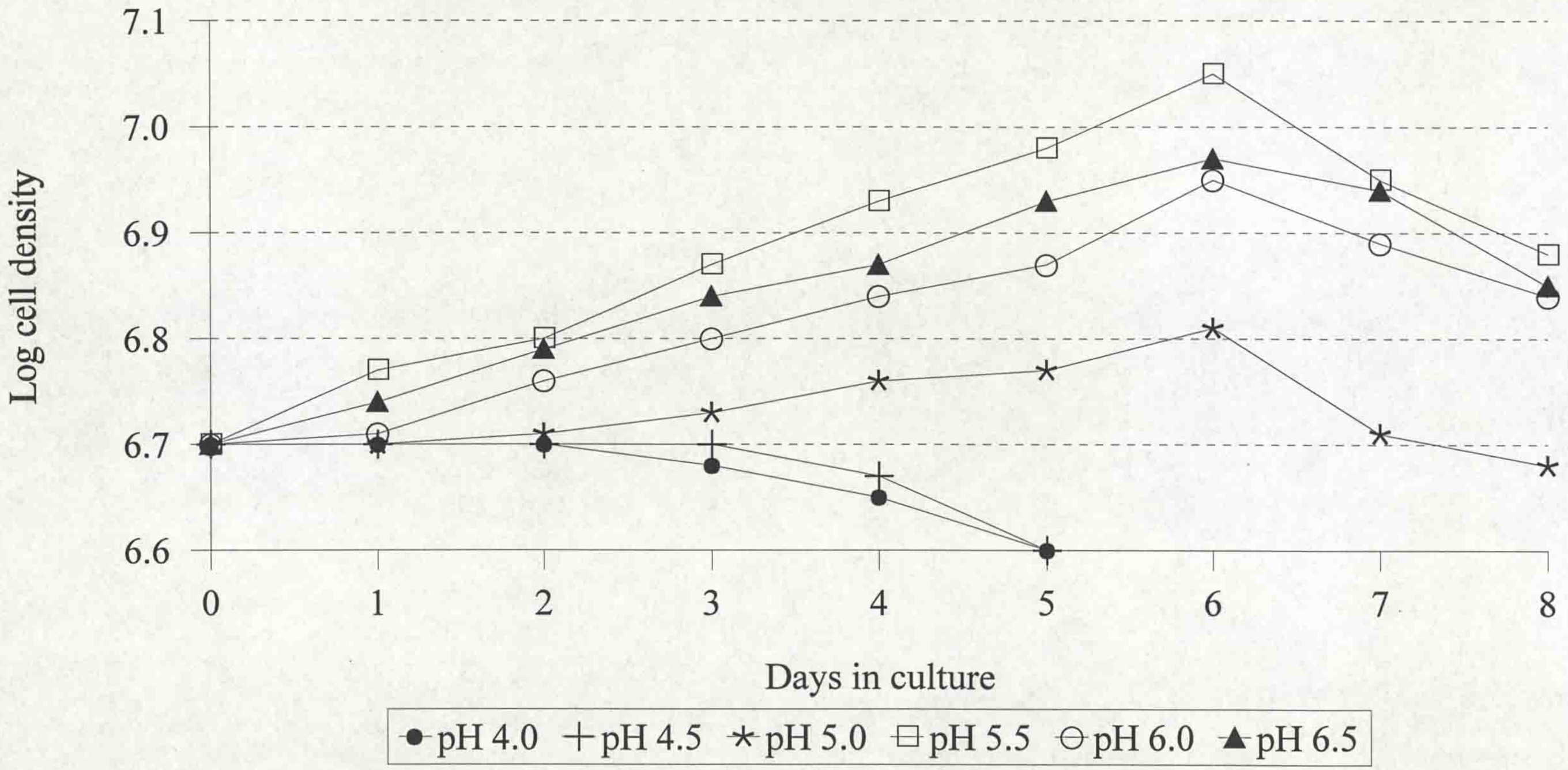
2.3.1 MANIPULATING THE TEMPERATURE FOR THE OPTIMUM TRANSFORMATION AND GROWTH OF AMASTIGOTE-LIKE FORMS

Transferring promastigotes directly from 26° C to 37° C was found to be better than gradually adapting them to grow at elevated temperatures. Promastigotes adapted to grow at 32° C and 35° C were able to transform to aflagellated, amastigote-like forms but were not able to grow or subpassage as amastigotes. Promastigotes transferred directly from 26° C to 37° C were able to grow and subpassage for a further 5 subpassages. In both cases, 99% of promastigotes transformed to amastigote-like forms within 72 hours.

2.3.2 DETERMINING THE OPTIMUM pH FOR THE TRANSFORMATION AND GROWTH OF AMASTIGOTE-LIKE FORMS

Axenic amastigotes were cultured under various initial pH conditions as described in 2.2.5.2. The results of a typical experiment are shown in figure 2.1. A pH of 5.5 for the culture medium was found to be optimum for the growth of amastigotes axenically as judged by higher cell densities and faster rate of growth. In fig. 2.1 the maximum density achieved on day 6 was 1.1×10^7 cells/ml and the population doubling time from day 0 to day 6 was 126.6 hours ie. the density doubled in 5.2 days.

Figure 2.1. Growth of *L.donovani* axenic amastigotes in HO-MEM medium supplemented with 20% FCS at different pH.


7.1
7.0
6.9
6.8
6.7
6.6
Log cell density
0
1
2
3
4
5
6
7
8
Days in culture
pH 4.0
pH 4.5
pH 5.0
pH 5.5
pH 6.0
pH 6.5

No growth was obtained at pH 4.0 or 4.5. Reasonable growth was observed at pH 6.0 and 6.5 as shown in fig. 2.1, but this was consistently lower than that obtained at pH 5.5.

2.3.3 EXAMINATION OF DIFFERENT CULTURE MEDIA FOR THE OPTIMUM GROWTH OF AMASTIGOTE-LIKE FORMS

Axenic amastigotes were cultured in HO-MEM medium, Schneider's *Drosophila* medium, and M199, each supplemented with 20% FCS, and 100% FCS as described in 2.2.5.3. In each experiment comparisons were made using FCS from the same batch. The results of some typical experiments are shown in figures 2.2 and 2.3. HO-MEM medium supplemented with 20% FCS at a pH of 5.5 was found to give optimum results. Amastigotes in HO-MEM medium consistently reached higher densities than those in Schneider's *Drosophila* medium or M199. Amastigotes grown in 100% FCS also reached high densities but such cultures experienced a more rapid fall in numbers after the peak cell density.

2.3.4 CULTIVATION OF AMASTIGOTE-LIKE FORMS IN DIFFERENT FCS CONCENTRATIONS

Axenic amastigotes were cultured in HO-MEM medium containing 10%, 20%, 25% and 30% FCS pH 5.5 as described in 2.2.5.4 (figure 2.4). Poor growth was obtained with 10% FCS and it seems that at least 20% FCS is required to support the growth of axenic amastigotes.

Figure 2.2. Growth of *L.donovani* axenic amastigotes in HO-MEM medium and Schneider's *Drosophila* medium both supplemented with 20% FCS.

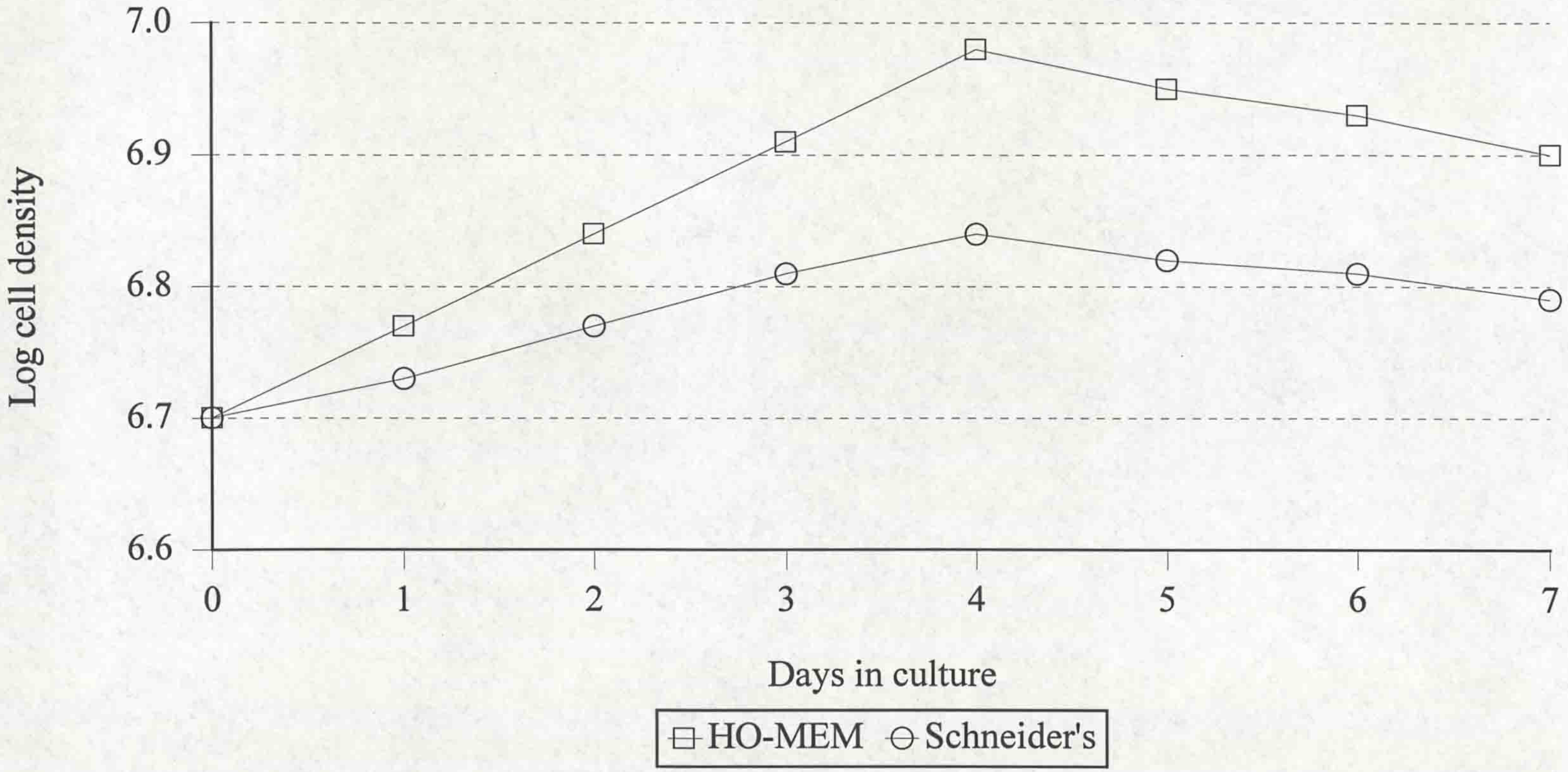

7.0
6.9
6.8
6.7
6.6
Log cell density
0
1
2
3
4
5
6
7
Days in culture
HO-MEM
Schneider's

Figure 2.3. Growth of *L.donovani* axenic amastigotes in HO-MEM medium, M199 both suplemented with 20% FCS, and in 100% FCS.

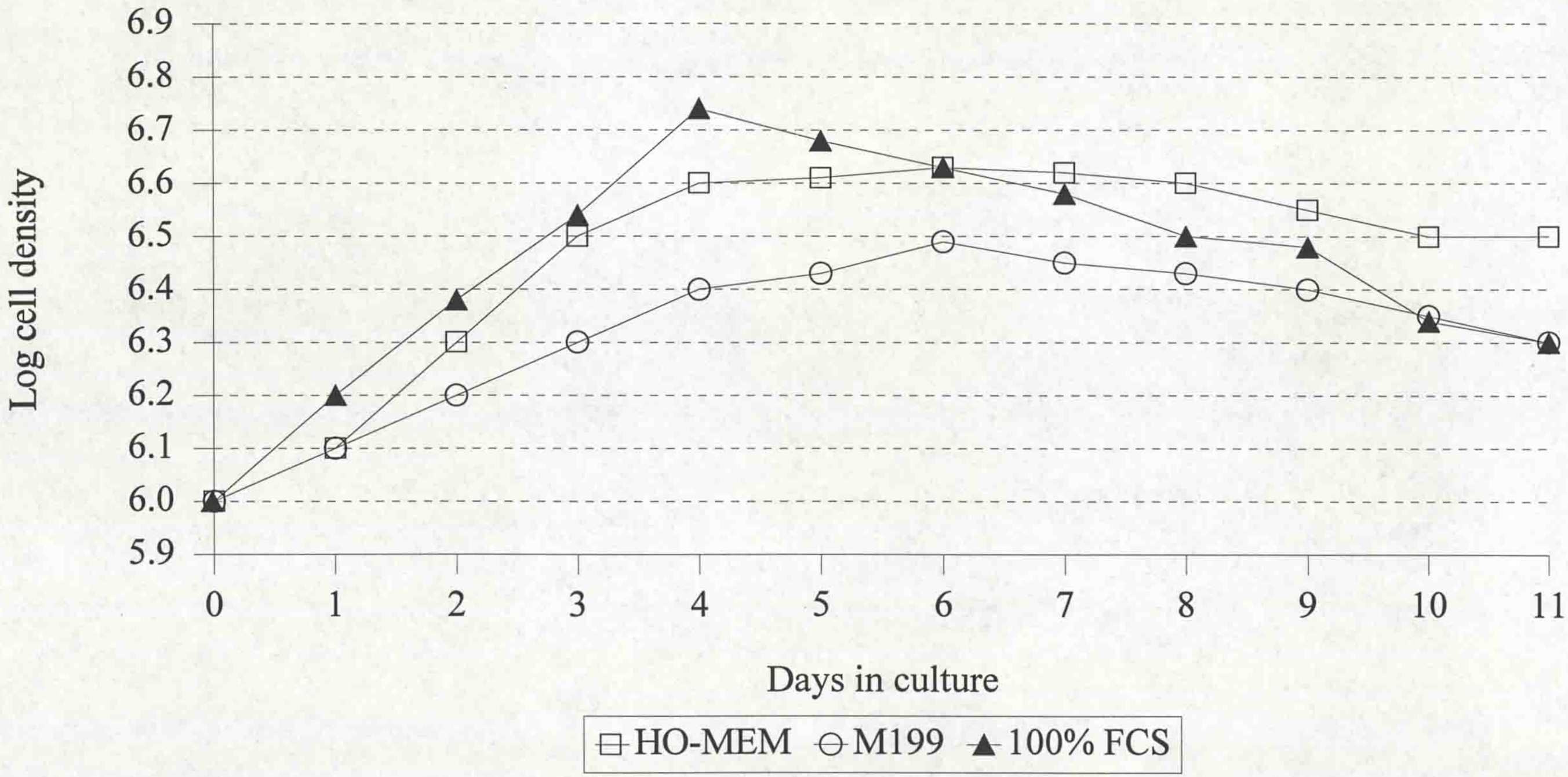
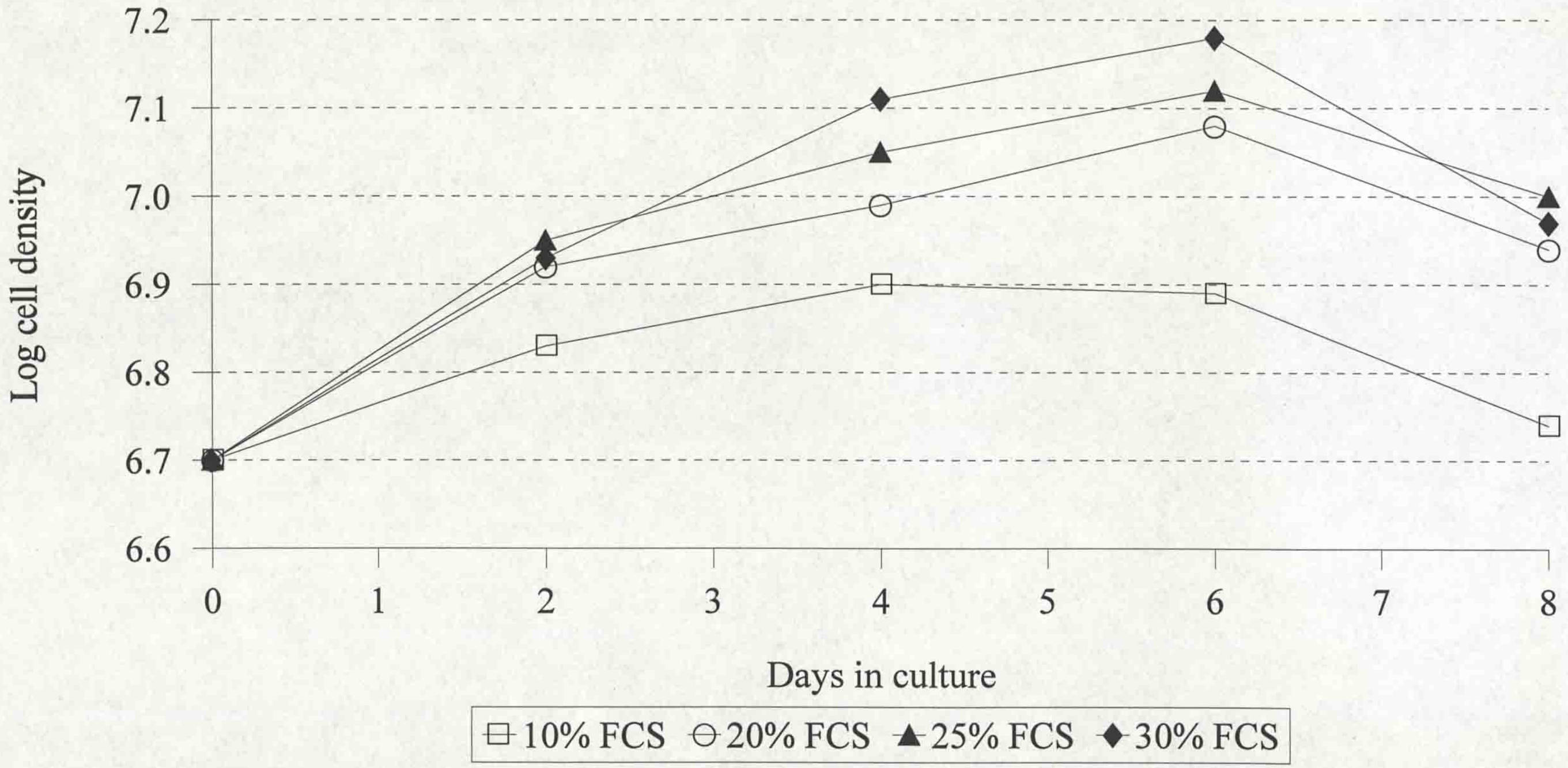

Log cell density
6.9
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6.0
5.9
0
1
2
3
4
5
6
7
8
9
10
11
Days in culture
HO-MEM
M199
100% FCS

Figure 2.4. Growth of *L.donovani* axenic amastigotes in different FCS concentrations.

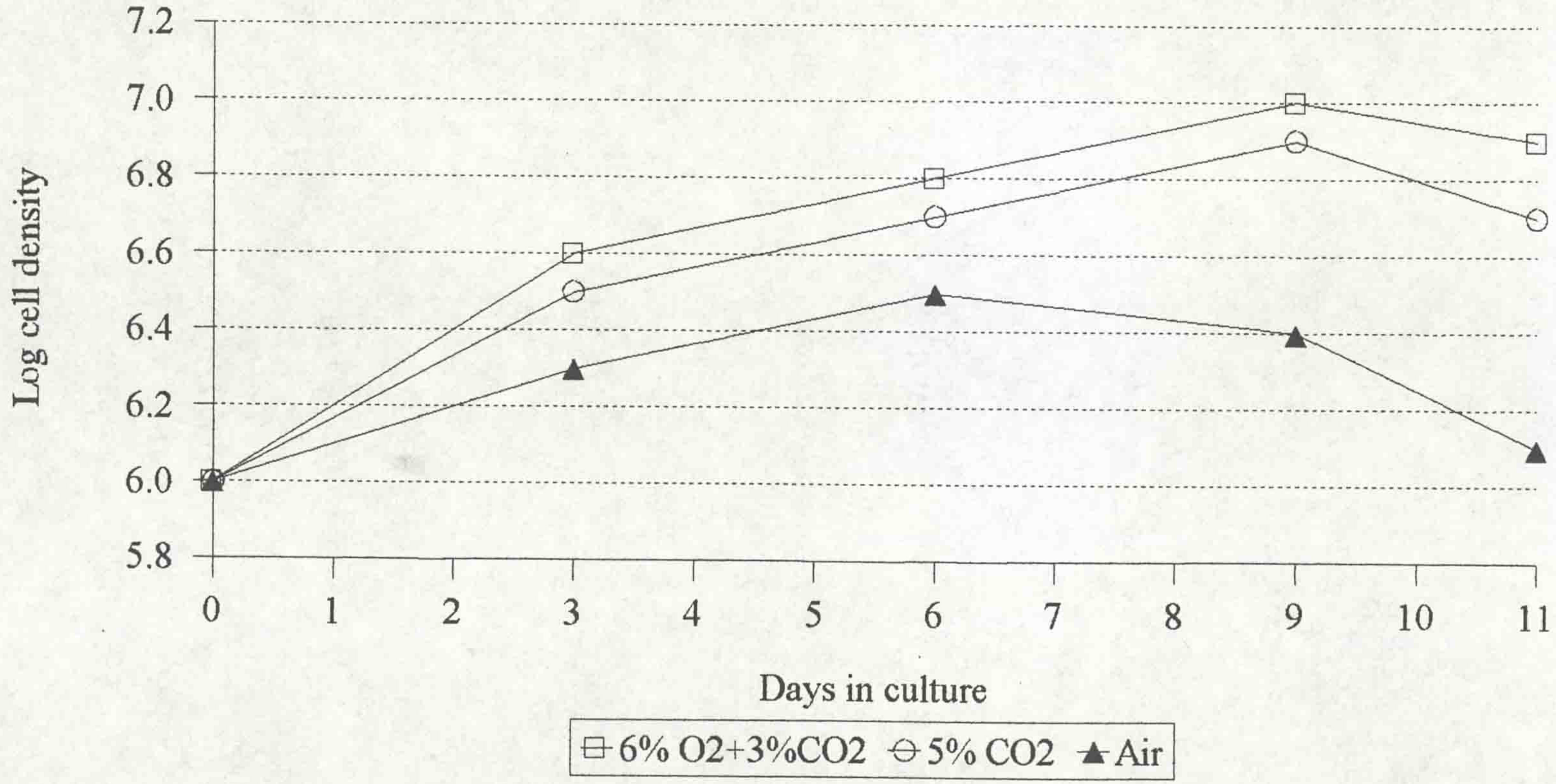

7.2
7.1
7.0
6.9
6.8
6.7
6.6
Log cell density
0
1
2
3
4
5
6
7
8
Days in culture
10% FCS
20% FCS
25% FCS
30% FCS

Cell densities were slightly higher in cultures containing 25 and 30% FCS. However, satisfactory cell densities were obtained from cultures containing 20% FCS, and the difference was not considered sufficient to justify the expense of using a higher concentration.

2.3.5. CULTIVATION OF AMASTIGOTE-LIKE FORMS UNDER DIFFERENT GAS CONDITIONS

Axenic amastigotes were cultured in HO-MEM medium supplemented with 20% FCS pH 5.5 under various gas conditions as described in 2.2.5.5. Amastigotes cultured in anaerobic conditions died after one day. The results of an experiment comparing the other three gas phases are shown in figure 2.5. Although cultures exposed to 6% O_2-3% CO_2 reached higher densities, the differences between these densities and those obtained by cultures exposed to 5% CO_2 were small. Amastigotes cultured in flasks exposed to air alone did not grow well.

Figure 2.5. Growth of *L.donovani* axenic amastigotes in different gas conditions.


Log cell density
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
0
1
2
3
4
5
6
7
8
9
10
11
Days in culture
6% O2+3%CO2
5% CO2
Air

2.4 DISCUSSION

Several studies have recently focused on the transformation and axenic cultivation of amastigotes (reviewed by Bates, 1993a). Different factors that contribute to the transformation and growth of axenic amastigotes have been investigated in this study. Currently, there is no single universal culture medium to grow all stages of different species of *Leishmania* and it is difficult to predict which culture medium will optimally support a specific isolate of *Leishmania* in advance (Evans, 1987). In this study, three culture media were used. HO-MEM medium and M199 are used in this laboratory for the continuous culture of promastigotes. Schneider's *Drosophila* medium is used for the axenic cultivation of *L. mexicana* amastigotes. These results show that HO-MEM medium supplemented with 20% FCS was the best of these tested for the transformation and growth of *L. donovani* amastigotes axenically. This was judged by the higher cell densities and faster rate of growth of amastigotes grown in this medium.

Temperature plays an important role in the transformation of promastigotes to amastigotes (Zilberstein and Shapira, 1994). A reversible transformation of *L. panamensis* promastigotes to amastigotes was observed upon increasing the temperature from 26° C to 34° C (Darling and Blum, 1987). Fehniger *et al.*, (1990) observed changes in the antigenic profile of *Leishmania* parasites following a shift in temperature. This agreed with the work of Pan and McMahon-Pratt (1988) who were able to characterize stage-specific antigens and raised antibodies against *L. pifanoi* amastigote-like forms. In all efforts to grow amastigotes axenically reported to date, the temperature was an important factor. In this study, the temperature for culturing axenic amastigotes was chosen to be 37° C, the temperature of the viscera of the

mammalian host. Temperature was not a factor that was optimised: it was considered that growth at 37° C was a prerequisite for axenic amastigotes of *L. donovani*. Gradual increase of temperature by adapting promastigotes to grow at 32° and then 35° C, before transferring them to 37° C was not successful whereas direct transfer to 37° C succeeded. This could be due to an adaptation to grow as promastigotes *in vitro* at 32° C and/or 35° C and subsequent loss of their ability to transform to amastigotes. The virulence of *L. major* promastigotes to BALB/c has been reported to decrease as they adapt to *in vitro* cultures (Segovia *et al.*, 1992).

Another important factor in the axenic cultivation of *Leishmania* amastigotes is the pH of the culture medium. Induction of transformation to the amastigote stage by low pH has also been reported in *Trypanosoma cruzi* (Tomlinson *et al.*, 1995). In this study we found that a pH of 5.5 is optimal for the transformation and subsequent growth of *L. donovani* amastigotes axenically. This agrees with the findings of Bates *et al.* (1992) who were able to grow amastigotes of *L. mexicana* axenically. The parasitophorous vacuoles of *L. amazonensis*-infected macrophages were found to maintain an acidic pH of 4.7 to 5.3 (Antoine *et al.*, 1990). Although unknown, the pH of the sandfly mid gut is likely to be relatively alkaline (Zilberstein, 1991) and, hence, the transformation of promastigotes to amastigotes, which occur during phagocytosis by host macrophages, could be due to a rapid exposure to an acidic environment. Amastigotes and promastigotes of *Leishmania* were shown to have developed mechanisms to sense changes in environmental pH (Zilberstein and Shapira, 1994). Furthermore, although *L. donovani* promastigotes metabolize glucose, proline, and nucleosides optimally at pH 7.0-7.5, amastigotes catabolize these substrates optimally at pH 4.5-5.0 (Mukkada *et al.*, 1985). Amastigote stage-specific proteins were

observed among promastigotes of *L. major* when cultured in acidic pH of 4.5 (Zilberstein *et al.*, 1991).

Gas requirements for *Leishmania* amastigote culture have not been investigated thoroughly before. In this study we found that amastigotes grew better in the presence of a high carbon dioxide concentration. A slight improvement in growth was observed when the oxygen concentration was also decreased to 6%. Gas conditions have also been shown to affect amastigote to promastigote transformation and promastigote metabolism. Hart and Coombs (1981) reported that a high carbon dioxide concentration increased the rate of amastigotes to promastigote transformation whereas lowering the oxygen tension slightly improved the rate of transformation. They concluded that amastigotes were adapted to grow in low oxygen tensions encountered *in vivo*. Keegan and Blum (1990) reported that glucose consumption in *L. major* promastigotes increased as the concentration of oxygen was reduced to 6%.

2.5. CONCLUSION

The optimum conditions to grow amastigotes of *L. donovani* axenically were:

- HO-MEM medium supplemented with 20% FCS.
- Temperature of 37° C.
- Acidic pH of 5.5.
- Low oxygen and high carbon dioxide concentrations.

CHAPTER THREE

3. CHARACTERIZATION OF AXENIC AMASTIGOTES

3.1 INTRODUCTION

Axenically cultured amastigotes should be proven not only to differ from promastigotes but, more importantly, to be similar to lesion amastigotes. A variety of criteria can be used to study these differences and similarities.

One of the most obvious ways to characterize axenically cultured amastigotes is by comparing them morphologically together with lesion amastigotes and cultured promastigotes and looking at their shapes and sizes. Many workers have used light microscopy for the morphological characterization of axenic amastigotes and in all cases these forms were similar to lesion amastigotes and different from promastigotes (Eperon and McMahon-Pratt, 1989b; Pan *et al.*, 1993; Bates, 1994a).

A higher degree of morphological characterization can be acheived at the ultrastructural level by examining ultrathin sections of parasites under the electron microscope. Several differences have been reported in the literature between promastigotes and amastigotes, from lesions or cultures. The extension of the flagellum beyond the cell surface in promastigotes but not in amastigotes is one of the obvious differences. Another difference is the presence of a paraxial rod, a paracrystalline lattice structure running parallel to the 9 + 2 microtubule axoneme in the promastigote stage, which is absent in amastigotes (Pan and Pan, 1986; Eperon and

McMahon-Pratt, 1989b; Pimenta *et al.*, 1991; Bates *et al.*, 1992; Bastin *et al.*, 1996). Afurther ultrastructural difference is that amastigotes of the *L. mexicana* complex have unique electron dense megasomes which are not present in promastigotes (Tetley, *et al.*, 1989; Galvao-Quintao *et al.*, 1990; Bates *et al.*, 1992).

Biochemical characterization of axenic amastigotes has also been used to differentiate them from promastigotes. *L. mexicana* lesion amastigotes have been shown to contain greater activities than promastigotes of the enzymes that catalyse the β-oxidation of fatty acids, but lower activities of several glycolytic enzymes especially pyruvate kinase (Coombs *et al.*, 1982).

Proteinases in *Leishmania*, especially cysteine proteinases, have been the subject of numerous studies (Coombs, 1982; Coombs *et al.*, 1991; North and Coombs, 1981; North *et al.*, 1990). Amastigotes of *L. mexicana* were found to have high activities of low molecular weight cysteine proteinases which were low or absent from exponential-phase promastigotes. Stationary-phase promastigotes had intermediate activities of these enzymes, some of which appear to have different molecular weights than those of amastigotes (Lockwood, *et al.*, 1987; Rainey *et al.*, 1991; Bates *et al.*, 1992; Bates, 1994a).

Bates (1993b) examined amastigotes and promastigotes of *L. mexicana* for the presence of nucleases. A 40 kDa nuclease was detected in promastigotes but not in amastigotes and a doublet of 29/31 kDa was found in both stages, but expressed at a 60-fold higher level in amastigotes.

Changes in the antigenic profile of *Leishmania* parasites following shifts in temperature have been reported in the literature. Fehniger *et al.*, (1990) examined the antigen profile of *Leishmania* parasites undergone shifts in temperature. They observed changes in immunoblot profiles of the parasites within one day of transferring the parasite from 25° C to 37° C. Furthermore, serum from patients with active infections reacted with antigens expressed in parasites at 37° C but failed to react with promastigotes cultured at 25° C.

Monoclonal antibodies raised against antigens from lesion and/or axenic amastigotes and promastigotes have been used to characterize these forms. Antibodies that recognized specific components on axenic amastigotes failed to recognize promastigotes and, at the same time, recognized amastigotes from lesions. Conversely, monoclonal antibodies specific for promastigote molecules failed to detect these molecules being expressed by the axenic amastigotes (reviewed by Pan *et al.*, 1993). For example, Eperon and McMahon-Pratt (1989a) raised monoclonal antibodies against membrane proteins of axenically grown amastigotes of *L. panamensis* and *L. braziliensis*. Six monoclonal antibodies recognized axenic and lesion amastigote antigens but not promastigote antigens by indirect immunofluorescence. Furthermore, the molecules recognized by these antibodies were found to be identical for both axenic amastigotes and amastigotes isolated from macrophages. This also agreed with previous work on *L. pifanoi* (Pan and McMahon-Pratt, 1988).

Working with *L. pifanoi*, Rainey *et al.* (1991) reported that rates of DNA, RNA, and protein synthesis in axenic amastigotes were different from those of promastigotes. In another study, Joshi *et al.* (1993) constructed cDNA libraries from poly $(A)^+$ RNA

isolated from promastigotes and axenic amastigotes of *L. donovani*. Three unique cDNA clones (P17, A41 and A45) were isolated using cDNA hybridization techniques and hybridized to RNA, isolated from promastigotes and amastigotes, in Northern and slot-blots. Levels of A41 and A45 RNAs were two fold higher in amastigotes compared to promastigotes. On the other hand, promastigotes showed two fold higher levels of the P17 RNA than amastigotes.

Axenically cultured amastigotes have been shown to be infective to and able to survive within macrophages *in vitro* (Eperon and McMahon-Pratt, 1989b) but, more importantly, they were shown to be significantly more infective *in vivo* than promastigotes(Al-Bashir *et al.*, 1992; Bates *et al.*, 1992).

In this study differences and similarities between promastigotes, lesion amastigotes and axenic amastigotes were investigated in terms of morphology, size, ultrastructure and gel electrophoresis of proteins.

3.2 MATERIALS AND METHODS

3.2.1 MORPHOLOGICAL CHARACTERIZATION

Thin smears of promastigotes and axenic amastigotes were prepared, air dried, fixed in absolute methanol and stained in 10% (v/v) Giemsa's stain/phosphate buffer for 10 minutes. Liver impression smears containing tissue amastigotes were prepared, air dried, fixed in absolute methanol and stained in 10% Giemsa's stain for 30 minutes. All slides were examined under the microscope by oil emmersion at 1000x magnification. The sizes (longest dimension of the cell body excluding flagellum) of 100 promastigotes, 100 axenic amastigotes and 100 lesion amastigotes were measured. Statistical tests to compare the sizes of promastigotes, axenic amastigotes and tissue amastigotes were performed using the Minitab programme.

3.2.2. ULTRASTRUCTURAL CHARACTERIZATION

Promastigotes, axenic amastigotes and tissue amastigotes were washed three times in HBSS by centrifugation at 2000g and resuspension of the cell pellet. Pellets were then fixed in 3% glutaraldehyde in 0.1*M* cacodylate buffer pH 7.4 for 3 hours at room temperature. The fixative was removed and replaced by buffer alone and samples were placed in a 4° C refrigerator until processing. Samples were embedded in propylene oxide for half an hour followed by 2 hours in propylene oxide and Epon-Araldite resin (1:1) then overnight in propylene oxide/resin (1:2) and finally in 2 changes of resin alone (4 hours each). Samples were placed in BEEM capsules, topped up with resin and were polymerised at 80° C for 48 hours. Sections of 90 nm thickness were cut using a Reichert "Ultracut E" microtome and placed onto 200 mesh hexagonal copper grids. These were stained in 2% uranyl acetate for 20 minutes at room temperature

followed by Reynold's lead citrate for 5 minutes. Specimens were examined under a Philips CM10 transmission electron microscope operated at 80kV.

For examination under scanning electron microscope, samples of promastigotes and axenic amastigotes were washed twice in HBSS and fixed in 3% glutaraldehyde in 0.1*M* cacodylate buffer pH 7.4 for 3 hours at room temperature, as described above, on a glass cover slip. Specimens were critical point dried from amyl acetate, mounted on stubs and sputter coated with gold prior to examination in Hitachi S-520 Scanning electron microscope (Hayat, 1989).

3.2.3 BIOCHEMICAL CHARACTERIZATION

Promastigotes, axenic amastigotes and tissue amastigotes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by total protein staining with Coomassie Blue. Stationary-phase promastigotes and stationary-phase axenic amastigotes were harvested by centrifugation at 2000g for 5 minutes in room temperature. Cells were washed three times in cold HBSS, the supernatant of the last wash was discarded and pellets were stored in -70° C until use. Tissue amastigotes were prepared as described in 2.2.1 and pellets were stored in -70° C until use. SDS-PAGE was performed as described in Hames and Rickwood (1990), see appendix 1.

3.3 RESULTS

3.3.1 MORPHOLOGICAL CHARACTERIZATION

Promastigotes, axenic amastigotes and tissue amastigotes were examined by light microscopy at a magnification of 1000x (Fig. 3.1). Axenic amastigotes were morphologically similar to tissue amastigotes, being round to oval and possessing a small round nucleus and a kinetoplast. Their average size was 3.7 μm (± 0.07 SE) compared to 3.0 μm (± 0.06 SE) which was the average size of tissue amastigotes. Promastigotes were elongated with a central, round nucleus, anterior kinetoplast and a flagellum. Their average size was 13.64 μm (± 0.43 SE) excluding flagellum.

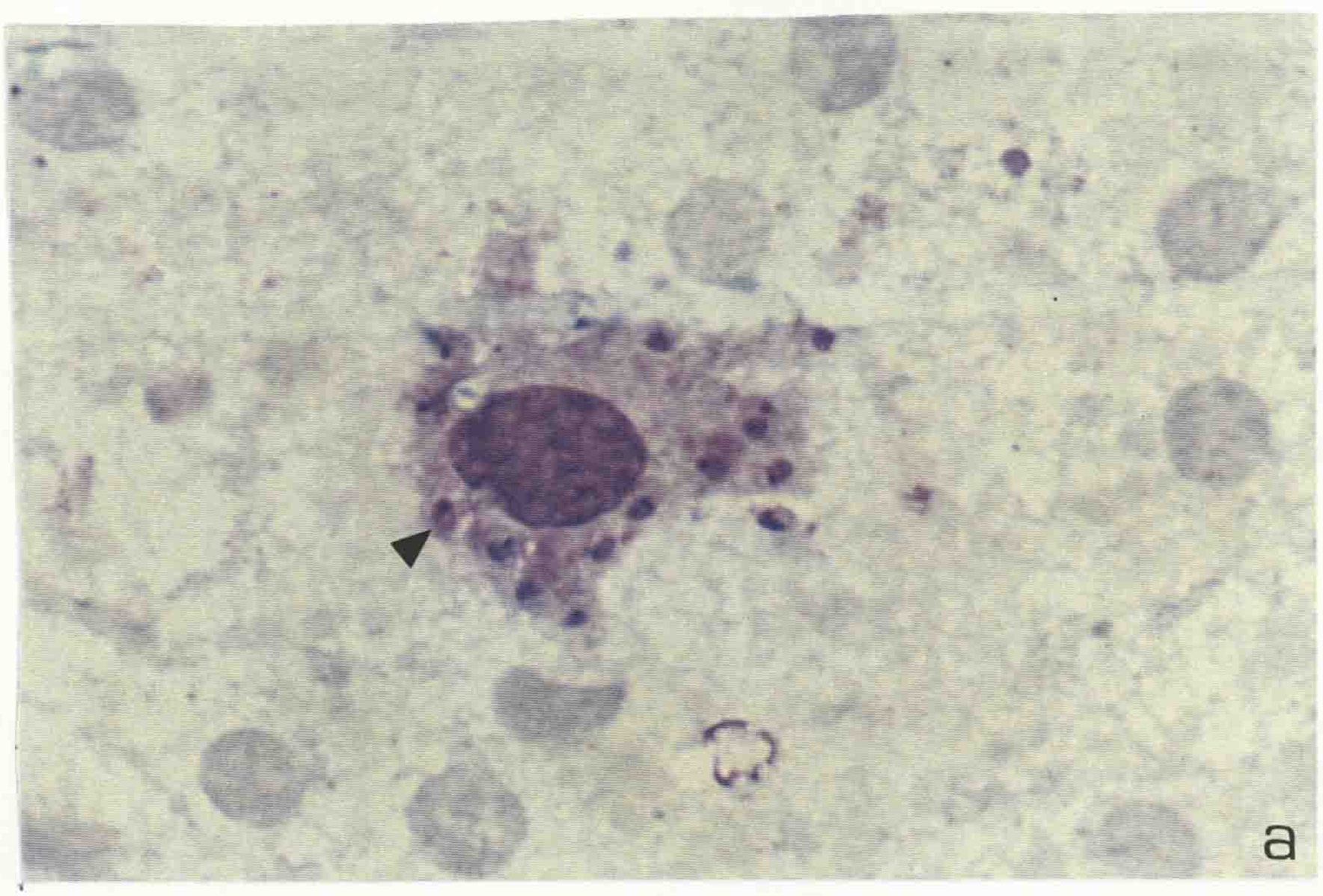
There was a statistical difference between the sizes of axenic amastigotes and tissue amastigotes ($t = 6.79$, $p < 0.001$, DF = 184). Differences in sizes between promastigotes and axenic amastigotes were statistically significant ($t = 22.68$, $p < 0.001$, DF = 104) as well as between promastigotes and tissue amastigotes ($t = 24.26$, $p < 0.001$, DF = 102).

3.3.2. ULTRASTRUCTURAL CHARACTERIZATION

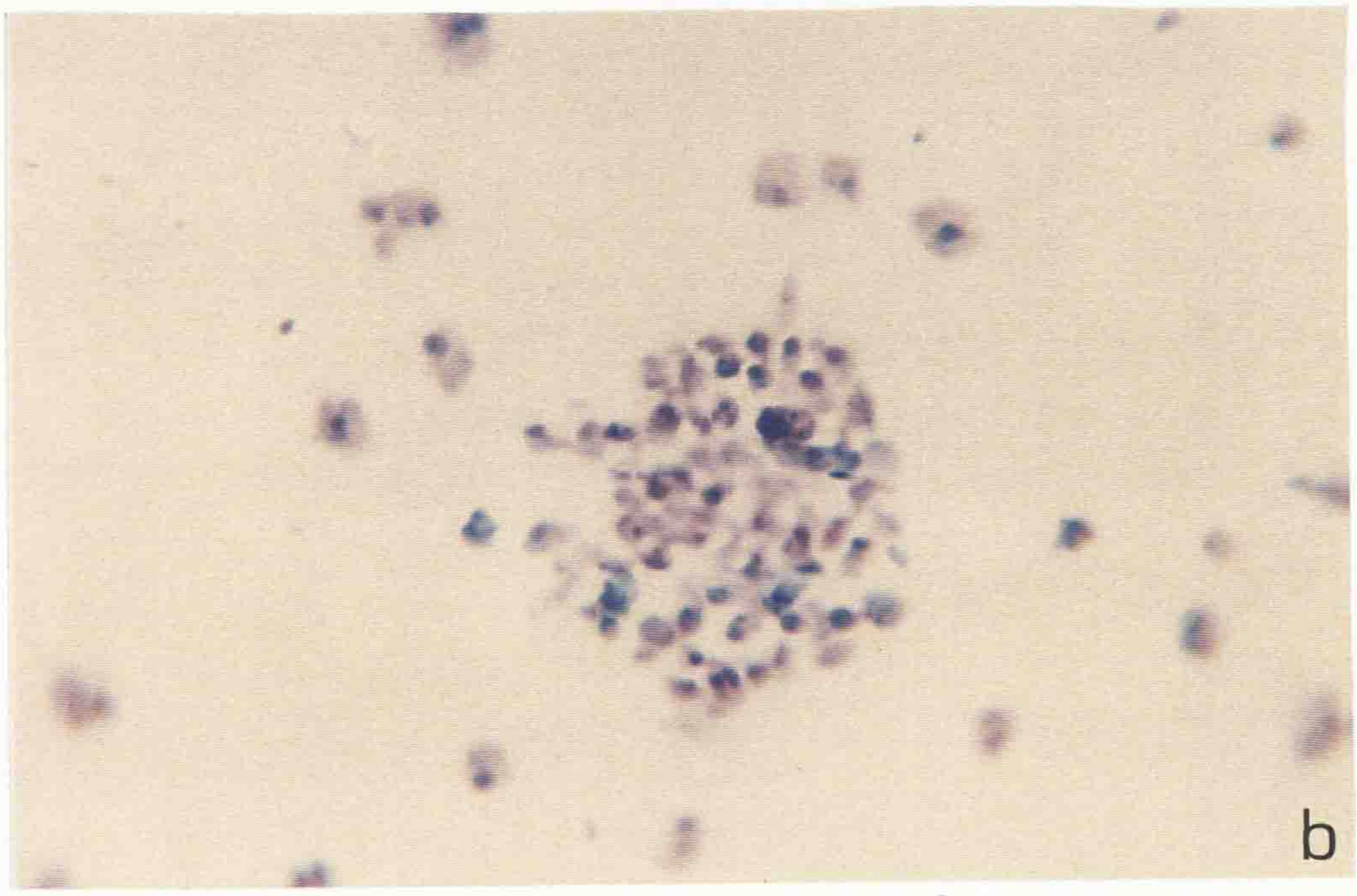
Promastigotes, axenic amastigotes and tissue amastigotes were examined under the transmission and scanning electron microscope as described in 3.2.2. Axenic amastigotes were similar to tissue amastigotes being round or oval, measuring 2.5 to 3.0 μm in diameter, and possessed a flagellum which did not extend beyond the cell body. Promastigotes were elongated with a body length ranging from 7.0 to 18.0 μm and possessed a paraxial rod which was not seen either in axenic amastigotes, or in tissue amastigotes. The flagellar pocket of axenic amastigotes was found to contain filamentous materials which were not seen in promastigotes (figures 3.2 to 3.9).

Figure 3.1. Light micrograph of (a) tissue amastigotes (arrow), (b) axenic amastigotes, and (c) promastigotes.

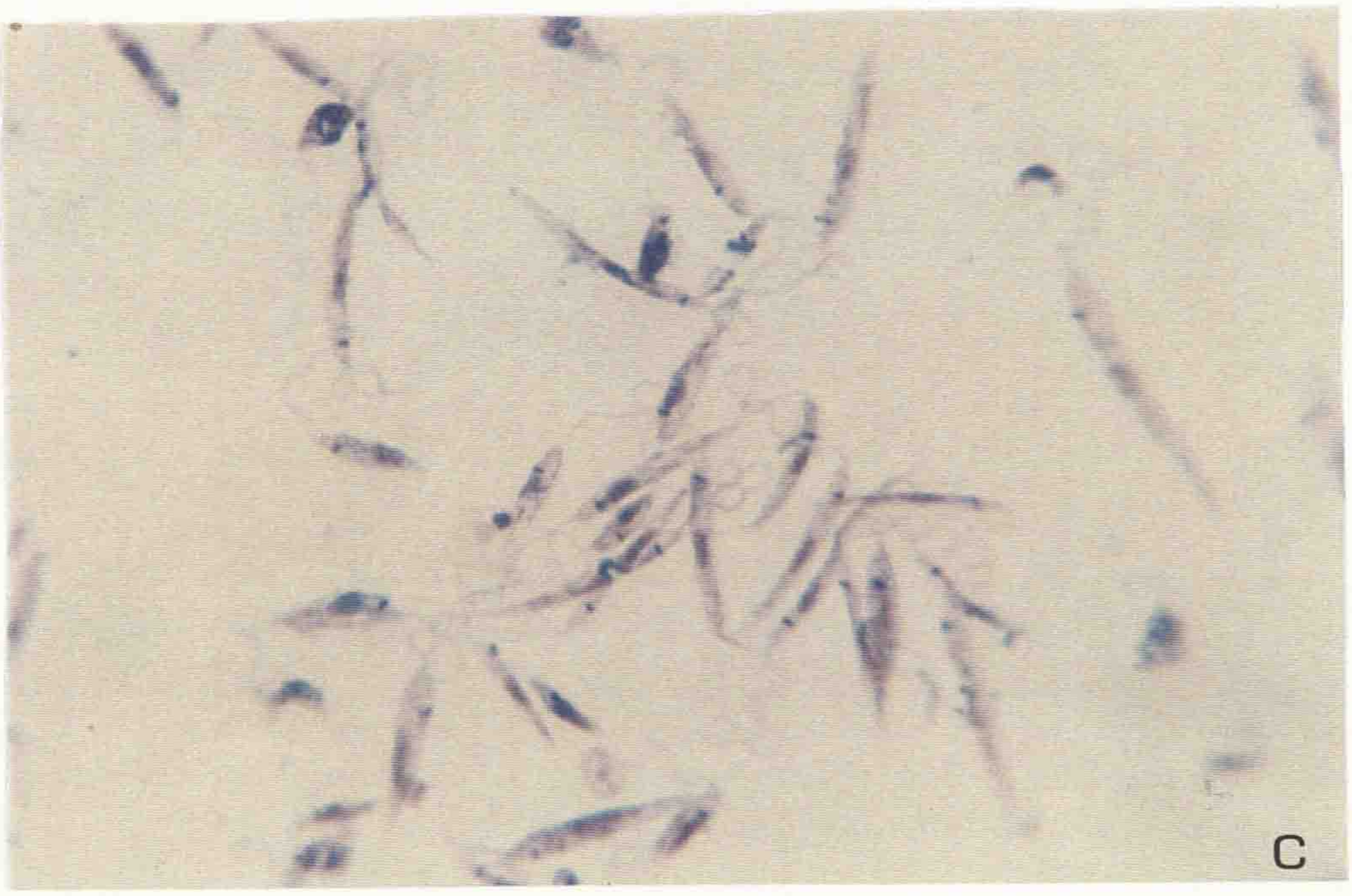
Bar = 5 μm.



a



b



C

Figure 3.2. Transmission electron micrograph of a longitudinal section of a promastigote showing flagellum extending beyond cell surface. N=nucleus, F=flagellum, K=Kinetoplast. Bar = 260 nm


N
K
F

Figure 3.3. Transmission electron micrograph of a cross section in a promastigote showing paraxial rod. F=flagellum, FP=flage!lar pocket, PR=paraxial rod.

Bar = 100 nm

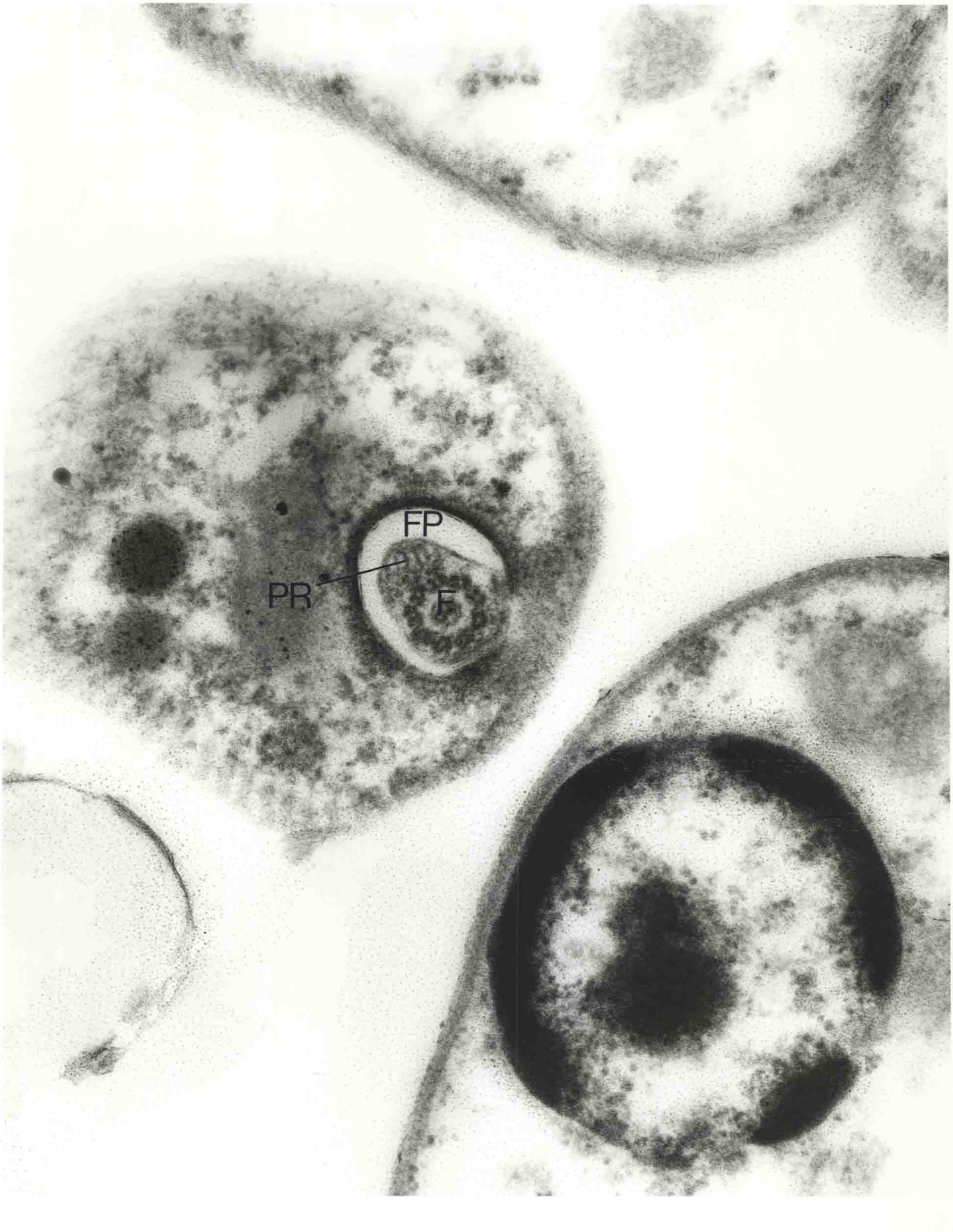

FP
PR
F

Figure 3.4. Transmission electron micrograph of tissue amastigote showing flagellum terminating at cell surface. F=flagellum, K=kinetoplast.

Bar = 170 nm


K
F

Figure 3.5. Transmission electron micrograph of tissue amastigote showing the absence of paraxial rod. N=neucleus, F=flagellum.

Bar = 140 nm

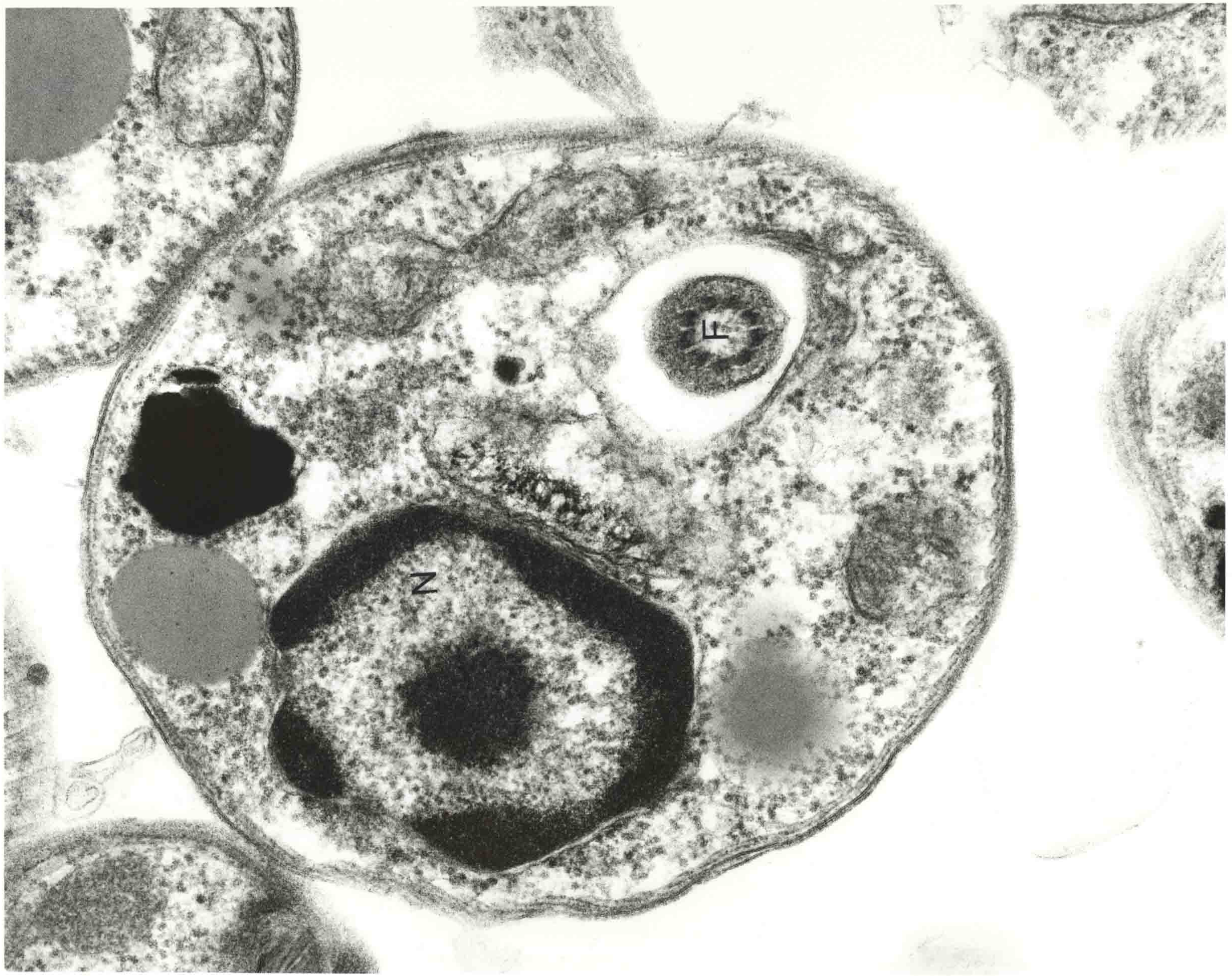

N
Z

Figure 3.6. Transmission electron micrograph of axenic amastigotes showing the round to oval shape and the flagellum does not extend beyond the cell surface.

Bar = 1.4 μm

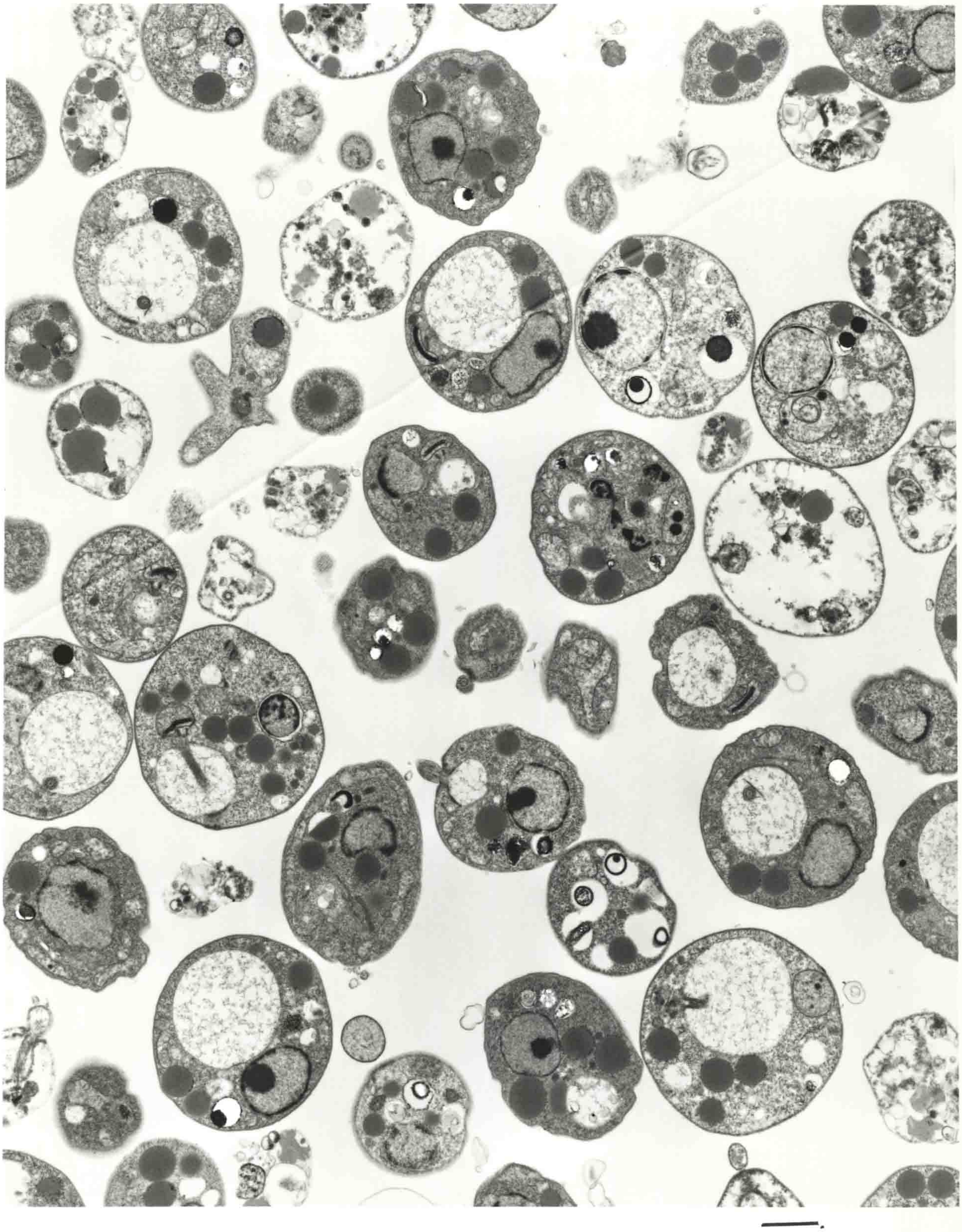


Figure 3.7. Transmission electron micrograph of axenic amastigote showing the absence of paraxial rod. N=nucleus, F=flagellum.

Bar = 200 nm

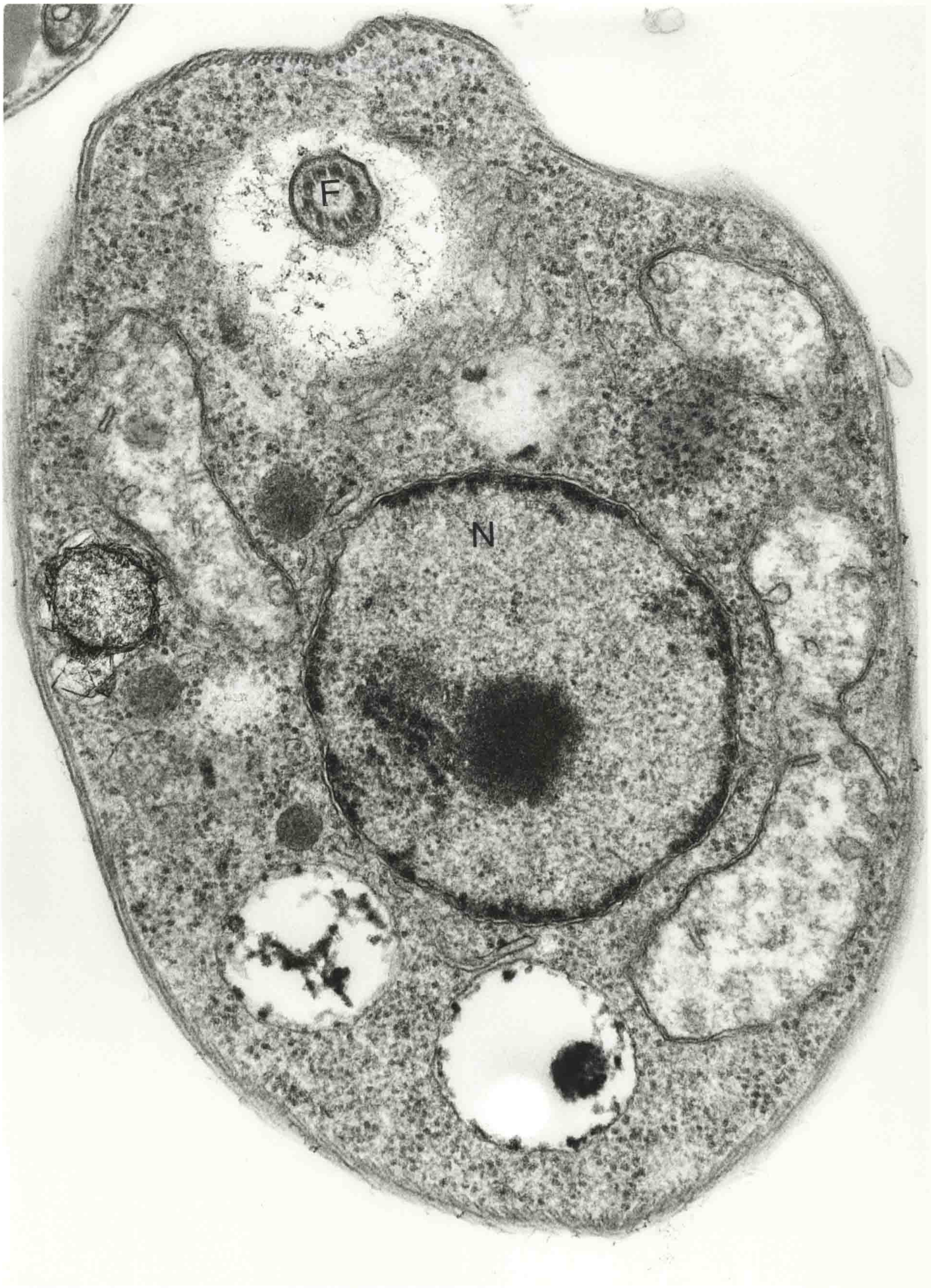

F
N

Figure 3.8. Scanning electron micrograph of promastigotes.

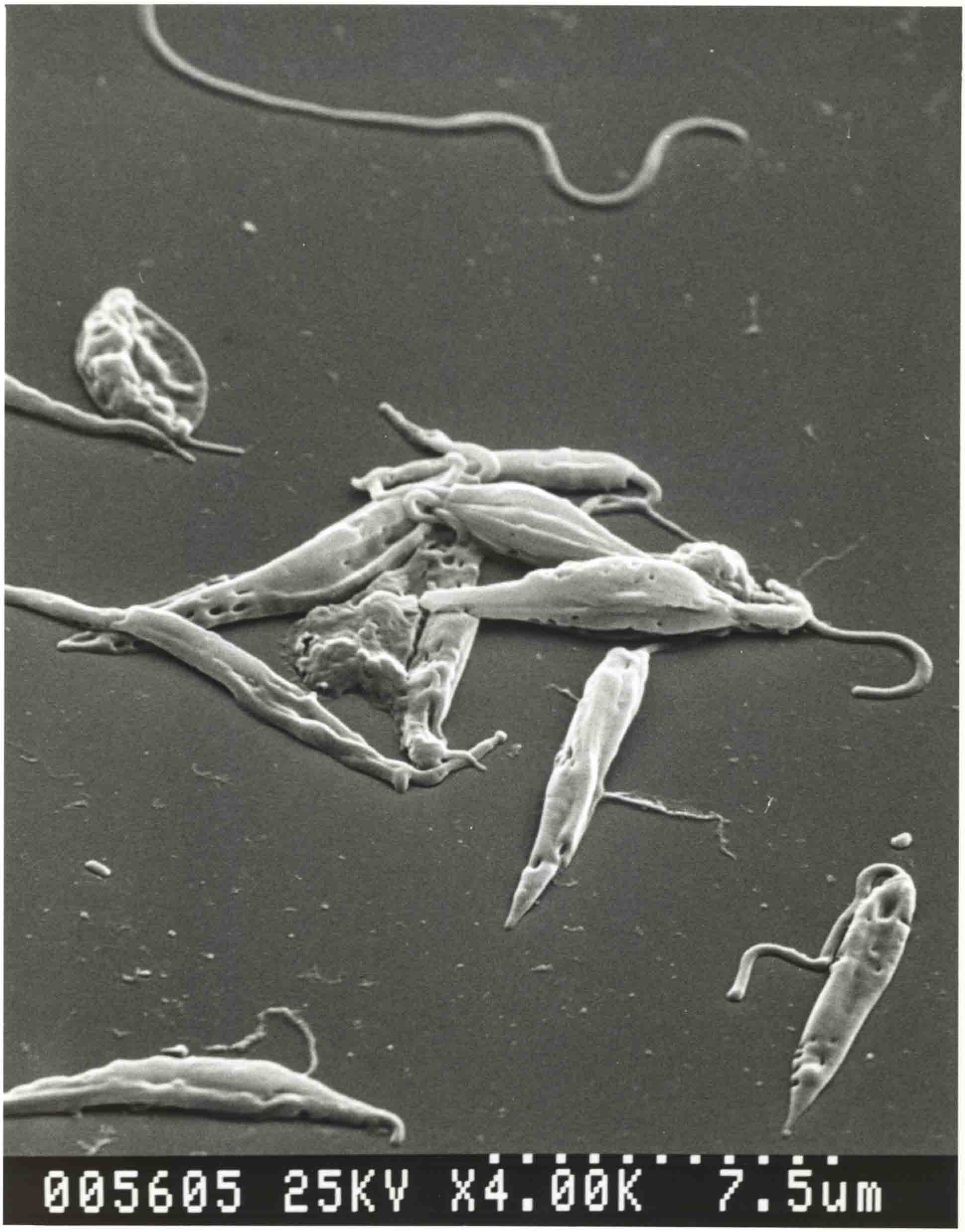
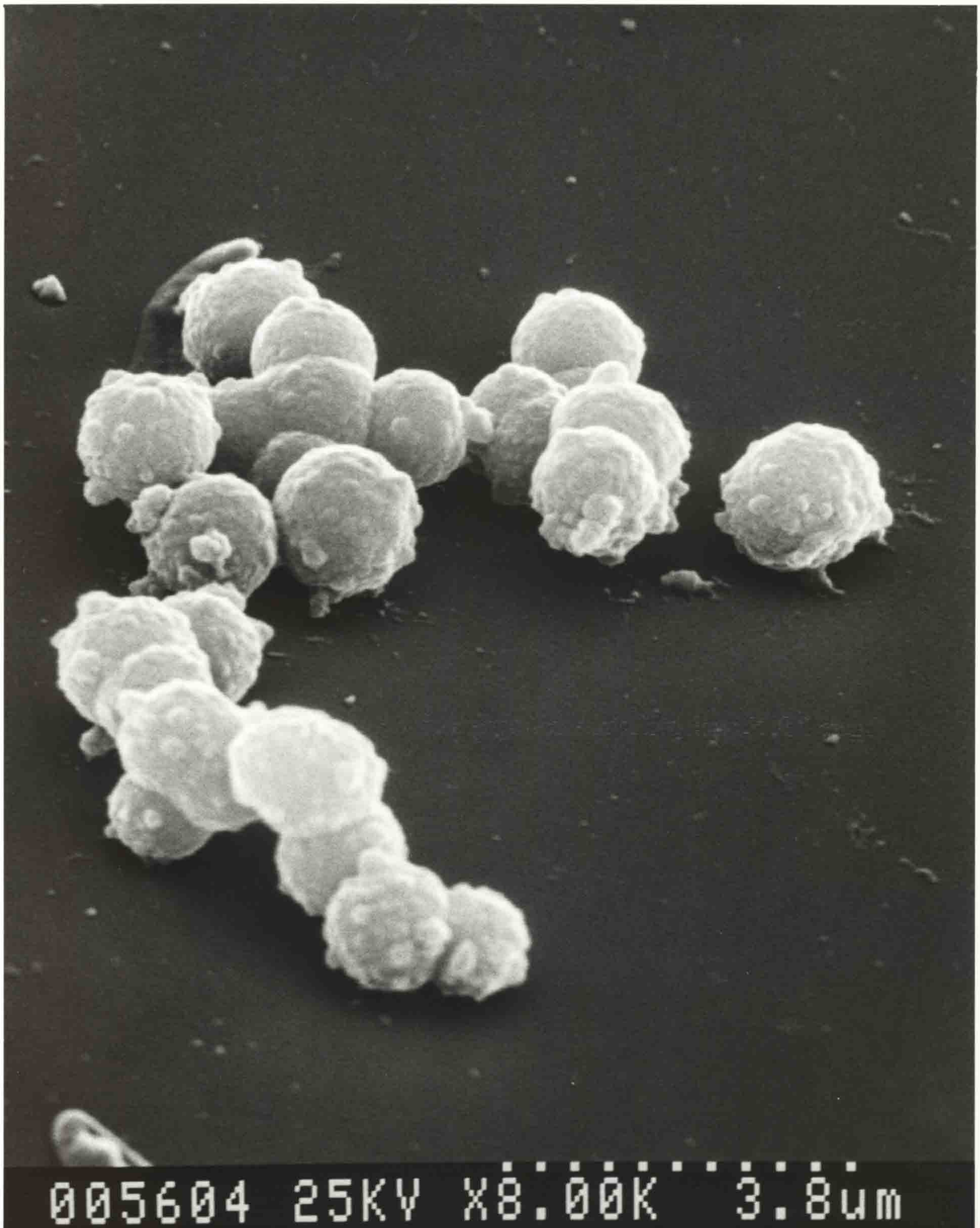

005605 25KV X4.00K 7.5um

Figure 3.9. Scanning electron micrograph of axenic amastigotes.

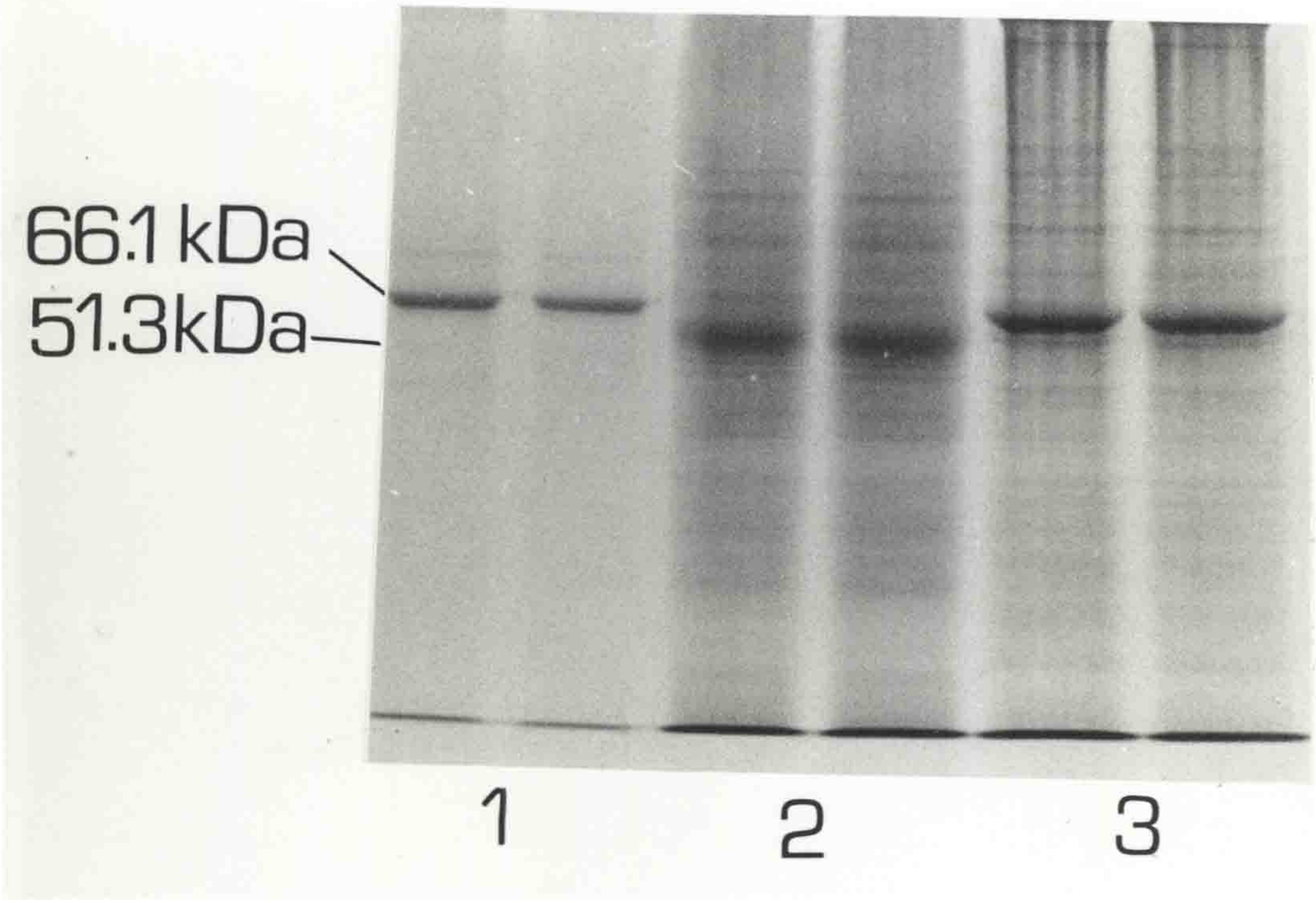

005604 25KV X8.00K 3.8um

In addition, the anticipated structure of *Leishmania* parasites was seen during examination. This include a bilayer cell membrane underlined by a palisade of uniformally spaced microtubules. The cytoplasm contained organelles such as ribosomes and small lipid droplets and a single mitochondrion. The cell membrane was invagenated to form the flagellar pocket and the kinetoplast, closely associated with the base of the flagellum, was generally oriented perpendicular to its axis. A relatively large nucleus was seen with the chromatin particles laying against the inner nuclear membrane.

3.3.3. BIOCHEMICAL CHARACTERIZATION

Promastigotes, axenic amastigotes and tissue amastigotes were analyzed by SDS-PAGE and gels were stained with Coomassie Blue as described in 3.2.3. A variety of protein bands were observed in all forms including one prominent band. Under non-reducing conditions, the molecular mass of this band was approximately 51.3 kDa in promastigotes and 66.1 kDa in axenic amastigotes and tissue amastigotes (figure 3.10). The same result was observed in samples reduced with 2% mercaptoethanol (not shown).

Figure 3.10. Coomassie Blue staining of SDS-PAGE of lysates of axenic amastigotes (1), promastigotes (2) and tissue amastigotes (3) using non-reducing sample buffer.


66.1 kDa
51.3kDa
1
2
3

3.4. DISCUSSION

Amastigote-like forms of *Leishmania donovani* were cultured axenically as described in chapter 2. Here, various criteria have been used to investigate whether these forms are different from promastigotes and, more importantly, are similar to true amastigotes.

The first obvious differences observed between axenic amastigotes and promastigotes were their morphologies and sizes. Morphologically, axenic amastigotes were very similar to tissue amastigotes in that they appeared as round to oval cells, having approximately the same size and lacking an extension of the flagellum beyond the cell body. This agrees with the findings of others who investigated axenic cultivation of amastigotes of other species of *Leishmania* (Eperon and McMahon-Pratt, 1989b; Bates *et al.*, 1992). There was a significant difference between the sizes of axenic amastigotes and tissue amastigotes. Several factors can contribute to this difference. The environment sorrounding the parasite in culture is very different from what would be expected in a macrophage's phagolysosome. Amastigotes in phagolysosomes experience a hostile environment. Another factor is that the multiplication rate for amastigotes in a phagolysosome is slower than that for amastigotes in axenic culture. Also, there was a significant difference between sizes of axenic or tissue amastigotes and that of promastigotes. Several studies reported that promastigotes round up in shape when incubated at elevated temperature (Darling and Blum, 1987; Shapira *et al.*, 1988) but, it is important to distinguish between heat-shocked promastigotes and true amastigotes. Unfortunately, this cannot be accomplished by looking at the morphology alone. Other criteria should be investigated.

Another way to distinguish amastigotes, axenic or tissue, from promastigotes is by studying their ultrastructure. This is a very useful tool, capable even of differentiating between some species of *Leishmania* (Gardener *et al.*, 1977). A very useful marker to differentiate between amastigotes and promastigotes is the presence or absence of a paraxial rod. This crystalline structure has been described in several members of the family Trypanosomatidae (Fuge, 1969; Farina *et al.*, 1986; Vickerman and Tetley, 1990; Bastin *et al.*, 1996). This study investigated the presence or absence of the paraxial rod in promastigotes and amastigotes. Promastigotes have been shown to possess a paraxial rod running parallel to the flagellar axoneme. Vickerman and Tetley (1990) reported that the paraxial rod is often not present until the flagellum has emerged from the flagellar pocket. In this study, it appeared that the paraxial rod can be found even in parts of the flagellum that are inside the flagellar pocket as well as in the part extending beyond the cell body. The paraxial rod was not seen in axenic or tissue amastigotes. This agrees with several reports on the ultrastructure of axenic amastigotes (Pan and Pan, 1986; Eperon and McMahon-Pratt, 1989b; Bates *et al.*, 1992). In addition, the flagellar pocket of axenic amastigotes was found to contain filamentous materials, which were not seen in promastigotes, which agrees with the work of Pimenta *et al.* (1991).

Biochemically, axenic amastigotes have been shown to be similar to tissue amastigotes and to differ from promastigotes in several aspects including proteinase activities (Galvao-Quintao *et al.*, 1990; Bates *et al.*, 1992; Robertson and Coombs, 1992), nucleases (Bates, 1993b) and several other enzymatic activities (Coombs *et al.*, 1982).

In this study it has been shown that axenic amastigotes are similar to tissue amastigotes and different from promastigotes in protein banding pattern as revealed by SDS-PAGE. A variety of bands were observed in all forms including one prominent band. The identity of these proteins has not been determined. However, they are likely to be major structural proteins. The molecular mass of this band was approximately 51.3 kDa in promastigotes and 66.1 kDa in axenic amastigotes and tissue amastigotes. The molecular weight of these bands is similar to that of β-tubulin (Fong and Chang, 1981). *L. mexicana* amastigotes have been shown to have a single β-tubulin mRNA while promastigotes have β-tubulin mRNA of three different sizes (Fong *et al.*, 1984).

3.5 CONCLUSION

Axenic amastigotes are similar to tissue amastigotes and different from promastigotes in their morphology, sizes, ultrastructure and protein banding pattern.

CHAPTER FOUR

4. METACYCLOGENESIS IN *LEISHMANIA*

4.1 INTRODUCTION

It has been proposed for some time, that promastigotes in the sandfly differentiate into mammal-infective forms, metacyclic promastigotes. These are adapted in some way to avoid destruction when entering the mammalian host, to bind and enter mononuclear phagocytes, and to transform into amastigotes and initiate the disease. Shortt, *et al.* (1931) and Adler and Ber (1941) provided the first evidence of transmission of leishmaniasis by the bite of sandflies. These investigators believed that there was one particular form of promastigote in the sandfly which was adapted for life in the vertebrate host. Subsequently other workers observed differences between dividing midgut forms and those found anteriorly in the sandfly.

Amastigotes taken by the sandfly during the acquisition of a blood meal transform into promastigotes. These forms of the parasite colonize the midgut or hindgut of the fly and attach to the gut by embedding their flagella between the microvilli of the gut (Chang *et al.*, 1985). As the colonization moves towards the anterior parts of the gut, promastigotes differentiate into metacyclic forms. The generation of these forms occurs as early as day 3 after obtaining the blood meal and optimum numbers of these metacyclic promastigotes can be recovered shortly after passage of the blood meal remnants (Sacks and Perkins, 1985) coinciding with the time for the second blood meal (Sacks, 1988). This is in agreement with the findings of Schlein and Jacobson

(1994) who demonstrated that the development of infective promastigotes was inhibited by haemoglobin.

Infective metacyclic promastigotes were found to lose their ability to attach to the midgut epithelial cells (Pimenta *et al.*, 1992). This enhances their migration to the anterior parts in order to gain entry to the vertebrate host when the sandfly obtains another blood meal.

Metacyclic promastigotes are small in size. Their body length is equal to or less than 8.4 μm, the width is approximately 1.5μm or less and they possess a discernable surface membrane coat seen by transmission electron microscopy (Bates and Tetley, 1993). They are more resistant to complement mediated lysis than procyclic promastigotes as well as more infective *in vitro* and *in vivo* (Howard *et al.*, 1987). Biochemically, there are several differences between procyclic and metacyclic promastigotes. Rizvi *et al.* (1985) demonstrated that infective promastigotes expressed surface antigenic determinants different from non-infective ones. The same result was also reported by Sacks and Da Silva (1987) and Kweider *et al.* (1989). The isoenzyme content of metacyclic promastigotes was also found to differ from that of procyclic promastigotes and to be more similar to that of amastigotes (Mallinson and Coombs, 1989). Also, metacyclic promastigotes expressed distinctive cysteine proteinase activities when analyzed by gelatin polyacrylamide gel electrophoresis (Bates *et al.*, 1994).

Several workers have reported the generation of metacyclic promastigotes *in vitro* and many of them characterized these forms (reviewed by Sacks, 1989 and Bates, 1994b). Almost all of this work involving metacyclogenesis reported on the abundance of metacyclic promastigotes in stationary-phase cultures, but did not describe methods

for their production. Sacks and Perkins (1984) reported that promastigotes taken from stationary-phase of growth are more infective *in vitro* than those taken from exponential-phase cultures. The same developmental changes were observed during growth of promastigotes in the sandfly.

Another criterion by which metacyclic promastigotes can be identified is the loss of their ability to bind certain lectins and their relative resistance to complement mediated lysis (Franke *et al.*, 1985; Bandyopadhyay *et al.*, 1991; Almeida *et al.*, 1993) which is thought to be due to the release of C5b-9 (Puentes *et al.*, 1990). The loss of their ability to bind peanut lectin was used by many investigators as a marker for metacyclic promastigotes (Wilson and Pearson, 1984; Franke *et al.*, 1985; Rizvi *et al.*, 1985; Howard *et al.*, 1987; Almeida *et al.*, 1993). With respect to exposure of promastigotes to the complement system of the mammalian host, it appears that promastigotes not only avoid destruction by complement, in addition, they use the complement components to gain entry into the macrophage (Joiner, 1988). Incubation of promastigotes with fresh serum resulted in an increase of their uptake by macrophages (Mosser and Edelson, 1984). Metacyclic promastigotes activate complement and bind C3b. This molecule is then recognized by CR1 receptors on macrophages and used in binding the parasite to the macrophage (Da Silva *et al.*, 1989).

The first well defined method for culturing a pure population of metacyclic forms was described by Bates & Tetley (1993). Here, freshly transformed exponential-phase *L.mexicana* promastigotes were passaged in an acidic (pH 5.5) Schneider's *Drosophila* medium supplemented with 20 % foetal calf serum. This resulted in an homogeneous stationary-phase population of metacyclic promastigotes, which were shown to be

resistant to complement-mediated lysis, possessed a discernable surface membrane coat, and were highly infective to macrophages *in vitro*.

This study investigated the generation of metacyclic promastigotes in *Leishmania donovani, L. mexicana, L. major* and *L. braziliensis* and these forms were characterized on basis of their morphology, sizes and resistance to complement-mediated lysis.

4.2 MATERIALS AND METHODS

4.2.1 THE PARASITE

Four species of *Leishmania* were studied for their ability to undergo metacyclogenesis. These were *L. donovani* (MHOM/ET/67/HU3;LV9), *L. mexicana* (MNYC/BZ/62/M379), *L. major* clone FV-1 (MHOM/IL/80/Friedlin) and *L. braziliensis* (MHOM/BR/84/LTB300). Tissue amastigotes were obtained from hamsters, for *L. donovani* and *L. braziliensis*, or from BALB/c mice, for *L. mexicana* and *L. major*, as described in 2.2.1. Amastigotes were transformed to promastigotes in HO-MEM medium supplemented with 20 % FCS and 25 µg gentamicin sulphate/ml, pH 7.2 at 26° C. Schneider's *Drosophila* medium supplemented with 20% (v/v) heat-inactivated FCS and 25µg gentamicin sulphate/ml was used to culture metacyclics at 26°C. The pH of the medium was adjusted to 7.2 or 5.5 using 1*M* HCl or 1*M* NaOH as required and 10 ml volumes were cultured in 25-cm^2 tissue culture flasks using air as the gas phase.

4.2.2 THE DETERMINATION OF THE OPTIMUM pH FOR METACYCLOGENESIS

Freshly transformed promastigotes of *L. donovani* were cultured in Schneider's *Drosophila* medium supplemented with 20% (v/v) heat-inactivated FCS and 25µg gentamicin sulphate/ml at different pH values ranging from 5.0 to 7.5. Thin smears were prepared from cultures on days 2, 5, and 8. Slides were air dried, fixed in absolute methanol and stained in 10 % Giemsa's stain for 10 minutes. Slides were examined under the microscope at a magnification of 1000x and the percentage of metacyclic promastigotes, judged by morphology and size, was estimated.

Measurements were made using an Olympus microscope fitted with a drawing tube. A ruler and a test slide were used to calibrate the microscope so that each 2μm on the test slide was equal to 1mm on the ruler. Cell body length (L) and width (W) were measured for 100 promastigotes in each slide. The value of L x W was calculated for each and any promastigote having a value of ≤ 12 was counted as a metacyclic promastigote.

Prior to any microscopic examination, all slides were coded so that all size measurements and enumeration was done blind.

4.2.3 EXAMINATION OF THE ABILITY OF PROMASTIGOTES TO UNDERGO METACYCLOGENESIS IN DIFFERENT CULTURE MEDIA WITH DIFFERENT FCS CONCENTRATIONS

Freshly transformed *L. donovani* promastigotes were cultured in HO-MEM medium or Schneider's *Drosophila* medium, supplemented with 10 % or 20 % FCS at pH 7.0 or 5.5. Thin smears of stationary-phase promastigotes were prepared, air dried, fixed in absolute methanol and stained in 10 % Giemsa's stain for 10 minutes. Slides were examined under the microscope at a magnification of 1000x and the percentage of metacyclic promastigotes, judged by morphology and size, was estimated.

4.2.4 EFFECT OF CULTURE VENTILATION ON THE pH OF THE MEDIUM

L.donovani promastigotes were used to initiate cultures in HO-MEM medium, Schneider's *Drosophila* medium and M199 (Kaneshiro, *et al.*, 1982) all media were supplemented with 20% (v/v) heat-inactivated FCS. The pH was adjusted to 7.2 or 5.5 using 1m*M* HCl or 1m*M* NaOH as required. Two sets of culture flasks were prepared.

In the first, ordinary 25-cm^2 culture flasks were used and these were opened daily for sampling for density and pH estimation. In the second set, vented 25-cm^2 flasks were used and the lid was covered by two layers of parafilm to prevent air exchange. One ml syringes and 21G needles were used to obtain samples daily for density and pH estimation. Cell counting was performed using an Improved Naubauer haemocytometer under phase-contrast microscopy. The pH was measured using a pH paper (range 4.0 to 10.0).

4.2.5 EFFECT OF LONG TERM CULTURE ON METACYCLOGENESIS

L. donovani promastigotes were maintained and subpassaged regularly in HO-MEM medium supplemented with 10 % FCS. Promastigotes from the second and the tenth subpassage were cultured in Schneider's *Drosophila* medium supplemented with 20 % FCS at pH 7.0 and 5.5. Thin smears of stationary-phase promastigotes were prepared, air dried, fixed in absolute methanol and stained in 10 % Giemsa's stain for 10 minutes. Slides were examined under the microscope at a magnification of 1000x and the percentage of metacyclic promastigotes, judged by morphology and size, was estimated.

4.2.6 COMPLEMENT MEDIATED LYSIS

Freshly transformed promastigotes of *L. donovani, L. mexicana, L. major* and *L. braziliensis* were cultured in neutral (pH 7.0) and acidic (pH 5.5) Schneider's *Drosophila* medium supplemented with 20 % FCS. Exponential-phase and stationary-phase promastigotes were washed in Hanks' balanced salt solution (HBSS) twice and resuspended to a density of 10^7 cells/ml in HO-MEM supplemented with 10% (v/v)

heat-inactivated FCS at a pH of 7.2. Serial dilutions of guinea-pig serum (source of complement) were made using the same medium in 96 well flat-bottom plates. Doubling dilutions were made from 1:1 to 1:128 with a total volume of 100µl. Promastigotes were added (100µl), the plate was covered with parafilm and incubated at 26°C for one hour. At the end of the incubation period, 40µl of 50 m*M* EDTA was added, giving a final concentration 8.3 m*M* EDTA, to stop complement-mediated lysis. Examination was performed by pipetting 5µl on a glass slide and covering it with a 22 X 22 mm cover slip. By this, promastigotes were trapped between the slide and the cover slip and movements of viable promastigotes were easily observed. Viability was estimated for each serum dilution by counting 100 promastigotes and estimating the percentage of viable ones judged by movement of their flagella. The concentration of serum that lysed 50 % of promastigotes was used to compare resistance to complement-mediated lysis.

4.2.7 EFFECT OF CONSTANT EXPOSURE OF PROMASTIGOTES TO CHANGES IN pH ON THEIR ABILITY TO UNDERGO METACYCLOGENESIS

Promastigotes of *L. donovani, L. mexicana, L. major* and *L. braziliensis* were regularly maintained in HO-MEM medium pH 7.2 supplemented with 10 % FCS. Two sets of cultures were maintained. In one set, vented culture flasks were used to allow gas exchange and ordinary, closed flasks were used in the other to prevent gas exchange other than at subpassage. Culture ventilation was shown to minimize pH changes in the medium during the culture period (See 4.2.4 and 4.3.3). Promastigotes from the fifth and tenth subpassages (SP5 and SP10) grown using both types of ventilation were subjected to complement mediated lysis as described in 4.2.6.

4.3 RESULTS

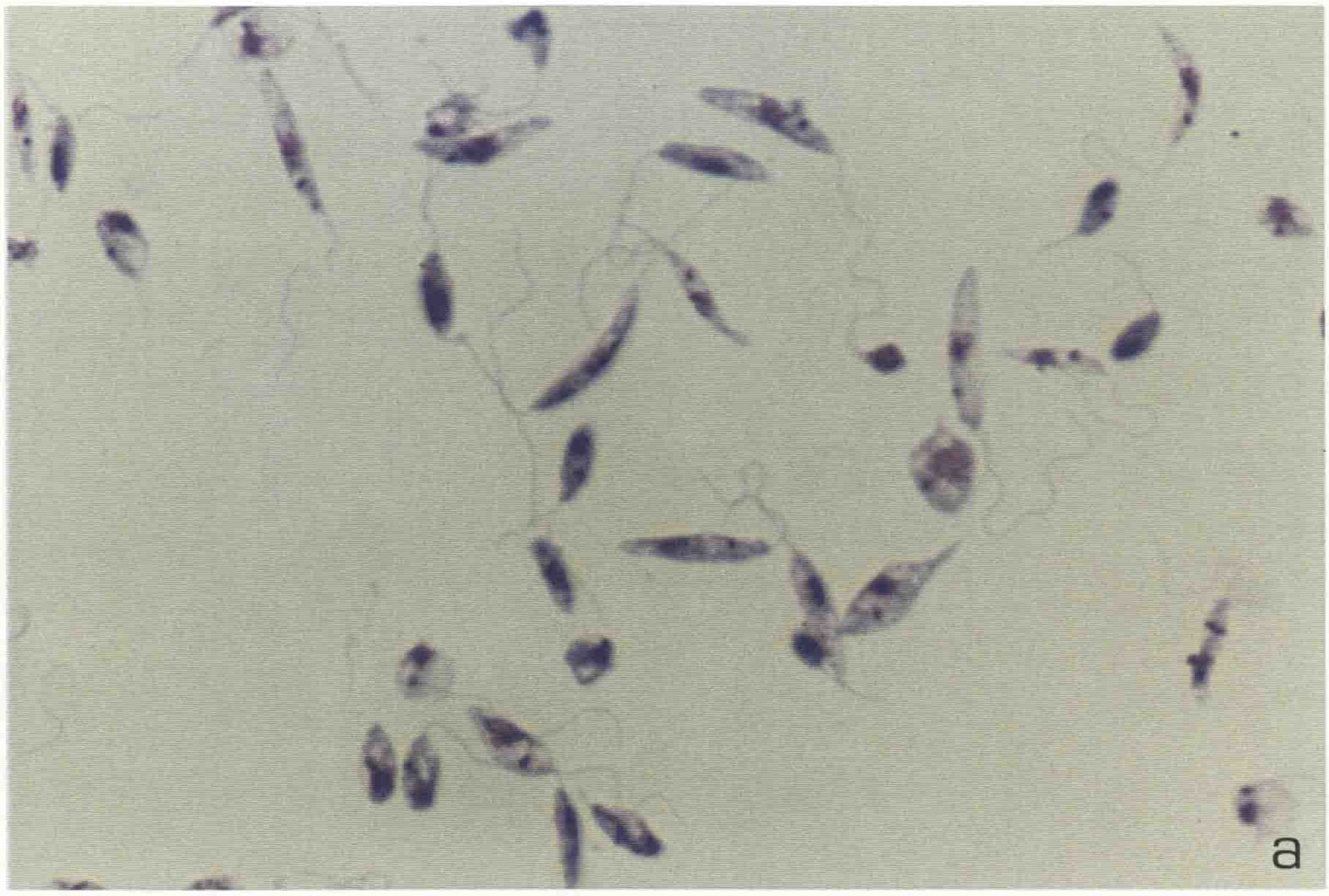
For each experiment involving measurement of sizes of promastigotes, the length and width of 100 promastigotes was measured as described in 4.2.2. The width (W) and length (L) were multiplied and any promastigote with a value of W X L equal to or less than 12 was considered as a metacyclic promastigote. This method was compared with visual identification of metacyclic promastigotes, which were defined as having a short, narrow body and a long flagellum at least twice as long as the cell body. Using a correlation test to compare the two methods. there was a good correlation between the two methods and the correlation coefficient was 0.996. A typical culture is shown in Figure 4.1 in which procyclic promastigotes (a), showing hetrogeniety in morphology and sizes, are compared to metacyclic promastigotes (b) with a short body length and a long flagellum.

4.3.1 THE DETERMINATION THE OPTIMUM pH FOR METACYCLOGENESIS

Promastigotes of *L. donovani* were cultured at different pH values and slides were prepared during exponential-phase, early stationary-phase and late stationary-phase as described in 4.2.2. The percentage of metacyclic promastigotes generated are shown in Figure 4.2. Acidic pHs of 5.5 and 5.0 were found to be optimal for metacyclogenesis. This was judged by the high number of metacyclic promastigotes in stationary-phase. Metacyclic promastigotes appeared as early as day 2 at these pHs.

Figure 4.1. *L. donovani* procyclic promastigotes cultured at neutral pH (a). *L. donovani* metacyclic promastigotes cultured at acidic pH (b).

Bar = 5 μm.


a

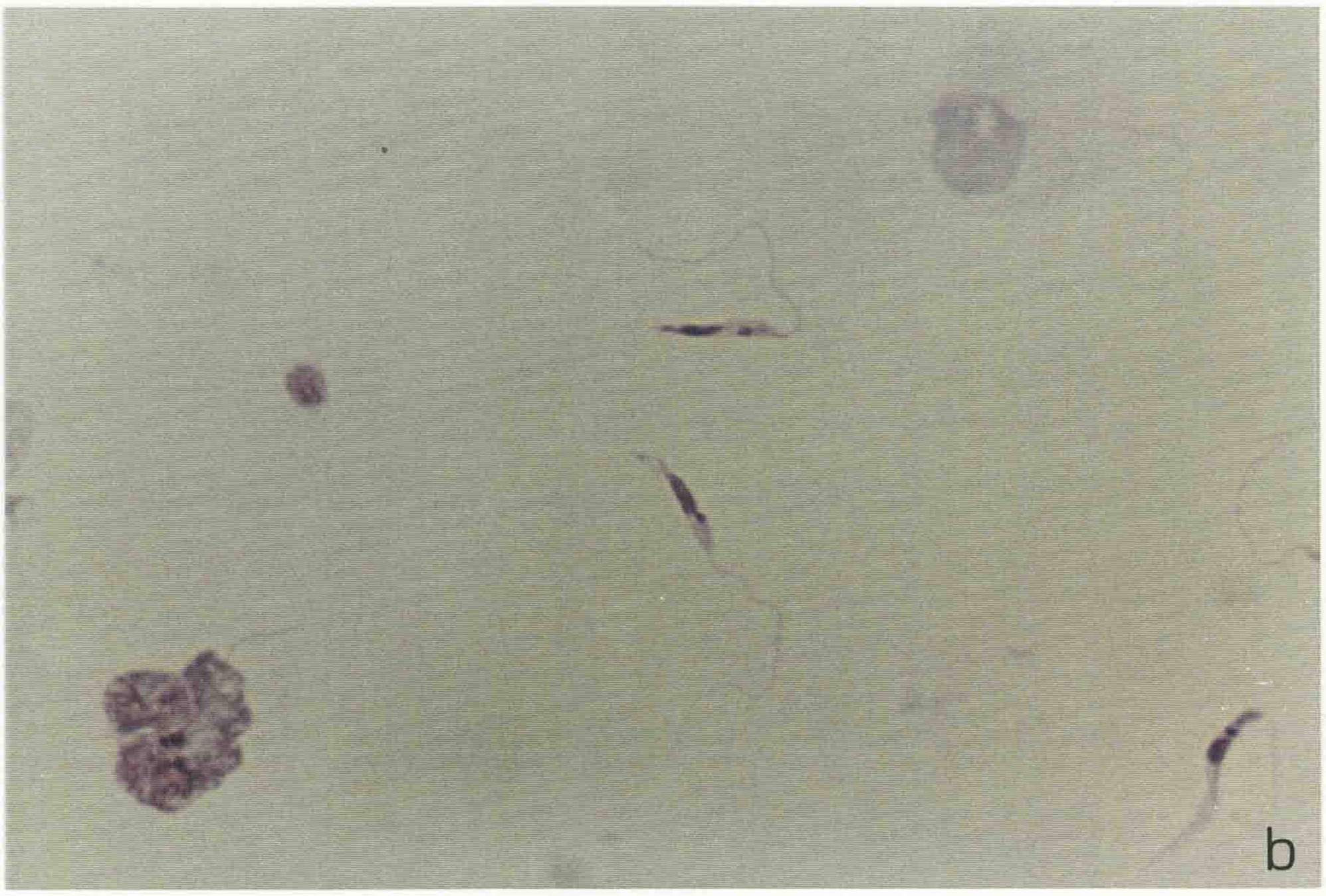
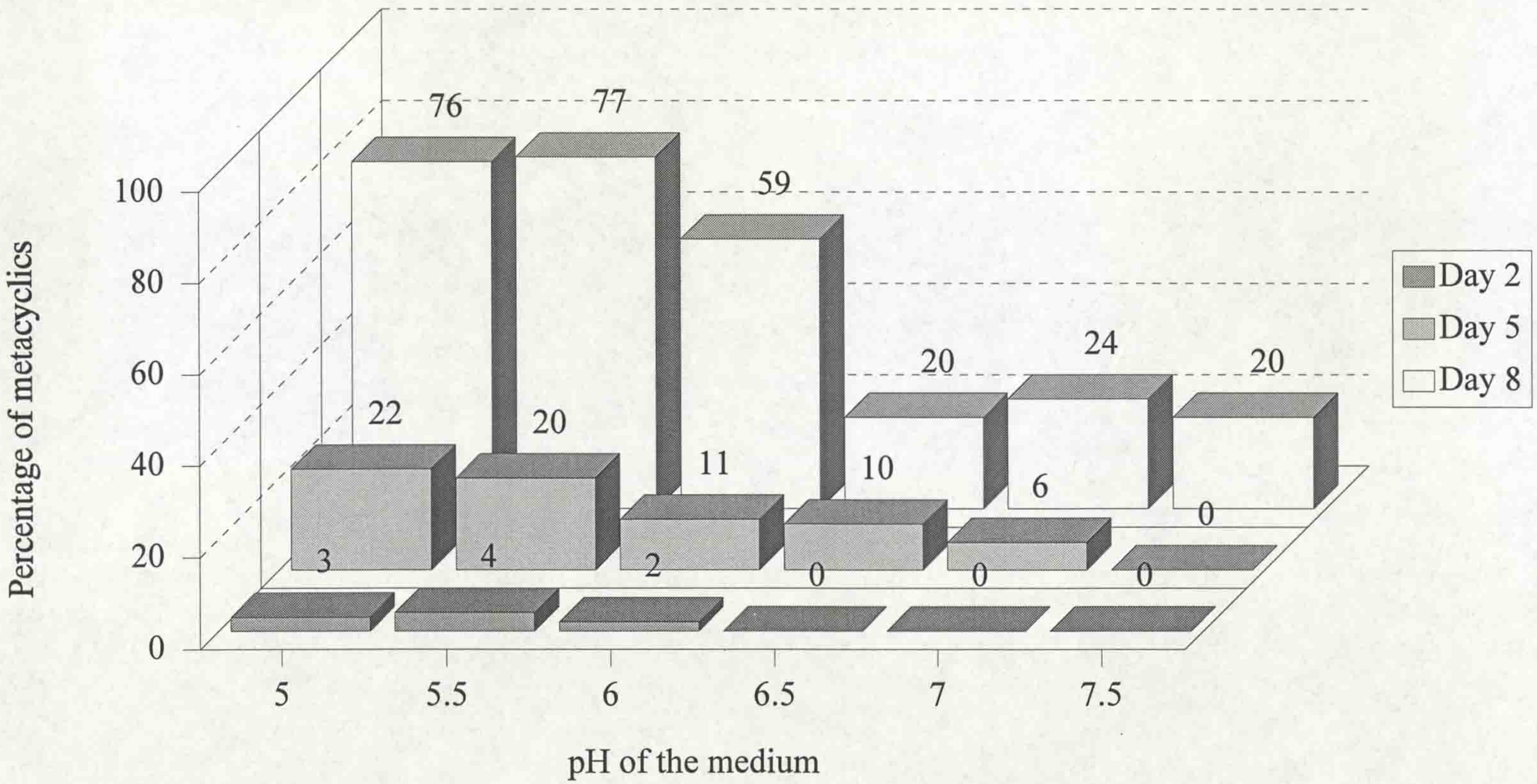

b

Figure 4.2. The percentage of *L. donovani* metacyclic promastigotes obtained in days 2, 5 and 8 from cultures at different pHs.


Percentage of metacyclics
100
80
60
40
20
0
5
5.5
6
6.5
7
7.5
pH of the medium
Day 2
Day 5
Day 8
22
20
11
10
20
24
20
3
4
2
0
0
0
76
77
59
6
0

However, metacyclic promastigotes only appeared on day 5 in cultures of neutral pH (7.0). The size distributions of exponential and stationary-phase promastigotes cultured in pH 7.0 and 5.5 are shown in Figure 4.3 in which it can be seen that cell sizes defined as metacyclics (i.e. W x L ≤12) were predominently seen in day 8 cultures at pH 5.5. Promastigotes cultured at neutral pH reached a higher cell density at stationary-phase. However, both neutral and acidic cultures reached stationary-phase at the same time.

4.3.2 METACYCLOGENESIS IN DIFFERENT CULTURE MEDIA WITH DIFFERENT FCS CONCENTRATIONS

L. donovani promastigotes were cultured in HO-MEM medium and Schneider's *Drosophila* medium both supplemented with 10 % or 20 % FCS at pH 7.0 and 5.5. Thin smears of stationary-phase promastigotes were prepared and examined as described in 4.2.3. In the two different culture media studied, the highest percentage of metacyclic promastigotes was obtained from stationary-phase cultures in Schneider's *Drosophila* medium supplemented with 20 % FCS. Results are shown in Figure 4.4.

4.3.3 EFFECT OF CULTURE VENTILATION ON THE pH OF THE MEDIUM

Promastigotes of *L. donovani* were cultured in different culture media and cell density and the pH of the culture medium were estimated daily as described in 4.2.4. The pH of all three culture media originally started at 7.2 decreased as cultures progressed towards stationary phase while that of cultures originally started at 5.5 increased. Among the three media used, Schneider's *Drosophila* medium appeared to maintain minimum changes in pH during the culture period. Results are shown in Figure 4.5.

Figure 4.3. The size distributions of exponential-phase (day 2) and stationary-phase (day 8) *L. donovani* promastigotes cultured in pH 7.0 and 5.5.

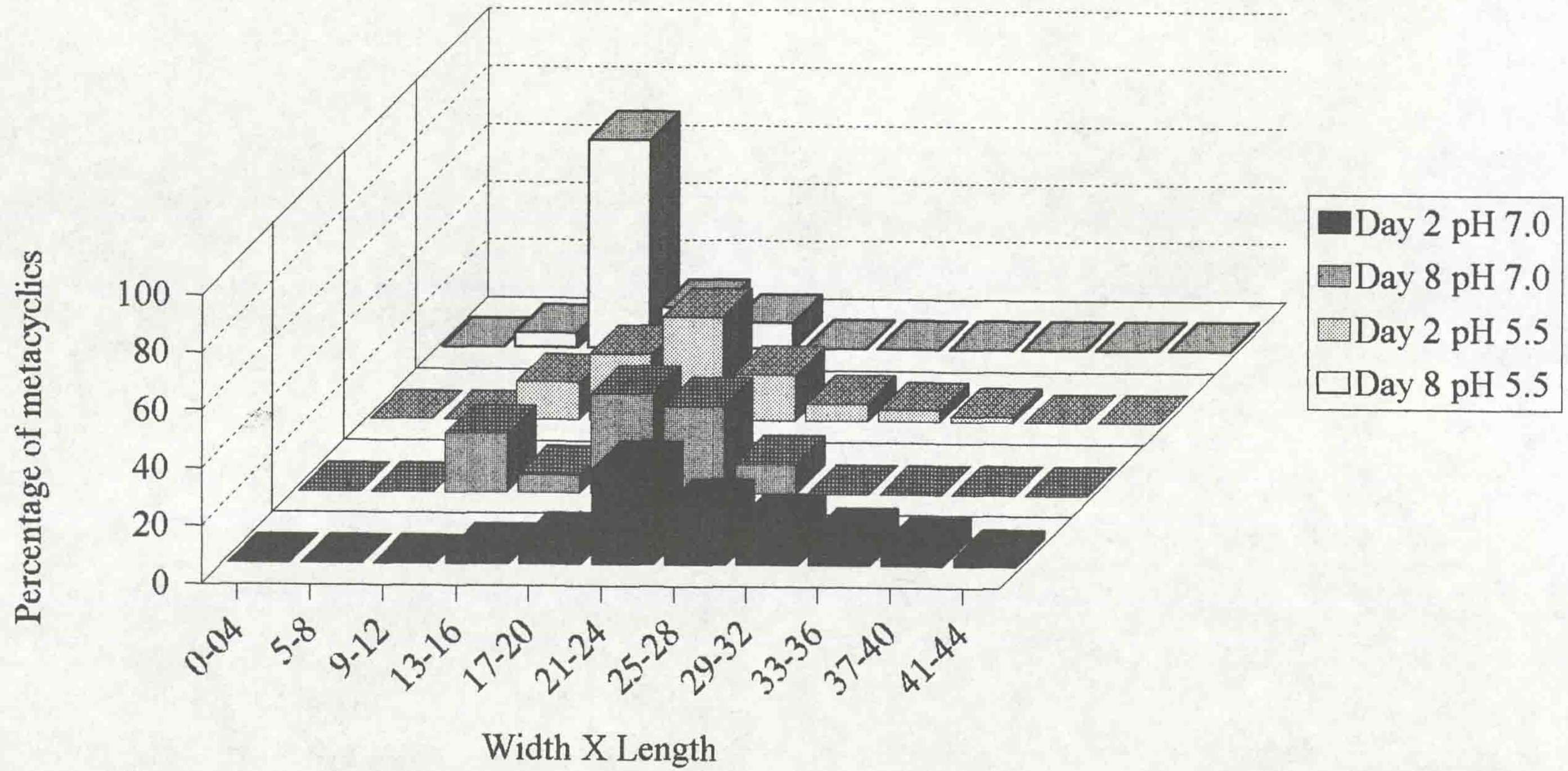

Percentage of metacyclics
100
80
60
40
20
0
0-04
5-8
9-12
13-16
17-20
21-24
25-28
29-32
33-36
37-40
41-44
Width X Length
Day 2 pH 7.0
Day 8 pH 7.0
Day 2 pH 5.5
Day 8 pH 5.5

Figure 4.4. The percentage of *L. donovani* metacyclic promastigotes obtained from HO-MEM and Schneider's *Drosophila* culture media containing different FCS concentrations.

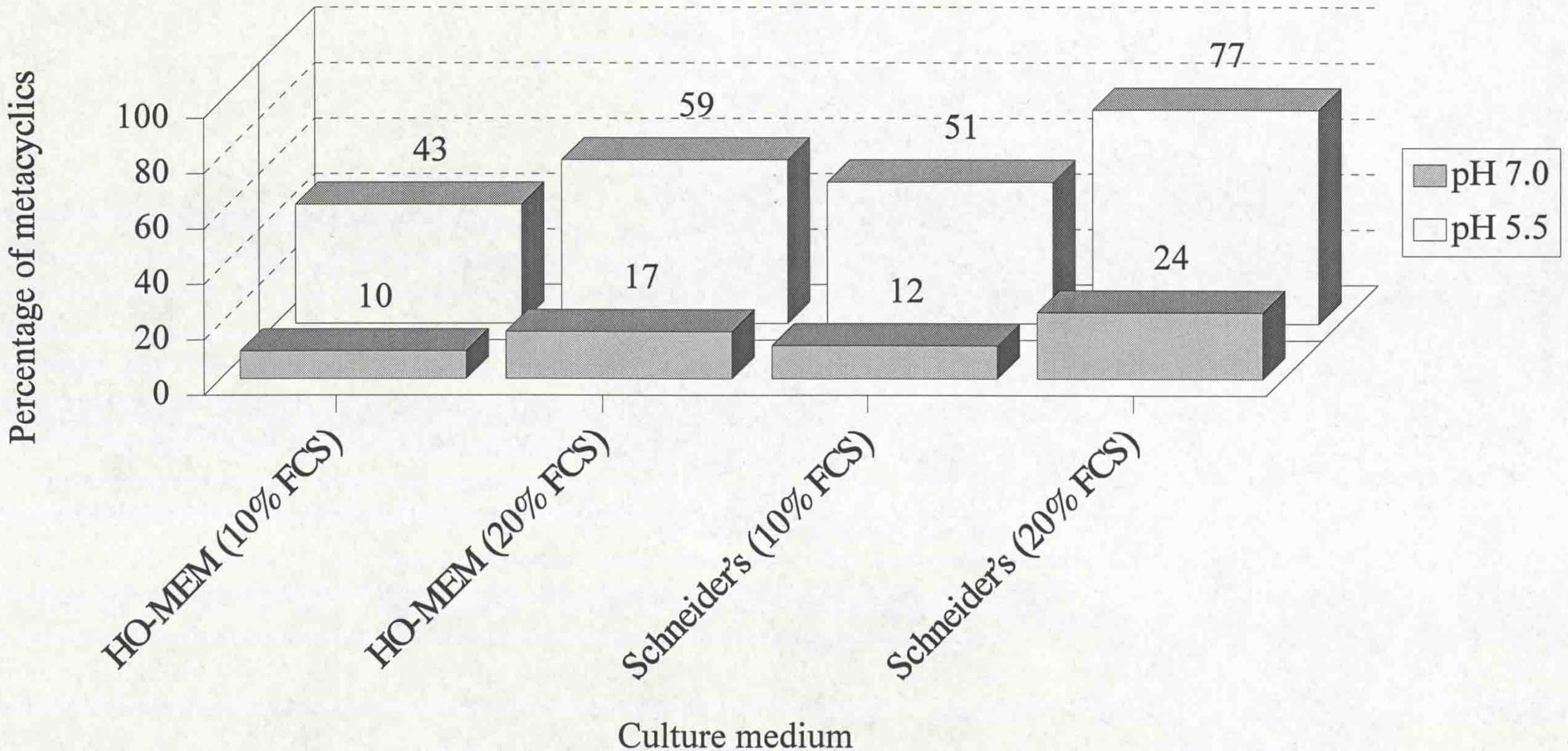
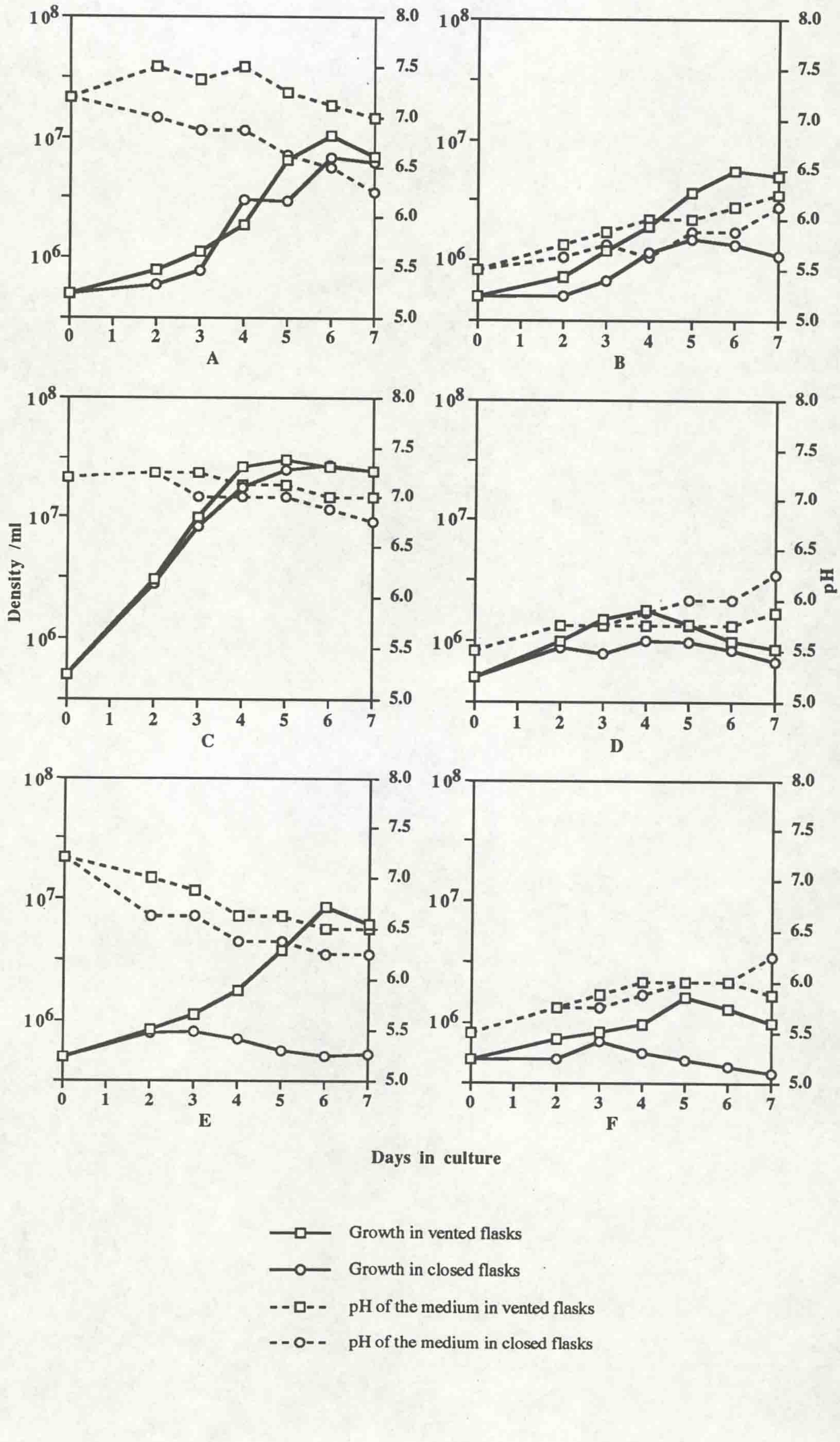

Percentage of metacyclics
100
80
60
40
20
0
43
10
59
17
51
12
77
24
pH 7.0
pH 5.5
HO-MEM (10% FCS)
HO-MEM (20% FCS)
Schneider's (10% FCS)
Schneider's (20% FCS)
Culture medium

Figure 4.5. Growth of *L. donovani* promastigotes in different culture media and the change in the pH of the culture media during growth. (A & B) HO-MEM medium, (C & D) Schneider's *Drosophila* medium, (E & F) M 199. (A, C & E) cultures started at pH 7.2. (B, D & F) cultures started at pH 5.5.


Density /ml
pH
Days in culture
A
B
C
D
E
F
Growth in vented flasks
Growth in closed flasks
pH of the medium in vented flasks
pH of the medium in closed flasks

4.3.4 EFFECT OF LONG TERM CULTURE ON METACYCLOGENESIS

Promastigotes of *L. donovani* from the second and the tenth subpassages were cultured in Schneider's *Drosophila* medium at pH 7.0 and 5.5. Thin smears of stationary-phase promastigotes were prepared and examined as described in 4.2.5. Freshly transformed promastigotes were more capable of undergoing metacyclogenesis than long term cultured ones. Results are shown in Figure 4.6.

4.3.5 RESISTANCE OF METACYCLIC PROMASTIGOTES TO COMPLEMENT MEDIATED LYSIS

Promastigotes were subjected to complement mediated lysis as described in 4.2.6. Stationary-phase promastigotes cultured in acidic pH showed a higher resistance to complement mediated lysis and a higher percentage of metacyclic forms than those grown in neutral pH. This was seen in all four species tested. The results are shown in Table 4.1. Different species showed different relative resistance to complement-mediated lysis. To enable comparison between species, the concentration of serum required to cause 50% lysis in acidic and neutral cultures was expressed as ratio (Table 4.2).

4.3.6 EFFECT OF CONSTANT EXPOSURE TO CHANGES IN THE pH ON METACYCLOGENESIS

Promastigotes of SP5 and SP10, cultured in vented and closed flasks, were passaged into neutral and acidic media and tested for complement mediated lysis as described in 4.2.7. Cultures maintained in vented flasks had more metacyclic promastigotes in the stationary-phase than those grown in closed flasks. Results are shown in table 4.3.

Figure 4.6. Effect of long term culture on metacyclogenesis. The percentage of *L. donovani* metacyclic promastigotes obtained from neutral and acidic cultures of subpassage 2 and subpassage 10.

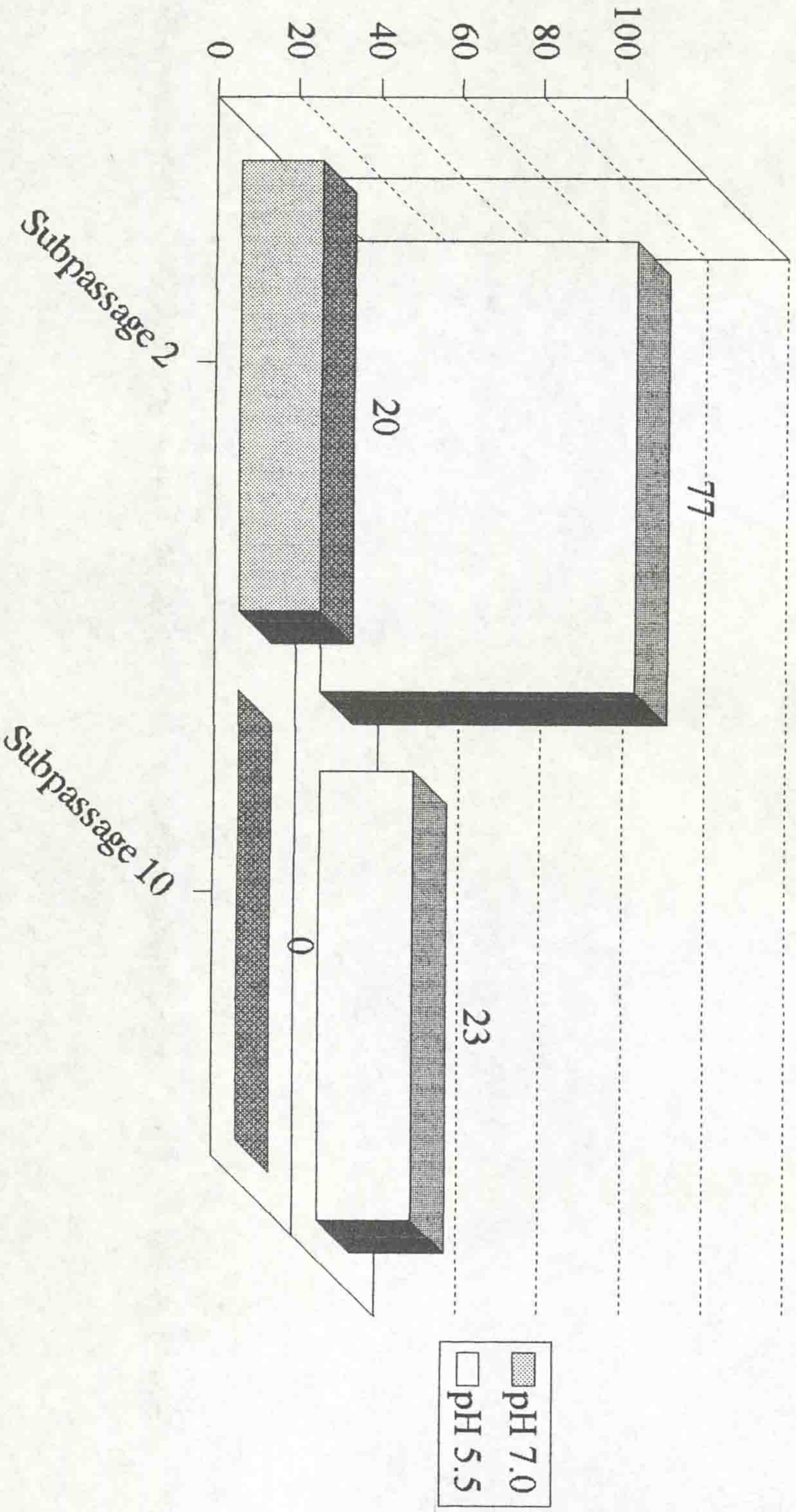

Percentage of metacyclics
0
20
40
60
80
100
Subpassage 2
20
77
Subpassage 10
0
23
pH 7.0
pH 5.5

Table 4.1. Resistance of promastigotes grown at different pH to complement mediated lysis.

Species	pH of culture	Phase	Serum concentration causing 50% lysis (%)	Percentage of metacyclic promastigotes (%)
Leishmania donovani	7.2	Exponential	25	3
	5.5		25	10
	7.2	Stationary	**6.25**	11
	5.5		**25**	77
Leishmania mexicana	7.2	Exponential	3.125	2
	5.5		6.25	16
	7.2	Stationary	**1.56**	29
	5.5		**12.5**	78
Leishmania major	7.2	Exponential	3.125	0
	5.5		3.125	9
	7.2	Stationary	**3.125**	10
	5.5		**50**	75
Leishmania braziliensis	7.2	Exponential	0.78	1
	5.5		1.56	4
	7.2	Stationary	**0.78**	12
	5.5		**3.125**	49

Table 4.2. Increase in resistance of promastigotes grown at acidic pH to complement mediated lysis

Species	Increase in resistance
L. donovani	4 - fold
L. mexicana	8 - fold
L. major	16 - fold
L. braziliensis	4 - fold

The ratio of the serum concentration required for 50% lysis of stationary-phase promastigotes in acidic and neutral cultures used as a measure of increased resistance to complement-mediated lysis. Data used for these calculations are shown in bold in Table 4.1.

Table 4.3. Effect of culture ventilation on resistance to complement mediated lysis and metacyclogenesis of promastigotes after long term culture.

Species	Subpass-age	pH	Vented		Closed	
			Serum conc. required to cause 50% lysis (%)	Percent metacyc-lic (%)	Serum conc. required to cause 50% lysis (%)	Percent metacyc-lic (%)
L.donovani	SP5	7.2	12.5	13	6.25	0
		5.5	25	25	12.5	3
	SP10	7.2	1.56	0	>0.4	0
		5.5	0.78	9	>0.4	0
L.mexicana	SP5	7.2	3.125	22	1.56	7
		5.5	6.25	45	3.125	23
	SP10	7.2	6.25	0	6.125	0
		5.5	6.25	0	1.56	0
L. major	SP5	7.2	3.125	19	3.125	5
		5.5	6.25	51	1.56	15
	SP10	7.2	0.78	0	0.78	0
		5.5	0.78	7	0.78	0
L.braziliensis	SP5	7.2	3.125	0	1.56	0
		5.5	6.25	0	0.78	0

4.4 DISCUSSION

The life cycle of *Leishmania* parasites is composed of two main parts. One part occurs in the vertebrate host where amastigotes parasitize macrophages and the other part occurs in the sandfly. Shortt *et al.* (1931) and Adler and Ber (1941) performed remarkable work in providing evidence that sandflies transmit *Leishmania.* They believed that promastigotes in the sandfly differentiate to produce a form of promastigote infective to mammals, later named metacyclic promastigotes. Subsequently, morphological differences between dividing mid-gut promastigotes and those found anteriorly were observed (reviewed by Killick-Kendrick, 1979).

In *in vitro* cultures, it is confirmed that some promastigotes differentiate into metacyclic promastigotes, infective promastigotes, as the growth of promastigotes reaches stationary-phase (Sacks and Perkins, 1984, 1985; Franke *et al.*, 1985; Rizvi *et al.*, 1985; Howard *et al.*, 1987; Da Silva and Sacks, 1987; Wozencraft and Blackwell, 1987; Bandyopadhyay *et al.*, 1991; Almeida *et al.*, 1993). Such promastigotes were smaller in size, highly active, resistant to complement mediated lysis and highly infective *in vitro* and *in vivo.*

Bates and Tetley (1993) revealed a method to induce metacyclogenesis and produce more metacyclic promastigotes of *L. mexicana* in stationary-phase populations. The method consisted of culturing promastigotes in an acidic culture medium at 26° C. Their use of acidic pH was because amastigotes of *L. mexicana* require an acidic pH of 5.5 for optimal growth in axenic cultures.

This seems to agree with other workers who reported on the production of metacyclic promastigotes in stationary-phase populations. Although most workers used a neutral

culture medium to grow promastigotes, the pH of the medium is likely to have reduced, as parasites multiply, to an acidic pH, thus, triggering metacyclogenesis.

In the sandfly, preliminary data suggest that the anterior part of the sandfly gut is more acidic than the posterior parts (Bates, pers. comm.).

In this study, metacyclogenesis was induced in different *Leishmania* species, producing different forms of the disease, by growing promastigotes in an acidic pH of 5.5. The decision to use Schneider's *Drosophila* medium was made after preliminary experiments showed that it maintained minimum changes in pH during the culture period (see 4.3.3). Foetal calf serum concentration was also examined and more metacyclic promastigotes were produced using 20 % FCS in the culture medium.

Metacyclic promastigotes produced were small in size measuring approximately 8µm or less in length and 1.5µm or less in width, they possessed a long flagellum which was twice or more the length of the body, highly active and more resistant to complement mediated lysis.

Although the percentage of metacyclic promastigotes produced varied between different species, consistency in producing similar percentages of metacyclic promastigotes can still be achieved. Different *Leishmania* species differ in several aspects other than metacyclogenesis such as their growth *in vitro*, the speed by which they produce the disease, the type of disease produced and their selectivity for the sandfly species.

Stationary-phase populations of promastigotes grown in neutral cultures contained metacyclic promastigotes, but, the percentage of metacyclic promastigotes in stationary-phase populations of acidic cultures was much higher. Metacyclic promastigotes appeared as early as day 2 in acidic cultures and on day 5 in neutral

cultures. During the exponential-phase of growth (day 2) lengths of promastigotes ranged from 9 to 22μm in neutral cultures and 7 to 18μm in acidic cultures. During stationary-phase of growth (day 8) there were 2 populations of promastigotes in neutral cultures, one with lengths of around 8μm and the other with lengths of around 15μm. In acidic cultures, there was a shift to the left in the sizes of promastigotes with a peak of about 8μm (fig. 4.2). This clearly indicate that metacyclogenesis does occur in neutral cultures but, when incubated at an acidic pH, more promastigotes undergo metacyclogenesis suggesting that acidity induces metacyclogenesis. This agrees with the hypothesis that metacyclic promastigotes are forms of promastigotes preadapted to life in the vertebrate host since they will be exposed to an acidic environment in the phagolysosomal vacuoles in macrophages (Antoine *et al.*, 1990).

The ability of promastigotes to undergo metacyclogenesis *in vitro* is negatively correlated with the age of promastigotes in cultures. That is, as promastigotes become adapted to *in vitro* cultivation they lose their ability to undergo metacyclogenesis and become less infective (Nolan and Herman, 1985; Segovia *et al.*, 1992). Freshly transformed promastigotes are usually considered optimal for metacyclogenesis (Bates and Tetley, 1993). More interestingly, the method of subculturing can affect the ability of promastigotes to undergo metacyclogenesis. *L. donovani* promastigotes that were subcultured during exponential-phase of growth were more able to initiate infections in hamsters than promastigotes subcultured after reaching stationary-phase of growth (Giannini, 1974). When growing promastigotes *in vitro*, the pH of the culture medium is usually reduced as cultures progress towards stationary-phase. Several factors can induce such a drop in pH including CO_2 (Davis, 1994). In this study, it has been found that promastigotes cultured in a constant neutral pH retained their ability to

differentiate into metacyclic promastigotes when transferred to acidic medium. The pH of the culture was maintained by allowing constant gas exchange at all times. Promastigotes cultured without gas exchange, where the pH of the medium was reduced to approximately 6, were less able to produce metacyclic promastigotes when transferred to an acidic environment. However, lower numbers of metacyclic promastigotes are produced as promastigotes becomes more adapted to *in vitro* cultures. This indicates that promastigotes exposed to acidic pH, for a longer period of time, become more adapted to such pH conditions and this may account for the loss of their ability to undergo metacyclogenesis.

One of the defence components of the mammalian host against *Leishmania* parasites is the complement system. Therefore, for a promastigote to be able to establish an infection, it has to be resistant to complement mediated lysis. Surprisingly, metacyclic promastigotes are not only resistant to complement mediated lysis, but in addition they use the complement components to gain entry into the macrophage (Alexander and Russell, 1992). Promastigotes of different *Leishmania* species have been shown to activate complement, either by the classical (Puentas *et al.*, 1988) or the alternative (Russell, 1987; Puentas *et al.*, 1990) pathway accumulating C3b on their surface. This is then recognized by the CR1 (Da Silva *et al.*, 1989) and the CR3 receptors on the macrophage (Russell and Talamas-Rohana, 1989).

This study has shown that stationary-phase promastigotes grown in acidic pH are more resistant to complement mediated lysis than those grown at neutral pH. A 4 to 16 fold increase in resistance was observed among the four *Leishmania* species studied.

It has been shown previously that metacyclic promastigotes of different *Leishmania* species are more resistant to complement mediated lysis than procyclic promastigotes (Mosser and Edelson, 1984; Franke, *et al.*, 1985; Howard *et al.*, 1987; Wozencraft and Blackwell, 1987; Bandyopadhyay *et al.*, 1991, Almeida *et al.*, 1993; Bates and Tetley, 1993). This resistance to complement mediated lysis is due to the presence of lipophosphoglycan (LPG) on the surface of promastigotes (Turco, 1991). The LPG molecule consist of four parts; a phosphatidylinositol lipid anchor, a phosphosaccharide core, repeating phosphorylated saccharides and a small oligosaccharide cap (Turco, 1990). It serves as a multifunctional molecule and one of its functions is the masking of promastigotes preventing them from lysis by complement. During metacyclogenesis the LPG is modified. It doubles in size due to a doubling of the number of repeating phosphorylated saccharide units. Also, the side chains on the repeat units of *L. major* LPG, which terminate with β-galactose and account for agglutinability by peanut agglutinin of exponential-phase promastigotes, terminate predominately with α-arabinose and to a lesser extent with β-glucose in metacyclic promastigotes (Sacks *et al.*, 1990; McConville, *et al.*, 1992; Turco and Descoteaux, 1992). The doubling in size of the LPG allows deposition of C3b on the surface but prevent further insertion of C5b-9, the lytic molecules, into the surface membrane (Puentas *et al.*, 1990).

4.5 CONCLUSION

As a conclusion, it is possible to induce metacyclogenesis in *L. donovani, L. mexicana, L. major* and *L. braziliensis* by culturing freshly transformed promastigotes in Schneider's *Drosophila* medium supplemented with 20% FCS, pH 5.5 at 26° C. Metacyclic promastigotes produced have the same morphological criteria observed by others, as being smaller in size and possessing a long flagellum, and are more resistant to complement mediated lysis.

CHAPTER FIVE

5. CHARACTERIZATION OF METACYCLIC PROMASTIGOTES

5.1 INTRODUCTION

Differentiation of promastigotes from procyclic to metacyclic forms involves several changes. Morphologically, metacyclic promastigotes measure approximately 8 μm in length and are smaller than procyclic promastigotes. Also, metacyclic promastigotes possess a long flagellum that is twice or more the length of the body as seen under light and electron microscopy (Howard *et al.*, 1987; Bates and Tetley, 1993).

Ultrastructural examination of *L major* metacyclic promastigotes revealed a thick surface coat measuring about 44 nm (Pimenta *et al.*, 1989 and 1991). This coat was also found on *L mexicana* metacyclic promastigotes but not on procyclic forms (Bates and Tetley, 1993). It is believed that this coat represents the modified metacyclic lipophosphoglycan (LPG). It is known that the LPG is modified during metacyclogenesis in *L. major*. It doubles in size and its repeat units terminate predominately with α-arabinose, while that of procyclic promastigotes terminate predominately with β-galactose (Sacks *et al.*, 1990; McConville, *et al.*, 1992; Turco and Descoteaux, 1992). Such modification to the LPG may render it visible under electron microscopy.

Another important difference between metacyclic and procyclic promastigotes is their infectivity. One of the early reports on the identification of infective promastigotes is that of Sacks and Perkins (1984). They observed an increase in the infectivity of cultured promastigotes as cultures progressed towards stationary phase, even though stationary-phase populations were not homogeneous. However, virulence of such stationary-phase populations decrease after long-term cultivation of the parasite (Nolan and Herman, 1985; Kallinikova *et al*., 1992).

Several molecules have been shown to contribute to *Leishmania* virulence. The lipophosphoglycan (LPG) is one of the most abundant molecules on the surface of *Leishmania* (Chang and Chaudhuri, 1990). This molecule has been shown to fulfil various roles including regulation of attachment and detachment of promastigotes in the sandfly midgut (Davis *et al*., 1990), protection against hydrolytic enzymes (El-On *et al*., 1980; Eilam *et al*., 1985; Handman *et al*., 1986;) and survival in the macrophage phagolysosome (Sacks and Da Silva, 1987; Sacks *et al*., 1990; Turco, 1990; Turco and Descoteaux, 1992).

The presence, abundance, surface localization and proteolytic activity of the glycoprotein gp63 supports the hypothesis that it is also a factor of *Leishmania* virulence (Chang and Chaudhari, 1990). It has been confirmed that changes in the infectivity of *L. infantum* by subculture correlated with the decrease in the proteolytic activity of gp63 (Santos-Gomes and Abranches, 1996). Other proteases, such as the cysteine proteinases, also contribute to the virulence of *Leishmania*. Cysteine proteinases were expressed by metacyclic promastigotes of *L. mexicana* and were not seen in non-metacyclic promastigotes (North *et al*., 1990; Coombs *et al*., 1991; Robertson and Coombs, 1992; Bates *et al*., 1994). Other molecules, such as acid

phosphatases and nucleases, have been demonstrated in *Leishmania* promastigotes and may play a role in survival of the parasite and/or controlling its virulence (Bates and Dwyer, 1987; Chang and Chaudhuri, 1990; Bates, 1993b).

Several workers have examined the infectivity of different *Leishmania* species *in vitro* and *in vivo*. Rey *et al.* (1990) reported that stationary-phase promastigotes of *L.b.braziliensis* and *L.b.guyanensis* were more infective to human macrophage cell line (U937) than log-phase promastigotes. Similarly, stationary-phase promastigotes of *L. mexicana* cultured at acidic pH, which possessed several metacyclic characters, were more infective to peritoneal macrophages than stationary-phase promastigotes cultured in neutral pH (Bates and Tetley, 1993). Stationary-phase promastigotes were also reported to be more infective than log-phase promastigotes when inoculated in laboratory animals (Giannini, 1974; Doran and Herman, 1981; Da Silva and Sacks, 1987, Howard *et al.*, 1987; Sacks, 1988; Almeida *et al.*, 1993).

This study investigated the ultrastructure of promastigotes grown in neutral and acidic pH and compared their infectivity to mice.

5.2 MATERIALS AND METHODS

5.2.1 ULTRASTRUCTURAL EXAMINATION

Samples of stationary-phase promastigotes of *L. donovani, L.mexicana, L. major* and *L. braziliensis* cultured at pH 7.0 and pH 5.5 were washed, fixed and processed as described in 3.2.2. Samples were examined under scanning and transmission electron microscopy.

5.2.2 INFECTIVITY OF *L. DONOVANI* PROMASTIGOTES TO BALB/c MICE

Stationary-phase promastigotes cultured at pH 7.0 and 5.5 were washed twice in HBSS and brought to a density of $1x10^8$ cells/ml. Two groups of 12 female BALB/c mice were used*. Mice were kept under a lamp for 5 minutes in order for their tail veins to dilate. Each mouse was inoculated intravenously, in the tail vein, with 0.2 ml containing $2x10^7$ promastigotes. Mice were killed two weeks after inoculation and their livers were removed. An impression smear was made from each liver after weighing it. Smears were fixed in absolute methanol for 1 minute, stained in 10% Giemsa/phosphate buffer for 25-30 minutes and examined under the microscope. A magnification of 400x was used to find an area on the slide with a uniform layer of hepatocyte nuclei. Amastigotes were counted against 1000 liver cell nuclei at 1000x magnification.

---

* Two independent experiments each done with two groups of 6 mice were combined.

The mean number of amastigotes per liver cell nuclei was multiplied by the weight of the liver (mg) to obtain the LDU (Leishman-Donovan units) index of infectivity (Bradley and Kirkley, 1977; Howard *et al.*, 1987). A *t* test of significance was used to statistically compare the LDU values using the Minitab program.

5.2.3 INFECTIVITY OF *L. MEXICANA, L. MAJOR* AND *L. BRAZILIENSIS* PROMASTIGOTES TO BALB/c MICE

Stationary-phase promastigotes cultured at pH 7.0 and 5.5 were washed twice in HBSS and brought to a density of $2x10^7$ cells/ml for *L. mexicana* and *L. major* and $4x10^8$ cells/ml for *L. braziliensis*. Two groups of 12 BALB/c mice were used*, one was inoculated with promastigotes cultured at pH 7.0 and the other with promastigotes cultured at pH 5.5. Hair at the base of the tail was shaved and each mouse was inoculated subcutaneously at the base of the tail with 0.05 ml containing 10^6 promastigotes for *L. mexicana* and *L.major* and $2x10^7$ promastigotes for *L. braziliensis*. Mice were examined weekly for the development of lesions. Length and width of lesions were measured weekly for 10 weeks and the average lesion size was estimated. A *t* test of significance was used to statistically compare mean lesion diameters using the Minitab program.

---

* Two independent experiments each done with two groups of 6 mice were combined.

5.3 RESULTS

5.3.1 ULTRASTRUCTURAL EXAMINATION

Samples of stationary-phase promastigotes of *L. donovani, L.mexicana, L. major* and *L. braziliensis* cultured at pH 7.0 and pH 5.5 were examined under the scanning and the transmission electron microscope as described in 5.2.1. In addition to the anticipated structure described in 3.3.2, promastigotes cultured at pH 5.5 were smaller in size than those cultured at pH 7.0. Their kinetoplast was positioned centrally in the cell and was adjacent to the nucleus (Figure 5.1 & 5.2).

The cell coat that was described by Pimenta *et al.*, (1989 and 1991) and Bates and Tetley (1993) was not seen in all species examined. It was seen in samples of *L. donovani* (18 nm) and, to a lesser extent, in samples of *L. mexicana* (11 nm) (Figures 5.3 to 5.6).

The surface coat was not seen in samples of *L. major* and *L. braziliensis* (Figures 5.7 to 5.10). This could be due to the difference in preparation procedures or could simply be that these species do not exhibit such coat material.

Under the scanning electron microscope, metacyclic promastigotes cultured at pH 5.5 had a short body measuring 4-7.5 μm in length and a long flagellum measuring approximately 14 μm in length (Figure 5.11). The cell length of procyclic promastigotes cultured at pH 7.0 measured more than 8 μm and the flagellum was about 10 μm in length (Figure 5.12).

Figure 5.1. Transmission electron micrograph of a longitudinal section in a metacyclic promastigote of *Leishmania donovani* cultured at pH 5.5. N=neucleus, K=kinetoplast, F=flagellum.

Bar= 350 nm

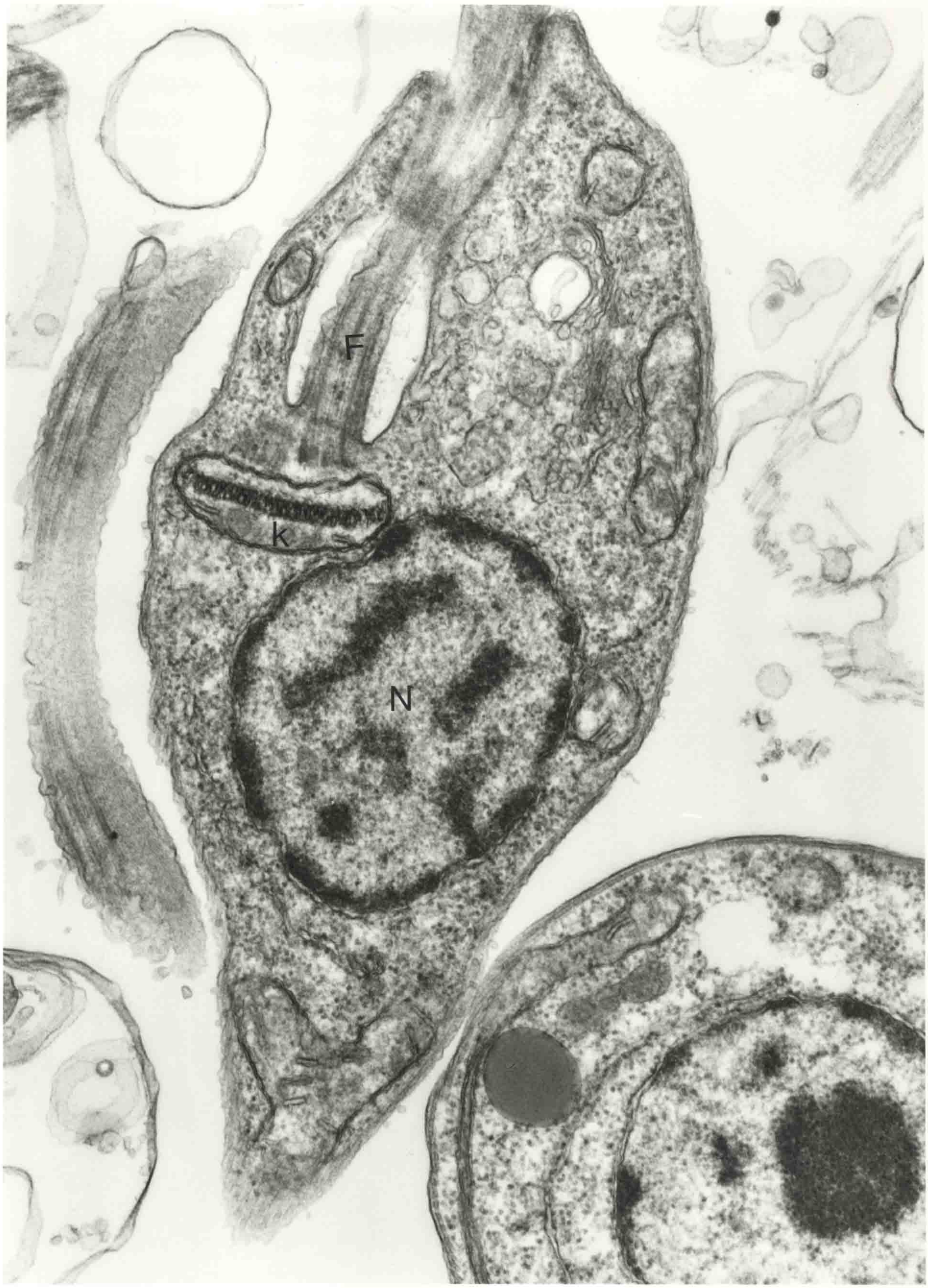

F
k
N

Figure 5.2. Transmission electron micrograph of a longitudinal section in a procyclic promastigote of *Leishmania donovani* cultured at pH 7.0. n=neucleus, k=kinetoplast, f=flagellum.

Bar= 350 nm

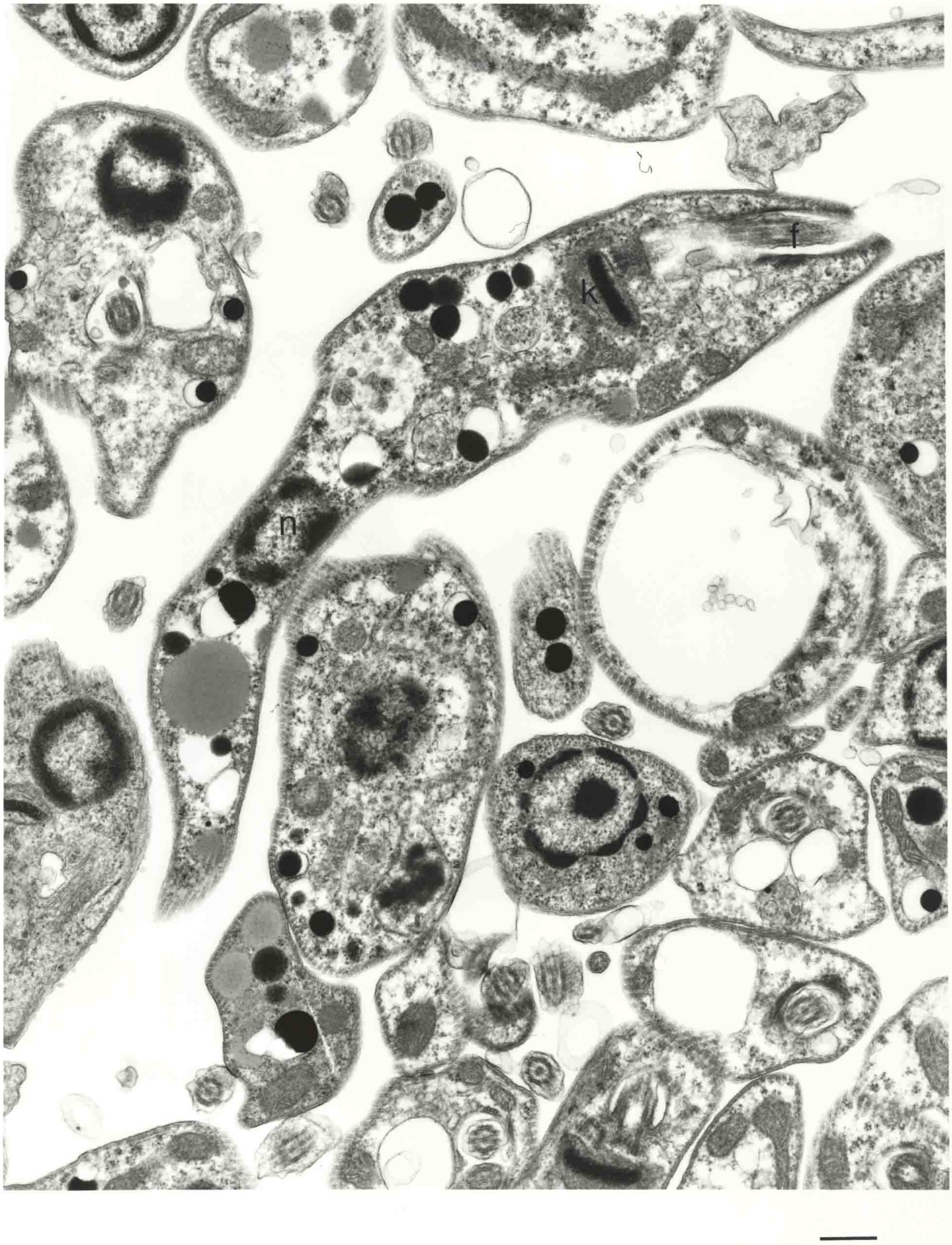

f
k
n

Figure 5.3. Transmission electron micrograph of a longitudinal section in the flagellar pocket of a metacyclic promastigote of *L. donovani* cultured at pH 5.5 showing the characteristic fuzzy coat (arrows). f=flagellum.

Bar= 74 nm

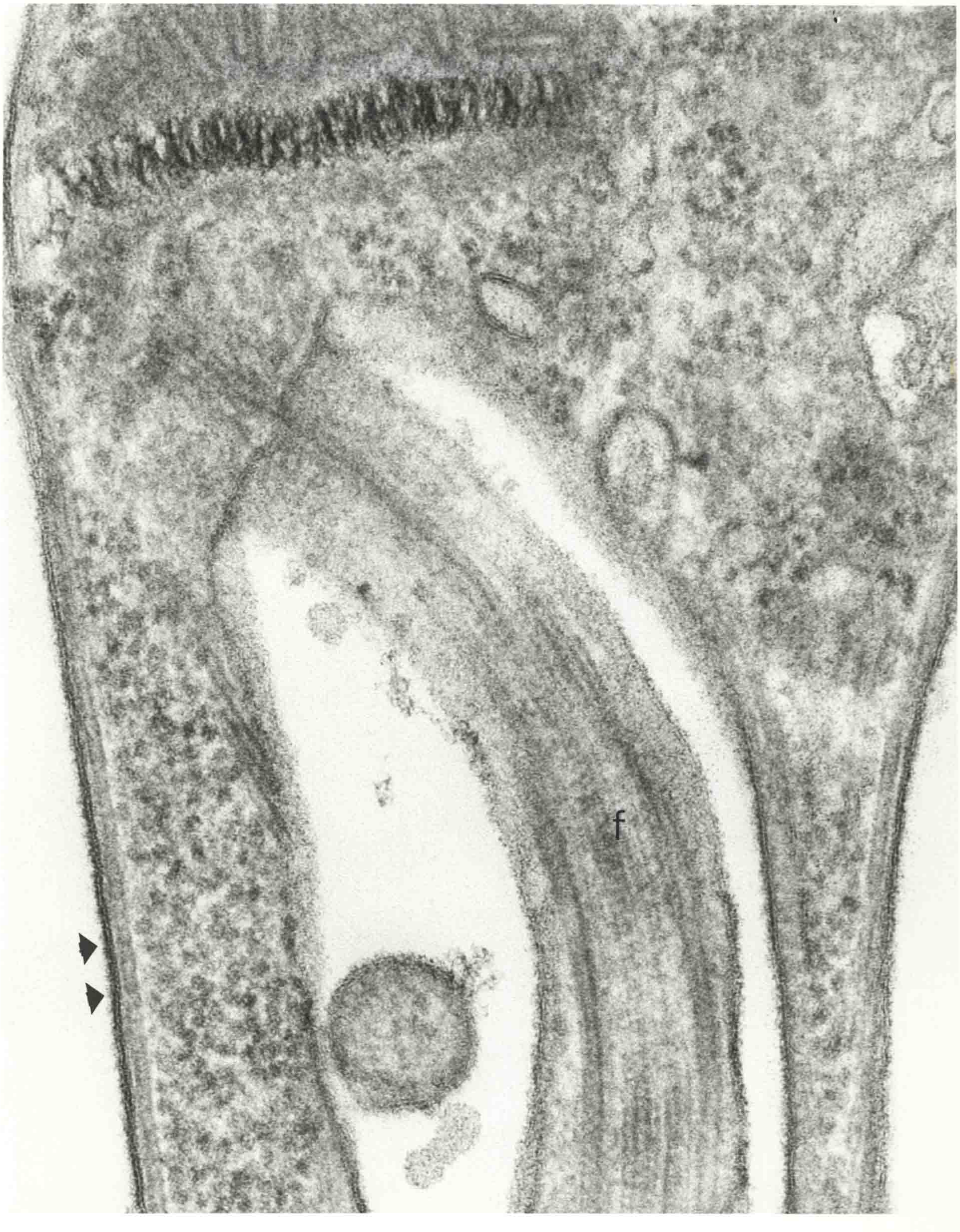

f

Figure 5.4. Transmission electron micrograph of a cross section in a procyclic promastigote of *L. donovani* cultured at pH 7.0. No visible cell coat is seen on the cell membrane underlined by microtubules f=flagellum.

Bar = 330 nm

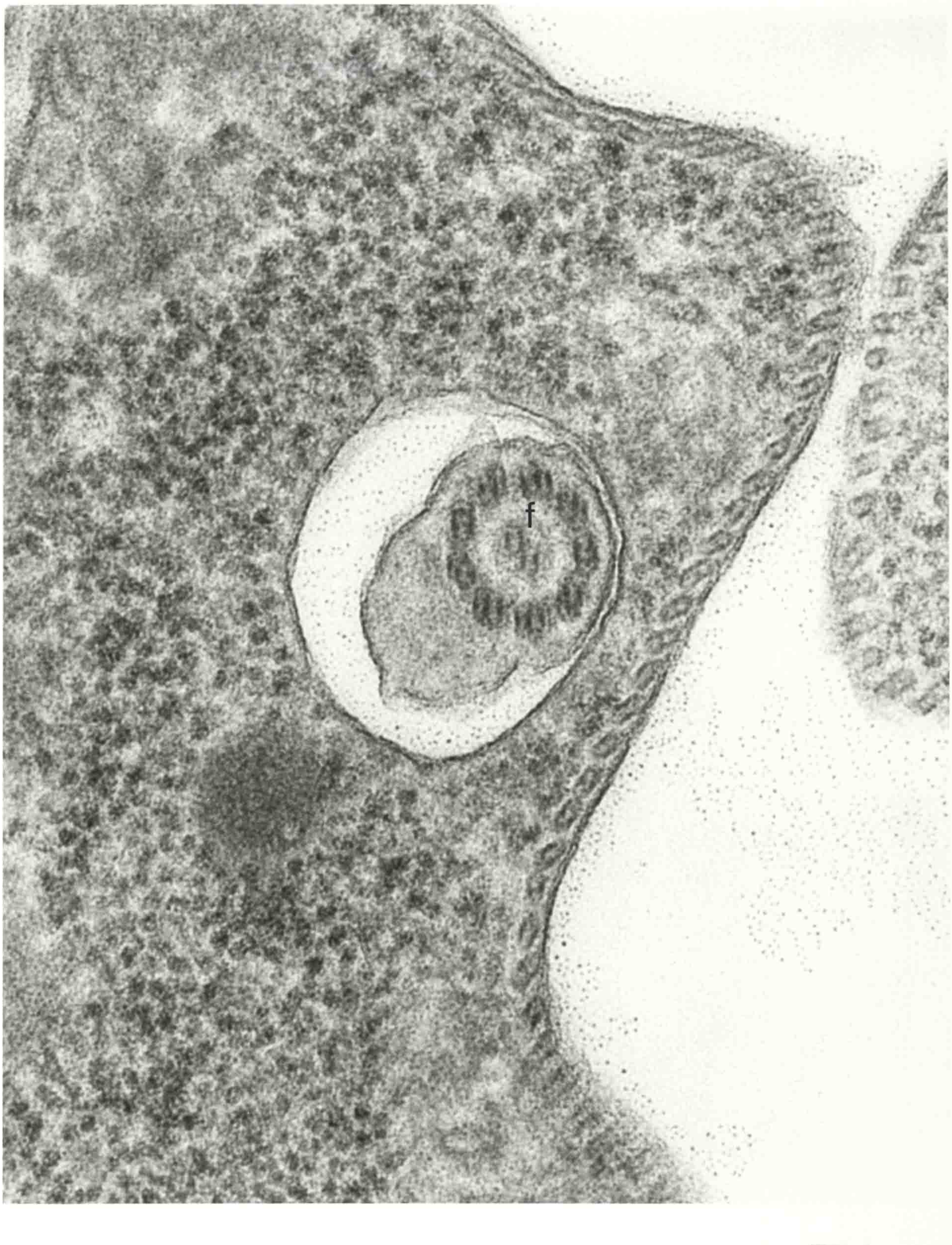

f

Figure 5.5. Transmission electron micrograph of a cross section in a metacyclic promastigote of *L. mexicana* cultured at pH 5.5 showing the characteristic fuzzy coat (arrows). N=neucleus.

Bar= 70 nm

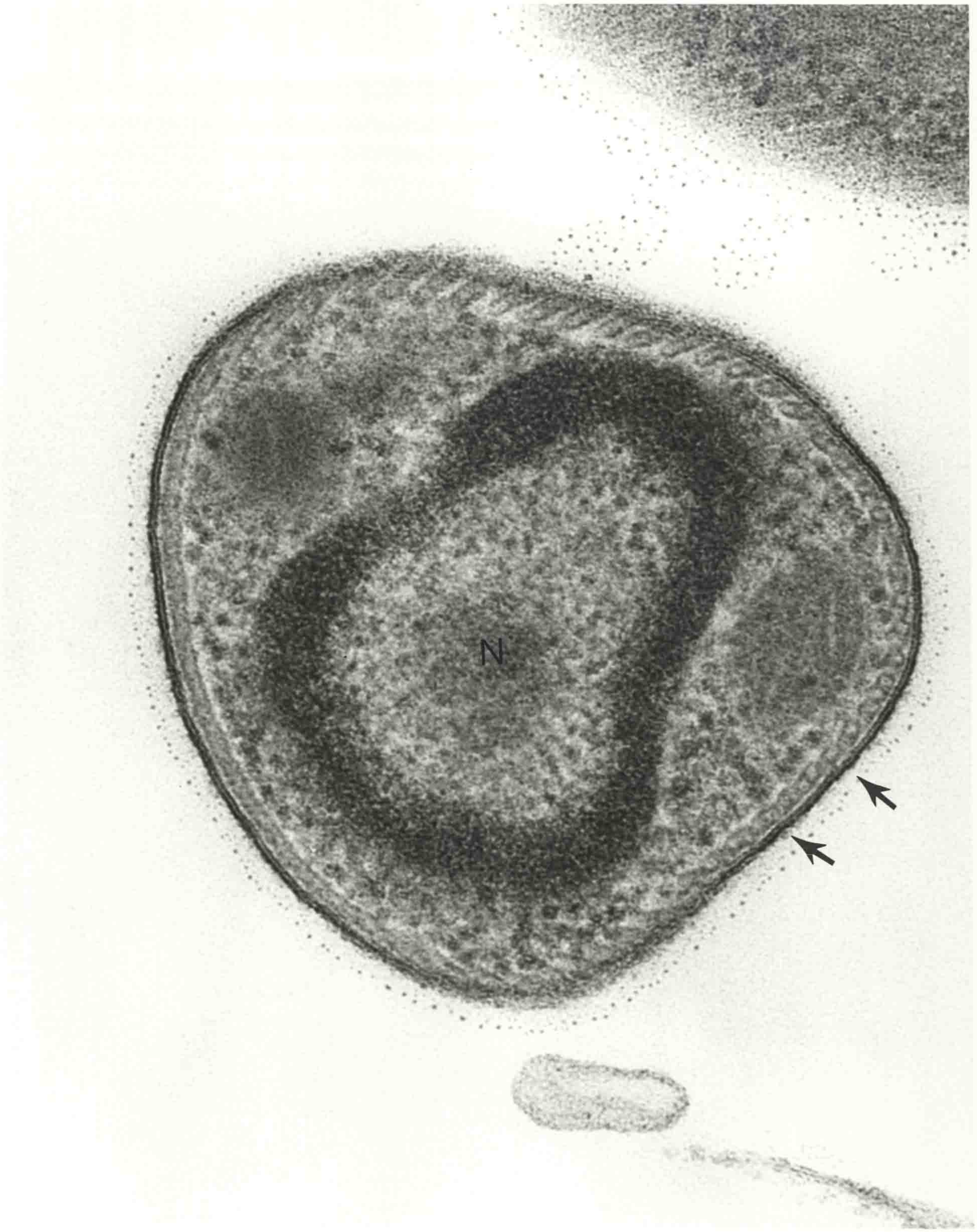

N

Figure 5.6. Transmission electron micrograph of a cross section in a procyclic promastigote of *L. mexicana* cultured at pH 7.0. No visible cell coat is seen on the cell membrane.

Bar = 85 nm

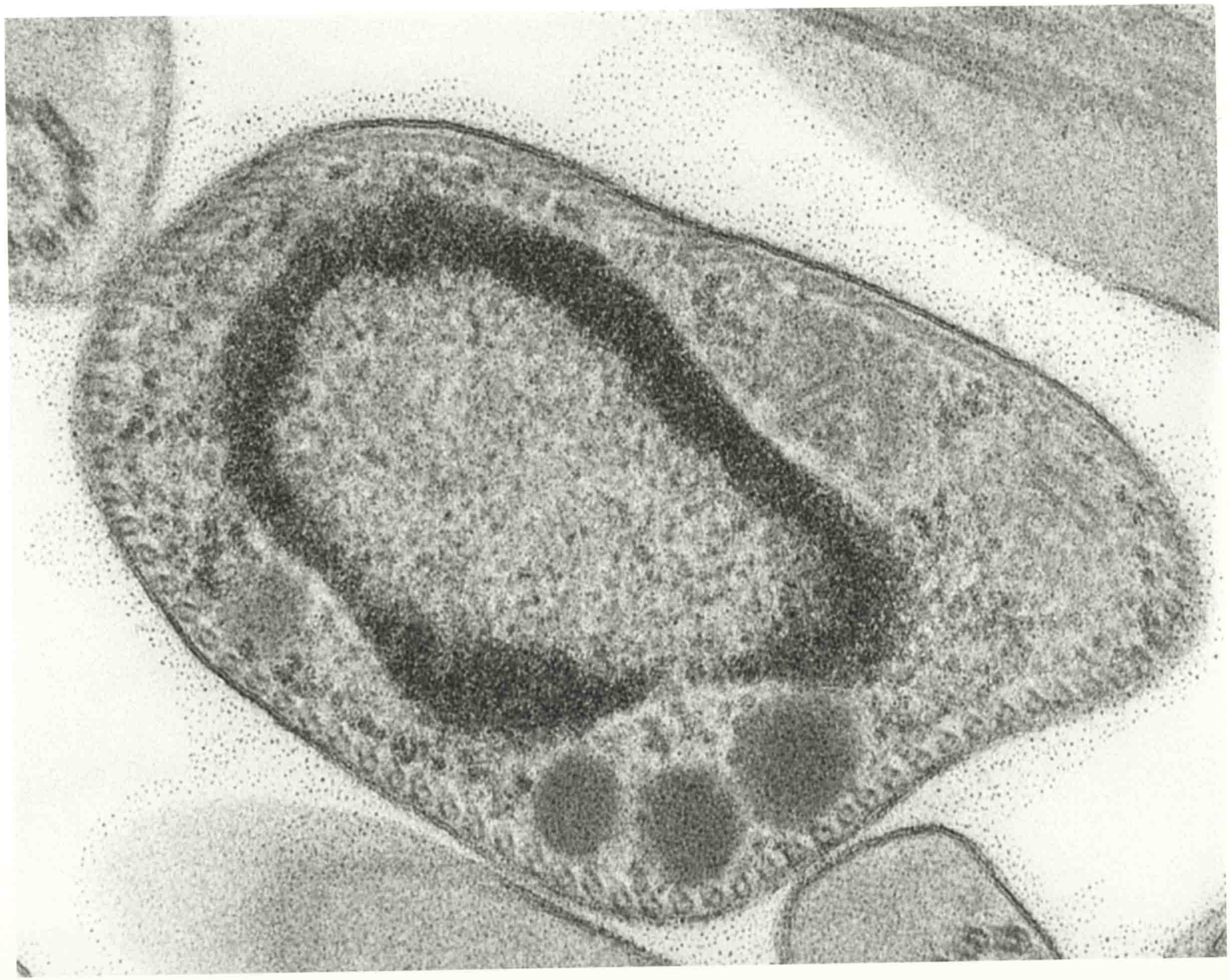


Figure 5.7. Transmission electron micrograph of a longitudinal section in the flagellar pocket of a metacyclic promastigote of *L. major* cultured at pH 5.5. f=flagellum.

Bar = 73 nm

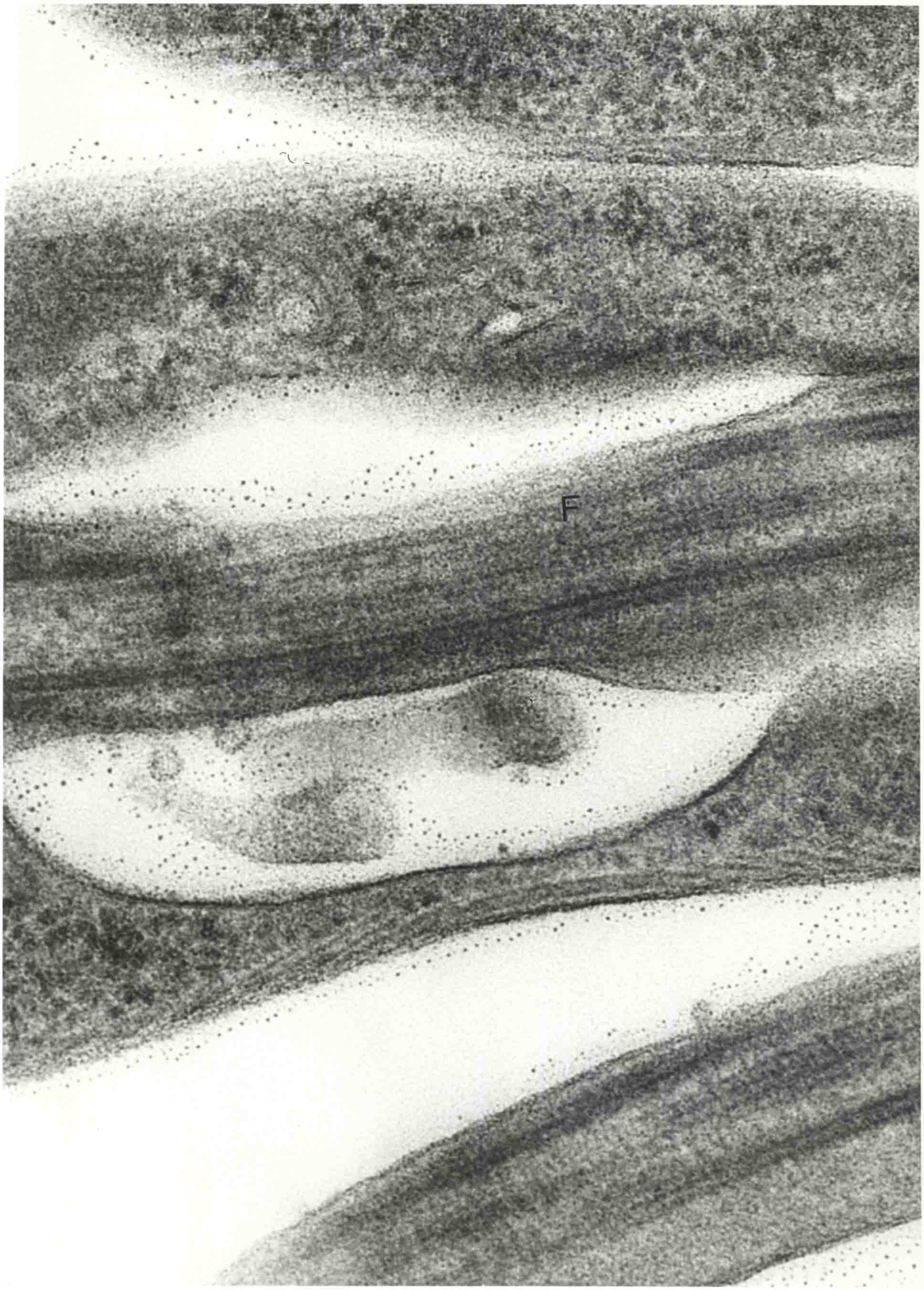

F

Figure 5.8. Transmission electron micrograph of a cross section in a procyclic promastigote of *L. major* cultured at pH 7.0.

Bar = 77 nm

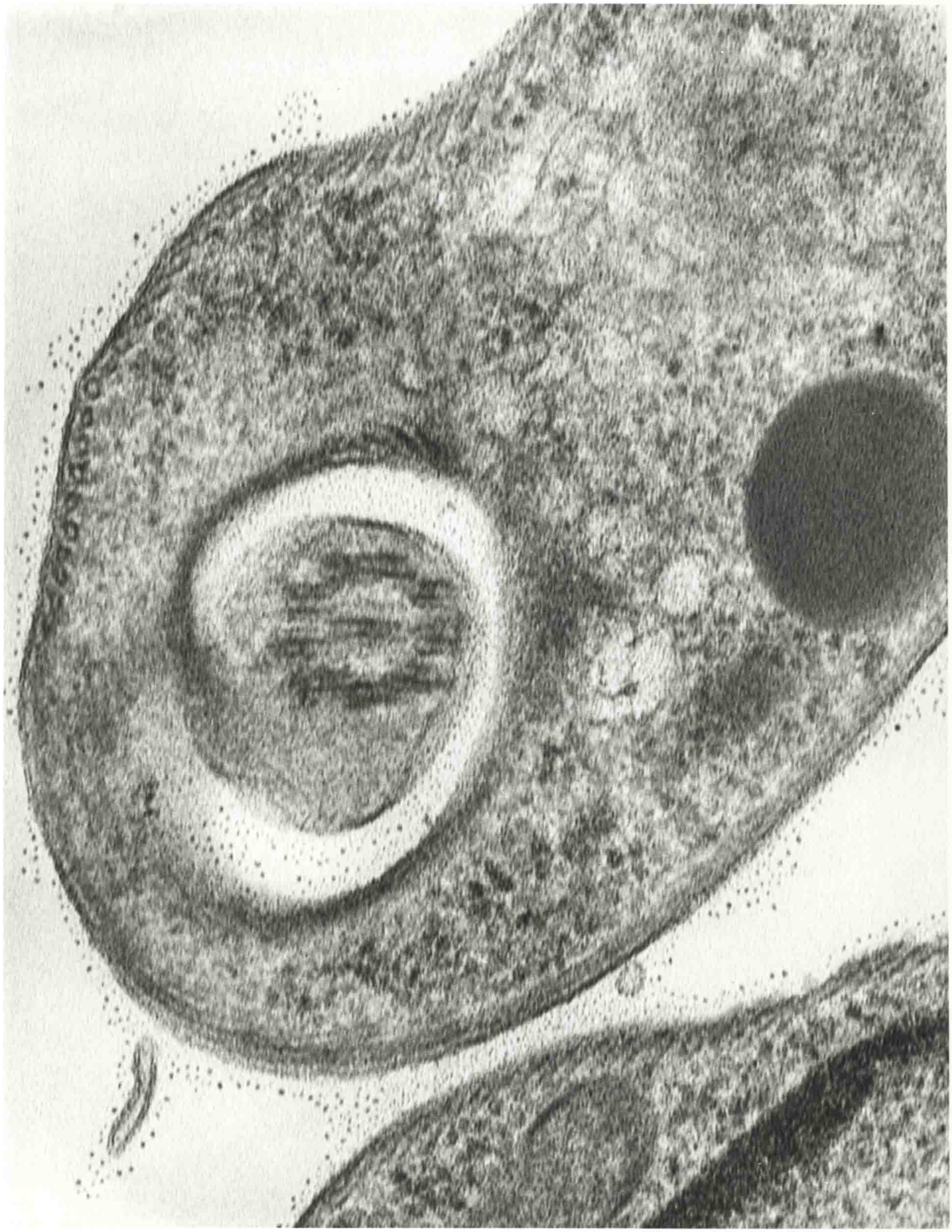


Figure 5.9. Transmission electron micrograph of a cross section in a metacyclic promastigote of *L. braziliensis* cultured at pH 5.5.

Bar = 80 nm

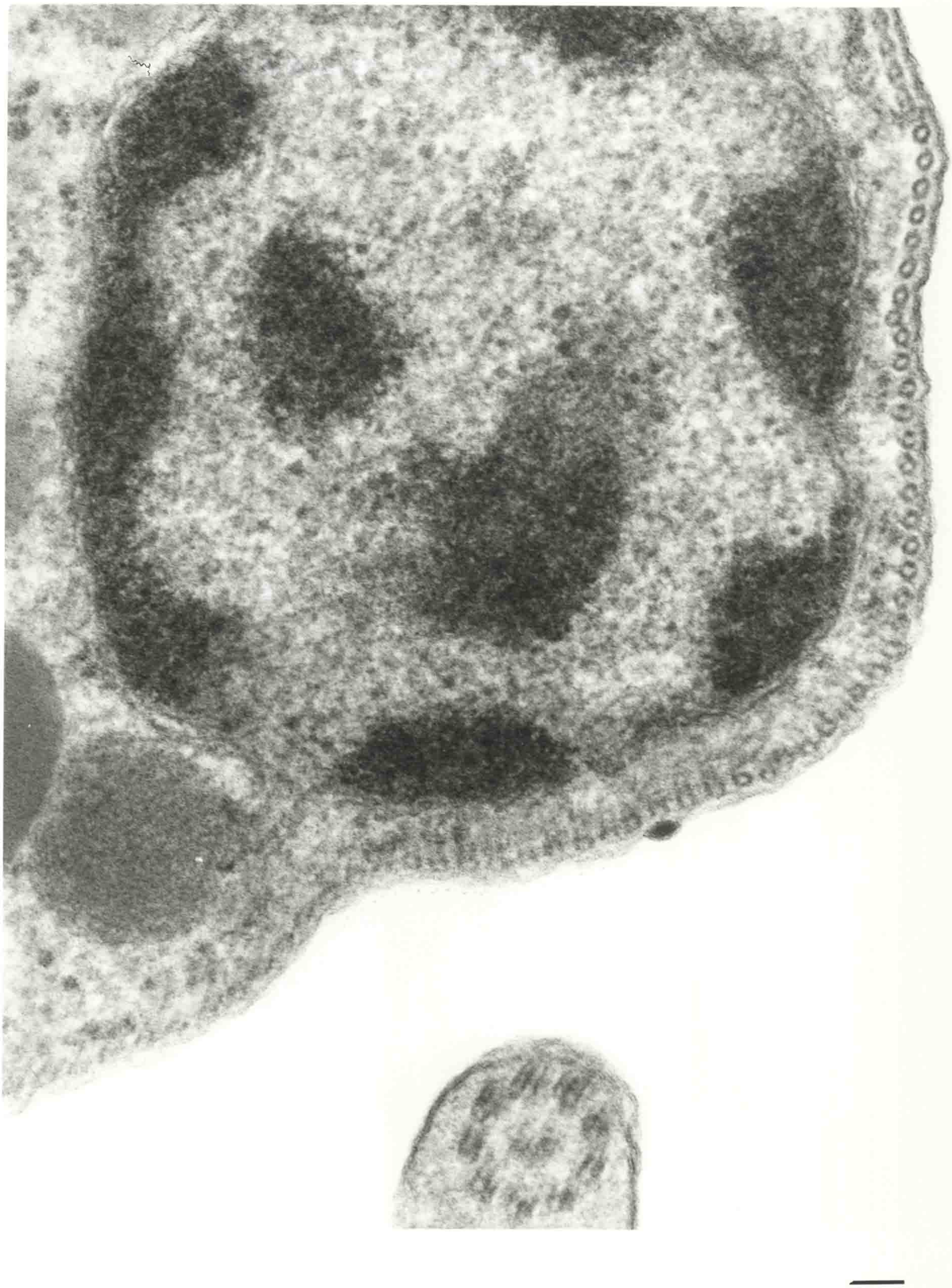


Figure 5.10. Transmission electron micrograph of a cross section in a procyclic promastigote of *L. brœziliensis* cultured at pH 7.0. F=flagellum, K=kinetoplast.

Bar = 80 nm

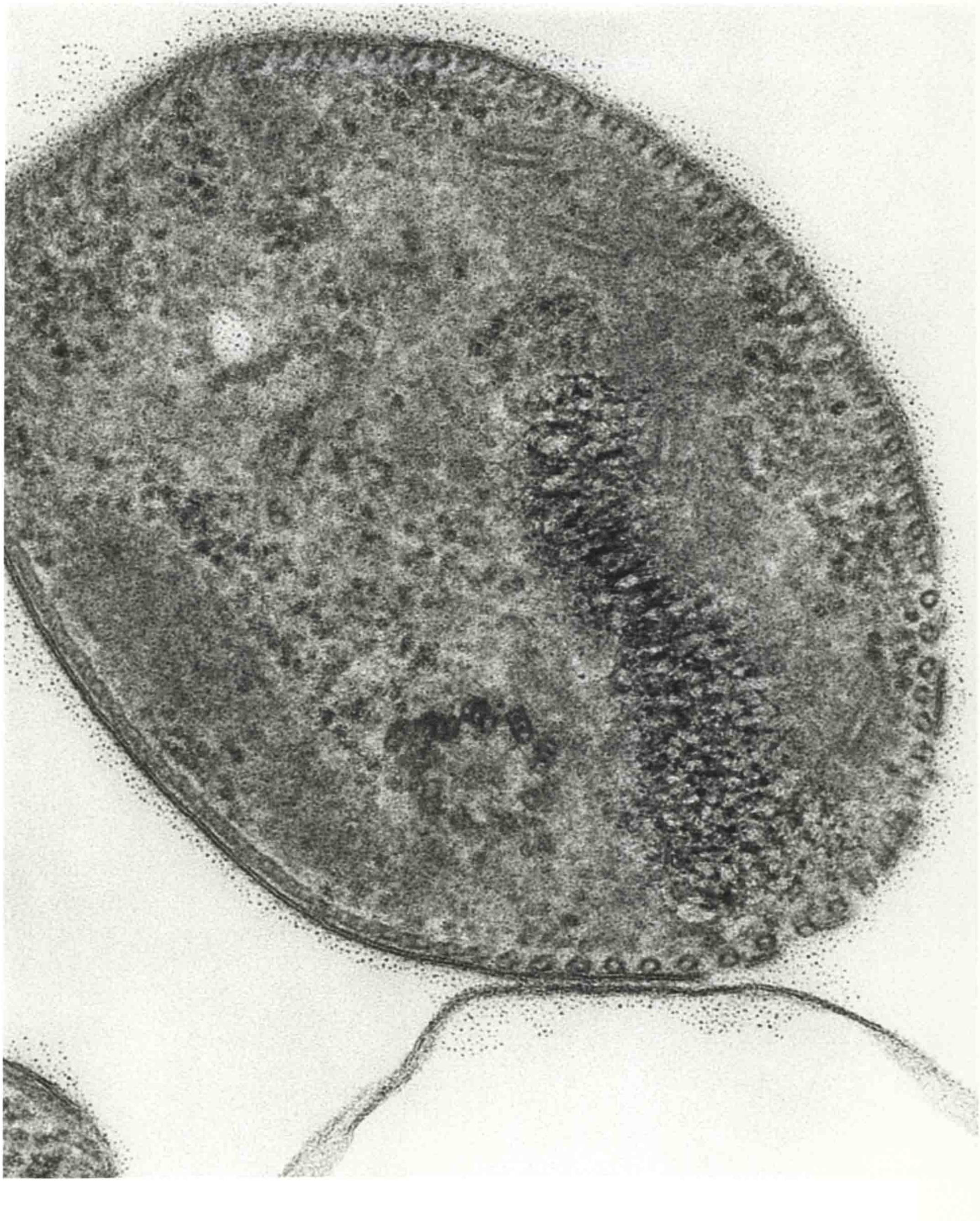


Figure 5.11. Scanning electron micrograph of a *L. donovani* metacyclic promastigote cultured at pH 5.5 showing the short body and the long flagellum.

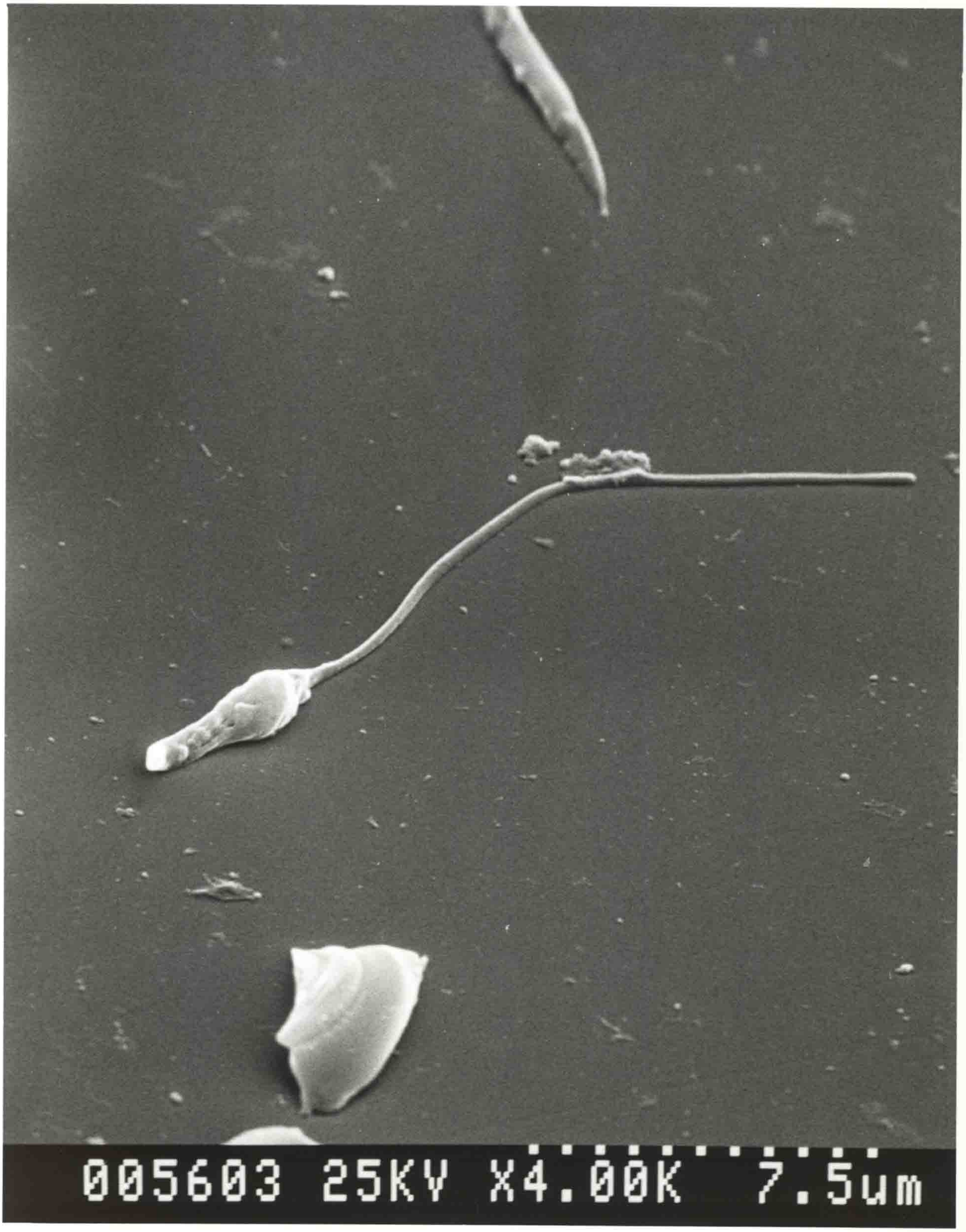
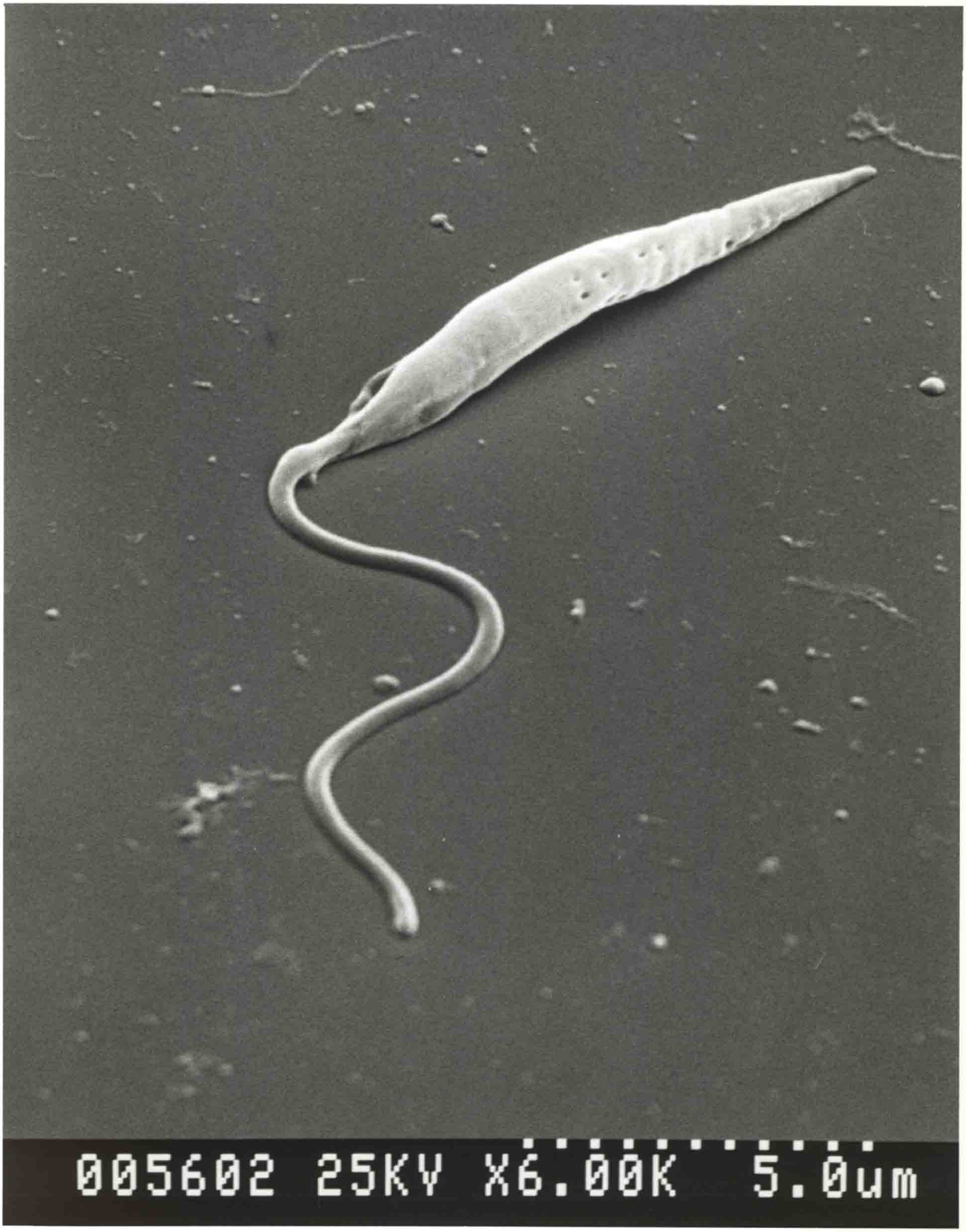

005603 25KV X4.00K 7.5um

Figure 5.12. Scanning electron micrograph of a *L. donovani* procyclic promastigote cultured at pH 7.0.


005602 25KV X6.00K 5.0um

5.3.2 INFECTIVITY OF *L. DONOVANI* PROMASTIGOTES TO BALB/c MICE

Stationary-phase promastigotes cultured at pH 7.0 and 5.5 were used to infect two groups of 12 female BALB/c mice. Mice were killed two weeks after inoculation and their livers were removed. An impression smear was made from each liver after weighing it. Smears were fixed, stained, and examined under the microscope as described in 5.2.2. The number of amastigotes per 1000 liver cell nuclei was counted. The mean number of amastigotes per liver cell was estimated and was multiplied by the liver weight (mg) to obtain the LDU.

Eleven mice originally inoculated with promastigotes cultured at pH 5.5 had amastigotes of *L. donovani* in the liver and their LDU was 276 (± 54 SE). On the other hand, only 3 out of 12 mice originally inoculated with promastigotes cultured at pH 7.0 had amastigotes of *L. donovani* in the liver and their LDU was 14.1 (± 7.4 SE) (Figures 5.13 & 5.14). When the LDU values were compared using a *t* test of significance, there was a significant statistical difference between the two values ($p=0.0007$). This clearly indicates that promastigotes cultured at acidic pH are more infective than those cultured at neutral pH.

Figure 5.13. A light micrograph of a liver impression smear stained with Giemsa's stain showing a liver cell containing amastigotes of *L. donovani* (arrow).

Bar = 5 μm.

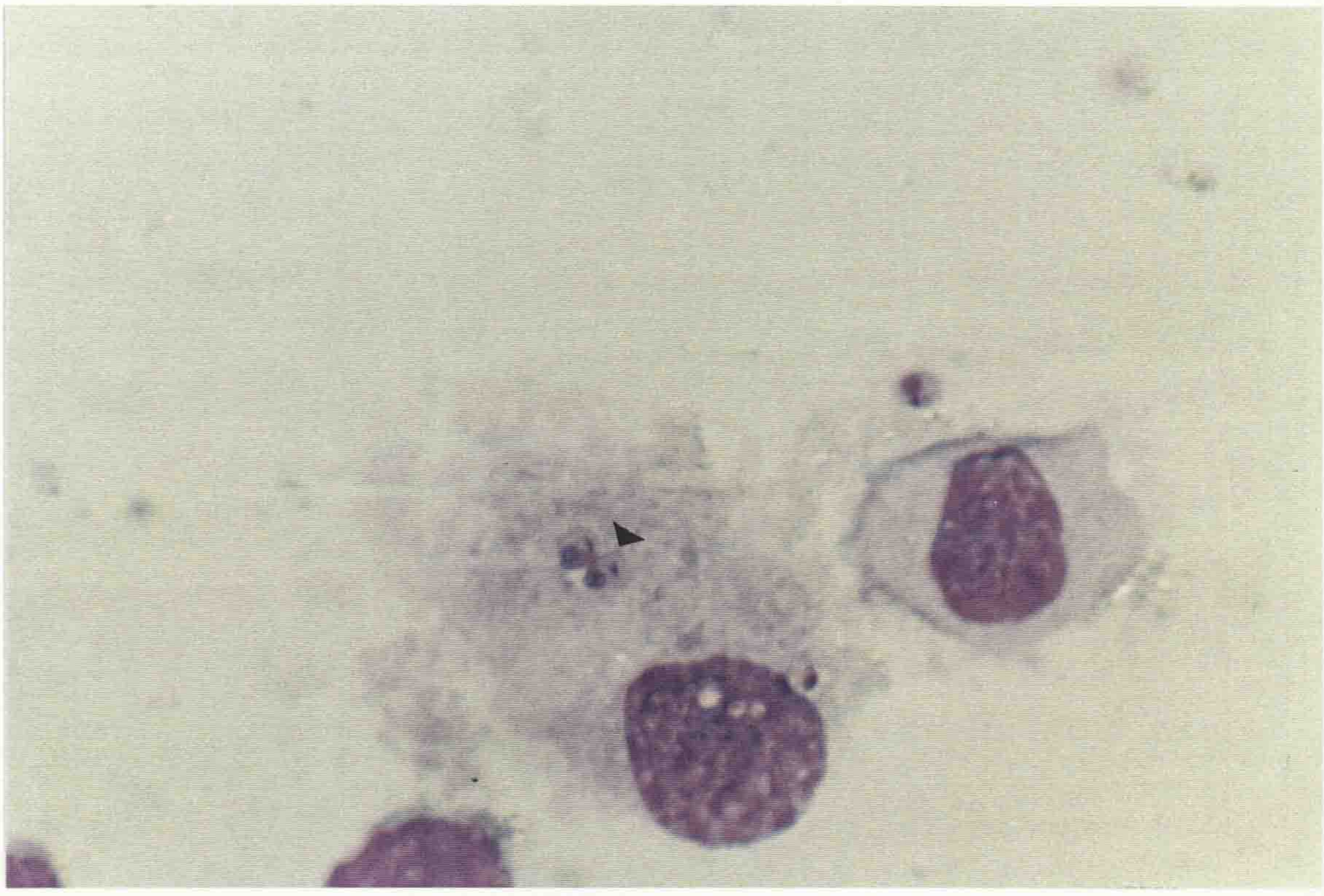
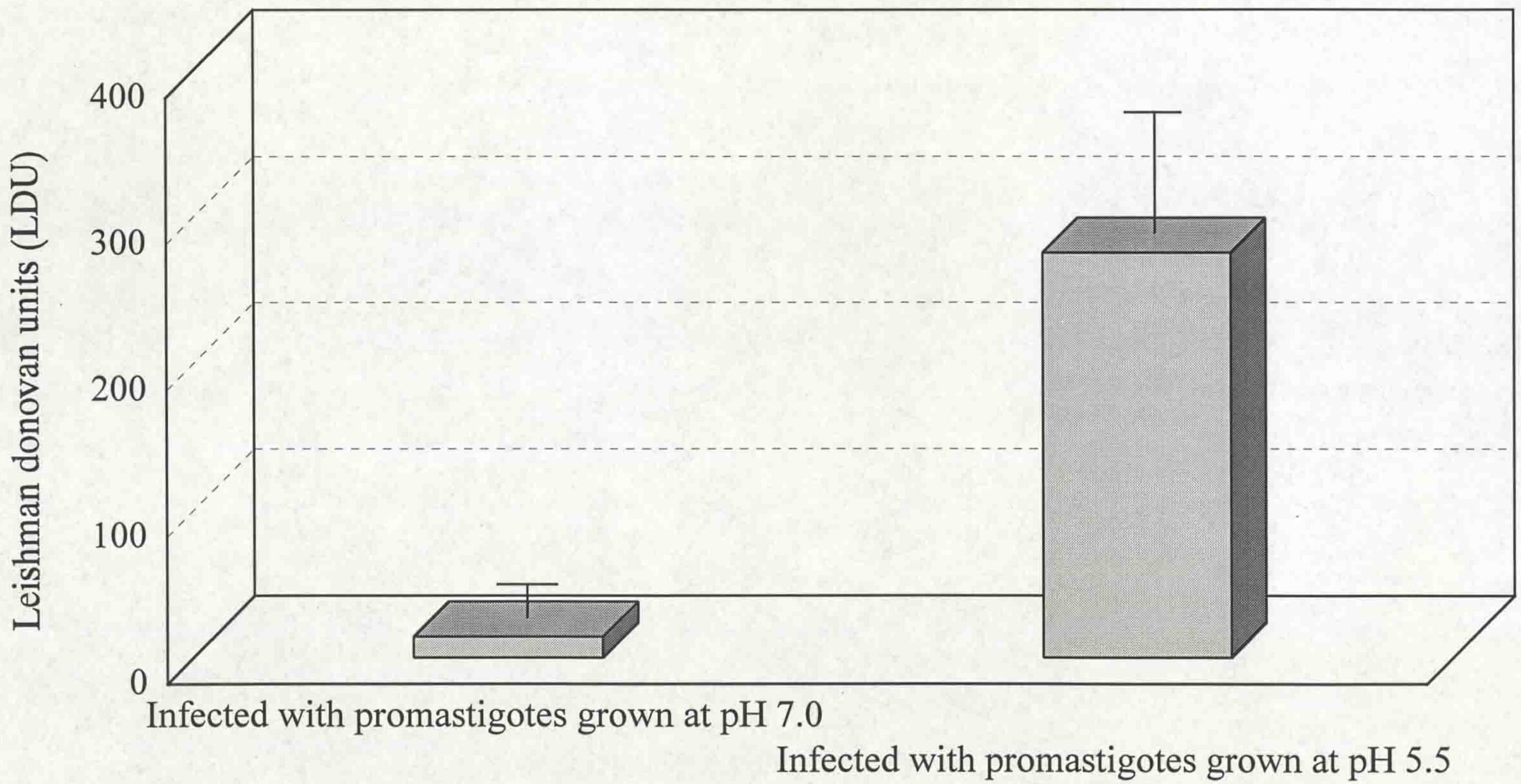


Figure 5.14. Infectivity, expressed in LDU, of metacyclic promastigotes, cultured at pH 5.5, and procyclic promastigotes, cultured at pH 7.0, of *L. donovani* .


400
300
200
100
0
Leishman donovan units (LDU)
Infected with promastigotes grown at pH 7.0
Infected with promastigotes grown at pH 5.5

5.3.3 INFECTIVITY OF *L. MEXICANA, L. MAJOR* AND *L. BRAZILIENSIS* PROMASTIGOTES TO BALB/c MICE

Stationary-phase promastigotes of *L. mexicana, L. major* and *L. braziliensis* cultured at pH 7.0 and 5.5 were used to infect two groups of 12 BALB/c mice for each species as described in 5.2.3. Mice were observed for 10 weeks and the sizes of cutaneous lesions were measured. In all cases of all of the 3 species, lesion development and size was faster in mice inoculated with promastigotes cultured at pH 5.5 than the ones inoculated with promastigotes cultured at pH 7.0. In the case of *L. mexicana* and *L. major*, a superficial, nonulcerating papule appeared first. This developed into a nodule with a larger borders and deeper centre. Three mice inoculated with *L. major* had centrally ulcerated nodules during the last couple of weeks of the experiment (Figure 5.15)

In the case of *L. braziliensis*, lesions appeared as small hairless patches of rough skin at the site of inoculation. Even though such lesions were not very distinctive, the sizes of lesions were larger and their appearance was earlier in mice inoculated with promastigotes cultured at pH 5.5 compared to mice inoculated with promastigotes cultured at pH 7.0. All results were reproducible. Results for all three species are shown in Figures 5.16 to 5.18 where the mean value of the lesion diameter was plotted against duration of infection.

The differences between the infectivity of promastigotes cultured at acidic and neutral pH were statistically significant in the case of *L.major* ($p < 0.01$) and *L. braziliensis* ($p < 0.05$) when comparing lesion developments at week 6 by means of a *t* test. In the case of *L. mexicana* there was no significant difference between the pattern of infection caused by the two promastigote population ($p < 0.1$).

Figure 5.15. A photograph of *L. mexicana* lesion in a BALB/c mouse.

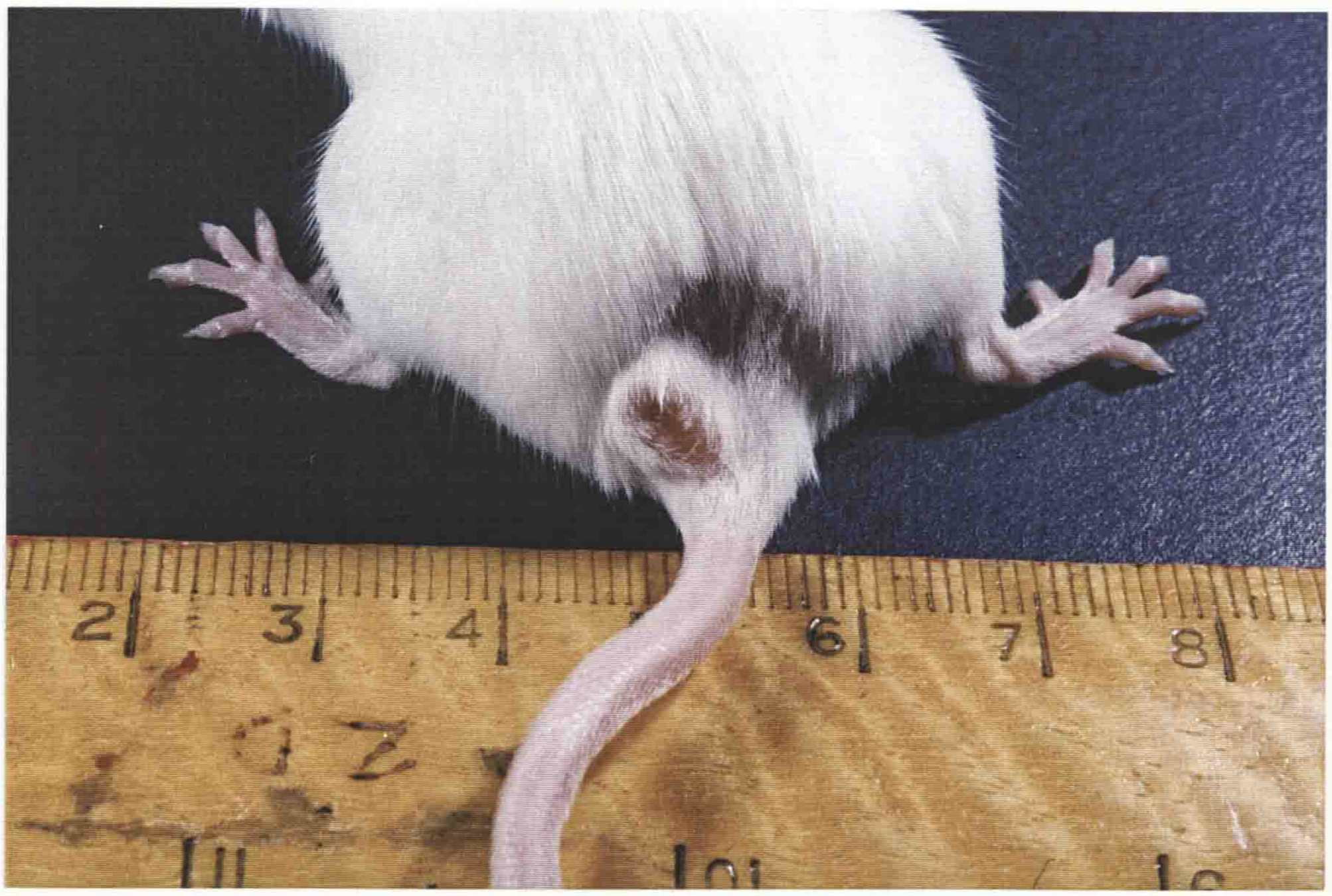


Figure 5.16. Development of *L. mexicana* lesions in BALB/c mice infected with promastigotes cultured at acidic and neutral pH.

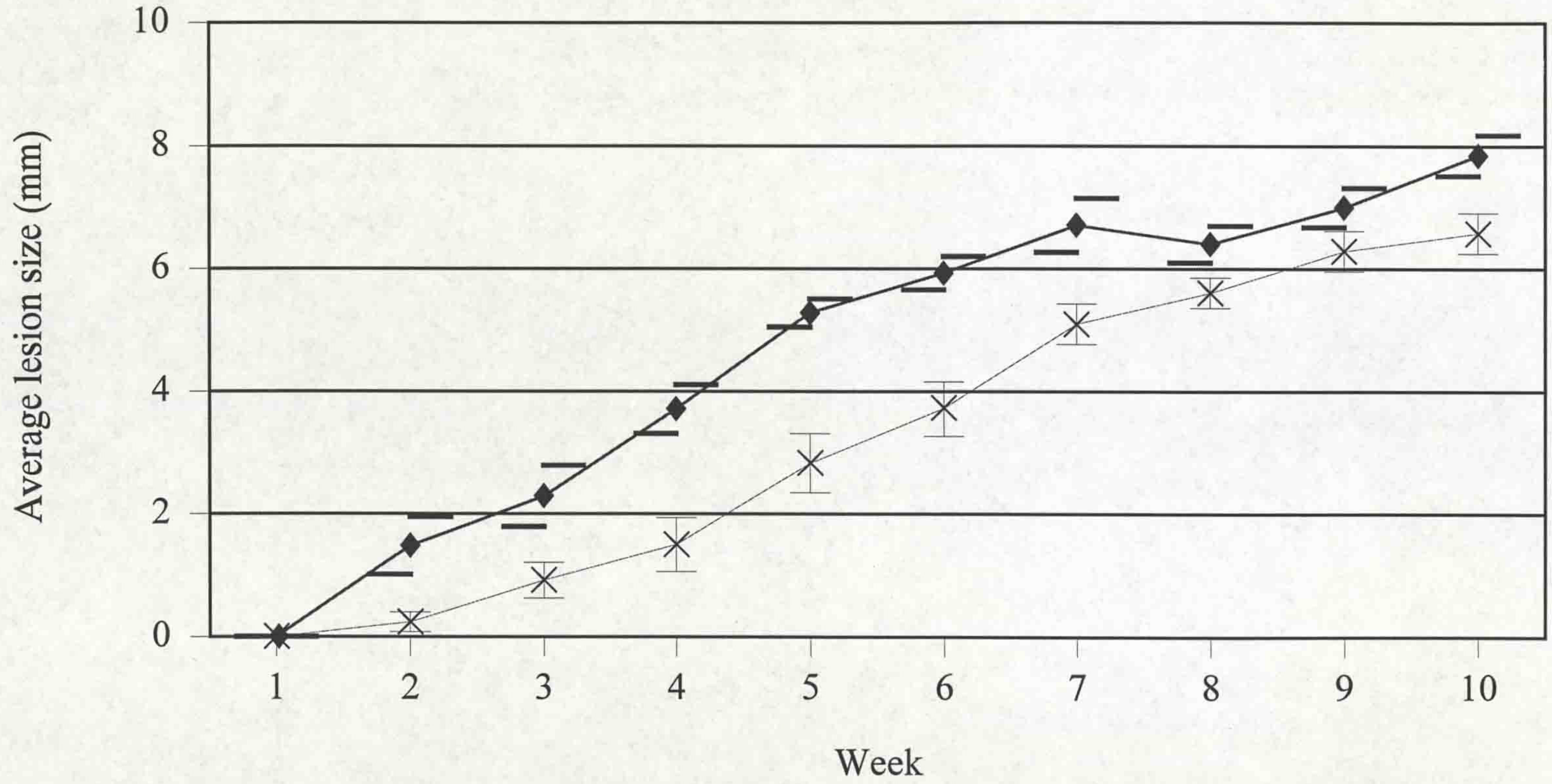

Average lesion size (mm)
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
Week
Infected with promastigotes cultured at pH 7.0
Infected with promastigotes cultured at pH 5.5

Figure 5.17. Development of *L. major* lesions in BALB/c mice infected with promastigotes cultured at acidic and neutral pH.

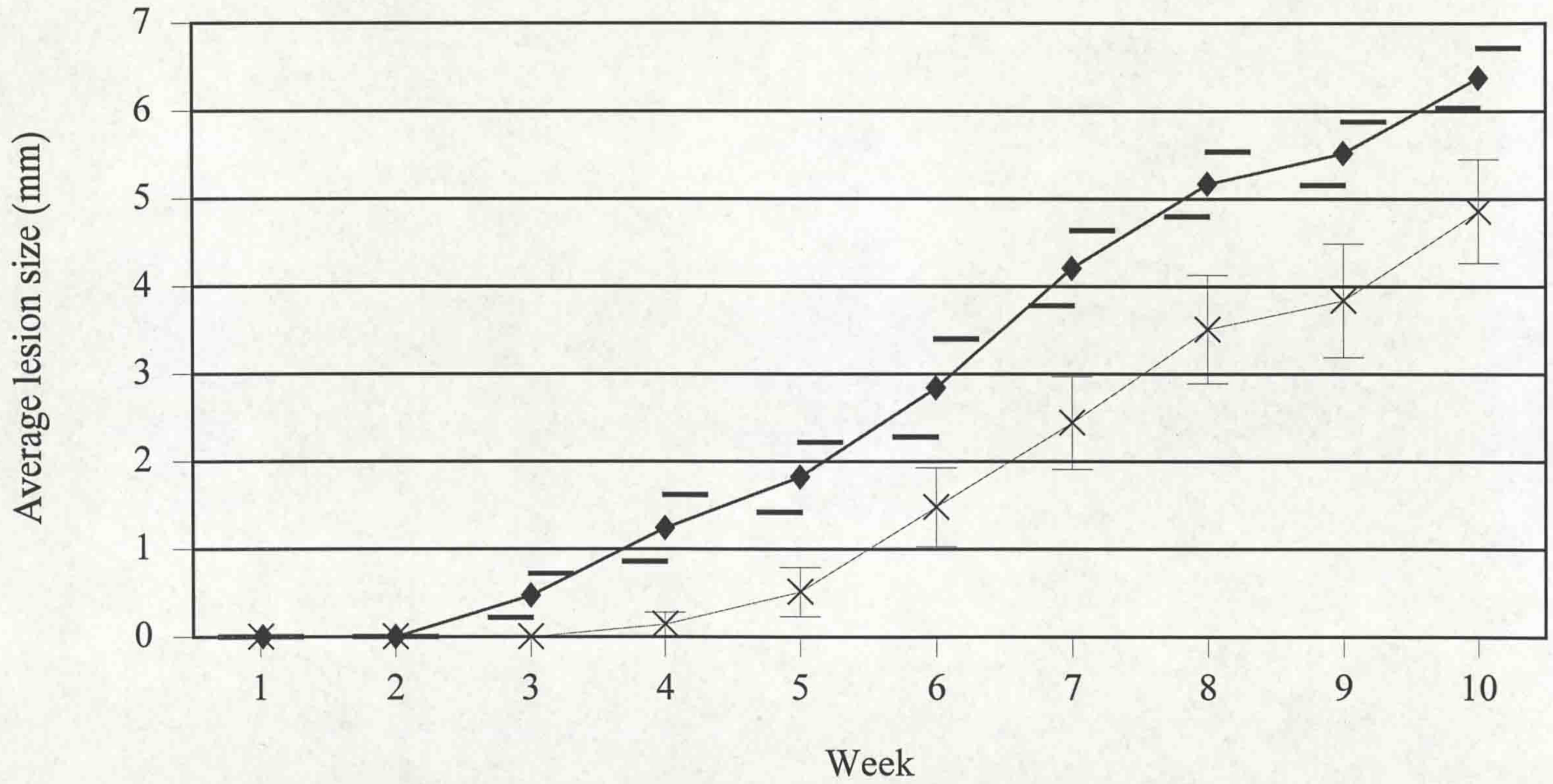
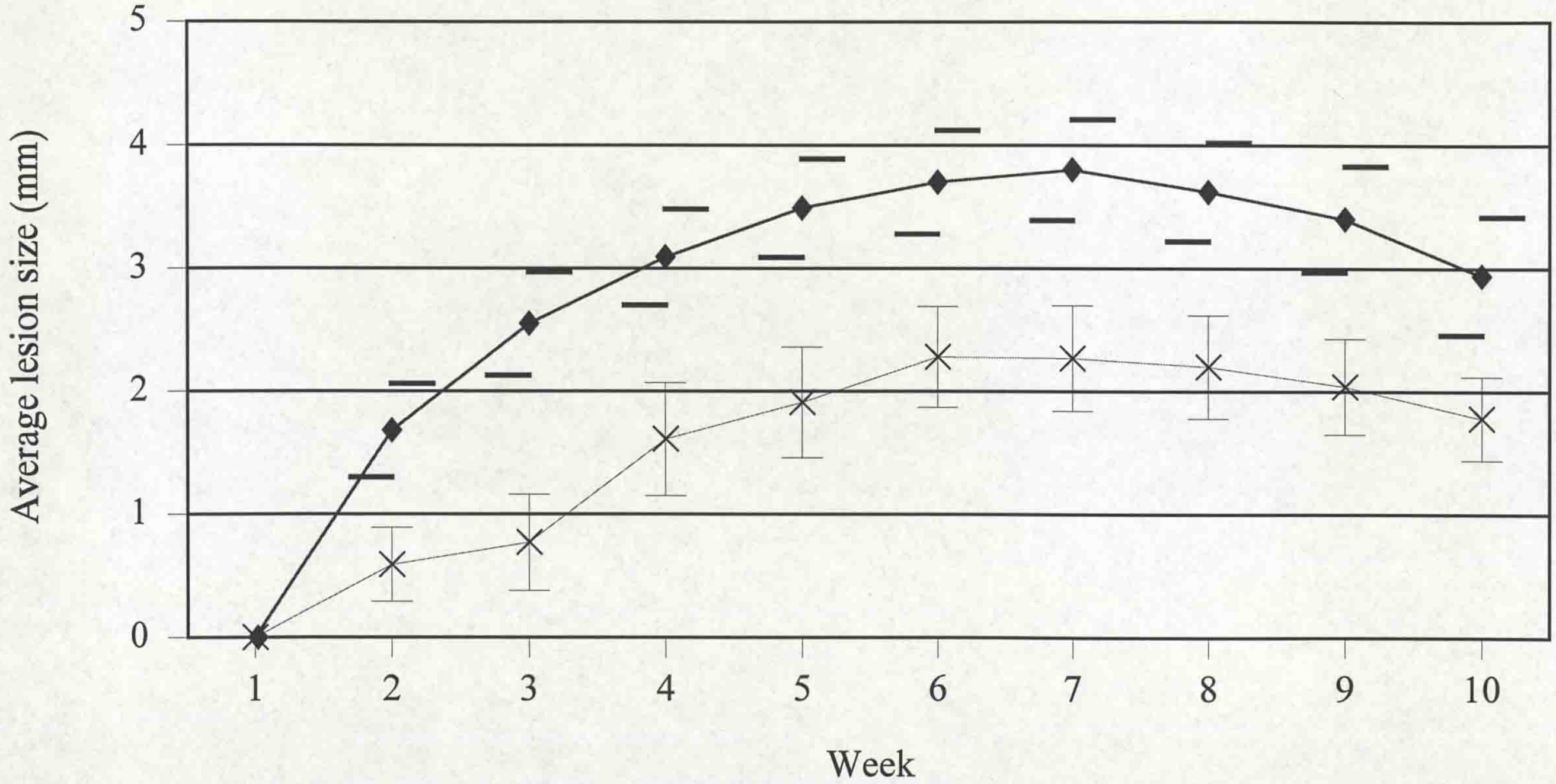

Average lesion size (mm)
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
9
10
Week
Infected with promastigotes cultured at pH 7.0
Infected with promastigotes cultured at pH 5.5

Figure 5.18. Development of *L. braziliensis* lesions in BALB/c mice infected with promastigotes cultured at acidic and neutral pH.


Average lesion size (mm)
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
Week
Infected with promastigotes cultured at pH 7.0
Infected with promastigotes cultured at pH 5.5

5.4 DISCUSSION

Metacyclogenesis is an important part of the life cycle of *Leishmania*. The maximum optimum numbers of metacyclic promastigotes can be recovered from infected sandflies shortly after the passage of the blood meal (Sacks, 1989). This coincides with the time at which the sandfly seeks another meal, therefore, enhancing the chances of disease transmission.

Several workers have reported on the production of metacyclic promastigotes in stationary-phase cultures (reviewed by Sacks, 1989 and Bates, 1994b). In most of these reports there was no effective method for the production of large numbers of metacyclic promastigotes. Bates and Tetley, 1993 were the first to describe a method to induce metacyclogenesis in *L. mexicana*. Their use of an acidic pH to grow *L. mexicana* promastigotes at 26°C led to the production of an homogenous stationary-phase population of metacyclic promastigotes.

In this study I have shown that this response is conserved among other species of *Leishmania* (see chapter 4).

Even though promastigotes produced in this study by cultivation at acidic pH were similar to metacyclic promastigotes in terms of morphology, sizes and complement resistance, it is important to use other methods of characterization to confirm the identity of these promastigotes.

Ultrastructural differences between procyclic and metacyclic promastigotes have been shown previously by several workers. Pimenta *et al.*, (1989 & 1991) demonstrated that *L. major* metacyclic promastigotes possessed a 44nm surface coat that was not seen in procyclic promastigotes. The same structure, measuring approximately 15 nm, was

seen by Bates and Tetley (1993) on stationary-phase promastigotes cultured at pH 5.5 but not on log-phase promastigotes.

In this study, a fuzzy surface coat was seen in stationary-phase promastigotes of both *L. donovani* and *L. mexicana* cultured at pH 5.5 which measured 18 nm and 11 nm respectively. It was not seen in samples of stationary-phase promastigotes of *L. major* or *L. braziliensis*. These results with *L. major* differ from those reported by Pimenta *et al.* (1991). This might be due to differences in methods of sample preparation for electron microscopy. Pimenta *et al.* (1991) used osmium tetroxide for fixation and the whole sample block stained instead of staining the ultrathin section.

Metacyclic promastigotes obtained from stationary-phase populations of acidic cultures had a small body and a long flagellum, twice or more the length of the body, under the scanning electron microscope (Figure 5.11). Under transmission electron microscopy, the shortening of the cell body brought the kinetoplast into close proximity with the nucleus (Figure 5.1) which agrees with the findings of Howard *et al.*, (1987).

Another criterion that was used to characterize metacyclic promastigotes was their infectivity *in vivo*. For a long time, animal models have been used to study leishmaniasis *in vivo*. Many experimental models have been used, each with its own features, but none of these models completely accurately reproduces what happens in humans. *Leishmania* parasites have been shown to infect several models (reviewed by Hommel *et al.*, 1995). The mouse and the hamster are considered as the ideal model for leishmaniasis due to their relative ease in handling and maintainance plus their relatively low cost. In this study, the decision to use mice to assess the infectivity of

metacyclic promastigotes was made because they are faster than hamsters in developing the disease. Inbred BALB/c mice have been used successfully to show the difference between the infectivity of stationary-phase *L. donovani* promastigotes cultured at pH 5.5 and those cultured at pH 7.0. Two weeks after inoculation, there was a statistically significant difference between the two types of inocula. In other words, mice infected with promastigotes cultured in acidic pH had approximately 5.5 x 10^7 amastigotes per liver while those infected with promastigotes cultured at pH 7.0 had approximately 2.8 x 10^6 amastigotes per liver (Bradley and Kirkley, 1977). This shows that metacyclic promastigotes obtained from stationary-phase of acidic cultures were approximately 20 x more infective than promastigotes obtained in stationary-phase from neutral cultures.

In the case of *L. major*, and using the same inoculum size, mice infected with stationary-phase promastigotes from acidic cultures developed lesions earlier then the ones infected with stationary-phase promastigotes from neutral cultures. The sizes of lesion increased consistently as the experiment progressed.

The same results were obtained in the case of *L. mexicana*, but there was no significant difference between the patterns of infections in the two groups. However, when the average lesion diameter was plotted against time the standard error bar of the 2 curves never overlapped. Also twice the standard error bar of each mean did not overlap to include the mean of the other group. This clearly show that the lesion development in one group was consistently greater than that of the other group.

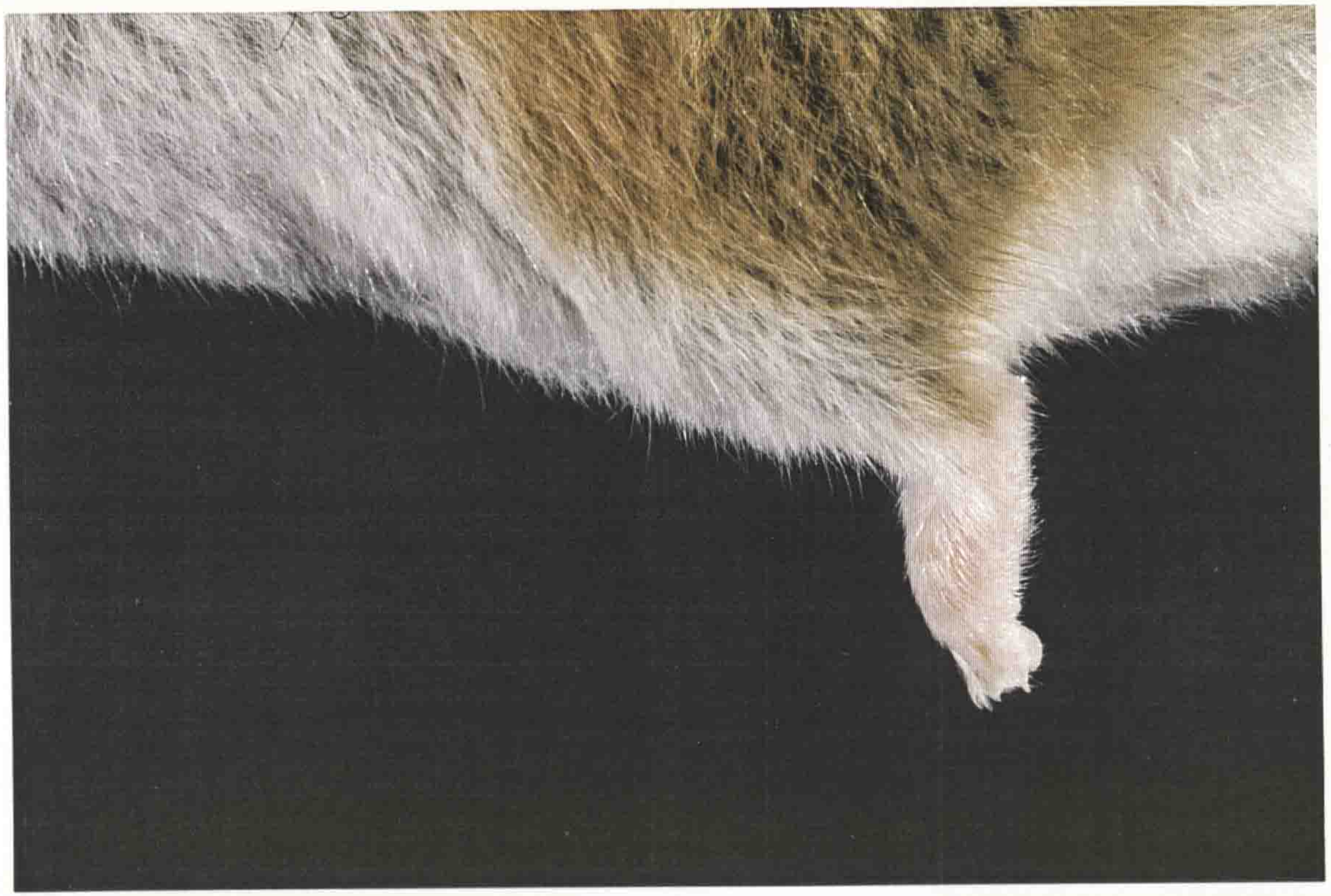
In the case of *L. braziliensis*, lesions appeared as small hairless rough skin at the site of inoculation. The sizes of lesions were larger and their appearance was earlier in

mice inoculated with promastigotes cultured at pH 5.5 and there was a significant difference between the patterns of infections observed in the two groups.

Another option explored was to use hamsters to compare the infectivity of *L. braziliensis* promastigotes cultured at acidic and neutral pH. Hamsters have been used previously to study the infectivity of *L. braziliensis* (Rey *et al.*, 1990; Almeida *et al.*, 1993; Hommel *et al.*, 1995). However, preliminary experiments showed that lesions in hamsters required around 3 months to appear and they appeared as a small swelling of the site of inoculation, usually the footpad, which is difficult to measure and assess (Figure 5.19).

Hommel (1977) observed 30 - 60 amastigotes/100 leucocytes in smears of tail blood of nude mice 4 - 8 weeks post infection with *L. braziliensis*. Another preliminary experiment was designed using severe combined immunodeficiency (SCID) mice to compare the infectivity of *L. braziliensis* promastigotes cultured at acidic and neutral pH. Three female mice were inoculated each with 10^6 promastigotes subcutaneously at the base of the tail and were followed up weekly for lesion development. Also a blood smear was made weekly from a tail snip, stained with 10% Giemsa/phosphate buffer and checked under the microscope for peripheral parasites. The experiment was continued for 9 weeks and every 3rd week one mouse was killed and its liver and spleen was checked for the parasite. This was not successful and none of the 3 mice used developed lesions or visceral infection.

Figure 5.19. A photograph of a *L. brœziliensis* lesion in the footpad of a hamster.



5.5 CONCLUSION

Promastigotes in stationary-phase population of acidic cultures have the ultrastructural criteria of metacyclic promastigotes and are more infective *in vivo* than those obtained from neutral cultures.

Table 5.1. Infectivity of *L. donovani* promastigotes cultured at neutral and acidic pH to BALB/c mice.

Mouse No.	Liver weight (mg)	Number of amastigotes /1000 liver cell nuclei	Number of amastigotes /liver cell nuclei	LDU
1	1270	39	0.039	49.5
2	1330	none	none	0.0
3	1220	51	0.051	62.2
4	1120	none	none	0.0
5	1270	none	none	0.0
6	1130	none	none	0.0
7	1110	none	none	0.0
8	1390	40	0.041	57.0
9	1450	none	none	0.0
10	1350	none	none	0.0
11	1490	none	none	0.0
12	1320	none	none	0.0
13	1980	107	0.107	211.9
14	1940	194	0.194	376.4
15	1890	177	0.177	334.5
16	2150	311	0.311	686.7
17	2060	181	0.181	372.9
18	1680	none	none	0.0
19	1510	309	0.309	466.6
20	1410	121	0.121	170.6
21	1410	155	0.155	218.6
22	1260	144	0.144	181.4
23	1320	110	0.11	145.2
24	1260	117	0.117	147.2

Mice 1-12 inoculated with promastigotes cultured at pH 7.0
Mice 13-24 inoculated with promastigotes cultured at pH 5.5

Table 5.2. Infectivity of *L. mexicana* promastigotes cultured at neutral and acidic pH to BALB/c mice.

No.	Week								
	2	3	4	5	6	7	8	9	10
1	0	1.5	4.6	5.8	6.95	8.15	Experiment was stopped after week 7		
2	0	0	0	4.5	4.55	5.9			
3	0	0	0	3.05	4.25	5.05			
4	0	0	0	2.3	3.55	4.65			
5	0	0	0	0	2.55	4.35			
6	0	0	0	0	0	3.5			
7	1.55	2	2.5	3.55	4.5	5.65	6.25	7	6.85
8	0	1.35	1.95	2.55	2.85	5.2	5.75	6.45	6.4
9	0	0	1.8	3.15	4.4	5	5.6	6.9	7.5
10	1.35	2.6	2.9	3.85	3.75	4.55	6.2	6.5	6.85
11	0	1.7	1.85	2.6	3.8	4.8	5.15	4.75	5.1
12	0	1.85	2.4	2.6	3.65	4.35	4.7	6.15	6.75
13	2	3.25	5.3	7	8.05	9.05	Experiment was stopped after week 7		
14	0	3.4	4.28	5.55	5.15	8.75			
15	0	0	2.78	4.35	5	7.35			
16	0	0	5.13	5.65	6.35	8.5			
17	0	0	3.55	5.43	6	4.8			
18	0	0	0	4.7	6.65	5.6			
19	3.8	3.85	4	5.2	5.2	5.45	6.35	7	8.4
20	3.3	3.75	3.5	4	Died				
21	2.95	3.4	4.15	4.95	5.25	5.6	6	6.35	7.35
22	2.15	2.75	4	5.65	5.7	6.15	6.7	7.05	7.5
23	3.55	4	4.7	5.85	6.2	6.55	7.35	8.1	8.85
24	0	3.05	3.1	5	5.7	6.05	5.6	6.4	7.15

Mice 1-12 inoculated with promastigotes cultured at pH 7.0
Mice 13-24 inoculated with promastigotes cultured at pH 5.5

Table 5.3. Infectivity of *L.major* promastigotes cultured at neutral and acidic pH to BALB/c mice

No.	Week							
	3	4	5	6	7	8	9	10
1	0	0	0	0	2.4	3.5	4.8	4.35
2	0	0	0	0	0	2.3	3.3	4.4
3	0	0	0	1.8	2.9	4.5	3.6	6
4	0	0	1.45	2.2	2.8	4	3.8	5.1
5	0	0	1.6	2.3	4.45	5.65	5.7	6.6
6	0	0	0	0	0	0	0	3
7	0	0	0	2.95	3.5	4.45	5.2	5.85
8	0	0	0	0	0	0	0	0
9	0	0	Died					
10	0	1.7	2.6	3.7	4.35	5.8	3.95	5.3
11	0	0	0	3.3	4.4	5.35	6.65	6.6
12	0	0	0	0	2	3.05	5.25	6.2
13	0	2.35	3.35	5.9	6.9	7.2	6.75	8.55
14	0	0	2.45	3.75	7.15	6.95	8.15	7.2
15	0	0	0	0	4.65	7.3	7.55	8.5
16	2.55	2.9	2.55	3.6	3.5	4.5	4.6	5
17	0	2.15	2.5	2	3.55	4	4.5	5.25
18	0	0	0	0	3.8	4.1	4.65	5.75
19	1.55	2.5	2.6	4.1	4.6	4.05	4.55	5.2
20	0	0	2.45	3.6	2.95	5	5.3	6.15
21	0	0	0	0	2.55	4.55	4.8	6
22	1.5	2.7	3.25	4.5	3.75	5.5	5.5	6.2
23	0	2.25	2.65	4	4.45	4.4	4.85	6.8
24	0	0	0	2.6	2.65	4.45	5	5.95

Mice 1-12 inoculated with promastigotes cultured at pH 7.0
Mice 13-24 inoculated with promastigotes cultured at pH 5.5

Table 5.4. Infectivity of *L.brœziliensis* promastigotes cultured at neutral and acidic pH to BALB/c mice.

No.	Week								
	2	3	4	5	6	7	8	9	10
1	0	0	2.95	2	2.5	1.95	2	1.75	1.5
2	0	0	2.1	2.95	2.75	2.3	2.45	2.4	1.9
3	0	0	0	0	0	0	0	0	0
4	2.25	2.5	3.1	2.05	2.6	2	2.2	2.15	2.1
5	1.7	2.4	2.5	2.8	3.2	2.65	2.2	2.05	1.7
6	0	0	0	3	2	3.15	2.25	2.1	1.75
7	Died								
8									
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	2.8	3.2	4	3.35	3
11	0	0	2	2.45	3.7	3.75	3.5	3.55	3
12	1.9	2.75	3.45	3.85	3.2	3.7	3.4	3	2.85
13	2.45	2.8	3	2.2	3.45	2.5	2.35	2.4	2.1
14	2.3	2	3.35	3.35	3.5	3	2.85	2.1	1.5
15	0	2.75	3	4.05	3.5	2.8	2.1	2.25	1.95
16	2.4	3.75	3.35	2.4	2.65	2.65	3.4	2.05	1.5
17	0	3.5	2	2.65	2.3	3.75	3.75	1.65	1.3
18	0	0	3	3.35	3.45	2.45	2.75	1.95	1.45
19	3.7	4.8	5.75	7.05	7.75	7.3	7	7.3	6.9
20	2.2	1.9	3.35	3.4	2.5	3.25	3	3.15	3
21	2.35	3.05	3.5	4.2	4.05	5	4.1	3.85	3.65
22	2.75	3.75	4.25	4.35	4.65	4.95	5	4.75	4.55
23	0	0	0	1.9	2.95	3.8	2.75	3.15	3.5
24	2	2.35	2.55	3	3.65	4.15	4.4	4.15	3.85

Mice 1-12 inoculated with promastigotes cultured at pH 7.0
Mice 13-24 inoculated with promastigotes cultured at pH 5.5

CHAPTER SIX

6. DISCUSSION

6.1 GENERAL DISCUSSION

Leishmaniasis is caused by infection with parasites of the genus *Leishmania*. This collection of diseases is widespread geographically and each geographical zone is characterized by its own complex of parasites. The severity of the disease ranges from self-healing cutaneous lesions to fatal visceral disease. The incidence of leishmaniasis is increasing world wide and the disease is seriously hampering productivity and vitally needed socio-economic progress (WHO, 1995). The latest WHO estimate of the prevalence of disease is 12 million people, with 350 million at risk of acquiring the infection. The annual incidence of new infections was estimated at 1 to 1.5 million cases of cutaneous leishmaniasis and 500 000 cases of visceral leishmaniasis (WHO, 1995).

In order to understand this disease which is creating serious public health problems in many countries in the world, it is essential to study the causative agent, the *Leishmania* parasites. *Leishmania* parasites can be found either as promastigotes in the sandfly and *in vitro* cultures or as amastigotes in the macrophages of the vertebrate host or from culture derived cell lines. Amastigotes are usually isolated from infected animals and/or from macrophage cultures. Their isolation is usually laborious and there is always a doubt about the purity of such amastigotes. The development of a

cell free culture system for the large scale cultivation of these amastigotes is attracting the attention of many workers. Large quantities of pure amastigotes can be obtained by such culture systems and the study of their biological, biochemical, immunological and molecular aspects can be performed more easily and with more confidence.

In this study, a method for the axenic cultivation of *L. donovani* amastigotes has been developed and the optimum conditions for this culture system are described in chapter 2. In this method, freshly transformed promastigotes of *L. donovani* are cultured in an acidic culture medium (preferably HO-MEM medium), pH 5.5, in the presence of 5% CO_2 at 37° C. Amastigotes produced by this method were very similar to amastigotes obtained from tissue in terms of morphology, sizes, ultrastructure and protein banding pattern.

In a culture system, it is very difficult and almost impossible to mimic the conditions in the phagolysosomes of macrophages. Even though very little is known about this environment, it is believed that it is a hostile environment for the *Leishmania* parasite. As metacyclic promastigotes enters the vertebrate host, they experience an increase in temperature. This has led many investigators to attempt to transform promastigotes to amastigotes using elevated temperature and this often resulted in the rounding of promastigotes to amastigote-like cells which rarely survived for more than a few days. Although *Leishmania* parasites and macrophages are involved in a marriage of inconvenience, the parasites have either adapted to such conditions and evolved means for survival in the presence of microbicidal agents or produced factors to destroy or neutralize the toxic molecules produced by the macrophage (Antoine *et al*., 1990). One of the key factors in developing axenic cultures for *Leishmania* is the pH.

Parasitophorous vacules of *L. amazonensis*-infected macrophages are known to maintain an acidic pH of 4.74-5.26 (Antoine *et al.*, 1990). This led to the development of a new method for the axenic cultivation of *L. mexicana* amastigotes (Bates *et al.*, 1992). In this study, a pH of 5.5 was found to be optimal for the axenic cultivation of *L. donovani* amastigotes. It has been shown that exposure of various *Leishmania* promastigotes to acidic pH induces the synthesis of amastigote-specific protein (Zilberstein *et al.*, 1991). Also, *L. donovani* amastigotes are metabolically more active in acidic pH and are able to regulate their internal pH to neutral when the external pH is acidic (Mukkada *et al.*, 1985; Glaser *et al.*, 1988). Further, trypomastigotes of *Trypanosoma cruzi* transformed into amastigotes when incubated at pH 5.0. The resultant amastigotes were ultrastructurally and biochemically indistinguishable from natural amastigotes (Tomlinson *et al.*, 1995). It seems that the pH, perhaps with other factors, triggers the transformation to the amastigote stage among Trypanosomatidae.

With regard to gas conditions, very little is known regarding the gas requirements of amastigotes. In this study, it has been shown that *L. donovani* amastigote require a high concentration of CO_2 (5%) in order to grow axenically. Also, a reduced concentration of oxygen (6%) slightly improved their growth axenically. Gas conditions have also been shown to affect amastigote to promastigote transformation and promastigote metabolism. High CO_2 concentration increased the rate of amastigote to promastigote transformation in *L. mexicana*, and glucose consumption in *L. major* promastigotes increased as the concentration of oxygen was reduced to 6% (Hart and Coombs, 1981; Keegan and Blum, 1990).

Several criteria have been used to characterize these axenic amastigotes. Morphologically, they were very similar to tissue amastigotes being round to oval cells with approximately similar sizes and lacking an extension of the flagellum beyond the cell body. However, sizes of axenic amastigotes were larger than those of tissue amastigotes and this difference in sizes was statistically significant. This difference was expected since the rate of multiplication of axenic amastigotes was far higher than that of amastigotes in the tissue. Also, the environment surrounding amastigotes in tissue is more hostile compared to the liquid culture medium in the culture flask.

Ultrastructural similarities between axenic and tissue amastigotes have been shown in this study. In addition to termination of the flagellum at the cell surface, axenic and tissue amastigotes lack the paraxial rod seen in promastigotes. Several workers reported that the paraxial rod in promastigotes is present in parts of the flagellum outside the flagellar pocket (Vickerman and Tetley, 1990; Bastin *et al*., 1996). In this study, it has been shown that the paraxial rod in promastigotes can be present and is seen in parts of the flagellum inside the flagellar pocket and this justifies the use of its presence or absence to differentiate between amastigotes and promastigotes.

Similarities between axenic and tissue amastigotes, with respect to their general protein banding pattern, have been shown in this study. A variety of bands recovered from lysate of axenic amastigotes, tissue amastigotes and promastigotes were observed on SDS-PAGE. These include a prominent band with a molecular weight of 51.3 kDa in promastigotes and 66.1 kDa in axenic and tissue amastigotes. This agrees with the findings of other workers who reported similarities between axenic and tissue amastigotes and differences from promastigotes (Coombs *et al*., 1982; Galvao-Quintao *et al*., 1990; Bates *et al*., 1992; Robertson and Coombs, 1992; Bates, 1993a).

Part of the life cycle of *Leishmania* parasites occurs in the sandfly. Here, *Leishmania* promastigotes undergo multiplication and differentiation into metacyclic promastigotes, the infective stage for mammals (reviewed by Molyneux and Killick-Kendrick, 1987). Two of the early reports on the transmission of *Leishmania* were those of Shortt *et al.* (1931) and Adler and Ber (1941). In these reports it was believed that promastigotes in the sandfly differentiate into infective, later named metacyclic, promastigotes. Many other workers have observed morphological differences between dividing midgut promastigotes and those found anteriorly (reviewed by Killick-Kendrick, 1979). Furthermore, several workers observed metacyclic promastigotes in axenic cultures and several studies have been conducted to identify different molecules expressed by metacyclic promastigotes and their role in the survival of the parasite *in vivo* (Sacks, 1988).

Metacyclogenesis is believed to be an important stage in the life cycle of *Leishmania* parasites. Therefore, to gain more knowledge about the transmission of the parasite, metacyclic promastigotes must be studied vigorously. This involves the acquisition of large quantities of pure metacyclic promastigotes. Up to date, and excepting the report on induction of metacyclogenesis in *L. mexicana* by Bates and Tetley (1993), all studies involving metacyclic promastigotes were conducted using promastigotes from stationary-phase cultures which include a variable percentage of metacyclic promastigotes. This indicates the need for a culture system to produce an homogenous population of metacyclic promastigotes. Bates and Tetley (1993) reported a method to produce a relatively homogeneous stationary-phase population of metacyclic promastigotes of *L. mexicana*. This novel method involves the cultivation of freshly transform promastigotes at an acidic pH of 5.5. Promastigotes obtained in stationary-

phase populations of such cultures expressed the structural and functional properties of metacyclic promastigotes. Also, the anterior parts of the gut of non-infected sandflies have a slightly acidic pH when compared with other parts of the gut (Bates, pers. comm.).

Metacyclic promastigotes are characterized by their short cell body, long flagellum, which is twice the length of the cell body, and high motility (Howard *et al.*, 1987; Bates and Tetley, 1993).

Biochemically, metacyclic promastigotes are different from procyclic promastigotes in several aspects. The isoenzyme content of metacyclic promastigotes differ from that of procyclic promastigotes and is more similar to that of amastigotes (Mallinson and Coombs, 1989). Also, *L.mexicana* metacyclic promastigotes expressed detectable cysteine proteinases activities which were lacking in procyclic promastigotes (Bates *et al.*, 1994). This supports the hypothesis that metacyclic promastigotes are more adapted to life in the vertebrate host than procyclic promastigotes. Also, metacyclic promastigotes expressed surface antigenic determinants different from those of non-infective procyclic promastigotes (Rizvi *et al.*,1985).

Another major difference between metacyclic and procyclic promastigotes is the relative resistance to complement mediated lysis (Howard *et al.*, 1987; Bates and Tetley, 1993). Metacyclic promastigotes do not only resist lysis by the complement components, they use the complement system to gain entry and parasitize macrophages (Mosser and Edelson, 1984; Joiner, 1988; Da Silva *et al.*, 1989).

The most important criteria expressed by metacyclic promastigotes is their relatively higher infectivity *in vitro* and *in vivo*. These criteria have been examined and

confirmed by several previous reports (Giannini, 1974; Doran and Herman, 1981; Da Silva and Sacks, 1987, Howard *et al.*, 1987; Sacks, 1988; Rey *et al.*, 1990; Almeida *et al.*, 1993; Bates and Tetley, 1993).

In this study, metacyclic promastigotes of *L. donovani, L. mexicana, L. major* and *L. braziliensis* were produced by culturing promastigotes at pH 5.5. The identity of these metacyclic promastigotes was confirmed after studying their characteristics with respect to size, morphology, resistance to complement-mediated lysis and infectivity *in vivo*.

Metacyclic promastigotes obtained by this method were smaller in size than procyclic promastigotes (≤ 8 µm x 1.5 µm). They possessed a long flagellum which was twice or more the length of the cell body and they were highly motile. Ultrastructural examination of such promastigotes of *L. donovani* and *L. mexicana* showed that they possess a fuzzy surface coat, measuring 18 and 11 nm respectively which was not seen in promastigotes cultured in neutral pH. This agrees with findings of Pimenta *et al.*, (1989 & 1991) and Bates and Tetley (1993). The shortening of their cell body brought the kinetoplast in close proximity to the nucleus. They also showed higher resistance to complement-mediated lysis. A 4 to 16 fold increase in resistance was observed among the different species studied. They were also more infective to BALB/c mice than promastigotes cultured at pH 7.0.

This study clearly indicates that metacyclogenesis can be triggered *in vitro* by acidity and that this phenomenon is conserved among the different species of *Leishmania* used in this study.

It was also shown in this study that adaptation of promastigotes to *in vitro* cultures can lead to the loss of their ability to undergo metacyclogenesis. The method by which they become adapted to cultures and, subsequently, lose their ability to undergo metacyclogenesis is not yet known. However, preliminary results suggest that routine exposure of promastigotes to acidic pH can contribute to their adaptation to such pH and, subsequently, acidity does not trigger metacyclogenesis in such promastigotes. This is supported by the findings that promastigotes cultured at a constant neutral pH were more able to undergo metacyclogenesis than those exposed to acidic pH each time the culture reached stationary-phase. Furthermore, the loss of the ability of promastigotes to undergo metacyclogenesis and, subsequently, the loss of their infectivity have been reported in the literature (Nolan and Herman, 1985; Segovia *et al.*, 1992).

6.2 FUTURE DIRECTIONS

Several lines of research can be conducted in the future as a continuation of this study. One of the most obvious developments is to improve the axenic culture system for *L. donovani* parasites. Also, to try and develop methods for the axenic cultivation of all *Leishmania* parasites. It is difficult, or even impossible, to develop a culture system that can be used to grow all *Leishmania* parasites axenically. However, it would be quite an achievement if the optimum conditions for the axenic cultivation of each species of *Leishmania* parasite are determined. This can eventually lead to ease in

obtaining amastigotes of different *Leishmania* species in large quantities to be able to undertake further studies regarding their biology, biochemistry, relation with the host and the molecular mechanism involved in their transformation.

Another major type of research that can benefit from such bulk production of amastigotes is chemotherapy research. Up to date, most of the research involving drugs and their effect on the *Leishmania* parasites are conducted either *in vivo* on animal models or *in vitro* on *Leishmania*-infected macrophages. Such research can be laborious and expensive and the use of axenic amastigotes in such research could be beneficial in several aspects including reducing the cost and time and observing the direct effect of a drug on amastigotes.

Manipulation of the culture conditions to reproduce the life cycle of *Leishmania* parasites in axenic culture can now be performed after describing methods for axenic culture of amastigotes and induction of metacyclogenesis. This *in vitro* life cycle can then be manipulated in different directions to compare with the natural *in vivo* life cycle. Such comparisons will provide more understanding of the relationship of the parasite with the vertebrate and the invertebrate host.

With regard to vaccines, there is no available vaccine for protection against leishmaniasis to date. Trials of a first generation vaccine, composed of killed parasites, have been and are still in progress (Modabber, 1995). Trials of a second generation vaccine, composed of genetically manipulated parasites or various molecules, are still in preclinical stages. Parasites used in these vaccines are *in vitro* cultured

promastigotes of different *Leishmania* species which may or may not contain metacyclic promastigotes. The use of metacyclic promastigotes in immunization or challenge is more sensible since these are the forms that are infective to mammals. The use of the metacyclogenesis induction method described in this study can be beneficial in providing large quantities of pure metacyclic promastigotes.

REFERENCES

Adler, S., and Ber, M. (1941). The transmission of *Leishmania tropica* by the bite of *Phelebotomus papatasii*. *Indian Journal of Medical Research*, **29**, 803-809.

Al-Bashir, N. T., Rassam, M. B., and Al-Rawi, B. M. (1992). Axenic cultivation of amastigotes of *Leishmania donovani* and *Leishmania major* and their infectivity. *Annals of Tropical Medicine and Parasitology*, **86**, 487-502.

Alexander, J., and Russell, D. G. (1989). A novel method of vaccination using parasite membrane antigens. *In*: Leishmaniasis: The Current Status and New Strategies for Control (D.T.Hart, Ed), pp. 359-366. Plenum: New York.

Alexander, J., and Russell, A. G. (1992). The interaction of *Leishmania* species with macrophages. *Advances in Parasitology*, **31**, 175-254.

Almeida, M. C., Cuba, C. A. C., Sa, C. M., Pharoah, M. M., Howard, K. M., and Miles, M. A. (1993). Metacyclogenesis of *Leishmania (Viannia) braziliensis in vitro*: evidence that lintil lectin is a marker of complemet resistance and enhanced infectivity. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **87**, 325-329.

Al-Zahrani, M. A., Peters, W., and Evans, D. A. (1988). Visceral leishmaniasis in man and dogs in south-west Saudi Arabia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **82**, 857.

Al-Zahrani, M. A., Peters, W., Evans, D. A., Smith, V., and Ching Chin (1989). *Leishmania* infecting man and wild animals in Saudi Arabia. 5. Diversity of parasites causing visceral leishmaniasis in man and dogs in the South-West. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **83**, 503-510.

Antoine, J., Prina, E., Jouanne, C., and Bongrand, P. (1990). Parasitophorous vacules of *Leishmania amazonensis*-infected macrophages maintain an acidic pH. *Infection and Immunity*, **58**, 779-787.

Ashford, R. W., and Bettini, S. (1987). Ecology and epidemiology: Old World. *In*: The Leishmaniases in Biology and Medicine (W.Peters, and R.Killick-Kendrick, Eds), Vol.1, pp. 365-424. Academic Press: London.

Ashford, R. W., Desjeux, P., and deRaadt, P. (1992). Estimation of population at risk of infection and number of cases of leishmaniasis. *Parasitology Today*, **8**, 104-105.

Azazy, A. A., Devaney, E., and Chance, M. L. (1994). A PEG-ELISA for the detection of *Leishmania donovani* antigen in circulating immune complexes. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **88**, 62-66.

Bandyopadhyay, P., Ghosh, D. K., De, A., Ghosh, K. N., Chaudhuri, P. P., Das, P., and Bhattacharya, A. (1991). Metacyclogenesis of *Leishmania* spp: species-specific *in vitro* transformation, complement resistance, and cell surface carbohydrate and protein profiles. *Journal of Parasitotlogy*, 77, 411-416.

Barker, D. C. (1989). Assessment of the use in the diagnosis of leishmaniasis of biotinylated kinetoplast derived DNA sequences. *In*: Leishmaniasis: The Current Status and New Strategies for Control (D.T.Hart, Ed), pp. 503-513. Plenum: New York.

Bastin, P., Matthews, K. R., and Gull, K. (1996). The paraflagellar rod of kinetoplastida: solved and unsolved questions. *Parasitology Today*, **12**, 302-307.

Bates, P. A. (1993a). Axenic culture of *Leishmania* amastigotes. *Parasitology Today*, **9**, 143-146.

Bates, P. A. (1993b). Characterization of developmentally-regulated nucleases in promastigotes and amastigotes of *Leishmania mexicana*. *FEMS Microbiology Letters*, **107**, 53-58.

Bates, P. A. (1994a). Complete developmental cycle of *Leishmania mexicana* in axenic culture. *Parasitology*, **108**, 1-9.

Bates, P. A. (1994b). The developmental biology of *Leishmania* promastigotes. *Expermintal Parasitology*, **79**, 215-218.

Bates, P. A., and Dwyer, D. M. (1987). Biosynthesis and secretion of acid phosphatase by *Leishmania donovani* promastigotes. *Molecular and Biochemical Parasitology*, **26**, 289-296.

Bates, P. A., Robertson, C. D., and Coombs, G. H. (1994). Expression of cysteine proteinases by metacyclic promastigotes of *Leishmania mexicana*. *Journal of Eukaryotic Microbiology*, **41**, 199-203.

Bates, P. A., Robertson, C. D., Tetley, L., and Coombs, G. H. (1992). Axenic cultivation and characterization of *Leishmania mexicana* amastigote-like forms. *Parasitology*, **105**, 193-202.

Bates, P. A., and Tetley, L. (1993). *Leishmania mexicana*: Induction of metacyclogenesis by cultivation of promastigotes at acidic pH. *Expermintal Parasitology*, **76**, 412-423.

Beaver, P. C., Jung, R. C., and Cupp, E. W. (1984). Clinical Parasitology. 9th edn., p. 825. Lea & Febiger: Philadelphia.

Berens, R. L., Brun, R., and Krassner, S. M. (1976). A simple monophasic medium for axenic cultivation of hemoflagellates. *The Journal of Parasitology*, **62**, 360-365.

Bradley, D. J., and Kirkley, J. (1977). Regulation of *Leishmania* populations within the host. I. The variable course of *Leishmania donovani* infections in mice. *Clinical and Experimental Immunology*, **30**, 119-129.

Bryceson, A. D. M. (1996). Leishmaniasis. *In*: Manson's Tropical Diseases (G.Cook, Ed), pp. 1213-1245. W.B. Saunders: London.

Castilla, J. J., Sanches-Moreno, M., Mesa, C., and Osuna, A. (1995). *Leishmania donovani: In vitro* culture and [1H] NMR charachterization of amastigote-like forms. *Molecular and Cellular Biochemistry*, **142**, 89-97.

Chang, K. P., and Chaudhuri, G. (1990). Molecular determinants of *Leishmania* virulance. *Annual Review of Microbiology*, **44**, 499-529.

Chang, K. P., Fong, D., and Bray, R. S. (1985). Biology of *Leishmania* and leishmaniasis. *In*: *Leishmaniasis* (K. P. Chang, and R. S. Bray, Eds), pp. 1-30. Elsevier: Amsterdam.

Childs, G. E., Foster, K. A., and McRoberts, M. J. (1978). Insect cell culture media for the cultivation of new world *Leishmania*. *International Journal for Parasitology*, **8**, 255-258.

Coombs, G. H. (1982). Proteinaes of *Leishmania mexicana* and other flagellate protozoa. *Parasitology*, **84**, 149-155.

Coombs, G. H., Craft, J. A., and Hart, D. I. (1982). A comparative study of *Leishmania mexicana* amastigotes and promastigotes. Enzyme activities and subcellular locations. *Molecular and Biochemical Parasitology*, **5**, 199-211.

Coombs, G. H., Robertson, C. D., and Mottran, J. C. (1991). Cystein proteinases of leishmanias. *In*: Biochemical Protozoology (G. H. Coombs, and M. J. North, Eds), pp. 208-220. Taylor & Francis: London.

Darling, T. H., and Blum, J. J. (1987). *In vitro* reversible transformation of *Leishmania braziliensis panamensis* between promastigotes and ellipsoidal forms. *Journal of Protozoology*, **34**, 166-168.

Da Silva, R. P., Hall, B. F., Joiner, K. A., and Sacks, D. L. (1989). CR1, the C3b receptor, mediated binding of infective *Leishmania major* metacyclic promastigotes to human macrophages. *The Journal of Immunology*, **143**, 617-622.

Da Silva, R., and Sacks, D. (1987). Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. *Infection and Immunity*, **55**, 2802-2806.

Davies, C. R., Cooper, A. M., Peacock, C., Lane, R. P., and Blackwell, J. M. (1990). Expression of LPG and GP63 by different developmental stages of *Leishmania major* in the sandfly *Phlebotomus papatasi*. *Parasitology*, **101**, 337-343.

Davis, J. M. (1994). Basic Cell Culture. p. 301. Oxford University Press: Oxford.

Doran, T. I., and Herman, R. (1981). Characterization of population of promastigotes of *Leishmania donovani*. *Journal of Protozoology*, **28**, 345-350.

Doyle, P. S., Engel, J. C., Pimenta, P. F. P , Pinto Silva, P., and Dwyer, D. M. (1991). *Leishmani donovani:* long-term culture of axenic amastigotes at 37° C. *Expermintal Parasitology*, **73**, 326-334.

Duarte, Z., Munguia, J., Orozco, M., and Gantier, J. (1994). First report of visceral leishmaniasis in Nicaragua. *Transaction of the Royal Society of Tropical Medicine and Hygiene*, **88**, 38.

Eilam, Y., El-On, J., and Spira, D. T. (1985). *Leishmania major:* excreted factors, calcium ions, and the survival of amastigotes. *Expermintal Parasitology*, **59**, 161-168.

Elbihari, S., Cheema, A. H., and El-Hassan, A. M. (1987). *Leishmania* infecting man and wild animals in Saudi Arabia. 4. Canine cutaneous leishmaniasis in the Eastern province. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **81**, 925-927.

El-On, J., Bradley, D. J., and Freeman, J. C. (1980). *Leishmania donovani:* action of excreted factor on hydrolytic enzyme activity of macrophages from mice with genetically different resistance to infection. *Expermintal Parasitology*, **49**, 167-174.

El Safi, S. H., and Evans, D. A. (1989). A comparison of the direct agglutination test and enzyme-linked immunosorbent assay in the sero-diagnosis of leishmaniasis in the Sudan. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **83**, 334-337.

Eperon, S., and MacMahon-Pratt, D. (1989a). Extracellular amastigote-like forms of *Leishmania panamensis* and *L. braziliensis* and species-specific monoclonal antibodies. *Journal of Protozoology*, **36**, 510-518.

Eperon, S., and MacMahon-Pratt, D. (1989b). I. Extracellular cultivation and morphological characterization of amastigote-like forms of *Leishmania panamensis* and *L. braziliensis*. *Journal of Protozoology*, **36**, 502-510.

Evans, D. A. (1987). Leishmania. *In*: *In vitro* methods for parasite cultivation (A. E. R. Taylor, and J. R. Baker, Eds), pp. 52-75. Academic Press: London.

Farina, M., Attias, M., Souto-Padron, T., and De Souza, W. (1986). Further studies on the organization of the paraxial rod of Trypanosomatids. *Journal of Protozoology*, **33**, 552-557.

Fehniger, T. E., Mongistu, G., Gessesse, A., Gabre-Mariam, H., and Akuffo, H. (1990). Changes in the antigenic profile of *Leishmania* parasites following shifts in temperature. *Acta Tropica*, **47**, 227-236.

Fong, D., and Chang, K. (1981). Tubulin biosynthesis in the developmental cycle of a parasitic protozoan, *Leishmania mexicana*: changes during differentiation of motile and nonmotile stages. *Proceedings of The National Academy of Science USA*, **78**, 7624-7628.

Fong, D., Wallach, M., Keithly, J., Melera, P. W., and Chang, K. P. (1984). Differential expression of mRNAs for alpha and beta tubulin during differentiation of the parasitic protozoan *Leishmania mexicana*. *Proceedings of The National Academy of Science USA*, **81**, 5782-5786.

Franke, E. D., McGreevy, P. B., Katz, S. P., and Sacks, D. L. (1985). Growth cycle-dependent generation of complement-resistance *Leishmania* promastigotes. *The Journal of Immunology*, **134**, 2713-2718.

Fuge, H. (1969). Electron microscopic studies on the intra-flagellar structure of trypanosomes. *Journal of Protozoology*, **16**, 460-466.

Galvao-Quintao, L., Alfeiri, S. C., Ryter, A., and Rabinovitch, M. C. (1990). Intracellular differentiation of *Leishmania amazonensis* promastigotes to amastigotes: presence of megasomes, cystein proteinase activity and susceptibility to leucine-methyl ester. *Parasitology*, **101**, 7-13.

Gardener, D. J., Shchory, L., and Chance, M. L. (1977). Species differentiation in the genus *Leishmania* by morphometric studies with the electron microscope. *Annals of Tropical Medicine and Parasitology*, **71**, 147-155.

Giannini, M. S. (1974). Effects of promastigote growth phase, frequancy of subculture, and host age on promastigote-initiated infection with *Leishmania donovani* in the golden hamster. *Journal of Protozoology*, **21**, 521-527.

Glaser, T. A., Baatz, J. E., Kreshman, G. P., and Makkada, A. J. (1988). pH Homeostasis in *Leishmania donovani* amastigotes and promastigotes. *Proceedings of The National Academy of Science USA*, **85**, 7602-7606.

Gupta, S., Srivastava, S., Ray, S., Chandra, R., Srivastava, V. K., and Katiyar, J. C. (1993). Evaluation of enzyme-linked immunosorbent assay in the diagnosis of kala-azar in malda district (West Bengal). *Indian Journal of Medical Research*, **97**, 242-246.

Hams, B. D., and Rickwood, D. (1990). Gelel Ectrophoresis of Proteins. 2nd edn., p. 383. Oxford University Press: Oxford.

Handman, E., Schnur, L. F., Spithill, T. W., and Mitchell, G. F. (1986). Passive transfer of *Leishmania* lipopolysaccharide confers parasite survival in macrophages. *The Journal of Immunology*, **137**, 3608-3613.

Harith, A. E., Chowdhury, S., Al-Masum, A., Semiao-Santos, S., Karim, E., El-Safi, S., and Haque, I. (1995). Evaluation of cleaving agents other that trypsin in direct agglutination test for further improving diagnosis of visceral leishmaniasis. *Journal of Clinical Microbiology*, **33**, 1984-1988.

Harith, A. E., Kolf, A. H. J., Kager, P. A., Leeuwenburg, J., Muigai, R., Kiugu, S., Kiugo, S., and Laarman, J. J. (1986). A simple and economical direct agglutination test for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80**, 583-587.

Harith, A. E., Kolk, A. H. J., Leeuwenburg, J., Muigai, R., Huigen, E., Jelsma, T., and Kager, P. A. (1988). Improvement of a direct agglutination test for field studies of visceral leishmaniasis. *Journal of Clinical Microbiology*, **26**, 1321-1325.

Hart, D. T., and Coombs, G. H. (1981). The effect of carbon dioxide and oxygen upon the growth and *in vitro* transformation of *Leishmania mexicana mexicana*. *Molecular and Biochemical Parasitology*, **4**, 117-127.

Hayat, M. A. (1989). Principles and Techniques of Electron Microscopy. 3rd edn., p. 469. Macmillan: London.

Hendricks, L. D., Wood, D. E., and Hajduk, M. E. (1978). Haemoflagellates: Commercially available liquid media for rapid cultivation. *Parasitology*, **76**, 309-316.

Hommel, M. (1977). New approaches to the experimental study of leishmaniasis. Ph.D. thesis, University of Liverpool, Liverpool

Hommel, M., Jaffe, C. L., Travi, B., and Milon, G. (1995). Experimantal models for leishmaniasis and for testing anti-leishmanial vaccines. *Annals of Tropical Medicine and Parasitology*, **89s1**, 55-73.

Howard, M. K., Sayers, G., and Miles, M. A. (1987). *Leishmania donovani* Metacyclic promastigotes: Transformation *in vitro*, lectin agglutination, complement resistance, and infectivity. *Expermintal Parasitology*, **64**, 147-156.

Howard, M. K., Sayers, G., and Miles, M. A. (1989). Characterization of *Leishmania donovani* infective (metacyclic) promastigotes from axenic cultures. *In*: Leishmaniasis: The Current Status and New Strategies for Control (D.T.Hart, Ed), pp. 267-270. Plenum: New York.

Ibrahim, E. A., Al-Zahrani, M. A., Al-Tuwaigri, A. S., AL-Shammary, F. J., and Evans, D. A. (1992). *Leishmania* infecting man and wild animals in Saudi Arabia. 9. The black rat (*Rattus rattus*) a probable reservoir of visceral leishmaniasis in Gizan province, south-west Saudi Arabia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **86**, 513-514.

Ibrahim, E. A., Mustafa, M. B., Al Amri, S. A., Al Seghayer, S. M., Hussein, S. M., and Gradoni, L. (1994). *Meriones libycus* (Rodentia: Gerbillidae), a possible reservoir

host of zoonotic cutaneous leishmaniasis in Riyadh province, Saudi Arabia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **88**, 39.

Joiner, K. A. (1988). Molecular basis for interactions between parasites and the complement cascade. *In*: The Biology of Parasitism (P.T.Englund, and A.Sher, Eds), pp. 309-328. Alan R.Liss: New York.

Joshi, M., Dwyer, D. M., and Nakhashi, H. L. (1993). Cloning and characterization of differentially exprssed genes from *in vitro*-grown amastigotes of *Leishmania donovani*. *Molecular and Biochemical Parasitology*, **58**, 345-354.

Kager, P. A., Leeuwenburg, J., Muigai, R., Kiugu, S., Iha, D. W., Mwaniki, J. K., Koech, D. K., and Harith, A. E. (1989). Field application of direct agglutination test for visceral leishmaniasis. *In*: Leishmaniasis: The Current Status and New Strategies for Control (D.T.Hart, Ed), pp. 89-94. Plenum: NewYork.

Kallinikova, V. D., Nasyrova, R. M., Nasyrov, F. S., and Safjanova, V. M. (1992). Morphogenesis and virulence of *Leishmania major* in the process of long-term cultivation. *Archiv fur Protistenkunde*, **141**, 327-334.

Kaneshiro, E. S., Gottlieb, M., and Dwyer, D. M. (1982). Cell surface origin of antigens shed by *Leishmania donovani* during growth in axenic culture. *Infection and Immunity*, **37**, 558-567.

Keegan, F., and Blum, J. J. (1990). Effect of oxygen concentration on the intermediary metabolism of *Leishmania major* promastigotes. *Molecular and Biochemical Parasitology*, **39**, 235-246.

Killic-Kendrick, R., Leaney, A. J., Peters, W., Rioux, J.-A., and Bray, R. S. (1985). Zoonotic cutaneous leishmaniasis in Saudi Arabia: the incrimination of *Phlebotomus papatasi* as the vector in the Al-HAssa oasis. *Transaction of the Royal Society of Tropical Medicine and Hygiene*, **79**, 252-255.

Killick-Kendrick, R. (1979). Biology of *Leishmania* in phlebotomine sand flies. *In*: Biology of the Kinetoplastida (W.H.R. Lumsden, and D.A. Evans, Eds), Vol.2, pp. 395-460. Academic Press: New York/Lnodon.

Killick-Kendrick, R., Molyneux, D. H., Rioux, J. A., and Leaney, A. J. (1980). Possible origins of *Leishmania chagasi*. *Annals of Tropical Medicine and Parasitology*, **74**, 563-565.

Kimsey, P. B., Theodos, C. M., Mitchen, T. K., Turco, S. J., and Titus, R. G. (1993). An avirulent lipophosphoglycan-deficient *Leishmania major* clone induces CD4 T cells which protect susceptible BALB/c mice against infection with virulent *L.major*. *Infection and Immunity*, **61**, 5205-5213.

Kweider, M., Lemesre, J. L., Santoro, F., Kusnierz, J. P., Sadigursky, M., and Capron, A. (1989). Development of metacyclic *Leishmania* promastigotes is associated with

the increasing expression of GP65, the major surface antigene. *Parasite Immunology*, **11**, 197-209.

Lemma, A., and Schiller, E. L. (1964). Extracellular cultivation of the leishmanial bodies of species belonging to the protozoan genus *Leishmania*. *Expermintal Parasitology*, **15**, 503-513.

Lockwood, B. C., North, M. J., Mallinson, D. J., and Coombs, G. H. (1987). Analysis of *Leishmania* proteinase reveals developmental changes in species-specific forms and a common 68 kDa activity. *FEMS Microbiology Letters*, **48**, 345-350.

Mallinson, D. J., and Coombs, G. H. (1989). Biochemical characteristics of the metacyclic forms of *Leishmania major* and *L. mexicana mexicana*. *Parasitology*, **98**, 7-15.

McConville, M. J., Turco, S. J., Ferguson, M. A. J., and Sacks, D. L. (1992). Developmental modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. *The EMBO Journal*, **11**, 3593-3600.

Modabber, F. (1995). Vaccines against leishmaniasis. *Annals of Tropical Medicine and Parasitology*, **89s1**, 83-88.

Mohareb, E. W., Boghdady, A. M., Bassiouny, G. A., and Eassa, A. H. (1995). Viscerotropic *Leishmania tropica*: transformation to amastigotes in cell-free media. *Journal of the Egyptian Society of Parasitology*, **25**, 883-891.

Molyneux, D. H. and Ashford, R. W., (1983). The Biology of *Trypanosoma* and *Leishmania* Parasites of Man and Domestic Animals. p. 294. Taylor and Francis: London.

Molyneux, D. H., and Killick-Kendrick, R. (1987). Morphology, ultrastructure and life cycles. *In*: The Leishmaniases in Biology and Medicine (W. Peters, and R. Killick-Kendrick, Eds), Vol.1, pp. 121-176. Academic Press: London.

Momen, H., and Grimaldi, G. (1989). Enzyme electrophoritic evidence for the importation of *Leishmania infantum* into the new world. *In*: Leishmaniasis: The Current Status and New Strategies for Control (D. T. Hart, Ed), pp. 911-916. Plenum: New York.

Morsy, T. A. (1996). Cutaneous leishmaniasis in Egypt (review and comment). *Journal of the Egyptian Society of Parasitology*, **26**, 105-130.

Mosser, D. M., and Edelson, P. J. (1984). Activation of the alternative complement pathway by *Leishmania* promastigotes: parasite lysis and attachement to macrophages. *The Journal of Immunology*, **132**, 1501-1505.

Mukkada, A. J., Meade, J. C., Glaser, T. A., and Bonventre, P. F. (1985). Enhanced metabolism of *Leishmania donovani* amastigotes at acid pH: an adaptation for intracellular growth. *Science*, **229**, 1099-1101.

Mustafa, M. B., Hussein, S. M., Ibrahim, E. A., Al Seghayer, S. M., Al Amri, S. A., and Gradoni, L. (1994). *Phlebotomus papatasi* (Scopoli), vector of zoonotic cutaneous leishmaniasis in Riyadh province, Saudi Arabia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **88**, 40.

Nolan, T. J., and Herman, R. (1985). Effect of long-term *in vitro* cultivation on *Leishmania donovani* promastigotes. *Journal of Protozoology*, **32**, 70-75.

North, M. J., and Coombs, G. H. (1981). Proteinases of *Leishmania mexicana* amastigotes and promastigotes: analysis by gel electrophoresis. *Molecular and Biochemical Parasitology*, **3**, 293-300.

North, M. J., Mottran, J. C., and Coombs, G. H. (1990). Cysteine proteinase of parasitic protozoa. *Parasitology Today*, **6**, 270-275.

Okong-Odera, E. A., Wamachi, A., Kagai, J. M., Kurtzhals, J. A. L., Githure, J. I., Hey, A. S., Were, J. B. O., Koech, D. K., Mitema, E. S., and Kharazmi, A. (1993). Field application of an ELISA using redefined *Leishmania* antigene for the detection of visceral Leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **87**, 423-424.

Pan, A. A. (1984). *Leishamnia mexican:* Serial cultivation of intracellular stages in a cell-free medium. *Expermintal Parasitology*, **58**, 72-80.

Pan, A. A., and MacMahon-Pratt, D. (1988). Monoclonal antibodies specific for amastigot stage of *Leishmania pifanoi*. I. Characterization of antigens associated with stage and species specific determinants. *The Journal of Immunology*, **140**, 2406-2414.

Pan, A. A., MonroeDuboise, S., Eperon, S., Rivas, L., Hodgkinson, V., Traub-Cseko, Y., and MacMahon-Pratt, D. (1993). Developmental life cycle of *Leishmania*-Cultivation and characterization of cultured extracellular amastigotes. *Journal of Eukaryotic Microbiology*, **40**, 213-223.

Pan, A. A., and Pan, S. C. (1986). *Leishmania mexicana*: Comperative fine structure of amastigotes and promastigotes *in vitro* and *in vivo*. *Expermintal Parasitology*, **62**, 254-265.

Peters, W., Elbihar, S., Ching Liu, Le Bianco, S. M., Evans, D. A., and Killic-Kendrick, R. (1985). *Leishmania* infecting man and wild animals in Saudi Arabia. 1. General survey. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **79**, 831-839.

Piarroux, R., Gambarelli, F., Dumon, H., Fontes, M., Dunan, S., Mary, C., Toga, B., and Quilici, M. (1994). Comparison of PCR with direct examination of bone marrow

aspiration, myeloculture, and serology for the diagnosis of visceral leishmaniasis in immunocompromised patients. *Journal of Clinical Microbiology*, **32**, 746-749.

Pimenta, P. F. P., Da Silva, R. P., Sacks, D. L., and Da Silva, P. P. (1989). Cell surface nanatomy of *Leishmania major* as revealed by fracture-flip. A surface meshwork of 44 nm fusiform filaments identifies infective developmental stage promastigotes. *European Journal of Cell Biology*, **48**, 180-190.

Pimenta, P. F. P., Saraiva, E. M. B., and Sacks, D. L. (1991). The comparative fine structure and surface glycoconjugate expression of three life stages of *Leishmania major*. *Expermintal Parasitology*, **72**, 191-204.

Pimenta, P. F. P., Turco, S. J., McConville, M. J., Lawyer, P. G., Perkins, P. V., and Sacks, D. L. (1992). Stage-specific adhesion of *Leishmanai* promastigote to the sandfly midgut. *Science*, **256**, 1812-1815.

Puentas, S. M., Dwyer, D. M., Bates, P. A., and Joiner, K. A. (1990). Binding and release of C3 from *Leishmania donovani* promastigotes during incubation in normal human serum. *The Journal of Immunology*, **143**, 3743-3750.

Puentas, S. M., Sacks, D. L., Da Silva, R. P., and Joiner, K. (1988). Complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. *The Journal of Experimental Medicine*, **167**, 887-902.

Qiao, Z., Miles, M. A., and Wilson, S. M. (1995). Detection of parasites of the *Leishmania donovani*-complex by a polymerase chain reaction-solution hybridization enzyme-linked immunoassay (PCR-SHELA). *Parasitology*, **110**, 269-275.

Rachamim, N., and Jaffe, C. L. (1993). Pure protein from *Leishmania donovani* protects mice against both cutaneous and visceral leishmaniasis. *The Journal of Immunology*, **150**, 2322-2331.

Rainey, P. M., Spithill, T. W., McMahon-Pratt, D., and Pan, A. A. (1991). Biochemical and molecular characterization of *Leishmania pifanoi* amastigotes in continuous axenic culture. *Molecular and Biochemical Parasitology*, **49**, 111-118.

Reed, S. G., Shreffler, W. G., Burns, J. M., Scott, J. M., Gloria Orge, M., Ghalib, H. W., Siddig, M., and Badaro, R. (1990). An improved serodiagnostic procedure for visceral leishmaniasis. *American Journal of Tropical Medicine and Hygiene*, **43**, 632-639.

Rey, J. A., Travi, B. L., Valencia, A. Z., and Saravia, N. G. (1990). Infectivity of the subspecies of *Leishmania braziliensis* complex *in vivo* and *in vitro*. *American Journal of Tropical Medicine and Hygiene*, **43**, 623-631.

Rizvi, F. S., Afchan, D., Sherlock, I., Sadigursky, M., Capron, A., and Santoro, F. (1985). Infectivity of *Leishmania* promastigotes is associated with surface antigenic expression. *Immunology Letters*, **11**, 317-323.

Robertson, C. D., and Coombs, G. H. (1992). Stage specific proteinases of *Leishmania mexicana mexicana* promastigotes. *FEMS Microbiology Letters*, **94**, 127-132.

Rodgers, M. R., Popper, S. J., and Wirth, D. F. (1990). Amplification of kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. *Expermintal Parasitology*, **71**, 267-275.

Russell, D. G. (1987). The macrophage-attachement glycoprotein gp63 is the predominant C3-acceptor site on *Leishmania mexicana* promastigotes. *European Journal of Biochemistry*, **164**, 213-221.

Russell, D. G., and Talmas-Rohana, P. (1989). *Leishmania* and the macrophage: a marriage of inconvenience. *Immunology Today*, **10**, 328-333.

Sacks, D. L. (1988). Developmental biology of *Leishmania* promastigotes. *In*: The Biology of Parasitism (P.T.Englund, and A.Sher, Eds), pp. 93-103. Alan R. Liss: New York.

Sacks, D. L. (1989). Metacyclogenesis in *Leishmania* promastigotes. *Expermintal Parasitology*, **89**, 101-103.

Sacks, D. L., and Da Silva, R. (1987). The generation of infective stage *Leishmania major* promastigotes is associated with the cell-surface expression and release of a developmentally regulated glycolipid. *The Journal of Immunology*, **139**, 3099-3106.

Sacks, D. L., Bordin, T. N., and Turco, S. J. (1990). Developmental modification of the lipophosphoglycan from *Leishmania major* promastigotes during metacyclogenesis. *Molecular and Biochemical Parasitology*, **42**, 225-234.

Sacks, D. L., and Perkins, P. V. (1984). Identification of an infective stage of *Leishmania* promastigotes. *Science*, **223**, 1417-1419.

Sacks, D. L., and Perkins, P. V. (1985). Development of infective stage of *Leishmania* promastigotes within Phlebotomine sand flies. *American Journal of Tropical Medicine and Hygiene*, **34**, 456-459.

Santos-Gomes, G., and Abranches, P. (1996). Comparative study of infectivity caused by promastigotes of *Leishmania infantum* MON-1, *L. infantum* MON-24 and *L. donovani* MON-18. *Folia Parasitologica*, **43**, 7-12.

Schlein, Y., and Jacobson, R. L. (1994). Haemoglobin inhibits the development of infective promastigotes and chitinase secretion in *Leishmania major* cultures. *Parasitology*, **109**, 23-28.

Schnur, L. F. (1989). Premunition, a possible limitation in the prevention of the leishmaniasis by vaccination. *In*: Leishmaniasis: The Current Status and New Strategies for Control (D.T.Hart, Ed), pp. 379-385. Plenum: New York.

Segovia, M., Artero, M., Mellado, E., and Chance, M. L. (1992). Effects of long-term *in vitro* cultivation on the virulence of cloned lines of *Leishmania major* promastigotes. *Annals of Tropical Medicine and Parasitology*, **86**, 347-354.

Service, M. W. (1986). Lecture Notes on Medical Entomology. p. 265. Blackwell Scientific Publications: Oxford.

Shapira, M., McEwen, J. G., and Jaff, C. L. (1988). Temperature effects on molecular processes which lead to stage differentiation in *Leishmania. The EMBO Journal*, 7, 2895-2901.

Shortt, H. E., Smith, R. O. A., and Swaminath, C. S. (1931). Transmission of indian kala-azar by the bite of *Phlebotomus argentipes*. *Indian Journal of Medical Research*, **18**, 1373-1375.

Steiger, R. F., and Steiger, E. (1976). A defiened medium for cultivatiing *Leishmania donovani* and *L. braziliensis*. *The Journal of Parasitology*, **62**, 1010-1011.

Steiger, R. F., and Steiger, E. (1977). Cultivation of *Leishmania donovani* and *Leishmania braziliensis* in defined media: Nutritional requirements. *Journal of Protozoology*, **24**, 437-441.

Tetley, L., Hunter, C. A., Coombs, G. H., and Vickerman, K. (1989). Generation of megasomes during the promastigote-amastigote transformation of *Leishmania*

mexicana mexicana in vitro. *In*: Leishmaniasis, the Current Status and New Strategies for Control (D. T. Hart., Ed), pp. 449-455. Plehum Press: New York.

Tomlinson, S., Vandekerckhove, F., Frevert, U., and Nussenzweig, V. (1995). The induction of *trypanosoma cruzi* trypomastigote to amastigote transformation by low pH. *Parasitology*, **110**, 547-554.

Turco, S. J. (1990). The leishmanial lipophosphoglycan: a multifunctional molecule. *Expermintal Parasitology*, **70**, 241-245.

Turco, S. J. (1991). Structural variations of the leishmanial lipophosphoglycan. *In*: Biochemical Protozoology (G. H. Coombs, and M. J. North, Eds), pp. 304-311. Taylor and Francis: London.

Turco, S. J., and Descoteaux, A. (1992). The lipophosphoglycan of *Leishmania* parasite. *Annual Review of Microbiology*, **46**, 65-94.

Van Eys, G. J. J. M., Schoone, G. J., Ligthart, G. S., Schalken, J. A., and Terpstra, W. J. (1989). Application of total and recombinant DNA probes in the diagnosis of leishmania infection. *In*: Leishmaniasis: The Current Status and New Strategies for Control (D.T.Hart, Ed), pp. 515-524. Plenum: New York.

Vickerman, K., and Tetley, L. (1990). Flagellar Surfaces of Parasitic Protozoa and Their Rile in Attachment. *In*: Ciliary and Flagellar Membranes (R.A.Bloodgood, Ed), pp. 267-304. Plenum Publishing Corporation: London.

Walton, B. C., Brooks, W. H., and Arjona, I. (1972). Serodiagnosis of American leishmaniasis by indirect fluorescent antibody test. *American Journal of Tropical Medicine and Hygiene*, **21**, 296-299.

Webster, P., and Russell, D. G. (1993). The flagellar pocket of trypanosomatids. *Parasitology Today*, **9**, 201-206.

Wenyon, C. M. (1911). Oriental sore in Baghdad, together with observations on a gregarine in *Stegomyia fasciata*, the haemogregrine of dogs and the flagellates of house flies. *Parasitology*, **4**, 273-330.

WHO (1990). Report of the WHO expert Committee. Control of leishmaniasis. *Technical Report Series*, **793**, .

WHO (1995). Leishmaniasis (the current situation). *The Internet, http://www.who*.ch/

Williams, J. E. (1995). *Leishmania* and *Trypanosoma*. *In*: Medical Parasitology (S.H.Gillespie, and P.M.Hawkey, Eds), pp. 151-176. Oxford University Press: Oxford.

Wilson, M. E., and Pearson, R. D. (1984). Stage-specific variations in lectin binding to *Leishmania donovani*. *Infection and Immunity*, **46**, 128-134.

Wozencraft, A. O., and Blackwell, J. M. (1987). Increased infectivity of stationary-phase promastigotes of *Leishmania donovani*: Correlation with enhanced C3 binding capacity and CR3-mediated attachement to host macrophages. *Immunology*, **60**, 559-563.

Zilberstein, D. (1991). Adaptation of *Leishmania* species to an acidic environment. *In*: Biochemical Protozoology (G. H. Coombs, and M. J. North, Eds), pp. 349-358. Tayolr & Francis: London.

Zilberstein, D., Blumonfeld, N., Liveanu, V., Gebstein, A., and Jaffe, C. L. (1991). Growth in acidic pH induces an amastigote stage-specific protein in *Leishmania* promastigotes. *Molecular and Biochemical Parasitology*, **45**, 175-178.

Zilberstein, D., and Shapira, M. (1994). The role of pH and temperature in the development of *Leishmania* parasites. *Annual Review of Microbiology*, **48**, 449-470.

APPENDIX 1.
ELECTROPHORESIS OF PROTEINS BY SDS-PAGE

PREPERATION OF STOCK SOLUTIOS

1. Stacking gel buffer stock.
   (0.5 M Tris-HCl pH 6.8)
   - 6g Tris
   - 40 ml water
   - Titrate to pH 6.8 with 1M HCl (≈48ml)
   - Bring to 100 ml final volume with water
   - Filter and store at 4°C

2. Resolving gel buffer stock.
   (3.0 M Tris-HCl pH 8.8)
   - 36.3 g Tris
   - 48ml of 1M HCl
   - Adjust pH to 8.8 if necessary
   - Bring to 100 ml final volume with water
   - Filter and store at 4°C

3. Reservoir buffer stock
   (0.25M Tris, 1.92 M glycine, 1% SDS pH 8.3) X 10
   - 30.3g of Tris
   - 144.0g glycine
   - 10g SDS
   - Dissolve and bring to 1 liter with water.
   - Store at room temperature.

4. 30 % Sucrose
   - 30g Sucrose
   - Dissolve and bring to 100 ml with water.

5. Gel sample buffer.
   1X Non-reducing (1XNR).
   (0.0625 M Tris/HCl pH 6.8, 2% SDS, 10% sucrose,
   0.002% Bromophenol blue BPB)
   - 12.5ml of 0.5 Tris pH 6.8
   - 20ml of 10%SDS
   - 33.3ml of 30% sucrose
   - 0.8ml of 0.25% BPB
   - 33.4 ml of water
   - Store at room temperature.

6. Overlaying buffer.
   - Resolving gel buffer stock 18.75ml
   - 10% SDS 1.5ml
   - Water 129.75ml
   - Store at room temperature.

7. Coomassie Blue staining solution
   (0.1% Coomassie Blue in water:methanol:glacial aceticacid [5:5:2]).
   - Glacial acetic acid 800 ml
   - Water 2000 ml
   - Methanol 2000 ml
   - Coomassie blue 5 g
8. Destaining solution
   (7% acetic acid)
   - 7 ml acetic acid
   - 93 ml water

SAMPLE PREPARATION FOR GEL ELECTROPHORESIS

1. Prepare all solutions.

2. Set the water bath temperature to boiling temperature.

3. When the water bath is ready, put some ice in a container and take the frozen cell pellets directly from the freezer into the ice.

4. Add sample buffer and mix. Final density of 10^9 parasites per ml is usually used.

5. Place in boiling water for 3 minutes.

6. Centrifuge for 2 minutes.

PROCEDURE FOR GEL ELECTROPHORESIS (MINIGELS)

1. Clean all glass plates with alcohol (absolute methanol or ethanol) or acetone.

2. Prepare all stock solutions. Check twice before starting.

3. Assemble glass plates into holders according to the manufacturer instructions.

4. Prepare the resolving gel and pour between the two plates with a pipette leaving a space for the stacking gel. Note that after polymerization the gel shrinks slightly.

5. Take a 1ml syringe with a 25G needle, fill with the overlaying buffer and place carefully between the two glass plates to overlay the gel.

6. Leave the gel to polymerize.

7. Wash with distilled deionized water and drain.

8. Prepare the stacking gel and pour on top of the resolving gel. Insert the comb and leave to polymerize.

9. During polymerization of the stacking gel prepare the samples.

10. After the stacking gel has polymerized, wash with reservoir buffer.

11. Place the gel into the electrophoresis chamber.

12. Add the reservoir buffer to the inner chamber and leave for 10 minutes to check for leaking.

13. Add 10 µl of samples and molecular weight markers and/or controls to each well.

14. Add the reservoir buffer to the outer chamber.

15. Run electrophoresis with a constant voltage of 200 volts. Check for bubbling from the electrode in the inner chamber.

16. Leave the electrophoresis running until the dye front reaches 3 mm from the bottom of the gel.

17. Stop electrophoresis. Pour off the buffer and deassemble the gel plates from the holder.

18. Open the two glass plates by inserting a spacer between the two plates and twisting it.

19. Remove the stacking gel using a razor blade.

20. Transfer the gel to a staining container.

21. Add the stain and leave on the shaker for a minimum of two hours (may be left over night).

22. Destain in 7 % acetic acid until the bands are clear.

RECIPE FOR GEL PREPARATION USING THE SDS DISCONTINUOUS BUFFER SYSTEM
40 % ACRYLAMID (37.5:1)

Stock solution	Stacking gel	Final acrylamide concentration in resolving gel						
		20 %	17.5%	15 %	12.5 %	10 %	7.5 %	5 %
Acrylamide-bisacrylamide	1.875	15.0	13.125	11.25	9.375	7.5	5.625	3.75
Stacking buffer	5.0	-	-	-	-	-	-	-
Resolving buffer	-	3.75	3.75	3.75	3.75	3.75	3.75	3.75
10 % SDS	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3
1.5 % ammonium per sulfate	1.0	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Distelled water	11.925	9.45	11.325	13.2	15.075	16.95	18.825	20.7
TEMED	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015
Total volume	20	30	30	30	30	30	30	30

Volumes are in ml.